

DIGESTION, EXTRACTION, AND PURIFICATION OF CORONAVIRUS SARS-COV-2 FROM WASTEWATER

Description

The aim of the protocol is to extract and purify the viral RNA from 40 mL of wastewater to a final volume of 80 μ L. Ideally, the wastewater sample should be a 24 h composite sample to be representative, though this method will also work for any aqueous environmental sample, and captures both DNA and RNA present in such a sample. The protocol is a modification based on the protocol for the Promega Wizard Enviro Total Nucleic Acid (TNA) Kit (Cat. No. A2991).

Required Reagents, Consumables, & Instruments

Reagents & Consumables

- Promega Wizard Enviro Total Nucleic Acid (TNA) Kit (Cat. No. A2991)
- Promega Eluator Vacuum Elution Device (Cat. No. A1071)
- 95-100% Ethanol (**EtOH**)
- 100% Isopropanol (**IPOH**)
- Murine Hepatitis Virus (**MHV**) viral stock (approximately 10^8 gc/mL) for internal control
- Sterile micropipette filter tips – nuclease-free grade (20, 200, and 1000 μ L)
- Sterile 50 mL plastic tube (e.g. BD Falcon)
- Sterile 5 mL plastic tube (e.g. Eppendorf)
- Sterile 1.5 – 2 mL plastic tube (e.g. Eppendorf)
- Sterile serological pipettes (5, 10, 25, 50 mL)
- Zymo One-Step PCR Inhibitor Removal Kit (Cat No. D6030V)

Instruments

- Micropipettor Set (20, 200, 1000, 5000 μ L sizes)
- Pipette-man (e.g. Drummond) and/or graduated cylinder
- Laboratory shaker
- Vacuum manifold and Air Pump rated for $\sim 10^2$ bar pressure.
- Luer-lock stop-cocks to insert into the vacuum manifold (if manifold system lacks them)
- High speed swinging-bucket centrifuge (max RCF ~ 4200 x g)
- Fixed-angle microcentrifuge (capable of at least 10000 x g)
- Thermal heating block capable of heating up to 60 °C and/or microwave oven
- Ice bucket with crushed ice and/or 4 °C refrigerator
- -80 °C Freezer

Method

A. Preparation

1. To Column Wash Buffer 1 (**CWE**) in the Promega kit add 57 mL of 100% **IPOH**, and mark the bottle to indicate that it was added.
2. To Column Wash Buffer 2 (**RWA**) in the Promega kit add 350 mL of 95-100% **EtOH**, and mark the bottle to indicate that it was added.
3. Mix Binding Buffer D (**BBD**) & Binding Buffer E (**BBE**) into a clean container at a ratio of 12:1, such that there are 13 mL of Binding Buffer Mixture (**BBM**) for each wastewater sample (ensuring some extra to account for pipetting loss).

4. Connect Volume extenders to Promega Midi-columns, label them, and then attach them to the Luer-lock stop cocks on top of the Vacuum Manifold.
5. Aliquot 45 mL of wastewater sample to a labelled 50 mL tube.
6. Pre-heat 1.2 mL-per-sample of Nuclease-Free water to 60 °C on Thermal heating block

B. Spiking in Murine Hepatitis Virus (MHV)

1. Add approximately 10⁶ gc of **MHV** stock for 40 mL wastewater to a subset of the samples. (This will vary based on stock concentration)
2. Shake the designated **MHV**-spiked control samples at room temperature at 220 rpm on a shaker for 20 minutes.
3. Remove samples from shaker and return them to the other non-**MHV**-spiked samples

C. Digestion of Proteins and Precipitation of Viral RNA

1. Add 500 µL of Promega Protease solution to each tube of wastewater sample and invert several times to mix. Allow to rest at room temperature for 30 minutes.
2. Centrifuge the tubes of wastewater sample in swinging-bucket centrifuge at maximum RCF for 15 min to pellet the solid fraction.
3. Using a serological pipette, remove 40 mL of clarified wastewater from each tube, being careful not to disturb the pellet, and dispense 20 mL into two separate clean and labelled 50 mL tubes. Optionally, pellet can be reserved for further downstream extraction (not detailed).
Caution! If pellet is disturbed and becomes mixed with the clarified wastewater, do not load onto columns and repeat Step C-2.
4. Pipette 6.5 mL of **BBM** from step A3 to each tube containing 20 mL wastewater and invert several times to mix.
5. Pipette 24 mL of **IPOH** into each tube containing the wastewater / **BBM** solution and invert several times to mix.

