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

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

Chan Zuckerberg Biohub Coronavirus Method Development Community

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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

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

STEPS MATERIALS

NAME	CATALOG #	VENDOR
QIAamp® Viral RNA Mini	52906	Qiagen

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NAME	CATALOG #	VENDOR
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QIAseq FastSelect rRNA Removal Kit	333180	Qiagen
High Sensitivity D5000 ScreenTape	5067-5592	Agilent Technologies

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
by Qiagen
Catalog #: 52906

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

DNase I Set
by 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.

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RNA Quantification

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

Qubit RNA HS Assay Kit
by 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
by 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
by Thermo Fisher
Catalog #: 4456740

** (optional) FastSelect is designed for removing human rRNA, omit this reagent if not using human derived samples and only

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incubate at 94°C and then directly to 4°C (omit ramping down steps).

QIAseq FastSelect rRNA Removal Kit
by 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

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Primer_CoV27	GAACCTTTAGTGTTATTAG
Primer_CoV28	GTTCAAATAGCCTTCTCTG
Primer_CoV29	CTTAAAAGAGGGTGTGTAG
Primer_CoV30	CTCACCTACTGTCTTATTAC
Primer_CoV31	CATTTAGATCGTTAAGTGTG
Primer_CoV32	GTGCGAACAGTATCTACAC
Primer_CoV33	CACAACACAGGCGAACTC
Primer_CoV34	CACCTTCCTTAAACTTCTC
Primer_CoV35	CTTCTGAATTGTGACATGCTG
Primer_CoV36	GTCTCACCACTACGACCG
Primer_CoV37	GTTCACGGCAGCAGTATACACC
Primer_CoV38	TCCACAAAAGCACTTGTGGAAGC
Primer_CoV39	TGTGGGAAGTGTTTCTCCCTC
Primer_CoV40	GTCTGAACAACTGGTGTAAGTTCC
Primer_CoV41	ATTTCAGTAGTGCCACCAGCC
Primer_CoV42	CATGTCCACAACTTGCGTGTG
Primer_CoV43	AGCACCGTCTATGCAATACAAAG
Primer_CoV44	ACAGCAGCTAAACCATGAGTAGC
Primer_CoV45	ACAACCGTCTACAACATGCAC
Primer_CoV46	GTCACGGGGTGTCATGTTTTC
Primer_CoV47	CGTGTGTCAGGGCGTAAACTTTC
Primer_CoV48	GAGCCTTTGCGAGATGACAAC
Primer_CoV49	AACGGCAATTCCAGTTTGAGC
Primer_CoV50	GCGGTTGAGTAAACAAAAGAGGC
Primer_CoV51	GGGAACACAACCATCTCTTGC
Primer_CoV52	ACGATGCACCACCAAAGGATTC
Primer_CoV53	AATACCAGCATTTCGCATGGCA
Primer_CoV54	TAGCAGCATTACCATCCTGAGC
Primer_CoV55	TGCATTAACATTGGCCGTGAC
Primer_CoV56	ACAACCTGGAGCATTGCAAAC
Primer_CoV57	TCACATAGTGCATCAACAGCGG
Primer_CoV58	TAAAGTTGCCACATTCCTACGTGG
Primer_CoV59	TAACAAAGCACTCGTGGACAGC
Primer_CoV60	CCTGTTGTCCATCAAAGTGTCCC
Primer_CoV61	GATGAACCTGTTTGCGCATCTG
Primer_CoV62	CTATTTGTTCGCGTGGTTTGCC
Primer_CoV63	ACCCTGTTTTCCTTCAAGGTCC
Primer_CoV64	TGCTACCGGCCTGATAGATTTC
Primer_CoV65	TGCTGCATTCAGTTGAATCACC
Primer_CoV66	CAGAAGCTCTGATTTCTGCAGC
Primer_CoV67	TTGCAGTAGCGCGAACAAAATC
Primer_CoV68	ACGCACACAATCGAAGCGCAG
Primer_CoV69	TGCCAATCCTGTAGCGACTGTATGC
Primer_CoV70	AGGACACGGGTCATCAACTAC
Primer_CoV71	TGCCAGCCATTCTAGCAGGAG
Primer_CoV72	TGTGGTGGCTCTTTCAAGTCC
Primer_CoV73	TTTTGTCATTCTCCTAAGAAGC

Enrichment primer sequences for SARS-CoV-2 genome

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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) NEBNext First Strand Synthesis Enzyme Mix	2
Total volume	20 uL/rxn

Thermocycler (heated lid set to 105℃):

- 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

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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 (8.6x)	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

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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) NEBNext Ultra II ligation master mix	30
(red) NEBNext 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.

