Preparation of SARS-CoV-2 Particles in Raw Wastewater for Sequencing on Illumina Platforms Using an ARTIC V4.1 Tiled Amplicon Approach

Reagents

- LunaScript RT Supermix (NEB, E3010L)
- Q5 Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- Water, Molecular Biology Grade (Fisher, BP2819-1)
- 1X TE Solution pH 8.0 (IDT, 11-05-01-09)
- SpeedBead Magnetic Carboxylate (Cytiva, 65152105050250)
- ARTIC V4.1 NCOV-2019 Panel (IDT, 10011442)
- ARTIC V4.1 oPool Primers (IDT, Custom Order)
- KAPA HiFi HotStart PCR Kit (Roche, 07958897001)
- TBE Buffer Premix Powder (Bio Basic, A0024)
- Agarose I™ (VWR, 97062-250)
- GelRed Nucleic Acid Gel Stain (Gold Bio, G-725-500)
- GeneRuler DNA Ladder Mix (Thermo Scientific, SM0333)
- Gel Loading Dye Purple 6X (NEB, B7024S)
- Qubit 1X dsDNA HS Assay Kit (Invitrogen, Q33231)
- Water, Deionized

Equipment

- Micropipettes (p10, p20, p200, p1000)
- Microcentrifuge
- Microplate centrifuge
- Vortex mixer
- PCR thermal cycler (Bio-Rad, T100)
- MagnaBot™ II Magnetic Separation Device (Promega, V8351)
- DynaMag™-2 Magnet (Invitrogen, 12321D)
- Analytical balance
- Graduated cylinder
- Plastic carboy with spigot
- 250 mL Flask
- Owl™ Wide Gel Electrophoresis System (Thermo Scientific, D3-14)
- PowerPac™ Basic Power Supply (Bio-Rad, 1645050)
- FastGene™ FAS-V Gel Imaging System (Nippon Genetics, GP-FAS-V)
- Qubit™ 4 Fluorometer (Invitrogen, Q33226)

Consumables

- 1.5 mL tubes, DNA/RNAse free
- 0.2 mL strip tubes, DNA/RNAse free
- 0.2 mL 96-well plates, DNA/RNAse free

- Reagent reservoirs, DNA/RNAse free
- TempPlate® Sealing Foil (USA Scientific, 2923-0100)
- Microseal® 'F' Foil Seal (Bio-Rad, MSF1001)
- Microseal® 'B' Seal (Bio-Rad, MSB1001)
- Micropipette tips, DNA/RNAse free (p10, p20, p200, p1000)

Store purified RNA at -20°C for short-term storage or at -80°C for long-term storage prior to the synthesis of cDNA

1. cDNA Synthesis

1.1. Gently mix and spin down LunaScript RT SuperMix reagent. Prepare the cDNA synthesis reaction as follows:

1.1.1. For purified RNA samples, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
RNA Sample	8
LunaScript RT SuperMix	2
Total Volume	*10*

1.1.2. For no template controls, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
Nuclease-free Water	8
LunaScript RT SuperMix	2
Total Volume	*10*

1.2. Pipet 10 times to mix and spin down.

1.3. Incubate reactions in a thermocycler with the following conditions:

CYCLE STEP	TEMP (°C)	TIME	CYCLES
Primer Annealing	25	2 minutes	1
cDNA Synthesis	55	20 minutes	1
Heat Inactivation	95	1 minute	1
Hold	4	∞	1

* Set heated lid to 105 °C

Safe to stop. Store cDNA at -20°C for short-term storage or at -80°C for long-term storage prior to the next step

2. Cleanup of cDNA

Note: See the SpeedBead Preparation Protocol for instructions on how to prepare the beads

2.1. Allow the bead solution to come to room temperature and vortex to resuspend.

2.2. Add 20 µl (2.0X) of resuspended beads to the 10 µl of cDNA. Mix well by pipetting up and down 10 times or vortex gently to mix. Quickly spin down the samples after mixing, stopping before the beads settle out.

2.3. Incubate at room temperature for at least 10 minutes.
2.4. Place the samples on an appropriate magnetic stand to separate beads from the supernatant.
2.5. Once the solution is clear, carefully remove and discard the supernatant. Be careful not to remove the beads.
2.6. While on the magnetic stand, add 100 µl of freshly prepared 80% ethanol to the tubes. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant without disturbing the beads.
2.7. While the beads are still dark brown and glossy, elute the samples in 12 µl of 0.1X TE buffer. Gently vortex and spin down the samples.

