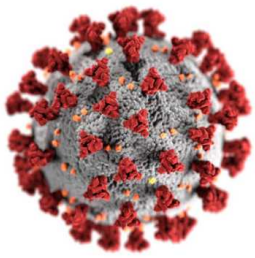
Apr 07, 2020 Version 3

SARS-CoV-2 Enrichment Sequencing by Spiked Primer MSSPE method V.3



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We use this protocol and it's working

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Abstract

This protocol was used to enrich for SARS-CoV2 sequencing reads from a confirmed COVID-19 swab sample and attain the full genome using an iSeq100. By using a spiked primer approach with 73 primers spanning the entire SARS-CoV2 genome, we were able to get an average of 15x genome coverage on an iSeq100 with 1.8 million paired end-reads. Here we overview all the steps, from sample extraction, library preparation with a spiked primer enrichment step, and sequencing on an iSeq100. The collaborative effort involved the Manning Lab, NIAID in Phnom Penh, Cambodia, Institut Pasteur Cambodge, Cambodia Ministry of Health, the Chan Zuckerberg Biohub, and the Chan Zuckerberg Initiative.

This approach was developed based on the work from Deng et al, Nature Microbiology, January 13, 2020.
https://www.nature.com/articles/s41564-019-0637-9

Materials

MATERIALS

- NEBNext Ultra II RNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs** **Catalog #**E7770L
- NEBNext Adaptor for Illumina **New England Biolabs**
- Qubit dsDNA HS Assay kit **Thermo Fisher Scientific** **Catalog #**Q32854
- QIAamp Viral RNA Mini Kit **Qiagen** **Catalog #**52904
- NEBNext USER Enzyme **New England Biolabs** **Catalog #**E7458
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)
- High Sensitivity D5000 ScreenTape **Agilent Technologies** **Catalog #**5067-5592
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
- TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina) **New England Biolabs** **Catalog #**E7500L
- ERCC RNA Spike-In Mix **Thermo Fisher** **Catalog #**4456740
- QIAseq FastSelect rRNA Removal Kit **Qiagen** **Catalog #**333180
- DNase I Set **Zymo Research** **Catalog #**E1010

STEP MATERIALS

- QIAamp® Viral RNA Mini **Qiagen** **Catalog #**52906
- DNase I Set **Zymo Research** **Catalog #**E1010
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
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Sample Collection

1 Nasopharyngeal and oropharyngeal swabs (combined into one tube) were collected from a symptomatic patient meeting case definition for possible infection with SARS-CoV-2.

RNA extraction

2 Extraction of viral nucleic acids from clinical sample was performed with a QIAamp Viral RNA Mini Kit (Qiagen #52906) as described by manufacturer

QIAamp® Viral RNA Mini **Qiagen Catalog #**52906

Extracted RNA samples were DNAse-treated using the Zymo DNAse I kit.

DNase I Set **Zymo Research Catalog #**E1010

The nucleic acid was tested via real-time polymerase chain reaction for COVID-19 using both the Drosden and HKU protocols published by the World Health Organization and confirmed positive for COVID-19 on January 27th, 2020.

Ct values for PUI = 24.

RNA Quantification

3 RNA was quantified using the Qubit High Sensitivity RNA kit as described by the manufacturer.

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32852

Qubit input RNA concentration for PUI = 6.9 ng/uL

Library Preparation

4 **Library Preparation was performed with the NEBNext Ultra II non-directional RNA kit.**

NEBNext Ultra II RNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #**E7770L

Fragmentation & SARS-CoV-2 primer spike in

Random primers at 1 µM are mixed with a 10 µM of SARS-CoV-2 primers (see below) at a 1:1 volume ratio.

		1 reaction
	Reagent	vol stock (uL)
	RNA (sample) (10ng – 100ng total)	3.5
	25pg ERCC Spike-in (50pg/uL stock) *	0.5
	(pink) First SS Reaction Buffer 5x	4
	(pink) Random Primers/Spiked primer mix	1
	QIAseq FastSelect (1:100) rRNA**	1
	Total volume	10
		uL/rxn

* (optional) ERCCs are internal synthetic RNA controls comprised of 92 synthetic RNAs that do not match to any known microbe in the NCBI NR/NT databases.

