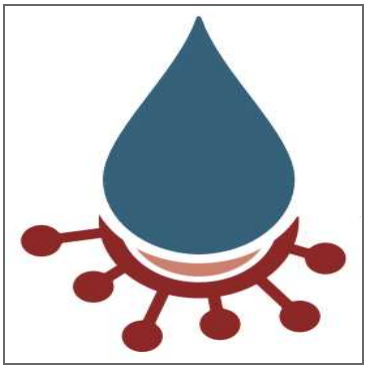

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Protocol status: Working
We use this protocol and it's working.

Effective Target Capture/Enrichment of Respiratory Viruses from Wastewater

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UNC Charlotte COVID WBE

Lauren Roppolo Brazell

ABSTRACT

Human respiratory viruses (HRVs) are highly communicable viral pathogens that present varying degrees of illness in infected individuals. The advent of the COVID-19 pandemic demonstrated the utility of wastewater-based epidemiology (WBE) methods that bypassed traditional clinical testing for the virus. This method yielded powerful results, informing communities of potential outbreaks and allowing them to make informed decisions for the health and well-being of their community. The success of WBE can be employed to screen wastewater for other HRVs. Herein we establish a protocol that prepares viral RNA from extracted wastewater for use in a HRV target capture/enrichment sequencing assay. This protocol addresses the unique challenge of low viral load input for library preparation by randomly priming and non-specifically amplifying viral RNA. By then enriching only the sequences of interest in subsequent target capture/hybridization reactions, this method conserves sequencing resources and results in high-quality sequence information. Following downstream bioinformatics analyses, this sequence information can be used to help communities take appropriate action in mitigating the negative effects of HRVs.

IMAGE ATTRIBUTION

Image made with BioRender.

MATERIALS

Sequence-Independent, Single Primer Amplification

1. SISPA A
- SOL Primer A: 5'-GTT TCC CAC TGG AGG ATA-(N9)-3'
- Molecular biology-grade H_2O
- SuperScript IV First-Strand Synthesis System

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Last Modified: Oct 04, 2023

PROTOCOL integer ID: 87105

- Sequenase Version 2.0 DNA Polymerase

2. SISPA B

- 2x LongAmp Taq Polymerase
- SOL Primer B: 5'-GTT TCC CAC TGG AGG ATA-3'

3. Purification

- AMPure XP Beads; equilibrate to RT for at least 30 minutes
- EtOH; dilute with H_2O to 70%
- Buffer EB
- Molecular biology-grade H_2O

Total Nucleic Acids Library Preparation

4. DNA Fragmentation, End Repair, and dA-Tailing

- From Twist Library Prep EF Kit 1, 2.0:
  - Frag/AT Buffer
  - Frag/AT Enzyme
- Molecular biology-grade H_2O

5. Ligate Twist Universal Adapters

- From Twist Universal Adapter System:
  - Twist Universal Adapters
- From Twist Library Prep EF Kit 1, 2.0:
  - Ligation Master Mix
- Molecular biology-grade H_2O

6. Purification

- From Twist Library Prep Kit 2:
  - DNA Purification Beads; equilibrate to RT for at least 30 minutes
- EtOH; dilute with H_2O to 80%
- Buffer EB

7. PCR Amplify Using Twist UDI Primers

- From Twist Library Prep EF Kit 1, 2.0:
  - Equinox Library Amp Mix (2x)
- From Twist Universal Adapter System:
  - Twist UDI Primers

8. Purification

- From Twist Library Prep Kit 2:
  - DNA Purification Beads; equilibrate to RT for at least 30 minutes
- EtOH; dilute with H_2O to 80%
- Buffer EB

9. Perform QC

- Qubit 1x dsDNA HS Assay Kit

Target Enrichment

10. Aliquot and Dry Down Library

11. Hybridize Capture Probes with Pools

- From Twist Hybridization Reagents:

- Hybridization Mix
- Hybridization Enhancer

- Twist Respiratory Viral Research Panel
- Molecular biology-grade H_2O
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution

12. Bind Hybridized Targets to Streptavidin Beads
- From Twist Wash Buffers
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- From Twist Binding and Purification Beads Kit; equilibrate to RT for at least 30 minutes
  - Streptavidin Binding Beads
  - DNA Purification Beads

13. Post-Capture PCR Amplify
- From Twist Hybridization Reagents:
  - Amplification Primers
  - Equinox Library Amp Mix (2x)

14. Purification
- From Twist Library Prep Kit 2:
  - DNA Purification Beads; equilibrate to RT for at least 30 minutes
- EtOH; dilute with H_2O to 80%
- Buffer EB

15. Perform QC
- Qubit 1x dsDNA HS Assay Kit

Miscellaneous Equipment
- Centrifuge
- Plate Centrifuge
- Vortex
- Thermocycler
- Magnetic Rack
- Qubit Fluorometer
- Vacuum Concentrator
- Heat Block
- Temperature-Programmable Mixer

Sequence Independent, Single-Primer Amplification [Moreno e..