D. Extraction of Viral RNA by Vacuum Filtration

1. Turn on Air Pump connected via tubing to the Vacuum Manifold, ensuring stop-cocks are in the closed position.
2. Decant both tubes of a sample into one labelled Midi-column per sample. Open the stop-cocks and allow all sample to pass through the column before closing the stop-cocks.
3. Add 5 mL of Column Wash 1 (**CWE**) to each Midi-column, open the stop-cock, allow all buffer to flow through, and then close again.
4. Add 20 mL of Column Wash 2 (**RWA**) to each Midi-column, open the stop-cock, allow all buffer to flow through.
5. Leave the stop-cock open for at least 1 minute to allow for any residual alcohol to evaporate out of the column/membrane, and then close the stop-cock.
6. Remove Midi-column and place 1.5-2 mL labelled tube into the Eluator device and attach them to the stop-cocks on the Manifold.
7. Place the Midi-column on top of the Eluator, such that the outlet of the column is directly over or inside of the labelled tube from step D-6.
8. Add 500 µL of Nuclease-Free water @ 60 °C directly to the silica membrane. Open the stop-cock and allow water to pass through the membrane into the labelled tube inside the Eluator. Close the stop-cock after 1 minute of vacuum application.
9. Repeat Step D-8 once, for a final elution volume of 1 mL.
10. Disconnect Midi-columns and remove labelled tubes from Eluators.
11. Using a 1 mL micropipette, transfer the eluated sample from the smaller 1.5-2 mL tube to a larger 5 mL tube with appropriate labelling. Samples can be stored on ice or at 4 °C.

12. Add 400 µL of **BBD** and 100 µL of **BBE** to each sample and mix by inverting the tubes. One can also mix BBD and BBE at a ratio of 4:1 ahead of time and add 500 µL of this mixture to each sample.
13. Add 1500 µL of **IPOH** to each sample and mix by inverting the tubes. Final volume should be ~3 mL. Samples can be stored on ice or at 4 °C for up to 24 hours.

E. Clean-Up Purification of Viral RNA

1. For each sample, load 750 µL onto its own labelled Mini-prep column in a flow-through tube and centrifuge the column at 10000 x g for 1 minute.
2. Repeat Step E-1 until all samples have passed through their respective columns.
3. Empty the flow-through tube into a waste container.
4. Add 300 µL of Column Wash 1 (**CWE**) to each column and centrifuge as before. Dispose of flow-through as in Step E-3.
5. Add 500 µL of Column Wash 2 (**RWA**) to each column and centrifuge as before. Dispose of flow-through as in Step E-3.
6. Repeat Step E-5 once.
7. Centrifuge columns one final time for 30 seconds to remove any residual wash buffer.
8. Remove Mini-prep columns from their flow-through tubes and place them in 1.5 mL sample tubes with caps (i.e. Eppendorf)
9. Add 40 µL of Nuclease-Free water @ 60 °C directly to the silica membrane, incubate for 1 minute, and centrifuge as before.
10. Repeat Step E-9 once, for a final elution volume of 80 µL. Remove and dispose of Mini-columns and cap the sample tubes, placing them on ice or at 4 °C.
11. Precondition the Zymo spin column by placing it in a flow-through tube and add 600 µL of -resin conditioning solution. Allow this solution to soak the resin in the column for at least 10 minutes, then centrifuge at 8000 x g for 3 min.
12. Discard the collection tube and place the Zymo spin column into a clean 1.5 ml tube.
13. Pipet the eluate from Step E-10 into the Zymo column and spin it at 16000 x g for 3 min.
14. Discard the Zymo column and place the tubes containing the nucleic acid extract on ice if doing analysis with 24 hours; otherwise place them in a -80 °C freezer.

Version History

Version	Updated By:	Date	Changes
1.0.0	Xavier Fernandez-Cassi, Carola Bänziger	2020-07-01	Protocol Development, Testing, and First Draft
1.0.1	Anina Kull	2020-10-05	Formalization of Protocol for Publishing
2.0	All	2020-10-09	Added centrifugation as pre-conditioning step
2.1	Anina Kull	2021-02-11	Removed filtration by using SteriCup
3.0b	A.J. Devaux	2021-03-12	Beta protocol removing glass bottles + stirrers
3.0	A.J. Devaux	2021-03-15	Incorporated beta protocol changes. Increase volume of concentrated wastewater to 70 mL.
3.1	A.J. Devaux	2021-03-19	Decreased Centricon loading volume from 70 to 68 mL
3.1.1	T. R. Julian	2021-06-02	Updating authorship and editing
4.0	A.J. Devaux, Federica Cariti	2021-12-01	Protocol switched to Promega Wizard Enviro Total Nucleic Acid (TNA) method relying on a Vacuum Manifold rather than a Centricon Ultrafilter, unifying the concentration and extraction phases, and implementing changes to decrease inhibition. This protocol recovers an estimated 2.5X more viral RNA than previous versions (based on comparison from a pilot study of 126 samples).

Appendix: Sample codification and labelling

Samples should be labelled following the format:
(WWTP code) _ year[YYYY] _ month[MM] _ day[DD]

Internal code for WWTP are provided in Table 1:

01	Vacallo/Chiasso
02	Rancate
03	Barbengo/Lugano
04	Croglio/Purasca
05	Bioggio/Lugano
06	Foce Ticino/Gordola
07	Giubiasco
08	Biasca
09	Locarno
10	Werdhölzli/Zürich
11.1	Kloten+Flughafen (KF)
11.2	Kloten (K)
12	Lausanne
13	Lenzburg
14	Bern
15	Basel
16	Genf/Geneva
17	Chur
18	Luzern
19	Altenrhein
20	Schaffhausen
21	Freienbach
22	Fribourg
23	Ergolz 1
24	Verbier
25	Laupen/Sensetal

e.g.: A sample from Schaffhausen collected the 2nd of February 2021 would be 20_2021_02_02.