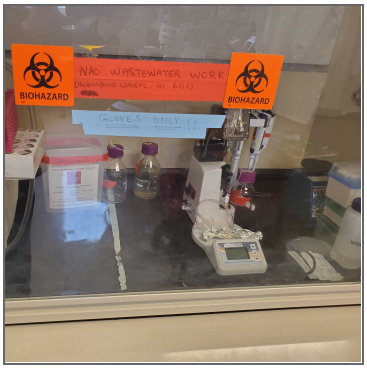

Concentration and nucleic acid extraction of viruses from wastewater influent V.2

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ABSTRACT

This protocol details our workflow for performing concentration and total nucleic acid extraction from wastewater influent for the purposes of untargeted RNA and DNA sequencing of viruses present in wastewater. In this protocol, 200 mL of raw influent wastewater is concentrated to a final volume of 400 uL using the InnovaPrep Concentrating Pipette Select. Prior to concentration, the wastewater sample is treated with Tween 20 and sonicated to dissociate viral particles from solids in the wastewater matrix. The sample is then centrifuged to remove larger solids. The pellet is discarded, and the supernatant is filtered with a 0.45 um PES 75 mm filtration unit to remove remaining suspended solids and bacteria. This filtrate is then concentrated with the Concentrating Pipette, using Ultra CPT tips and recommended device settings for the InnovaPrep modified wastewater processing protocol. Nucleic acids are then extracted from the concentrated product using the Zymo quick-DNA/RNA Viral kit using the manufacturer protocol with a few modifications that we have found to be helpful.

GUIDELINES

- **RNA processing and handling**: Please review Protocol Note: Working with RNA Samples before handling RNA samples.

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MATERIALS

Materials:
(for one sample replicate and one negative control)

- 2 x InnovaPrep Ultra CPT (Unirradiated) tips for the InnovaPrep Concentrating Pipette Select
- 2 x 0.45um PES 75 mm vacuum filtration tops (VWR No. 10040-470)
- 2 x 250mL pyrex bottles
- 9 x 50mL falcon tubes (VWR No. 21008-178)
- 2 x 5mL centrifuge tubes
- 2 x 50 mL serological pipettes (VWR No. 170358N)
- 1.5 mL microcentrifuge tubes (VWR No. 1420-2600)
- PREempt wipes (VWR No. 10822-456)
- Parafilm (Millipore Sigma No. HS234526C)
- RNaseZap
- Kimwipes (VWR No. 34120)
- Paper towels
- Filtered micropipette tips

Reagents:

- Tween 20
- 1x Phosphate Buffered Saline (w/o Ca & Mg)
- Ice
- CP Select Elution buffer (Tris)
- CP Select Storage Fluid
- All buffers in the Zymo quick-DNA/RNA Viral Kit

Equipment:

- InnovaPrep Concentrating Pipette Select
- Bransonic M 1800 Sonicator (filled ¾ with tap water)
- Attachment for vortexer (Cole-Parmer No. UX-04724-89)
- Vortexer
- Vacuum Line
- Micropipettes (1000 uL, 200 uL, 10 uL, 2 uL, 1 uL) and holder
- Timer
- Floor Centrifuge (Ex: Beckman Coulter Avanti J series)
- Rotor compatible with 50 mL tubes (Ex: Beckman Coulter JA-14.50)

SAFETY WARNINGS

Biosafety precautions: All raw wastewater samples will be received and stored with primary and secondary containment. The primary container (the bottle or falcon tube) should remain in the secondary container (a Ziploc bag containing paper towels to absorb spills) until processed. All raw samples must be handled within a dedicated fume hood or biosafety cabinet. All laboratory personnel handling these samples must use safety glasses, gloves, and lab coats. Samples will be transported between processing stations within a secondary container that has been cleaned with PreEmpt. All surfaces (outside of the fume hood/sash, centrifuge lid and rotor, etc.) will also be wiped down with PreEmpt. All autoclave-able bottles that are in contact with wastewater samples will be cleaned by filling with 10% bleach for at least 20 minutes before disposal.