1 SISPA-A: Reverse Transcription & 2nd Strand cDNA Synthesis

1.1 Make a working stock of your SOL Primer A. Stock should be 100 pmol/1µL. Add 4µL of stock + 6µL molecular biology-grade H_2O; you now have 10µL of a 40pmol/µL working stock.

1.2 Add 1µL SOL Primer A working stock to 4µL extracted RNA. Heat to 65°C for 5 mins in a thermocycler and let cool at 4°C for 5 mins.

1.3 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of the following:

Reagent	Volume per Reaction
5x RT Buffer	2µL
10mM dNTP	1µL
Molecular Biology-Grade H_2O	1µL
0.1M DTT	0.5µL
SSIV RT	0.5µL
TOTAL	5µL

1.4 Add 5µL master mix to reaction. Incubate at 50°C for 10 mins.

1.5 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of the following:

Reagent	Volume per Reaction
5x Sequenase	1µL
Molecular Biology-Grade H_2O	3.8µL
Sequenase	0.15µL
TOTAL	4.95µL

1.6 After 10 minute incubation, add 5µL of Sequenase Mix #1 to the reaction.

1.7 Incubate at 37°C for 8 min.

1.8 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of the following:

	Reagent	Volume per Reaction
	Sequenase dilution buffer	0.45µL
	Sequenase	0.15µL
	TOTAL	0.6µL

1.9 After 8 minute incubation, add 0.6µL of Sequenase Mix #2 to the reaction.

1.10 Incubate at 37°C for 8 min.

1.11 After 8 min incubation, add 1µL RNAse H to each sample.

1.12 Incubate at 37°C for 20 min. SISPA A is now complete and samples can be stored at -20°C.

2 SISPA B: PCR Amplification of Randomly Primed cDNA

2.1 Make a master mix for 1 reaction (scale up as needed) consisting of the following:

	Reagent	Volume per Reaction
	2X LongAmp Taq Master Mix	44µL
	SOL Primer B	1µL
	TOTAL	45µL

2.2 Add 5µL of product from SISPA A to 45µL master mix.

2.3 Run a thermocycler set with the following parameters:

	Step	Temperature	Time	Number of Cycles
	Initialization	98°C	30 seconds	1
	Denaturation	94°C	15 seconds	30
	Annealing	50°C	20 seconds	
	Extension	68°C	2 minutes	
	Final Extension	68°C	10 minutes	1
	Hold	4°C	∞	--

3 Purification of SISPA Product

3.1 Add 50µL of AMPure XP beads to 50µL SISPA product for a 1:1 purification ratio.

3.2 Incubate at RT for 10 minutes.

3.3 Spin down briefly and place on magnetic rack. Remove and discard supernatant once solution turns clear. Be sure not to disturb the bead pellets.

3.4 Wash beads with 200µL of freshly prepared 70% EtOH.

3.5 Remove EtOH without disturbing beads.

3.6 Repeat wash for a total of 2 washes. Remove EtOH without disturbing beads.

3.7 Remove samples from magnet and spin down briefly.

3.8 Remove residual EtOH.

3.9 Let air dry briefly. Do not overdry the bead pellet.

3.10 Resuspend samples in 50µL of buffer EB.

3.11 Incubate samples at RT for 5 minutes.

3.12 Transfer samples to magnetic rack. Transfer 48µL of eluted product to a new tube. Proceed to library prep protocol of your choice, or the samples may be stored at -20°C.

Total Nucleic Acids Library Preparation [2]

4 DNA Fragmentation, End Repair, and dA-Tailing

4.1 Program a thermocycler set with the following parameters:

Step	Temperature	Time	Number of Cycles
Hold	4°C	--	1
Incubation 1	37°C	30 minutes	1
Incubation 2	65°C	30 minutes	1
Hold	4°C	∞	--

Set the heated lid to 105°C, and start the program to pre-chill the thermocycler.