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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.

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12 **SPRI Cleanup**

*allow beads to sit in RT for 30 mins prior

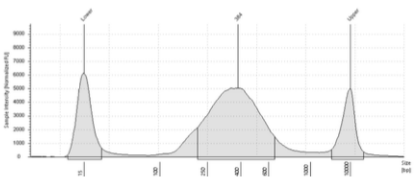
- Use SPRI Beads at 0.8x ratio of beads-to-total volume of sample. Prep 80% EtOH.
- Add 43uL of room temperature Ampure Beads (0.8x) to barcoded DNA. Mix well.
- Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
- Place samples on magnetic rack, and incubate 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
by Agilent Technologies
Catalog #: 5067-5592



Location B1
Concentration 15.6
Description
Observations

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	PeakMolarity [nmol/l]	% Integrated Area	PeakComment	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.

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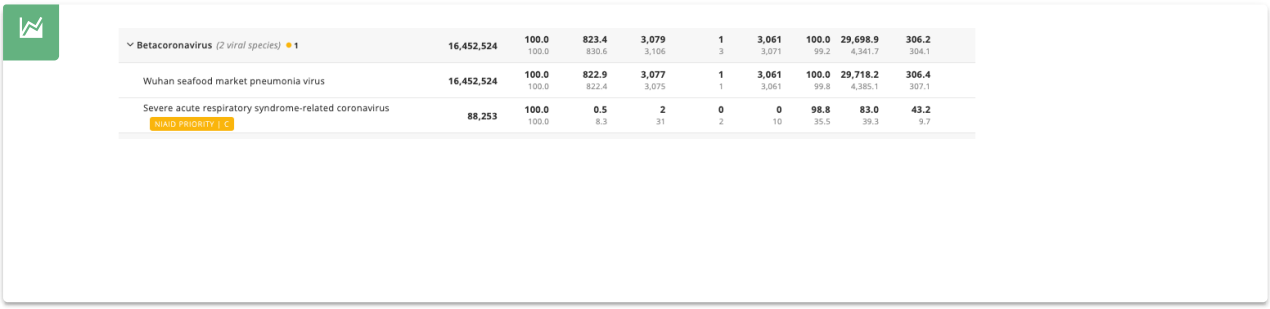
iSeq Loading

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

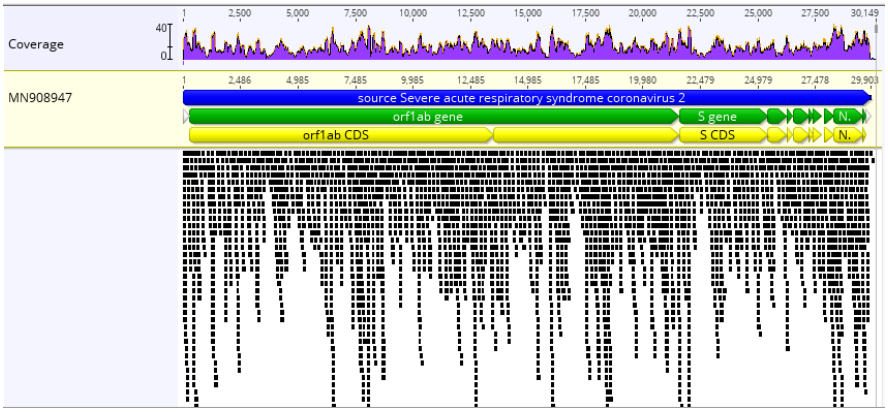
Analyses

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.



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.



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