BEFORE START INSTRUCTIONS

Read the 'Safety Warnings' section for biosafety precautions necessary for handling raw wastewater samples. Prepare the fume hood for wastewater handling, gather materials and reagents (centrifuge tubes, serological pipettes, pipette tips, micropipettes, a marker, strips of parafilm, sterile-filtered 10% Tween 20, PBS). Label centrifuge tubes (five tubes for each influent sample, and two for a negative control sample). Ensure proper PPE.

Reagent Preparation

7m

1 Prepare the [M] 10 % volume Tween 20 stock solution. Use the following materials: 7m

- Tween 20
- 1x PBS
- 2 x 50 mL falcon tube
- 0.22 um vacuum filtration unit

1.1 Dispense 4 mL of Tween 20 into a 50 mL Falcon tube. Bring the volume of the Falcon tube to 40 mL with 1x PBS. Vortex briefly. 5m

1.2 Filter sterilize with a 0.22 um vacuum filtration unit into a new falcon tube. 2m

Part 1: Influent Handling, Dissociation, Centrifugation, Filtrat...

1h

2 5m

Safety information

Refer to the 'Safety Warnings' section for biosafety precautions necessary for handling raw wastewater samples.

Transfer influent sample (with secondary container) from the refrigerator to the fume hood. Remove the sample bottle from the secondary container and unseal the bottle by removing the affixed Parafilm.

3 Aliquot influent into centrifuge tubes. 10m

- Invert the bottle of influent several times to resuspend contents, then carefully open it.
- Using a fresh 50 mL serological pipette, aspirate and dispense 40 mL of influent into a 50 mL centrifuge tube. Repeat until 200 mL of influent has been dispensed across 5 centrifuge tubes.
- Repeat for desired number of sample replicates.

Note

The 200 mL sample is separated into five 50-mL tubes due to supply and equipment limitations.

Additionally, EHS recommended limiting sample volume to 40 mL in each tube to reduce the risk of leaks.

4 Prepare the negative control. 2m

- Using a fresh 50 mL serological pipette, add 40 mL of 1x PBS to two centrifuge tubes, for a total volume of 80 mL.
- For each step in this protocol, handle the negative control tubes in the same manner as the influent sample tubes.

5 3m

Add 400 µL of [M] 10 % (v/v) Tween 20 stock solution to each centrifuge tube for a final concentration of [M] 1 % (v/v) Tween 20.

6 Cap and parafilm all bottles and centrifuge tubes. 5m

Safety information

Return the influent bottle to secondary containment, wipe exposed surfaces with pre-empt wipes, and return the remaining influent to refrigeration.

7 Place all centrifuge tubes on a vortexer using a 50 mL tube adapter. Shake for 1000 rpm, 00:01:00 2m

8 Transfer all centrifuge tubes from the vortexer to a sonication bath, and sonicate for 00:01:00 at 1m kHz. Use a paper towel to dry the tubes when finished.

9 Transfer all centrifuge tubes from the fume hood to a centrifuge equipped with the appropriate rotor 15m and/or adapters (ex: Beckman Coulter Avanti J series with JA-14.50 rotor) . Centrifuge the samples at 10000 rpm, 4°C, 00:05:00 . After centrifugation is complete, wait 00:10:00 (as recommended by EHS) to allow aerosols to settle before opening the centrifuge. Remove samples from the centrifuge and return them to the fume hood.

10 In the fume hood, prepare a separate vacuum filtration apparatus for each sample and control by 5m attaching the filtration unit to a clean pyrex bottle.

Note

The centrifuge tubes for each sample will be combined during this step. For example:

- The contents of the five influent tubes (~ 200 mL) will be combined using one filtration unit
- The contents of the two control tubes (~ 80 mL) will be combined into the second filtration unit.

11 Decant the supernatant from all five influent tubes directly into a vacuum filtration top attached to the 2m influent sample collection bottle, taking care not to dislodge the pellet.

12 Begin vacuum filtration by capping the vacuum filtration top and opening the vacuum line. When complete, cap the pyrex bottle and set aside. 5m

> **Note**
>
> The filtrate volume will vary depending on the amount of solid material in the sample.