ERCC RNA Spike-In Mix **Thermo Fisher Catalog #**4456740

** (optional) FastSelect is designed for removing human rRNA, omit this reagent if not using human derived samples and only incubate at 94°C and then directly to 4°C (omit ramping down steps).

QIAseq FastSelect rRNA Removal Kit **Qiagen Catalog #**333180

Thermocycler (heated lid set to 105°C):

- 8 min at 94°C (Adjust this fragmentation time depending on the quality of extracted RNA)

- 2 min 75°C
- 2 min 70°C
- 2 min 65°C
- 2 min 60°C
- 2 min 55°C
- 5 min 37°C
- 5 min 25°C

The 73 primers tile across the entire SARS-CoV2 genome at a spacing of ~400bp.
The PDF below shows the approximate location of the primer binding sites.

enrichmentprimers.pdf

	Name	Sequence
	Primer_CoV1	GTGACTTCCATGCCAATG
	Primer_CoV2	CTGATTTTGGGGTCCATTATC
	Primer_CoV3	GAAATGGTGAATTGCCCTC
	Primer_CoV4	GATAGCAATTCCACCGGTG
	Primer_CoV5	CAGTATAACCACCAATCTG
	Primer_CoV6	CATTAATGCCAGAGATGTC
	Primer_CoV7	GTATTTGTAATGCAGCAC

	Primer_CoV8	CTTCTGTGCAGTTAACATC
	Primer_CoV9	GATTCTGTTGGTTGGAC
	Primer_CoV10	GAATGTAAAACTGAGGATCTG
	Primer_CoV11	CAGCTGTACCTGGTGCAAC
	Primer_CoV12	CACTACCTTCTGTAATAAG
	Primer_CoV13	CATACAAACTGCCACCATC
	Primer_CoV14	GTCCTTTGTACATAAGTG
	Primer_CoV15	GAGCTGATTTGTCTTTATGTG
	Primer_CoV16	CAGCATCACCATAGTCAC
	Primer_CoV17	CGAACCGTTCAATCATAAG
	Primer_CoV18	CACCATAGAATTTGCTTGTTC

	Primer_CoV19	CTAGCTCTCTGAAGTGGTATC
	Primer_CoV20	GTTTCTTCATGTTGGTAG
	Primer_CoV21	CTAGCCCATTTCAAATCCTG
	Primer_CoV22	GTTGTCCAGCATTTCTTCAC
	Primer_CoV23	GACAAACTAGTATCAACCATATC
	Primer_CoV24	CTGTCCTGGTTGAATGCGAAC
	Primer_CoV25	CAGAGTACAGTGAATGAC
	Primer_CoV26	GTAGATGCTATGTCACGAG
	Primer_CoV27	GAACCTTTAGTGTTATTAG
	Primer_CoV28	GTTCAAATAGCCTTCTCTG
	Primer_CoV29	CTTAAAAGAGGGTGTGTAG

	Primer_CoV 30	CTCA CCTAC TGTCT TATTA C
	Primer_CoV 31	CATTT AGATC GTTAA GTGTG
	Primer_CoV 32	GTGC GAAC AGTAT CTACA C
	Primer_CoV 33	CACA ACACA GGCG AACT C
	Primer_CoV 34	CACC TTCCT TAAAC TTCTC
	Primer_CoV 35	CTTCT GAATT GTGA CATGC TG
	Primer_CoV 36	GTCTC ACCA CTACG ACCG
	Primer_CoV 37	GTTCA CGGC AGCA GTATA CACC
	Primer_CoV 38	TCCA CAAA AGCA CTTGT GGAA GC
	Primer_CoV 39	TGTG GGAA GTGTT TCTCC CTC
	Primer_CoV 40	GTCTG AACA ACTG

