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SARS-CoV-2 Whole Genome Amplicon Sequencing from Liquid Wastewater V.1

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We use this protocol and it's working

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Abstract

This process instruction describes steps for whole genome amplicon sequencing of SARS-CoV-2 from wastewater, including concentration and RNA extraction steps, purification steps, reverse transcription and PCR with ARTIC primers, library construction, and Illumina sequencing. Sequencing data produced using this protocol may be used for a variety of analyses including estimation of variant and mutation abundance. The read lengths are sufficient for read merging and analysis of intact amplicons. This protocol has been used for sequencing activities as part of the WastewaterSCAN program.

Materials

- ARTIC v5.3.2 primers (IDT 10016495 or custom order based on https://github.com/quick-lab/SARS-CoV-2/tree/main/400/v5.3.2_400)
- ATCC Quantitative genomic RNA from Severe acute respiratory syndrome-related coronavirus 2 strain 2019-nCoV/USA-WA1/2020 (VR-1986D)
- Molecular biology grade ethanol
- Molecular biology grade water
- Qiagen elution buffer (19086)
- Ceres Nanotrap Microbiome A Particles (44202-30)
- Ceres Nanotrap Enhancement Reagent 1 Solution (10111-30)
- Thermo MagMAX Microbiome Lysis Solution (A42361)
- chemagic Viral DNA/RNA 300 Kit H96 (CMG-1033-S)
- Zymo OneStep PCR Inhibitor Removal Kit (D6035)
- Turbo DNase (AM2239)
- KAPA HyperPure (08963843001)
- NEB LunaScript MM (M3010E)
- NEB Q5 Hot Start High-Fidelity 2X Master Mix (M0494)
- NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645L)
- NEBNext Indexing primers (E6440, E6442, E6444, E6446, E6448)
- KAPA Illumina Library Quantification Kit (KK4824)
- Rainin 1000 uL tips (30389213)
- Rainin 200 uL tips (30389240)
- Rainin 20 uL tips (30389226)
- Fisher scientific Reagent reservoirs (89094-662)
- Eppendorf Skirted Twin.Tec LoBind PCR plates (E0030129512)
- Centrifuge with 96-well PCR plate rotor attachment capable of generating ≥ 2000 rcf
- Thermal cycler capable of 2°C/s ramp rate
- Magnet for magnetic SPRI bead separation
- Optical cycler for library qPCR
- Illumina sequencer capable of ≥250 PE reads

Troubleshooting

Before start

Pool and dilute primers

1. ARTIC 5.3.2 primers can be purchased premixed from IDT (10016495) or custom ordered and mixed per the instructions provided by the Quick lab (https://github.com/quick-lab/SARS-CoV-2/tree/main/400/v5.3.2_400).
2. Make sufficient working stock of each primer mix at (on average) ~150 nM per primer by diluting the stock tubes or mixtures from ~1040 nM. For a full 96-well plate at the ARTIC PCR step, 150 µL of 150 nM working stock of each pool is sufficient.

Dilute gRNA

1. SARS-CoV-2 gRNA (ATCC VR-1986D) is used as a positive control. The positive control sample is 500 copies of the gRNA in 40 µL of water (12.5 copies / µL) added to the plate before the digestion of DNA. Dilute the stock gRNA in water according to the concentration provided by ATCC.

Viral Particle Concentration

1

Note

Viral particles are concentrated from 9.75 mL of clarified influent using **Ceres Nanotrap Microbiome A particles**. Note: include at least one concentration/extraction negative control (water instead of sample).

2 Spin down 50 mL of influent at 5,250 xg for 5 min to clarify the influent.

3 Add 4.875 mL of influent sample to one well (one well per sample) of a new KingFisher 24 Well Deep Well Plate.

4 Add another 4.875 mL of influent sample to the same well location on a second KingFisher 24 Well Deep Well Plate.

5 Add 50 µL of Nanotrap Enhancement Reagent 1 (ER1) Solution to each well used.

6 Add 75 µL of Nanotrap Microbiome A Particles to each well used on the two KingFisher 24 Well Deep Well sample plates.

7 Prepare the “Lysis Plate”: Add 350 µL of MagMAX Microbiome Lysis Solution to each well of a new KingFisher 24 Well Deep Well Plate.

