Nov 04, 2020

COVID-19 ARTIC v3 Illumina library construction and sequencing protocol - tailed method

Forked from COVID-19 ARTIC v3 Illumina library construction and sequencing protocol

DNA Pipelines R&D[1], Benjamin Farr[1], Diana Rajan[1], Emma Betteridge[1], Lesley Shirley[1], Michael Quail[1], Naomi Park[1], Nicholas Redshaw[1], Iraad F Bronner[1], Louise Aigrain[1], Scott Goodwin[1], Scott Thurston[1], Stefanie Lensing[1], James Bonfield[1], Keith James[1], Nicholas Salmon[1], Charlotte Beaver[1], Rachel Nelson[1], David K. Jackson[1], Alex Alderton[1], Ian Johnston[1]

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1 Works for me dx.doi.org/10.17504/protocols.io.bky5kxy6

COG-UK

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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) in multiplex PCR. Illumina-compatible sequencing libraries are then made directly from these amplicons in a second PCR step, obviating the need for conventional library preparation. The products of these PCRs are then equivolume pooled and quantitated, prior to sequencing on the Illumina NovaSeq.

It is an adaptation of the COVID-19 ARTIC v3 amplicon protocol which can be found here: https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bibtkann

Both the above protocols were adapted from the nCov-2019 sequencing protocol: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w

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PROTOCOL CITATION

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COVID-19, SARS-Cov-2, amplicon sequencing, ARTIC, Illumina library construction, coronavirus

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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 TEXT

MATERIALS

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602** Step 13

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

Illumina Library Quantitation Complete kit (Universal) **Kapa Biosystems Catalog #KK4824**

NEB Q5® Hot Start High-Fidelity 2X Master Mix **New England Biolabs Catalog #M0494L** Step 8

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

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) in multiplex PCR. Illumina-compatible sequencing libraries are then made directly from these amplicons in a second PCR step, obviating the need for conventional library preparation. The products of these PCRs are then equivolume pooled and quantitated, prior to sequencing on the Illumina NovaSeq.

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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** .

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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/384 RXN (µL) inc. excess
LunaScript Super Mix	4	1843
Nuclease-free water	6	2765
Total	**10**	**4608**

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 plate and place on a BioShake plate shaker for 30 seconds at 1500rpm to mix. 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 (PCR1)

8

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

Where an alt primer is available, the non alt version is omitted.

Achieving more even genome coverage

A hypothetical 'ideal' multiplex primer pool would generate the same number of reads from each amplicon, so

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the fraction of reads due to each amplicon would be 1/n, where n is the number of primer pairs in the multiplex pool. In reality this is not achievable, and the fraction of reads observed for each amplicon varies widely.

The ratio [actual observed read fraction/'ideal' read fraction] can be calculated for each individual amplicon, as indicated by the differently-coloured dots on the box-and-whisker plots below. This tells us whether a particular amplicon is under-represented (ratio <1x) or over-represented (>1x).

By changing the weights of each primer pair within the primer pool ('*rebalancing*') the number of reads obtained for each amplicon can be modified, and the effect of the process is illustrated below. The plots show the distribution per amplicon prior to rebalancing primer pair concentrations (above) and after (below). More amplicons cluster around 1x after rebalancing and the distance between the maximum and minimum ratios is also markedly reduced.