4.2 Add 25µL of each cDNA pool into a well of a 96-well plate.

4.3 Spin down to ensure all solution is at the bottom of the tube and place on ice.

4.4 Vortex the Frag/AT Buffer for 5 seconds. Spin down to collect all liquid in the bottom of the tube.

4.5 Invert the Frag/AT Enzymes a minimum of 10 times to homogenize or briefly vortex to ensure complete mixing. Spin down to collect all liquid in the bottom of the tube.

4.6 Prepare an enzymatic fragmentation mix consisting of the following (scale up as needed):

Reagent	Volume per Reaction
Molecular Biology-Grade H_2O	15µL
Frag/AT Buffer	4µL
Frag/AT Enzymes	6µL
TOTAL	25µL

Homogenize the master mix with moderate vortexing for 5 seconds or pipetting a minimum of half the total volume up and down 10 times, avoiding the formation of bubbles.

4.7 Add 25µL enzymatic fragmentation mix to each 25µL sample. Homogenize with moderate vortexing for 5 seconds or by pipetting a minimum of half the total volume up and down 10 times, avoiding the formation of bubbles. Seal the samples and keep the reactions on ice.

4.8 Spin down the samples and immediately transfer to the pre-chilled thermocycler.

4.9 Initiate the remaining steps of the thermocycler program (from step 4.1). While thermocycler is running, prepare reagents for step 5.

4.10 When the thermocycler has completed and the sample block has returned to 4°C, remove the samples and place them on ice. Proceed to ligate adapters and purification.

5 Ligate Twist Universal Adapters

5.1 Add 2.5µL of Twist Universal Adapters into each sample containing the dA-tailed DNA fragments from the above step. Mix gently by pipetting and keep on ice.

5.2 Invert the Ligation Master Mix a minimum of 10 times until homogenized and place on ice. Do not vortex the Ligation Master Mix.

5.3 Add 2.5µL molecular biology-grade H_2O and 20µL Ligation Master Mix to each sample. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Seal the samples and spin down to ensure all solution is at the bottom of the tube.

5.4 Incubate the ligation reaction at 20°C for 15 minutes in a thermocycler with the heated lid off, then move the samples to the bench top and proceed to purify.

6 Purification of Product

6.1 Vortex the pre-equilibrated, RT DNA purification beads until well mixed.

6.2 Add 60µL of homogenized (0.8x) DNA purification beads to each ligation sample from the previous step. Mix well by vortexing.

6.3 Incubate the samples for 5 minutes at RT.

6.4 Place the samples on a magnetic rack for 1 minute. The DNA beads form a pellet, leaving a clear supernatant.

6.5 Without removing the samples from the magnetic rack, remove and discard the supernatant.

6.6 Wash the bead pellet by gently adding 200µL of freshly prepared 80% EtOH, being careful not to disturb the pellet. Incubate for 1 minute, then remove and discard the EtOH.

6.7 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic rack.

6.8 Carefully remove all remaining EtOH with a 10µL pipette, making sure not to disturb the bead pellet.

6.9 Air dry the bead pellet on the magnetic rack for 5 minutes or until the bead pellet is dry. Do not overdry the pellet.

6.10 Remove the samples from the magnetic rack and add 17µL of buffer EB to each sample. Mix by pipetting until homogenized.

6.11 Incubate at RT for 2 minutes.

6.12 Place the samples back on a magnetic rack and let stand for 3 minutes or until beads form a pellet.

6.13 Transfer 15µL of the clear supernatant containing the ligated libraries to a clean 96-well plate, making sure not to disturb the bead pellet. Proceed to index and amplify library.

7 PCR Amplify Using Twist UDI Primers

7.1 Program a thermocycler set with the following parameters:

	Step	Temperature	Time	Number of Cycles
	Initialization	98°C	45 seconds	1
	Denaturation	98°C	15 seconds	12
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
	Final Extension	72°C	1 minute	1
	Hold	4°C	∞	--

Set the heated lid to 105°C.

7.2 Add 10µL of Twist UDI Primers from the provided 96-well plate to each of the cDNA libraries from the previous step. Mix well by gentle pipetting.

7.3 Invert, do not vortex, Equinox Library Amp Mix (2x) 5 times prior to use. Add 25µL Equinox Library Amp Mix (2x) to the cDNA libraries and mix well by gentle pipetting.

7.4 Sin samples down and immediately transfer to thermocycler, and start the program.

7.5 Remove the samples from the thermocycler block when the program is complete, and proceed to purification.

8 Purification

8.1 Vortex the pre-equilibrated DNA purification beads until mixed.

8.2 Add 50µL (1x) homogenized DNA purification beads to each ligation sample from the above step. Mix well by vortexing.

8.3 Incubate the samples for 5 minutes at RT.

8.4 Place the samples on a magnetic rack for 1 minute.

8.5 The DNA purification beads form a pellet, leaving a clear supernatant. With plate still on the magnetic rack, remove and discard the supernatant.