		GTGTA AGTTC C
	Primer_CoV 41	ATTTC AGTAG TGCC ACCA GCC
	Primer_CoV 42	CATGT CCAC AACTT GCGT GTG
	Primer_CoV 43	AGCA CCGT CTATG CAATA CAAA G
	Primer_CoV 44	ACAG CAGC TAAAC CATGA GTAGC
	Primer_CoV 45	ACAA CCGT CTACA ACATG CAC
	Primer_CoV 46	GTCAC GGGG TGTCA TGTTT TC
	Primer_CoV 47	CGTGT GTCA GGGC GTAAA CTTTC
	Primer_CoV 48	GAGC CTTTG CGAG ATGAC AAC
	Primer_CoV 49	AACG GCAAT TCCA GTTTG AGC
	Primer_CoV 50	GCGG TTGAG TAAAC

		AAAA GAGG C
	Primer_CoV 51	GGGA ACAC AACC ATCTC TTGC
	Primer_CoV 52	ACGAT GCAC CACC AAAG GATTC
	Primer_CoV 53	AATAC CAGC ATTTC GCATG GCA
	Primer_CoV 54	TAGCA GCATT ACCAT CCTG AGC
	Primer_CoV 55	TGCAT TAACA TTGG CCGT GAC
	Primer_CoV 56	ACAA CCTG GAGC ATTGC AAAC
	Primer_CoV 57	TCACA TAGTG CATCA ACAG CGG
	Primer_CoV 58	TAAAG TTGC CACAT TCCTA CGTG G
	Primer_CoV 59	TAACA AAGC ACTC GTGG ACAG C

	Primer_CoV 60	CCTG TTGTC CATCA AAGT GTCC C
	Primer_CoV 61	GATGA ACCT GTTTG CGCA TCTG
	Primer_CoV 62	CTATT TGTTC GCGT GGTTT GCC
	Primer_CoV 63	ACCC TGTTT TCCTT CAAG GTCC
	Primer_CoV 64	TGCTA CCGG CCTG ATAGA TTTC
	Primer_CoV 65	TGCT GCATT CAGTT GAATC ACC
	Primer_CoV 66	CAGA AGCT CTGAT TTCTG CAGC
	Primer_CoV 67	TTGCA GTAGC GCGA ACAA AATC
	Primer_CoV 68	ACGC ACAC AATCG AAGC GCAG
	Primer_CoV 69	TGCC AATCC TGTAG CGAC TGTAT GC

	Primer_CoV 70	AGGA CACG GGTC ATCAA CTAC
	Primer_CoV 71	TGCC AGCC ATTCT AGCA GGAG
	Primer_CoV 72	TGTG GTGG CTCTT TCAA GTCC
	Primer_CoV 73	TTTTG TCATT CTCC TAAGA AGC

Enrichment primer sequences for SARS-CoV-2 genome

5 **First Strand Synthesis**

Mix the following by pipetting up and down.

		1 rxn
	Reagent	vol stock (uL)
	Fragmented & primed RNA	10
	Nuclease-free water	8
	(pink) NEB Next	2

	First Strand Synthesis Enzyme Mix	
	Total volume	20 uL/rxn

Thermocycler (heated lid set to 105°C):

- 10 mins at 25°C
- 15 mins at 42°C
- 15 mins at 70°C
- Hold at 4°C

6 **Second Strand Synthesis**

		1 rxn
	Reagent	vol stock (uL)
	First strand synthesized DNA	20
	(orange) 2nd SS Reaction buffer (10X)	8

	(orange) 2nd SS enzyme mix	4
	Nuclease-free water	48
	Total volume	80 uL/rxn

Thermocycler (heated lid off):

- 1 hour at 16°C
- Hold at 4°C

7 **SPRI cleanup**

*allow beads to sit in RT for 30 mins prior

- Use SPRI Beads1.8x ratio of beads-to-total volume of sample. Prep 80% EtOH.
- Add 144uL of room temperature beads to 2nd Strand Synthesis Rxn. Mix well by pipetting gently.
- Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
- Place samples on magnetic rack, and incubate for 5 mins on the rack.
- Remove supernatant.
- Add 200uL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 30s then remove the supernatant.
- Repeat EtOH wash step for a total of 2 timess
- Air dry the beads for 5 mins while on the magnetic rack.
- Remove tube from magnetic rack. Elute DNA from beads into **53uL** of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
- Vortex to mix. Spin tubes and incubate for 2 mins at room temperature off the magnetic rack.
- Place on magnetic rack until solution is clear ~ 5 mins.
- Remove 50uL of the supernatant and transfer to a clean nuclease free PCR tube.