13 For the negative control centrifuge tubes, decant both directly into a vacuum filtration top. There shou 2m not be a pellet. Perform vacuum filtration as was done for the influent, cap the pyrex bottle, and set aside.

Part 2: Concentration via InnovaPrep Concentrating Pipette S... 40m

14 Perform the "Start-up" protocol for the InnovaPrep Concentrating Pipette Select. 5m

- Turn on the Concentrating Pipette, and navigate to "Maintenance" and then "Start-up". Follow the prompts.
- Check that the maintenance tip is in place.
- Place the waste line in the proper position.
- Remove the storage fluid line and insert the foam elution canister.
- Ensure that the screen reads "WWULTRA".

15 Run the concentration protocol for the filtered influent sample. 25m

15.1 Remove the maintenance tip and place a fresh Ultra CPT into the tip port. Lower the tip into the sample. 1m

> **Note**
>
> Ensure that the tip is as close to the bottom of the sample bottle as possible. The bottle can be balanced on its edge while a weighted object holds down the top of the Concentrating pipette. A bottle of PBS can be used as the weighted object.

15.2 Press "Start Run". 20m

Note

A timer will run on the display during the concentration. The Concentrating Pipette will stop and make beeping sounds when the Tip detects air instead of liquid sample.

15.3 While holding a 5 mL centrifuge tube under the Tip, press "Elute" and catch the foam that is dispensed. 2m

15.4 After the foam bubbles down to a liquid, add 400 µL of the Zymo DNA/RNA shield reagent. 2m

Note

The eluate should be roughly 400 uL. The Zymo DNA/RNA shield reagent is added at a 1:1 ratio of sample:reagent.

16 Run the concentration protocol for the negative control. Perform as is done for the influent sample. 5m

17 If samples are sitting for longer than 30 minutes then store at 4 °C 2m

18 Perform the "Shut Down" protocol for the InnovaPrep Concentrating Pipette Select. 5m

- Navigate to "Maintenance" and then "Shut Down".
- Place the maintenance tip into the tip port.
- Remove the elution canister.
- Check to ensure that there is adequate storage fluid and insert the storage fluid line.
- Turn off the device and remove the waste line.

Part 3: Nucleic Acid Extraction - Zymo quick-DNA/RNA Viral Kit 20m

19 Gather the materials and reagents for the Zymo quick-DNA/RNA Viral Kit in the Biosafety Cabinet. Equilibrate samples to room temperature. 5m

Note

Refer to the "Guidelines" section for instructions on processing and handling RNA samples.

20 Add 1600 µL of Viral DNA/RNA buffer to the sample (a 2:1 ratio) and vortex briefly to mix. 3m

21 Transfer up to 700 µL of lysate into a Zymo-SpinTM IIC-XLR Column in a collection Tube and centrifuge for 12.000 x g, 00:02:00. Discard the flow-through in the miniprep waste container. 2m

Repeat until full lysate volume is processed.

22 Add 500 µL of Viral Wash Buffer to the column, centrifuge for 12.000 x g, 00:00:30 and disc 30s
the flow-through.

23 Repeat the previous step. 2m

24 Add 500 µL ethanol (95-100%) to the column and centrifuge for 12000 x g, 00:01:00 to ensu 1m
complete removal of the wash buffer.

25 Transfer the column to a clean collection tube, and centrifuge at 12.000°C, 00:01:00 to remove a 1m
remaining ETOH.

Note

This step is not included in the Zymo manual, but reduces the chance of ETOH carryover and subsequent inhibition of downstream enzymatic steps.

26 Carefully, transfer the column into a 1.5 mL nuclease-free tube. 1m

27 Add 50 µL DNase/RNase-Free Water directly to the column matrix and incubate at RT for 00:01:00. Centrifuge for 12000 x g, 00:00:30 to collect the eluate. 1m 30s

28 Place all extracted nucleic acids in a freezer set to -80 °C 3m

Note

Optional: Perform nucleic acid quantification prior to freezing. Perform RNA or DNA quantification using the Qubit HS RNA assay kit or the Qubit 1X dsDNA Assay kit.