Note: Do not over-dry the beads, as this may result in lower recovery

2.8. Incubate at room temperature for at least 10 minutes before placing the samples back on the magnetic rack. Remove the supernatant and transfer to a clean 96-well plate.
Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

3. Tiled Amplicon PCR 1

3.1. Gently mix and spin down reagents. Prepare the split pool amplification reactions as follows:

3.1.1. For Pool Set 1, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
cDNA	4.5
Q5 Hot Start High-Fidelity 2X Master Mix	6.25
IDT Artic V4.1 NCOV-2019 Pool 1 (10 µM)	1.75
Total Volume	*12.5*

3.1.2. For Pool Set 2, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
cDNA	4.5
Q5 Hot Start High-Fidelity 2X Master Mix	6.25
IDT Artic V4.1 NCOV-2019 Pool 2 (10 µM)	1.75
Total Volume	*12.5*

3.2. Pipet 10 times to mix and spin down.
3.3. Incubate reactions in a thermocycler with the following conditions:

CYCLE STEP	TEMP (°C)	TIME	CYCLES
Initial Denaturation	98	30 seconds	1
Denature	95	15 seconds	25
Annealing/Extension	63	5 minutes	
Hold	4	∞	1

*Set heated lid to 105 °C

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

4. Cleanup of Tiled Amplicon PCR 1 Product

Note: Do not combine split reactions

Note: See the SpeedBead Preparation Protocol for instructions on how to prepare the beads

4.1. Allow the bead solution to come to room temperature and vortex to resuspend.
4.2. Add 10 µl (0.8X) of resuspended beads to the 12.5 µl of PCR 1 product. Mix well by pipetting up and down 10 times or vortex gently to mix. Quickly spin down the samples after mixing, stopping before the beads settle out.
4.3. Incubate at room temperature for at least 10 minutes.
4.4. Place the samples on an appropriate magnetic stand to separate beads from the supernatant.
4.5. Once the solution is clear, carefully remove and discard the supernatant. Be careful not to remove the beads.
4.6. While on the magnetic stand, add 100 µl of freshly prepared 80% ethanol to the tubes. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant without disturbing the beads.
4.7. While the beads are still dark brown and glossy, elute the samples in 12 µl of 0.1X TE buffer. Gently vortex and spin down the samples.

Note: Do not over-dry the beads, as this may result in lower recovery

4.8. Incubate at room temperature for at least 10 minutes before placing the samples back on the magnetic rack. Remove the supernatant and transfer to a clean 96-well plate.

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

5. Tiled Amplicon PCR 2

5.1. Gently mix and spin down reagents. Prepare the split pool amplification reactions as follows:

5.1.1. For Pool Set 1, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
Clean PCR 1 Product	5
Nuclease-free Water	3.75
KAPA dNTPs (red)	0.45
KAPA HiFi Enzyme (green)	0.3
5X KAPA Buffer (yellow)	3
Custom ARTIC V4.1 Fusion oPool **1** (10 µM)	2.5
Total Volume	*15*

5.1.2. For Pool Set 2, add the following components to a 96-well plate:

COMPONENT	VOLUME (µl)
PCR 1 Product	5
Nuclease-free Water	3.75
KAPA dNTPs (red)	0.45
KAPA HiFi Enzyme (green)	0.3

5X KAPA Buffer (yellow)	3
Custom ARTIC V4.1 Fusion oPool **2** (10 µM)	2.5
Total Volume	*15*

5.2. Pipet 10 times to mix and spin down.

5.3. Incubate reactions in a thermocycler with the following conditions:

CYCLE STEP	TEMP (°C)	TIME	CYCLES
Initial Denaturation	98	2 minutes	1
Denature	95	30 seconds	8
Annealing	55	30 seconds	
Extension	72	1 minute	
Final Extension	72	5 minutes	1
Hold	4	∞	1

*Set heated lid to 105 °C

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

6. Quality Control of Tiled Amplicons

6.1. Prepare a 1.5% TBE agarose gel

6.1.1. Follow manufacturer instructions to prepare a 10X TBE stock solution from TBE premix powder.

6.1.2. Prepare 1 L of 0.5X TBE solution by combining the following reagents in a carboy:

COMPONENT	AMOUNT
10X TBE stock	50 mL
DI water	950 mL

6.1.3. Shake or mix until homogenous.

6.1.4. Prepare a 1.5% TBE agarose gel large enough to fit 2x50-well combs (enough for 96 samples, plus ladders) by combining the following reagents in a flask:

COMPONENT	AMOUNT
0.5X TBE solution	150 mL
Agarose gel powder	2.25 g

6.1.5. Mix gently to combine.

6.1.6. Microwave the flask in short intervals, until the agarose powder is fully dissolved.

6.1.7. Allow the flask to cool before adding 1.70 µl of the GelRed Nucleic Acid Gel Stain and mixing gently to combine.

Note: Don't cool the agarose for too long or it will begin to solidify

6.1.8. Place the gel casting rig on the benchtop and slowly pour the liquid agarose into the rig, add the well combs, and allow the gel to fully solidify.

6.2. **Run gel electrophoresis**

6.2.1. Remove the well combs and place the prepared gel in an appropriately sized electrophoresis rig and fill with 0.5X TBE solution until the gel is completely submerged.

6.2.2. Follow manufacturer instructions for preparing the DNA loading dye.

6.2.3. To prepare the samples for the gel, combine 3 µl of each sample with 3 µl of prepared DNA loading dye and pipette to mix.

6.2.4. Use a p10 pipette to load the 6 µl of sample-dye mixture into the prepared wells.

6.2.5. Connect the gel rig to the power supply and run gel electrophoresis at 100V for 40 minutes

6.2.6. Visualize the DNA using a gel imager.

6.3. **Use Qubit fluorometer to check sample concentrations (OPTIONAL)**

6.3.1. Follow manufacturer instructions for how to prepare samples and calculate dsDNA concentrations using the 1X dsDNA HS Assay.