Weight to apply per primer pair

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Pool1 primer pair	Weight (rounded to 1dp)		Pool2 primer pair	Weight (rounded to 1dp)
21L alt2 & 21R alt0	1		44L alt3 & 44R alt0	1
45L alt2 & 45R alt7	1.3		76L alt3 & 76R alt0	1.1
93L & 93R	1.4		14L alt4 & 14R alt2	1.4
47L & 47R	1.4		40L & 40R	1.7
77L & 77R	1.4		52L & 52R	1.8
37L & 37R	1.4		58L & 58R	1.8
43L & 43R	1.5		88L & 88R	1.8
53L & 53R	1.5		6L & 6R	1.9
49L & 49R	1.5		34L & 34R	1.9
15L alt1 & 15R alt3	1.5		42L & 42R	1.9
75L & 75R	1.5		62L & 62R	1.9
59L & 59R	1.5		72L & 72R	1.9
27L & 27R	1.6		82L & 82R	1.9
57L & 57R	1.6		84L & 84R	1.9
83L & 83R	1.6		90L & 90R	1.9
51L & 51R	1.7		2L & 2R	2
3L & 3R	1.7		16L & 16R	2
61L & 61R	1.7		30L & 30R	2
35L & 35R	1.7		32L & 32R	2
39L & 39R	1.7		56L & 56R	2
1L & 1R	1.7		92L & 92R	2
69L & 69R	1.7		46L alt1 & 46R alt2	2
87L & 87R	1.7		48L & 48R	2.1
11L & 11R	1.8		60L & 60R	2.1
79L & 79R	1.8		94L & 94R	2.1
41L & 41R	1.8		38L & 38R	2.2
65L & 65R	1.8		78L & 78R	2.2
63L & 63R	1.9		80L & 80R	2.2
25L & 25R	1.9		4L & 4R	2.3
7L alt0 & 7R alt5	1.9		28L & 28R	2.3
19L & 19R	2		8L & 8R	2.4
73L & 73R	2.1		10L & 10R	2.4
13L & 13R	2.2		22L & 22R	2.4
85L & 85R	2.2		98L & 98R	2.4
97L & 97R	2.3		20L & 20R	2.5
81L & 81R	2.3		96L & 96R	2.6
33L & 33R	2.4		18L alt2 & 18R alt1	3
95L & 95R	2.4		26L & 26R	3
29L & 29R	2.4		36L & 36R	3.1
31L & 31R	2.7		54L & 54R	3.1
89L alt2 & 89R alt4	2.8		66L & 66R	3.5
71L & 71R	3.1		68L & 68R	3.5
55L & 55R	3.2		24L & 24R	3.6
9L alt4 & 9R alt2	3.2		50L & 50R	4.1
5L & 5R	3.4		12L & 12R	4.4
17L & 17R	3.5		86L & 86R	5.7
23L & 23R	3.8		64L & 64R	6
91L & 91R	3.9		74L & 74R	6.7
67L & 67R	6.2		70L & 70R	7.8
mean weight	*2.1*		*mean weight*	*2.6*
cumulative weight	*104.3*		*cumulative weight*	*129.5*

A more detailed description of the process is provided in this document:

Improving the evenness of SARS-CoV-2 genome coverage by titration of primer concentrati

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

Prepare the following mastermixes:

Weighted PCR Primer Pool 1 Master Mix	**Vol/PCR RXN (µl)**	**Vol/384 plate (µl) inc. excess**
Q5 Hotstart 2X Master Mix	12.5	5760
Primer **Pool 1** (mean 102nM)	3.6	1659
Nuclease-free water	2.9	1336
Total	**19**	**8755**

Weighted PCR Primer Pool 2 Master Mix	**Vol/PCR RXN (µl)**	**Vol/384 plate (µl) inc. excess**

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Q5 Hotstart 2X Master Mix	12.5	5760
Primer **Pool 2** (mean 102nM)	3.6	1659
Nuclease-free water	2.9	1336
Total	**19**	**8755**

The equivolume primer pools used in the standard protocol are of **[M]10 Micromolar (µM)** cumulative concentration, therefore each of the 98 primers in each pool is at **[M]102 Nanomolar (nM)** in the pool and at **[M]15 Nanomolar (nM)** in the final reaction. With the rebalanced primer pools, for equivalency we dilute them such that the **average** primer concentration is **[M]102 Nanomolar (nM)** , and therefore the **average** concentration of each primer in the final reaction is also **[M]15 Nanomolar (nM)** .

Mix thoroughly by vortexing.

9 Use the SPT Labtech Dragonfly Discovery to dispense **19 µl** mastermix per well into 2x384 well plates.

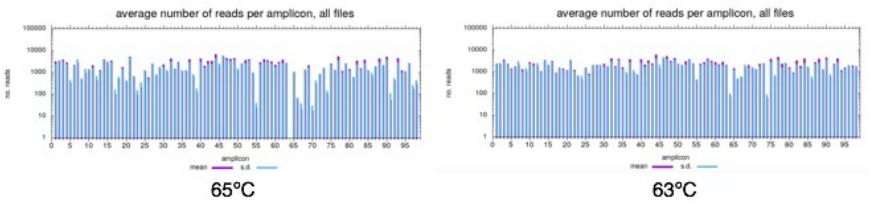
10 Use the Agilent Bravo to add **6 µl** of cDNA template to each primer pool reaction and mix.

It is recommended to use filtered tips for this transfer to reduce risk of cross sample contamination via aerosolisation.

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.

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.



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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).

Library construction from amplified cDNA (PCR2)

13

Illumina-compatible libraries are generated from a small aliquot of the amplified cDNA using KAPA HiFi HotStart ReadyMix, unique dual indexed (UDI) barcoding primers and pools of tailed versions of the primers used for the cDNA amplification.

