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COVID-19 ARTIC v3 Illumina library construction and sequencing protocol - high throughput 384 format V.2

Version 1 is forked from COVID-19 ARTIC v3 Illumina library construction and sequencing protocol

DOI

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Coronavirus Method De...

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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 offers the benefit of higher density sample processing in 384 format, whilst matching the data quality achieved in 96 format described in the original protocol:
https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bgxjjxkn

Both the above protocols were adapted from the nCov-2019 sequencing protocol:
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

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

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

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

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

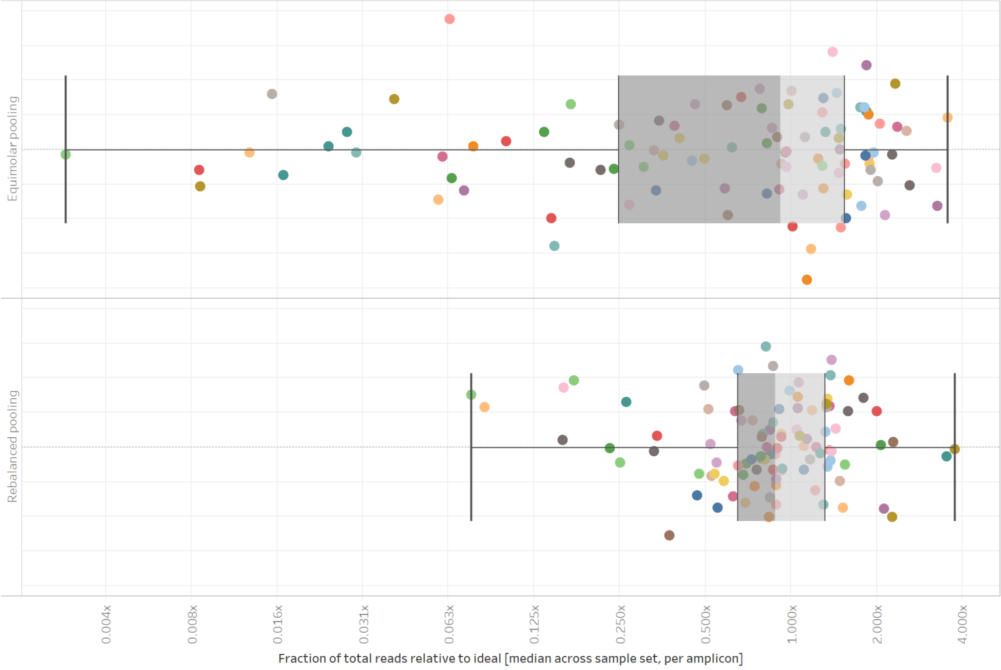
Expected result

Achieving more even genome coverage

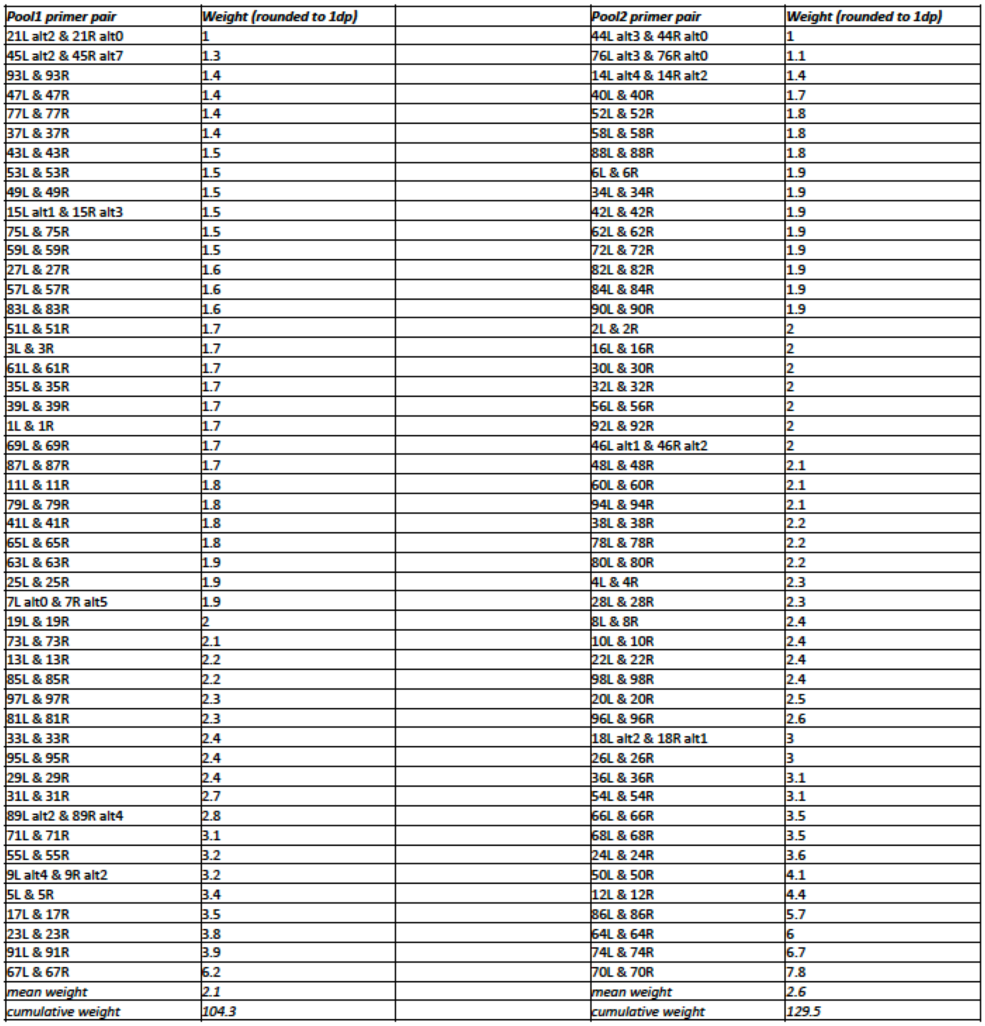
A hypothetical 'ideal' multiplex primer pool would generate the same number of reads from each amplicon, so the fraction of reads due to each amplicon would be 1/n, where n is the number of primer pairs in the multiplex pool. In reality this is not achievable, and the fraction of reads observed for each 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



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-C...