8 Run KF-008-WW-Nanotrap-24.bdz on the King Fisher Flex to concentrate the viral particles.

9 Once the protocol is completed, the “Lysis Plate” will contain ~350 µL lysate that is ready to proceed to nucleic acid extraction.

Nucleic Acid Extraction

10

Note

This total nucleic acid extraction is automated using the Chemagic 360 instrument using the **chemagic Viral DNA/RNA 300 Kit H96**.

11 Transfer 300 µL of lysate to each well of the Chemagic Deepwell Plate

12 Add 300 µL of Lysis Buffer to each well.

13 Add 10 µL of Proteinase K to each well.

14 Add 60 µL of Elution Buffer to each well of an Elution Plate.

15 Set up, load, and run the Chemagic for the Viral DNA/RNA 300 Kit H96.

16 Once the protocol is complete, the nucleic acid (~60 µL) will be in the Elution Plate and ready to proceed to the inhibitor removal steps.

Inhibitor Removal

20m

17

Note

Additional PCR inhibitors are removed from the extracted and purified nucleic acid using the **Zymo OneStep PCR Inhibitor Removal Kit**.

18 Mount a Zymo Silicon-A-HRC plate (included in kit) onto an Elution Plate (included in kit)

19 Add 150 µL Prep Solution to the wells by piercing the cover foil in the middle with a pipette tip.

20 Incubate at room temperature for 5 min.

21 Centrifuge the plate at 3,500 xg for 5 min.

22 Transfer 60 µL of eluate from the Chemagic Elution Plate into the wells of a prepared Silicon-A-HRC Plate mounted on a new Elution Plate.

23 Centrifuge the plate at 3,500 x g for 3 minutes.

24 The cleaned up nucleic acid is now in the Zymo Elution Plate.

DNA Digestion

1h 30m

25

Note

DNA is digested from the total nucleic acid solution using Turbo DNase and then cleaned up and concentrated using KAPA HyperPure SPRI beads. Include at least one well of gRNA as a positive control and one of water as a negative control for the whole process after extraction.

26 Transfer 40 µL of nucleic acid into a new plate.

27 To each sample add: 1 µL Turbo DNase and 4 µL 10X Turbo DNase Buffer

28 Incubate:

	A	B	C	D
	Table 1. Thermal Cycler Program for Turbo DNase			
	Steps	Cycles	Temp (°C)	Time
	1	1	37	30 min
	2	-	4	Hold

Lid Temp = 47°C, Total Volume = 45 µL

29 Add 90 µL Kapa HyperPure Beads to each well.

30 Mix well by pipetting up and down at least 10 times.

31 Incubate at room temperature for 5 minutes.

32 Place the plate on a magnet to capture the beads.

33 Incubate until the liquid is clear.

34 Carefully remove and discard the supernatant.

35 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

36 Incubate the plate on the magnet at room temperature for 30 seconds.

37 Carefully remove and discard the ethanol.

38 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

39 Incubate the plate on the magnet at room temperature for 30 seconds.

40 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

41 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

42 Remove the plate from the magnet.

43 Resuspend the beads in 13 µL of elution buffer.

44 Incubate the plate at room temperature for 2 minutes.

45 Place the plate on a magnet to capture the beads.

46 Incubate until the liquid is clear.

47 Transfer the 11 µL of clear supernatant to a new plate for reverse transcription.

Reverse Transcription

1h 45m

48

Note

The remaining RNA is reverse transcribed into cDNA with the NEB LunaScript RT SuperMix then cleaned up and split in preparation for the two ARTIC PCRs.

49 Add 2.75 µL LunaScript RT SuperMix to each well containing RNA.

50 Mix well by pipetting up and down at least 10 times.

51 Incubate:

	A	B	C	D
	Table 2. Thermal Cycler Program for LunaScript RT			
	Steps	Cycles	Temp (°C)	Time
	1	1	25	10 min
	2	1	50	50 min
	3	1	85	5 min
	4	-	4	Hold

