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

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Leah Lariscy[1], Amanda Sullivan[1], Katie Dillon[1], Megan Lott[1], Travis Glenn[1], Erin Lipp[1]

[1]University of Georgia

Leah Lariscy
University of Georgia

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We use this protocol and it's working

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Abstract

This protocol describes how to prepare purified SARS-CoV-2 RNA from raw wastewater samples for short-read sequencing. The initial targeted amplification utilizes the commercially available IDT ARTIC V4.1 tiled amplicon panel, followed by amplification with IDT custom-ordered fusion primers that follow the same amplicon scheme, allowing for further amplification of target molecules and eliminating the need for end repair and adapter ligation. Completion of this protocol should result in a clean, multiplexed DNA library pool ready for sequencing.

Materials

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)

cDNA Synthesis

1 Thaw reagents appropriately prior to use.

- Thaw purified RNA samples on ice
- LunaScript RT Supermix does not require thawing and should remain at -20°C

2 Gently vortex and spin down reagents and samples

3 Prepare the cDNA synthesis reactions as follows:

3.1 For purified RNA samples, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	RNA Sample	8
	LunaScript RT SuperMix	2
	Total Volume	10

3.2 For no template controls, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	Nuclease-free Water	8
	LunaScript RT SuperMix	2
	Total Volume	10

4 Pipet up and down 10 times to mix, then spin down.

5 Incubate reactions in a thermocycler under the following conditions:

	A	B	C	D
	Step	Temp (°C)	Duration	Cycles
	Annealing	25	2 minutes	1
	Synthesis	55	20 minutes	1
	Inactivation	95	1 minute	1
	Hold	4	∞	1

Set heated lid to 105°C

Note

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.

Cleanup of cDNA

20m 30s

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

Note

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

7 Add 20 µL (2.0X) of resuspended beads to the 10 µL of cDNA.

8 Mix well by pipetting up and down 10 times or vortex gently. Quickly spin down the samples after mixing, stopping before the beads settle out.

9 Incubate at room temperature for 00:10:00 .

10m

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

11 Once the solution is clear, carefully remove and discard the supernatant.

Note

Take care not to remove any beads, otherwise product may be lost.

12 While on the magnetic stand, add 100 µL of freshly prepared 80% ethanol to the tubes. Briefly incubate at room temperature for 00:00:30 , then carefully remove and discard the supernatant without disturbing the beads.

30s

13 While the beads are still dark brown and glossy, elute the samples in 10 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.

14 Incubate at room temperature for at least 00:10:00 before placing the samples back on the magnetic rack. Once solution is clear, remove the supernatant and transfer to a clean 96-well plate.

10m

Note

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

Tiled Amplicon PCR 1

15 Thaw reagents appropriately prior to use.

- Thaw amplicon primer pools at room temperature
- Thaw cDNA on ice
- Q5 Hot Start High-Fidelity 2X Master Mix does not require thawing and should remain at -20°C

16 Prepare the ARTIC V4.1 NCOV-2019 Panel by diluting each 100 µM primer pool 1:10 in Nuclease-free Water for a 10 µM working stock.

17 Gently vortex and spin down reagents and samples.

18 Prepare the split pool amplification reactions as follows:

18.1 For **Pool Set 1**, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	cDNA	4.5
	Q5 Hot Start Master Mix	6.25

	A	B
	ARTIC V4.1 Pool 1	1.75
	Total Volume	12.5

18.2 For **Pool Set 2**, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	cDNA	4.5
	Q5 Hot Start Master Mix	6.25
	ARTIC V4.1 Pool 2	1.75
	Total Volume	12.5

19 Pipet up and down 10 times to combine.

20 Add plate seal and spin down.

21 Incubate reactions in a thermocycler under the following conditions:

	A	B	C	D
	Step	Temp (°C)	Duration	Cycles
	Initial Denaturation	98	30 seconds	1
	Denaturation	95	15 seconds	25
	Annealing/ Extension	63	5 minutes	25
	Hold	4	∞	1

Set heated lid to 105°C

Note

Do not combine split reactions. This will be done after Tiled Amplicon PCR 2 and subsequent gel electrophoresis.