The tailed primer pools used in this stage correspond to those used in the cDNA amplification stage, with the following modifications:

- All primers are used at the same concentration in the pools; from the individual **[M]500 Micromolar (µM)** primer stocks we create pool 1 and pool 2 stocks with each of the 98 primers in each pool @ **[M]5 Micromolar (µM)**
- The penultimate DNA base at the 3' end of the primer is replaced with its 2'-*O*-Methyl RNA equivalent (this reduces the formation of primer-dimers). Typically we use this modification for tailed multiplex PCR in the first (non barcoding) PCR. The use of non 2'-*O*-Methyl RNA modified tailed primers for use as detailed within this protocol is undergoing evaluation.
- ACACTCTTTCCCTACACGACGCTCTTCCGATCT appended to the 5' end of all LEFT primers (this is the Illumina Multiplexing Read 1 sequence)
- TGACTGGAGTTCAGACGTGTGCTCTTCCGATCT appended to the 5' end of all RIGHT primers (this is the Illumina Multiplexing Read 2 sequence)

Both UDI barcoding primers and tailed primer pools are predispensed to plates and frozen down in advance for ease of processing. Starting from stock plates of the UDI barcoding primers (**5 µl** at **[M]10 Micromolar (µM)**), we dilute the UDIs and add the tail primers to create plates with a volume of **6.25 µl** per well, with i5 and i7 indexing primers at **[M]2 Micromolar (µM)** each and the tail primers at **[M]4 Nanomolar (nM)** .

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

14 Defrost two UDI tag plates (one containing each tail primer pool), both of which should contain the same i5 and i7 barcodes per well.

15 Use the SPT Labtech Mosquito LV to transfer **100 nl** of amplified pool 1 cDNA into the UDI tag plate containing the pool 1 tailed primers and **100 nl** of amplified pool 2 cDNA into the UDI tag plate containing the pool 2 tailed primers, maintaining the same well locations throughout. Immediately proceed to the next step.

16 Use the SPT Labtech Dragonfly Discovery to dispense **6.25 µl** of Kapa HiFi 2X Mastermix into each well of both UDI tag plates, and place **On ice** immediately. The dispense is sufficient to mix all the reagents.

The final PCR volume is **12.5 µl**

The final concentration of each tailing primer in the reaction will be **2 Nanomolar (nM)**

The final concentration of each barcoding primer in the reaction will be **1 Micromolar (µM)**

The amplified cDNA template forms **0.8 % (v/v)** of the total PCR volume

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

Step	Temperature	Time
1	95°C	5 minutes
2	98°C	30 seconds
3	61°C	20 minutes
4	72°C	2 minutes
Repeat steps 2-4 once more		
5	98°C	30 seconds
6	65°C	30 seconds
7	72°C	2 minutes
Repeat steps 5-7 six more times		
8	72°C	5 minutes
9	4°C	∞

The long annealing times of the first two cycles of PCR ensure efficient annealing of the tailed primers to their targets in the amplified cDNA (and therefore incorporation of the tail sequences) in spite of their very low concentration in the PCR. In the following seven cycles of PCR the much shorter annealing time and increased annealing temperature make the annealing of the tailed primers inefficient, therefore only the UDI barcoding primers participate in the PCR. This ensures that the vast majority of products formed at the end of the PCR are of

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full length.

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

Construction of equivolume pool

19 In a post-PCR lab, use the Agilent Bravo to combine and mix **5 µl** of pool 1 and pool 2 PCR2 reactions per sample into one plate.

20 Use the Hamilton STAR to combine **3 µl** of each sample to form an equivolume pool of 384 samples.

Equivolume pool SPRI bead cleanup

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

The Hamilton STAR will perform a **0.8X** SPRI clean-up and elute the final pool in 1ml elution buffer as follows:

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

21.2 Incubate for **00:06:00** at **Room temperature** . 6m

21.3 Transfer the tube to a magnet, allow **00:04:00** for the beads to form a pellet. 4m

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

21.5 Wash the beads with **500 µl** 75% ethanol for **00:00:15** then carefully remove ethanol and discard. 15s
(First wash)

21.6 Wash the beads with **500 µl** 75% ethanol for **00:00:15** then carefully remove ethanol and discard. 15s
(Second wash)

21.7 Wash the beads with **500 µl** 75% ethanol for **00:00:15** then carefully remove ethanol and discard. 15s

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(Third wash)

21.8 Allow beads to dry for **00:05:00** .

21.9 Remove tube from magnet and resuspend beads in **200 µl** elution buffer, mix well by pipetting.

21.10 Incubate for **00:03:00** at **Room temperature**

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

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

21.13 Add **800 µl** elution buffer to bring the pool to a final volume of 1ml, mix well by pipetting.

Equivolume pool quantification

22

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

23

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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.

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

25 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.

26 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.

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

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

29 **18 µl** of each **1 Nanomolar (nM)** pool is required per SP lane.
Denature pools by adding **4 µl** 0.2N NaOH per 18µl. Vortex briefly to mix.

30 Incubate at **Room temperature** for **00:08:00**

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

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

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

Prepare ExAmp mastermix on ice:

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ExAmp Master Mix	Volume 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**

33 Add **63 µl** ExAmp mastermix to each denatured pool, mix well by pipetting.

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

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

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

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

36.2 Load the SBS and cluster reagent cartridges.

36.3 Start sequencing run (250PE).

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