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

7. Combine Split Reactions

7.1. In a new 96-well plate, combine 6 µl of PCR 2 Pool 1 and 6 µl of PCR 2 Pool 2 for a total volume of 12 µl.

7.2. Vortex gently to mix and spin down.

8. Cleanup of Combined Tiled Amplicon PCR 2 Product

Note: See the SpeedBead Preparation Protocol for instructions on how to prepare the beads

8.1. Allow the bead solution to come to room temperature and vortex to resuspend.

8.2. Add 9.6 µl (0.8X) of resuspended beads to the 12 µl of combined PCR product. Mix well by pipetting up and down 10 times or vortex gently to mix. Quickly spin down the samples after mixing, stopping before the beads settle out.

8.3. Incubate at room temperature for at least 10 minutes.

8.4. Place the samples on an appropriate magnetic stand to separate beads from the supernatant.

8.5. Once the solution is clear, carefully remove and discard the supernatant. Be careful not to remove the beads.

8.6. While on the magnetic stand, add 100 µl of freshly prepared 80% ethanol to the tubes. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant without disturbing the beads.

Note: Do not over-dry the beads, as this may result in lower recovery

8.7. While the beads are still dark brown and glossy, elute the samples in 12 µl of 0.1X TE buffer. Gently vortex and spin down the samples.

8.8. Incubate at room temperature for at least 10 minutes before placing the samples back on the magnetic rack. Remove the supernatant and transfer to a clean 96-well plate.

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

9. PCR Enrichment with Illumina TruSeq Primers

9.1. Gently mix and spin down reagents.

9.2. In a new 96-well plate, prepare the combined pool amplification reactions as follows:

COMPONENT	VOLUME (µl)
Clean Combined PCR 2 Product	10
Nuclease-free Water	3.75
KAPA dNTPs (red)	0.75
KAPA HiFi Enzyme (green)	0.5
5X KAPA Buffer (yellow)	5
i5 Primer (5 µM)	2.5
i7 Primer (5 µM)	2.5
Total Volume	*25*

9.3. Pipet 10 times to mix and then spin down.

9.4. Incubate reactions in a thermocycler with the following conditions:

CYCLE STEP	TEMP (°C)	TIME	CYCLES
Initial Denaturation	98	2 minutes	1
Denature	95	30 seconds	8
Annealing	55	30 seconds	
Extension	72	1 minute	
Final Extension	72	5 minutes	1
Hold	4	∞	1

*Set heated lid to 105 °C

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

10. Quality Control of Tagged Libraries

10.1. **Prepare a 1.5% TBE agarose gel**

Note: See steps under 6.1 for how to prepare the agarose gel

10.2. **Run gel electrophoresis**

Note: See steps under 6.2 for how to run the gel and visualize the tagged libraries

10.3. **Use Qubit fluorometer to check sample concentrations (OPTIONAL)**

10.3.1. Follow manufacturer instructions to prepare samples and calculate dsDNA concentrations using the 1X dsDNA HS Assay.

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

11. Pooling of Tagged Libraries for Sequencing

11.1. Create a library pooling spreadsheet with the following information included for each sample to be pooled:

11.1.1. Sample ID

11.1.2. Average sample size (bp)

11.1.3. Sample concentration (estimated from gel or calculated using Qubit)

11.1.4. Desired number of reads

11.2. Using the above information, calculate the appropriate volume (µl) from each sample to be added to the final pool so that each sample receives the desired number of reads.

11.3. Combine the appropriate volume of each sample into a clean 1.5 mL tube.

11.4. Vortex to combine and then spin down.

Safe to stop. Store at 4°C overnight or -20°C for short-term storage prior to the next step.

12. Final Cleanup of Pooled Libraries

Note: See the SpeedBead Preparation Protocol for instructions on how to prepare the beads

1.1. Add 50 µl of the final pool to a clean 1.5 mL tube.

12.1. Allow the bead solution to come to room temperature and vortex to resuspend.

12.2. To the 50 µl of final pool, add 45 µl (0.9X) of resuspended beads. Mix well by pipetting up and down 10 times or vortex gently to mix. Quickly spin down the samples after mixing, stopping before the beads settle out.

12.3. Incubate at room temperature for at least 10 minutes.

12.4. Place the samples on an appropriate magnetic stand to separate beads from the supernatant.

12.5. Once the solution is clear, carefully remove and discard the supernatant. Be careful not to remove the beads.

12.6. While on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant without disturbing the beads.

Note: Do not over-dry the beads, as this may result in lower recovery

12.7. While the beads are still dark brown and glossy, elute the beads in 50 µl of 0.1X TE buffer. Gently vortex and spin down the samples.

12.8. Incubate at room temperature for at least 10 minutes before placing the plate back on the magnetic rack. Remove the supernatant and place in a clean 1.5 mL tube.

Libraries are now ready to be sequenced. Store at -20°C for short-term storage or -80°C for long-term storage prior to sequencing.