**Checkpoint: Samples can be stored frozen at -20 °C and library prep resumed the next day.

8 **End Repair**

Mix all the following by pipetting up and down

		1 rxn
	Reagent	vol stock (uL)
	Purified ds-cDNA	50
	(green) Ultra II End Prep reaction buffer (8.6 x)	7
	(green) Ultra II End Prep enzyme mix	3
	Total volume	60 uL/rxn

Thermocycler (heated lid set to 105°C):

- 30 mins at 20°C
- 30 mins at 65°C
- Hold at 4°C

9 **Adapter Ligation**

- dilute adaptor to dilution prior to making master mix. Adaptor concentration depends on the amount of input; 1:100 dilution for samples <5ng, 1:25 for input of >5ng
- **Add adaptor separately after ligation master mix and ligation enhancer to avoid adaptor dimers.**

		1 rxn
	Reagent	vol stock (uL)
	End Prep reaction mixture	60
	(red) NEB Next Ultra II ligation master mix	30
	(red) NEB Next ligation enhancer	1
	1:100 Adaptor (Cat No. E7337AA)	2.5
	Total volume	93.5 uL/rxn

Thermocycler (heated lid off):

- 15 mins at 20°C with heated lid **off**

- Proceed immediately to Bead Purification.

10 **SPRI Cleanup**

*allow beads to sit in RT for 30 mins prior

- Use SPRI bead 0.9x ratio of beads-to-total volume of sample. Prep 80% EtOH.
- Add 87uL of room temperature beads (0.9x) to Adaptor Ligation reaction. Mix well.
- Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
- Place samples on magnetic rack, and incubate for 5 mins on the rack.
- Remove supernatant.
- Add 200uL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 30s then remove the supernatant.
- Repeat EtOH wash step for a total of 2 times.
- Air dry the beads for 5 mins while on the magnetic rack.
- Remove tube from magnetic rack. Elute DNA from beads into 17uL of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
- Vortex to mix. Spin tubes and incubate for 2 mins at room temperature off the magnetic rack.
- Place on magnetic rack until solution is clear ~ 5 mins.
- Remove 15uL of the supernatant and transfer to a clean nuclease free PCR tube.

**Checkpoint: Samples can be stored frozen at -20 °C and library prep resumed the next day.

11 **USER/Q5 Indexing PCR**

Mix the following components by pipetting up and down.

***Technical Note from NEB: The TruSeq adaptor and primer strategy from Illumina uses a barcoded adaptor and universal primer. If someone used the universal NEBNext adaptor and Illumina TruSeq universal primer, the result would be a lack of amplification. It is of course possible to use non- NEB adaptors and primers. We support it and we do have an FAQ about this: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb**

**** In this new version, we have reduced the purified, adaptor-ligated cDNA to 12uL rather than 15uL to assure that the reaction is not underbuffered. Alternatively, the previous SPRI reaction can concentrate the cDNA further prior to setting up the PCR reaction.**

		1 rxn
	Reagent	vol stock (ul)
	Purified, adaptor-ligated cDNA	12**
	(white) USER Enzyme (Cat no. M5505L, 250uL)	3
	(blue) NEBNext Ultra II Q5 master mix	25
	5uM i7 barcoded primer (NEB index primer/ TruSeq/or similar)*	5uL
	5uM i5 barcoded primer (NEB Universal primer/ TruSeq/or similar)*	5uL
	Total volume	50uL

Cycling conditions:

	Thermocycler (heated lid at 105° C):	Cycles
	37 °C for 15 mins	1
	98 °C for 30s	1
	98 °C for 10s	6-15**
	65 °C for 75s	
	65 °C for 5 mins	1
	Hold at 4 °C	

** PCR cycles are dependent on the input RNA. For libraries with <5ng input, perform 15-19 cycles of PCR. For 5-20ng input, perform 10-14 cycles of PCR. For >20ng input, perform 6-8 cycles of PCR.