Note

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

Cleanup of Tiled Amplicon PCR 1 Product

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

Note

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

23 Add 10 µL (0.8X) of resuspended beads to the 12.5 µL of PCR 1 product.

24 Mix well by pipetting up and down 10 times or vortex gently. Quickly spin down the samples after mixing, stopping before the beads settle out.

25 Incubate at room temperature for 00:10:00 .

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

27 Once the solution is clear, carefully remove and discard the supernatant.

Note

Take care not to remove any beads, otherwise product may be lost.

28 While on the magnetic stand, add 100 µL of freshly prepared 80% ethanol to the tubes. Briefly incubate at room temperature for 00:00:30 , then carefully remove and discard the supernatant without disturbing the beads.

29 While the beads are still dark brown and glossy, elute the samples in 12.5 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.

30 Incubate at room temperature for at least 00:10:00 before placing the samples back on the magnetic rack. Once solution is clear, remove the supernatant and transfer to a clean 96-well plate.

Note

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

Tiled Amplicon PCR 2

31 Thaw reagents appropriately prior to use.

- Thaw amplicon primer pools at room temperature
- If needed, thaw PCR 1 products on ice
- KAPA HiFi Enzyme does not require thawing and should remain at -20°C
- All other KAPA components should be thawed on ice

32 Prepare the Custom ARTIC V4.1 Fusion oPool Panel by reconstituting each primer pool with Nuclease-free Water to a final concentration of 10 µM.

33 Gently vortex and spin down reagents and samples.

34 Prepare the split pool amplification reactions as follows:

34.1 For **Pool Set 1**, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	PCR 1 Pool 1 Product	5
	Nuclease-free Water	5.5
	KAPA dNTPs	0.45
	KAPA HiFi Enzyme	0.3

	A	B
	5X KAPA Buffer	3
	Custom ARTIC V4.1 oPool 1	0.75
	Total Volume	15

34.2 For **Pool Set 2**, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)
	PCR 1 Pool 2 Product	5
	Nuclease-free Water	5.5
	KAPA dNTPs	0.45
	KAPA HiFi Enzyme	0.3
	5X KAPA Buffer	3
	Custom ARTIC V4.1 oPool 2	0.75
	Total Volume	15

35 Pipet up and down 10 times to combine.

36 Add plate seal and spin down.

37 Incubate reactions in a thermocycler under the following conditions:

	A	B	C	D
	Step	Temp (°C)	Duration	Cycles
	Initial Denaturation	98	2 minutes	1
	Denaturation	95	30 seconds	6
	Annealing	55	30 seconds	6
	Extension	72	1 minute	6
	Final Extension	72	5 minutes	1
	Hold	4	∞	1

Set heated lid to 105°C

Note

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

Quality Control of Tiled Amplicons

40m

38 Prepare a 1.5% TBE agarose gel.

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

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

A	B
Component	Amount
10X TBE	50 mL
DI Water	950 mL

38.3 Shake or mix the 0.5X TBE solution until homogenous.

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

Component	Amount
0.5X TBE	150 mL
Agarose gel powder	2.25 g

38.5 Mix components in flask gently to combine.

38.6 Microwave the flask in short intervals, until the agarose powder if fully dissolved.

38.7 Allow the flask to cool briefly before adding 1.70 µL of the GelRed Nucleic Acid Stain and mixing gently to combine.

> **Note**
>
> Do not cool the agarose for more than a few minutes or it will begin to solidify.

38.8 Place the gel casting rig on a flat surface and slowly pour the liquid agarose into the rig, then add the well combs and allow the gel to fully solidify.

39 Run gel electrophoresis .

39.1 Remove the well combs and place the prepared gel in an appropriately sized electrophoresis rig.

39.2 Fill the rig with 0.5X TBE solution until the gel is completely submerged.

39.3 Follow manufacturer instructions for preparing the DNA loading dye.

39.4 To prepare the samples for loading, combine 3 µL of each sample with 3 µL of prepared DNA loading dye and pipette gently to mix.