Lid Temp = 95°C, Total Volume = 14 µL

52 Add 27.5 µL Kapa HyperPure Beads to each well.

53 Mix well by pipetting up and down at least 10 times.

54 Incubate at room temperature for 5 minutes.

55 Place the plate on a magnet to capture the beads.

56 Incubate until the liquid is clear.

57 Carefully remove and discard the supernatant.

58 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

59 Incubate the plate on the magnet at room temperature for 30 seconds.

60 Carefully remove and discard the ethanol.

61 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

62 Incubate the plate on the magnet at room temperature for 30 seconds.

63 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

64 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

65 Remove the plate from the magnet.

66 Resuspend the beads in 12 µL of elution buffer.

67 Incubate the plate at room temperature for 2 minutes.

68 Place the plate on a magnet to capture the beads.

69 Incubate until the liquid is clear.

70 Transfer the 5 µL of clear supernatant to two new plates for the two ARTIC PCRs.

SARS-CoV-2 genome amplification with ARTIC PCR

4h 15m

71

Note

SARS-CoV-2 cDNA is amplified in two reactions using the ARTIC 5.3.2 primers (15 nM each primer in the PCR) and NEB Q5 Hot Start High-Fidelity 2X Master Mix then combined and cleaned up. Include at least one well on each plate as a negative control for the ARTIC PCR.

72 Make two master mixes, one for ARTIC Pool A and one for ARTIC Pool B. Per sample there should be: 6.25 µL Q5 Hot Start High-Fidelity 2X Master Mix and 1.25 µL of ARTIC PCR primer pool working stock (150 nM).

73 Add 7.5 µL of Pool A master mix to each well of one plate containing 5 µL cDNA.

74 Add 7.5 µL of Pool B master mix to each well of the plate other containing 5 µL cDNA.

75 Mix well by pipetting up and down at least 10 times.

76 Incubate:

	A	B	C	D
	Table 3. Thermal Cycler Program for ARTIC PCR			
	Steps	Cycles	Temp (°C)	Time
	1	1	98	30 sec
	2	35	95	15 sec
	3		63	5 min
	4	-	4	Hold

Lid Temp = 105°C, Total Volume = 13 µL

77 Combine 12.5 µL of each of the Pool A and Pool B PCR reactions for a total of 25 µL.

78 Add 20 µL Kapa HyperPure Beads to each well.

79 Mix well by pipetting up and down at least 10 times.

80 Incubate at room temperature for 5 minutes.

81 Place the plate on a magnet to capture the beads.

82 Incubate until the liquid is clear.

83 Carefully remove and discard the supernatant.

84 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

85 Incubate the plate on the magnet at room temperature for 30 seconds.

86 Carefully remove and discard the ethanol.

87 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

88 Incubate the plate on the magnet at room temperature for 30 seconds.

89 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

90 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

91 Remove the plate from the magnet.

92 Resuspend the beads in 27 µL of elution buffer.

93 Incubate the plate at room temperature for 2 minutes.

94 Place the plate on a magnet to capture the beads.

95 Incubate until the liquid is clear.

96 Transfer the 25 µL of clear supernatant to a new plate.

Library Construction: End Prep

1h 10m

97

Note

The ends of the DNA amplicons are prepared for adapter ligation, this includes end repair, 5' phosphorylation and dA-tailing. This step uses the **NEBNext Ultra II DNA Library Prep Kit for Illumina**.

98 Add 1.5 µL NEBNext Ultra II End Prep Enzyme Mix to each well.

99 Add 3.5 µL NEBNext Ultra II End Prep Reaction Buffer to each well.

100 Mix well by pipetting up and down at least 10 times.

101 Incubate:

	A	B	C	D
	Table 4. Thermal Cycler Program for End Prep			
	Steps	Cycles	Temp (°C)	Time

	A	B	C	D
	1	1	20	30 min
	2	1	65	30 min
	3	-	4	Hold

Lid Temp = 75°C, Total Volume = 30 µL

Library Construction: Adaptor Ligation

45m

102

Note

NEBnext adapters are ligated to the end-repaired amplicons and then the uracil is excised via User enzyme incubation. This step uses the **NEBNext Ultra II DNA Library Prep Kit for Illumina**.

