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RNA Extraction and Quality Assessment targeting SARS-CoV-2 from Wastewater Concentrates using Zymo Environ Water RNA Kit

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The Zymo Environ Water RNA kit has been tested and evaluated for the application of SARS-CoV-2 wastewater surveillance. This kit was designed for water, wastewater, sludge, sewage, biofilm and other similar sample sources. As such, minimal optimization was required for this application. The kit incorporates an enrichment step that is accomplished with the use of Water Concentrating Buffer (WCB), which promotes the pelleting of viral capsids and protein-associated RNA, increasing SARS-CoV-2 RNA yields by more than 8-fold compared to without WCB. DNA/RNA Shield™ allows for safe handling as it has been validated for inactivation of pathogens including SARS-CoV-2. The kit yields highly concentrated, PCR inhibitor-free high quality RNA, applicable for many downstream applications including RT-qPCR, RT-ddPCR, and NGS.

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SARS-Cov-2, wastewater, RNA extraction

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Zymo Environ™ Water RNA Kit (contents):
ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)
DNA/RNA Shield™
Water Concentrating Buffer™
RNA Binding Buffer
RNA Prep Buffer
RNA Wash Buffer
DNase/RNase-Free Water
Prep Solution
Zymo-Spin™ IC Columns
Zymo-Spin™ IIICG Columns
Zymo-Spin™ III-HRC Columns
Collection Tubes

Provided by user:
100% or 95% ethanol, enough to add appropriate amount to RWB concentrate
Microcentrifuge
Vortex genie with horizontal tube holder assembly
pipettors and tips
RNase-free microcentrifuge tubes

Add 96 mL of 100% ethanol (or 104 mL of 95% ethanol) to the bottle labeled, RNA Wash Buffer, which contains 24 mL of RWB concentrate.

Sample Homogenization

1

Product: Zymo Environ Water RNA Kit | ZYMO RESEARCH
Protocol from Zymo: https://files.zymoresearch.com/protocols/r2042-zymo_environ_water_rna_kit.pdf
Collect all flow through/waste in a safe storage bottle until proper disposal, check with your institution's policy.

Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml RNA Wash Buffer concentrate before starting the extraction if this is a new kit.

Add 750 µl of DNA/RNA Shield™ to the concentrated wastewater sample (~200 µl, or to 250 µl

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liquid) sample to obtain a total of 1 ml of mixture. Mix well by pipetting up and down.

This kit is capable of concentrating raw wastewater for viral enrichment, if that is the input sample type. In this case, follow the "Viral Enrichment" section of the manufacturer's protocol, and proceed to Sample Homogenization, step 2.

2 Add the 1 ml mixture to a ZR BashingBead™ Lysis Tube.

3 Secure in a vortex genie fitted with a 2 ml tube holder assembly and process for 5 minutes at level 4.

4 **12000 x g, 25°C, 00:02:00** Centrifuge the tube at 12,000 x g for **00:02:00** 2 minutes 4m to reduce foam.

5 Transfer 400 µl of the supernatant into an RNase-free tube. Proceed to RNA Purification.

RNA Purification

6 Add 1 volume of RNA Binding Buffer to the supernatant. Mix well.

Perform all steps at room temperature and **10000-160000 x g, 25°C, 00:00:30** centrifugation at 10,000-16,000 x g for 30 seconds, unless specified.

7 Transfer the mixture into a Zymo-Spin™ IIICG Column in a Collection Tube and centrifuge. If sample volume is greater than 800 µl, reload column. **Save the flow-through!**

8 Add 1 volume of ethanol (95-100%) to the flow-through in the Collection Tube from Step 2 and mix well by pipetting up and down.

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9 Transfer the mixture into a new Zymo-Spin™ IIICG Column in a Collection Tube and centrifuge. If sample volume is greater than 800 µl, reload column. **Discard the flow-through**. Repeat again until all of the mixture is gone.

10 Add 400 µl of RNA Prep Buffer to the column and centrifuge. Then, transfer the column into an RNase-free microcentrifuge tube.

11 Add 100 µl of DNase/RNase-Free Water directly to the column matrix and centrifuge.

12 Place a Zymo-Spin™ III-HRC Filter into a new Collection Tube and add 600 µl of Prep Solution. Centrifuge at 8,000 x g for 3 minutes and discard the flow-through.

13 Transfer the eluted RNA from step 6 into a prepared Zymo-Spin™ III™ HRC Filter in a new RNase-free tube and centrifuge **at exactly** 16,000 x g for 3 minutes.

14 Add 200 µl of RNA Binding Buffer to the filtrate and mix well by pipetting up and down.

15 Add 300 µl of ethanol (95-100%) and mix well by pipetting up and down.

16 Transfer the mixture into a Zymo-Spin IC Column in a new Collection Tube and centrifuge. Discard the flow-through.

17 Add 400 µl of RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

18

If this is a new kit and you have not added 96 ml 100% ethanol (or, 104 ml 95% ethanol) to the 24 ml RNA Wash Buffer concentrate before starting the extraction, do so now before proceeding.