8.6 Gently wash the bead pellet by adding 200µL of freshly prepared 80% EtOH, careful not to disturb the bead pellet. Incubate for 1 minute, then remove and discard the EtOH.

8.7 Repeat this wash for a total of two washes, while keeping the samples on the magnetic rack.

8.8 Carefully remove all remaining EtOH with a 10µL pipette, making sure not to disturb the bead pellet.

8.9 Air dry the pellet on the magnetic rack for 5-10 minutes, or until it is dry. Do not overdry the pellet.

8.10 Remove the samples from the magnetic rack and add 22µL of buffer EB to each sample. Mix by pipetting until homogenized.

8.11 Incubate at RT for 2 minutes.

8.12 Place the plate or tubes on a magnetic rack and let stand for 3 minutes or until the beads form a pellet.

8.13 Transfer 20µL of the clear supernatant containing the amplified indexed libraries to a clean PCR tube, making sure not to disturb the pellet.

9 Perform QC

9.1 Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay.

Target Enrichment [3]

10 Aliquot and Dry Down Library

10.1 Use the concentration of each amplified, indexed library to calculate the volume (in µL) of each library needed for hybridization.

	Number of Indexed Samples per Pool	Amount of Each Indexed Library per Pool	Total Mass per Pool
	1	500 ng	500 ng
	2	500 ng	1,000 ng
	3	500 ng	1,500 ng
	4	375 ng	1,500 ng
	8	187.5 ng	1,500 ng

10.2 Transfer the calculated volumes from each amplified, indexed library to an indexed library pool reaction tube for **each** hybridization reaction being performed.

10.3 Spin down the library tubes to minimize the amount of bubbles present.

10.4 Dry the indexed library pools using a vacuum concentrator on a low or no heat setting. If not immediately proceeding to next step, store the dried indexed library pools at -20°C for 24 hours.

11 Hybridize Capture Probes with Pools

11.1 Heat the Hybridization Mx at 65°C in the heat block for 10 minutes, or until all precipitate is dissolved.

11.2 Prepare a probe solution for each pool in a PCR tube consisting of the following (scale up as needed):

Reagent	Volume per Pool
Hybridization Mix	20µL
Twist Respiratory Panel	4µL
Molecular Biology-Grade H_2O	4µL
TOTAL	28µL

Mix by flicking tubes.

11.3 Resuspend the dried, indexed library pools by preparing the reagents as follows (scale up as needed):

Reagent	Volume per Pool
Dried, Indexed Library Pool	---
Blocker Solution	5µL
Universal Blockers	7µL
TOTAL	12µL

11.4 Heat the probe solution to 95°C for 2 minutes in a thermocycler with the lid at 105°C, then immediately cool on ice for 5 minutes.

11.5 While probe solution is cooling on ice, heat the tube containing the resuspended, indexed library pool at 95°C for 5 minutes in a thermocycler with the lid at 105°C, then equilibrate both

the probe solution and resuspended, indexed library pool to RT on the bench top for 5 minutes.

11.6 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.

11.7 Spin down again to ensure all solution is at the bottom of the tube(s).

11.8 Incubate the hybridization reaction at 70°C for 16 hours in a thermocycler with the lid at 85°C.

12 Bind Hybridized Targets to Streptavidin Beads

12.1 Vortex the pre-equilibrated streptavidin binding beads until mixed.

12.2 Add 100µL streptavidin binding beads to a 1.5mL micro centrifuge tube. Prepare one tube for each hybridization reaction.

12.3 Add 200µL binding buffer to the tube(s) and mix by pipetting.

12.4 Place the tube(s) on a magnetic rack for 1 minute, then remove and discard the clear supernatant. Make sure not to disturb the bead pellet. Remove the tube from the magnetic rack. Repeat the wash two more times for a total of three washes.

12.5 After removing the clear supernatant from the third wash, add a final 200µL binding buffer and resuspend the beads by vortexing until homogenized.

12.6 Heat the resuspended beads at 68°C for at least 10 minutes before continuing to the next step.

12.7 Once the hybridization is complete, open the thermocycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of preheated streptavidin binding beads from the above step. Mix by pipetting and flicking.

12.8 Incubate the tube(s) of the hybridization reaction with the streptavidin binding beads for 5 minutes on a heated mixer set to 68°C.