103 Add 1.25 µL NEBNext Adaptor for Illumina to each well. Do not premix adaptor with the Ligation Master Mix.

104 Add 15uL NEBNext Ultra II Ligation Master Mix to each well.

105 Mix well by pipetting up and down at least 10 times.

106 Incubate:

	A	B	C	D
	Table 5. Thermal Cycler Program for Adaptor Ligation			
	Steps	Cycles	Temp (°C)	Time
	1	1	20	15 min
	2	-	4	Hold

Lid Temp = 30°C, Total Volume = 47 µL

107 Add 1.5 µL USER Enzyme to each well.

108 Mix well by pipetting up and down at least 10 times.

109 Incubate:

	A	B	C	D
	Table 6. Thermal Cycler Program for USER			
	Steps	Cycles	Temp (°C)	Time
	1	1	37	15 min
	2	-	4	Hold
	Lid Temp = 47°C, Total Volume = 48 µL			

Lid Temp = 47°C, Total Volume = 48 µL

Library Construction: Cleanup (0.9X SPRI) 45m

110

Note

Libraries are cleaned up using **KAPA HyperPure** SPRI beads to remove enzymes and salts in preparation for indexing PCR.

111 Add 43 µL Kapa HyperPure Beads to each well.

112 Mix well by pipetting up and down at least 10 times.

113 Incubate at room temperature for 5 minutes.

114 Place the plate on a magnet to capture the beads.

115 Incubate until the liquid is clear.

116 Carefully remove and discard the supernatant.

117 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

118 Incubate the plate on the magnet at room temperature for 30 seconds.

119 Carefully remove and discard the ethanol.

120 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

121 Incubate the plate on the magnet at room temperature for 30 seconds.

122 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

123 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

124 Remove the plate from the magnet.

125 Resuspend the beads in 12 µL of elution buffer.

126 Incubate the plate at room temperature for 2 minutes.

127 Place the plate on a magnet to capture the beads.

128 Incubate until the liquid is clear.

129 Transfer the 10 µL of clear supernatant to a new plate.

Library Construction: Indexing by PCR

1h

130

Note

The uniquely dual-indexed forward and reverse (i7 and i5) primer pairs are added to the adapter ligated DNA via PCR, this enables high throughput sample multiplexing. This step uses the **NEBNext Ultra II DNA Library Prep Kit for Illumina** and one of the sets of **NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs)**.

131 Add 12.5 µL Q5 NEBNext Library PCR Master Mix to each well.

132 Add 2.5 µL Index Primer Mix to each well.

133 Mix well by pipetting up and down at least 10 times.

134 Incubate:

	A	B	C	D
	Table 7. Thermal Cycler Program for Indexing PCR			
	Steps	Cycles	Temp (°C)	Time
	1	1	98	30 sec
	2	4	98	10 sec

	A	B	C	D
	3		65	75 sec
	4	1	65	5 min
	5	-	4	Hold

Lid Temp = 105°C, Total Volume = 25 µL

135 Add 22.5 µL Kapa HyperPure Beads to each well.

136 Mix well by pipetting up and down at least 10 times.

137 Incubate at room temperature for 5 minutes.

138 Place the plate on a magnet to capture the beads.

139 Incubate until the liquid is clear.

140 Carefully remove and discard the supernatant.

141 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

142 Incubate the plate on the magnet at room temperature for 30 seconds.

143 Carefully remove and discard the ethanol.

144 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

145 Incubate the plate on the magnet at room temperature for 30 seconds.

146 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

147 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

148 Remove the plate from the magnet.

149 Resuspend the beads in 27 µL of elution buffer.

150 Incubate the plate at room temperature for 2 minutes.

151 Place the plate on a magnet to capture the beads.

152 Incubate until the liquid is clear.

153 Transfer the 25 µL of clear supernatant to a new plate. Libraries are ready for pooling and sequencing.

Library Pooling

154 Measure the library concentration by qPCR using the KAPA Illumina Library Quantification kit.

155 Pool samples, aiming for equimolar amounts of each. Negative control libraries may not yield enough material to be pooled in the same quantity as actual samples.

Sequencing and Analysis

156 Sequence the pool on an Illumina system using a kit that will yield ≥250PE reads.

157 The resulting sequences can be used for various analyses, including estimation of variant and mutation abundance. The read lengths are sufficient for read merging and analysis of intact amplicons.

Our pipelines for mutation and variant abundance estimation rely on:

- viralrecon for processing and mutation abundance (https://github.com/nf-core/viralrecon),
- VLQ for variant abundance (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-022-02805-9 and https://github.com/baymlab/wastewater_analysis), and
- Freyja for variant abundance (https://www.nature.com/articles/s41586-022-05049-6 and https://github.com/andersen-lab/Freyja).