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19 Add 700 µl of RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

20 Add 400 µl of RNA Wash Buffer to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube.

21 Add 50 µl of DNase/RNase-Free Water directly to the column matrix and centrifuge. The eluted RNA can be used immediately or stored at ≤ -70°C. If using immediately, keep RNA on ice at all times.

Quality Assessment

22 Qubit 3.0 High Sensitivity RNA Quantification
Qubit Protocol: [Document Connect (thermofisher.com)]

22.1 Take reagents out 30 minutes before use to reach room temperature.

22.2 Calculate the total amount of buffer needed ((190 buffer*2 standards)+(199 buffer * x number of samples)= y). Add an additional 10% to the volume calculated for y, to ensure there is enough buffer for all of your samples. Mix the appropriate amount of HS buffer with the concentrated dye (divide the total amount of buffer needed by 200, the result z is the amount of dye you need. Subtract y-z and that is the amount of buffer needed, pipette z amount of dye in to the buffer. Vortex.

22.3 Pipette the correct amount of buffer to a corresponding Qubit tube. (190 µL for standards, 199 µL for samples.) Pipette 10 µL of Standards 1 and 2 in to their respective tubes and 1 µL of sample in to the respective tubes. Vortex all the tubes.

22.4 Incubate all samples for at least 2 minutes.

After this incubation period, the fluorescence signal is stable for 3 hours at room temperature, in the dark.

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22.5 Place standards in to the Qubit reader, moving on to your samples after. Record results.

23 Femto Analyzer (or if using Fragment Analyzer, follow that protocol)
See Femto protocol here: Agilent FP-1201 Ultra Sensitivity RNA Quick Guide for Femto Pulse Systems

Take all needed reagents out at least 30 minutes before use to adjust to room temperature. Set up the gel and various buffers needed to operate the Femto before running samples. (Page 7 of the above protocol.) Running a weekly maintenance flush is also recommended.

23.1 If this is a new kit, set up the ladder as described in the protocol:

a. Thaw the RNA ladder on ice, agitate solution to ensure it is properly mixed and centrifuge vial prior to dispensing. Using the provided Eppendorf LoBind 0.5 mL tubes, aliquot 3µL of the RNA ladder per tube into 5 tubes and store the aliquots at -80°C.
b. Thaw an RNA ladder aliquot on ice.
c. Heat-denature the entire 3 µL aliquot of the RNA ladder at 70°C for 2 min, immediately cool to 4°C and keep on ice.
d. Transfer 2 µL of denatured RNA ladder to a fresh Lo-Bind tube and add 18 µL of the provided FP RNA Dilution Buffer (FP-6501-0003); mix thoroughly. This is now the working RNA ladder solution.
e. Store any unused portion of the working RNA ladder at -80°C. The working RNA ladder should not need to be heat denatured again. Each diluted aliquot is good for 5 freeze/thaw cycles.

If ladder is diluted, take out of the freezer and keep on ice until use.

23.2 Calculate the dilution needed for the samples based on the Qubit result. The Femto takes 15 pg/µL - 250 pg/µL input RNA. Make appropriate dilutions. DO NOT heat-denature sample RNA as described in the protocol.

Dilution set up:
(x ng/µL * 1000)= y pg/µL
y pg/µL* dilution factor (either 0.1,0.01,0.001)= final concentration in pg/µL,

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should be between 15 pg/µL - 250 pg/µL
Then take 1 µL of sample in to 9/99/999 µL of NF water. Mix by flicking and spin down.

23.3 Using a clean RNase-free 96-well sample plate, pipette 18 µL of FP US RNA Diluent Marker (DM) solution (FP-8201) to each well in a row that is to contain sample or RNA ladder. Fill any unused wells within the row of the sample plate with 20 µL of BF-P25 Blank Solution.

23.4 RNA ladder: The RNA ladder must be run in parallel with the samples for each experiment to ensure accurate quantification. a) Pipette 2 µL of working RNA ladder Solution (prepared above) into the 18 µL of DM solution in the designated ladder well (Well #12) of each row to be analyzed. b) Mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip

23.5 Samples: Pipette 2 µL of each DNA sample into the 18 µL of DM Solution in the respective wells of the Sample Plate; mix the contents of the well using the pipette by aspiration/expulsion in the pipette tip.

23.6 After mixing sample/RNA ladder and DM solution in the sample plate, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.

23.7 For best results, run the plate as soon as possible. If the sample plate will not be used immediately, cover the sample plate with a RNase-free plate seal, store at 2-8°C and use within the same day. Bubbles may develop in the sample wells while sitting at 2-8°C, make sure to centrifuge the plate again and remove the seal before placing the plate into the instrument. The sample plate should be analyzed within a day after preparation. To run the samples, place the plate in one of the three sample plate trays (Drawers 4-6 from the top) of the Femto Pulse System. Run using the total RNA method.

24 Assessment of RNA quality using NanoDrop Microvolume Spectrophotometer

24.1 Select RNA as the sample type.

24.2 Blank the NanoDrop with 1 µl of nuclease free water. Once blanked, wipe clean with a KimWipe.

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24.3 Pipette 1 µl of sample on to the NanoDrop. Measure and record 260/280 and 260/230. The closer each are to 2, the better the quality. Save the image if you want as well. Wipe the sample off the machine with a Kim Wipe before pipetting the next sample on.

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