12 **SPRI Cleanup**

*allow beads to sit in RT for 30 mins prior

- Use SPRI Beads at 0.8x ratio of beads-to-total volume of sample. Prep 80% EtOH.
- Add 43uL of room temperature Ampure Beads (0.8x) to barcoded DNA. Mix well.

- Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
- Place samples on magnetic rack, and incubate for 5 mins on the rack.
- Remove supernatant.
- Add 200uL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 30s then remove the supernatant.
- Repeat EtOH wash step for a total of 2 times.
- Air dry the beads for 5 mins while on the magnetic rack.
- Remove tube from magnetic rack. Elute DNA from beads into **23uL**of 0.1x TE Buffer, 10mMTris-HCL, or Nuclease free water.
- Vortex to mix. Spin tubes and incubate for 2 mins at room temperature off the magnetic rack.
- Place on magnetic rack until solution is clear ~ 5 mins.
- Remove 20uL of the supernatant and transfer to a clean nuclease free PCR tube.

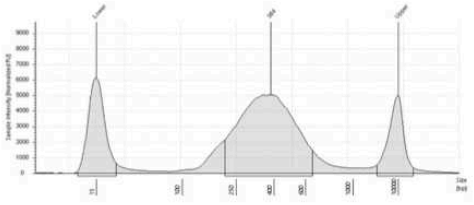
** Checkpoint: Libraries are complete, samples can be stored frozen at -20 °C until ready for quantification and pooling.

Library Quality Control

13 Libraries were quantified by the Qubit High Sensitivity DNA kit and the Agilent High Sensitivity D500 DNA Tapestation assay. If adapter dimers are found, library should be size selected using a SPRI ratio of 0.8x.

High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #**5067-5592

Expected result



Location B1
Concentration 15.6
Description
Observations

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
15	4.84	-	497	-		Lower Marker
384	15.6	-	62.5	100.00		
10000	3.25	3.25	0.500	-		Upper Marker

Tapestation HS D5000 assay shows an average size library of 384bp at a concentration of 62.5 nM.

Quantified library is then diluted down to the loading concentration for the iSeq, 100pM. This value will vary depending on the type of sequencer.

iSeq Loading

14 The Illumina iSeq was loaded with 20uL of a 100pM library with a 5% PhiX spike in.

Analyses

15 Metagenomic sequencing results were uploaded onto IDseq.net directly from the Illumina Basespace Sequence Hub. The open-source cloud-based pipeline analyzed 1.8 million reads. The majority of reads mapped to the nasal and oral microbiome. Analyses of the viral components resulted in 3,077 single end reads aligning to the SARS-CoV2 taxon.

Expected result

Betacoronavirus *(2 viral species)* 1	16,452,524	100.0 100.0	823.4 830.6	3,079 3,106	1 3	3,061 3,071	100.0 99.2	29,698.9 4,341.7	306.2 304.1
Wuhan seafood market pneumonia virus	16,452,524	100.0 100.0	822.9 822.4	3,077 3,075	1 1	3,061 3,061	100.0 99.8	29,718.2 4,385.1	306.4 307.1
Severe acute respiratory syndrome-related coronavirus NIAID PRIORITY | C	88,253	100.0 100.0	0.5 8.3	2 31	0 2	0 10	98.8 35.5	83.0 39.3	43.2 9.7

Resulting reads from the Betacoronovirus genus were downloaded from IDseq and mapped to the NCBI genome accession number: MN908947.1. The geneious alignment displayed even coverage across the genome with an average coverage of 14.9x. One SNP was noted at position 25,654 in ORF3a resulting in a valine to leucine substitution when compared to NCBI accession MN908947.1.

Expected result