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**
	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**

Note

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 2×384 well plates.

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

Note

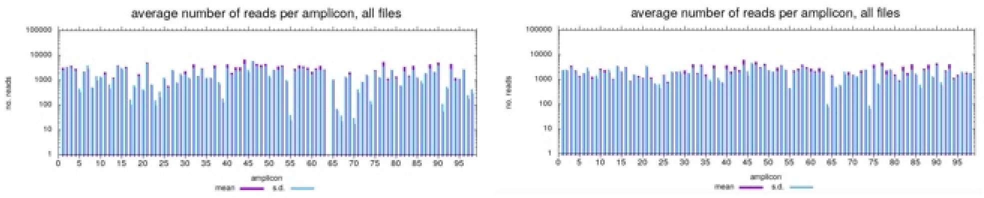
It is recommended to use filtered tips for this transfer to reduce risk of cross sample contamination via aerosolisation.

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.

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.



65°C 63°C

	Step	Temperature	Time
	1	98°C	30 seco

			nds
	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 the SPT LabTech HV Mosquito to combine 5 µL of primer 1 PCR and 5 µL of primer 2 PCR reactions per sample into a new plate. Store the unused portion of primer 1 and primer 2 PCR plates at -20 °C . Proceed as follows with the recombined plate.

16 Use the Hamilton STAR with a 384 well multichannel head to perform the following steps:

16.1 Add 10 µL nuclease-free water to each sample and mix well by pipetting.

16.2 Add **0.8X** volume of SPRI beads per sample (16 µL SPRI : 20 µL amplified cDNA), mix well by pipetting.

16.3 Incubate for 00:05:00 at Room temperature

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

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

16.6 Wash the beads with 45 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(First wash)

16.7 Wash the beads with 45 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(Second wash)

16.8 Allow beads to dry 00:05:00

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

16.10 Incubate for 00:03:00 at Room temperature

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

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

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

Amplified cDNA quantification

18

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

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

20 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.

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

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

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

24 Confirm known standards are performing as expected.

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

26 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

27

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

28

Prepare end prep mastermix On ice :

	NEBNext End Prep	**Vol/PCR RXN (µl)**	**Vol/384 plate (µl) inc. excess**
	NEBNext Ultra II End Prep Enzyme Mix	1.2	552
	NEBNext Ultra II End Prep Reaction Buffer	2.8	1288
	Total	**4**	**1840**

Mix well by pipetting.

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

30 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	∞

31 Prepare adapter ligation mastermix 🌡 On ice :

Adapter Ligation	Vol/PCR RXN (µl)	Vol/384 plate (µl) inc. excess
NEBNext Ultra II Ligation Master Mix	12	5520
NEBNext Ligation Enhancer	0.4	184
TruSeq adapter (10µM)	1	460
Total	**13.4**	**6164**

Mix well by pipetting.

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

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

34

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.

35 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:

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

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

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

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

35.5 Wash the beads with 45 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(First wash)

35.6 Wash the beads with 45 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(Second wash)

35.7 Allow beads to dry for 00:05:00

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

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

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

35.11 Carefully transfer supernatant into a new plate, taking care not to disturb the bead pellet. 10 µL of this eluate is used as input for library PCR.

Library PCR

36

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

6900 µL KAPA HiFi HotStart ReadyMix is required per 384 plate (including excess).

37 The Bravo will add 15 µL KAPA HiFi HotStart ReadyMix and 10 µL sample into a 5 µL plate of UDIs and mix thoroughly by pipetting. The final concentration of each UDI in the PCR reaction is 2µM.

38 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

39 In a post-PCR lab, use the Hamilton STAR to combine 3 µL of each sample per plate to form an equivolume pool of 384 samples.

Equivolume pool SPRI

40 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 **200µl** elution buffer as follows:

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

40.2 Incubate for 00:06:00 at Room temperature .

40.3 Transfer the tube to a magnet, allow 00:04:00 for the beads to form a pellet.

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

40.5 Wash the beads with 500 µL 75% ethanol for 00:00:15 then carefully remove ethanol and discard.
(First wash)

40.6 Wash the beads with 500 µL 75% ethanol for 00:00:15 then carefully remove ethanol and discard.
(Second wash)

40.7 Wash the beads with 500 µL 75% ethanol for 00:00:15 then carefully remove ethanol and discard.
(Third wash)

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

40.9 Remove tube from magnet and resuspend beads in 200 µL elution buffer, mix well by pipetting.

40.10 Incubate for 00:05:00 at Room temperature

40.11 Transfer tube to magnet, allow 00:00:45 for the beads to form a pellet.

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

Equivolume pool quantification

41

Note

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

qPCR
Quantify pools 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

42

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.

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

44 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.

45 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.

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

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

48 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.

49 Incubate at Room temperature for 00:08:00

50 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.

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

Prepare ExAmp mastermix on ice:

	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

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

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

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

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

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

55.2 Load the SBS and cluster reagent cartridges.

55.3 Start sequencing run (250PE).