12.9 Remove the tube(s) containing the hybridization reaction with the streptavidin binding beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).

12.10 Place the tube(s) on a magnetic rack for 1 minute.

12.11 Remove and discard the clear supernatant including the hybridization enhancer. Do not disturb the bead pellet.

12.12 Remove the tube(s) from the magnetic stand and add 200µL 68°C standard wash buffer 1, and mix by pipetting.

12.13 Incubate the tubes for 5 minutes at 68°C.

12.14 Spin down to ensure all solution is at the bottom of the tube(s).

12.15 Transfer the entire volume from the above step (about 200µL) into a new 1.5 mL micro centrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic rack for 1 minute.

12.16 Remove and discard the clear supernatant. Make sure not to disturb the bead pellet. Remove the tube(s) from the magnetic rack and add 200µL of 48°C wash buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).

12.17 Incubate the tube(s) for 5 minutes at 48°C.

12.18 Place the tube(s) on a magnetic rack for 1 minute.

12.19 Remove and discard the clear supernatant. Make sure not to disturb the bead pellet.

12.20 Repeat the wash two more times, for a total of three washes.

12.21 After the final wash, use a 10µL pipette to remove all traces of supernatant. Proceed immediately to the next step, and do not allow the beads to dry.

12.22 Remove the tube(s) from the magnetic rack and add 45µL molecular biology-grade H_2O. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the streptavidin binding bead slurry, on ice.

13 Post-Capture PCR Amplify

13.1 Program a thermocycler set with the following parameters:

	A	B	C	D
	Initialization	98°C	45 seconds	1
	Denaturation	98°C	15 seconds	15
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
	Final Extension	72°C	1 minute	1
	Hold	4°C	∞	--

Set the heated lid to 105°C.

13.2 If the streptavidin binding bead slurry has settled, mix by pipetting.

13.3 Transfer 22.5µL of the streptavidin binding bead slurry to a PCR tube. Keep on ice until ready to use in the next step.

13.4 Prepare a PCR mixture for each streptavidin binding bead aliquot consisting of the following (scale up as needed):

	Reagent	Volume per Reaction

	Reagent	Volume per Reaction
	Streptavidin Binding Bead Slurry	22.5µL
	Amplification Primers, ILMN	2.5µL
	Equinox Library Amp Mix (2x)	25µL
	TOTAL	50µL

Mix by pipetting.

13.5 Spin down the tubes, and transfer them to the thermocycler. Start the thermocycling program.

13.6 When the thermocycler program is complete, remove the tube(s) from the block and immediately proceed to the next step.

14 Purification

14.1 Vortex the pre-equilibrated DNA purification beads until well mixed.

14.2 Add 90µL (1.8x) homogenized DNA purification beads to the tube(s) from the previous step. Mix well by vortexing.

14.3 Incubate at RT for 5 minutes.

14.4 Place the tubes on a magnetic rack for 1 minute or until the supernatant is clear.

14.5 The DNA purification beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic rack, remove and discard the clear supernatant.

14.6 Wash the bead gently by adding 200µL of freshly prepared 80% EtOH, careful not to disturb the pellet. Incubate for 1 minute, then remove and discard the EtOH.

14.7 Repeat this wash, for a total of two washes while keeping the tube on the magnetic rack.

14.8 Carefully remove all remaining EtOH using a 10µL pipette, making sure not to disturb the bead pellet.

14.9 Air Dry the bead pellet on the magnetic rack for 5 minutes, or until the bead pellet is dry. Do not overdry the bead pellet.

14.10 Remove the tubes from the magnetic rack and add 32µL buffer EB to each capture reaction. Mix by pipetting until homogenized.

14.11 Incubate at RT for 2 minutes.

14.12 Place tubes on a magnetic rack and let stand for 3 minutes or until beads fully pellet.

14.13 Transfer 30µL of the clear supernatant containing the enriched library to a clean PCR tube, making sure not to disturb the bead pellet. If not proceeding to sequence immediately, the enriched library may be stored at -20°C for up to 24 hours.

15 **Perform QC**

15.1 Quantify and validate the concentration and/or fragment size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay or an Agilent DNA 7500 assay.

Sequence Prepared Libraries [4]

16 **Sequencing on an Illumina NextSeq 2000 Platform**

16.1 Subsequent sequencing preparation steps, including thawing of flow cell/reagent cartridge, diluting libraries to starting concentration, and setting up/loading a sequencing run into the NextSeq 2000 instrument can be found in Illumina's NextSeq 1000/2000 Product Documentation in the Sequencing Protocol section.