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

39.6 Connect the gel rig to the power supply and run gel electrophoresis at 100V for 00:40:00 . 40m

39.7 Visualize the DNA using a gel imager.

40 Check DNA concentrations using a Qubit Fluorometer (**OPTIONAL**).

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

Note

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

Combine Split Reactions

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

42 Add a plate seal, then vortex gently and spin down.

Note

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

Cleanup of Combined Tiled Amplicon PCR 2 Product

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

Note

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

44 Add 9.6 µL (0.8X) of resuspended beads to the 12 µL of PCR 1 product.

45 Mix well by pipetting up and down 10 times or vortex gently. Quickly spin down the samples after mixing, stopping before the beads settle out.

46 Incubate at room temperature for 00:10:00 .

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

48 Once the solution is clear, carefully remove and discard the supernatant.

Note

Take care not to remove any beads, otherwise product may be lost.

49 While on the magnetic stand, add 100 µL of freshly prepared 80% ethanol to the tubes. Briefly incubate at room temperature for 00:00:30 , then carefully remove and discard the supernatant without disturbing the beads.

50 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.

51 Incubate at room temperature for at least 00:10:00 before placing the samples back on the magnetic rack. Once solution is clear, remove the supernatant and transfer to a clean 96-well plate.

Note

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

PCR Enrichment with Illumina TruSeq Primers

52 Thaw reagents appropriately prior to use.

- Thaw TruSeq primers at room temperature
- If needed, thaw clean PCR 2 products on ice
- KAPA HiFi Enzyme does not require thawing and should remain at -20°C
- All other KAPA components should be thawed on ice

53 Gently vortex and spin down reagents and samples.

54 For TruSeq PCR, combine the following components in a 96-well plate:

	A	B
	Component	Volume (µl)

	A	B
	Clean PCR 2 Product	10
	Nuclease-free Water	3.75
	KAPA dNTPs	0.75
	KAPA HiFi Enzyme	0.5
	5X KAPA Buffer	5
	i5 Primer (5 µM)	2.5
	i7 Primer (5 µM)	2.5
	Total Volume	25

55 Pipet up and down 10 times to combine.

56 Add plate seal and spin down.

57 Incubate reactions in a thermocycler under the following conditions:

	A	B	C	D
	Step	Temp (°C)	Duration	Cycles
	Initial Denaturation	98	2 minutes	1
	Denaturation	95	30 seconds	6
	Annealing	55	30 seconds	6
	Extension	72	1 minute	6
	Final Extension	72	5 minutes	1
	Hold	4	∞	1

Set heated lid to 105 °C

Note

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

Quality Control of TruSeq Libraries

58 Prepare a 1.5% TBE agarose gel.

> **Note**
>
> See steps 38.1 through 38.8 for instructions on how to prepare the gel.
> go to step #38

59 Run gel electrophoresis and visualize results.

> **Note**
>
> See steps 39.1 through 39.7 for instructions on how to run gel electrophoresis and visualize the libraries. go to step #39

60 Check DNA concentrations using a Qubit Fluorometer (**OPTIONAL**).

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

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

Pooling of TruSeq Libraries for Sequencing

61 Create a library pooling spreadsheet with the following information included from each sample to be pooled:

1. Sample ID
2. Average sample size (bp)
3. Sample concentration (estimated from gel or calculated using Qubit)
4. Desired number of reads

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

63 Combine the appropriate volume of each library into a clean 1.5 mL tube.

64 Vortex to combine and spin down.

Note

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

Final Cleanup of Pooled Libraries

20m 30s

65 Add 50 µL of the final pool to a clean 1.5 mL tube.

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

Note

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

67 To the 50 µL of final pool, add 45 µL (0.9X) of resuspended beads.

68 Mix well by pipetting up and down 10 times or vortex gently. Quickly spin down after mixing, stopping before the beads settle out.

69 Incubate at room temperature for 00:10:00 .

10m

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

71 Once the solution is clear, carefully remove and discard the supernatant.

Note

Take care not to remove any beads, otherwise product may be lost.

72 While on the magnetic stand, add 200 µL of freshly prepared 80% ethanol to the tubes. Briefly incubate at room temperature for 00:00:30, then carefully remove and discard the supernatant without disturbing the beads.

30s

73 While the beads are still dark brown and glossy, elute the samples in 50 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.

74 Incubate at room temperature for at least 00:10:00 before placing the samples back on the magnetic rack. Once solution is clear, remove the supernatant and transfer to a clean 1.5 mL tube.

10m

Note

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.