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RNA Extraction from Wastewater Concentrates Using RNeasy and Zymo Kits

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[1]Method Developer

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GenomeTrakr APHL Wastewater Surveillance Community of Practice

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This method was developed at the FDA's Center for Food Safety and Applied Nutrition for GenomeTrakr's pandemic response project, monitoring SARS-CoV-2 variants in wastewater. Protocols developed for this project cover wastewater collection, concentration, RNA extraction, RT-qPCR detection, library prep, genome sequencing, quality control checks, and data submission to NCBI. This method describes the extraction of RNA from viral concentrates using the RNeasy and Zymo kits.

DOI

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GenomeTrakr, wastewater, SARS-CoV-2, RNA, Extraction, RNeasy, Zymo

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A. EQUIPMENT AND SUPPLIES (Mini KIt)

1. Centrifuge capable of speeds of ≥16,000 x g (e.g, Eppendorf 5424 or 5415 D), and with rotors capable of holding 1.5ml and 2.0ml microcentrifuge tubes (Eppendorf 2231000655 or equivalent)
2. Adjustable Calibrated Micropipettors (0.2 – 1000 µl), dedicated for RNA work only.
3. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
4. Hype-Wipe Disinfecting Towelettes (Fisher Scientific 14-412-56 or equivalent)
5. DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450) OR DNase/RNase-free microcentrifuge tubes 2.0 mL, non-stick, low retention, siliconized (Life Technologies AM12475)
6. Qiagen RNeasy Mini Kit (Qiagen 74104) OR
7. OneStep™ PCR Inhibitor Removal Kit (Zymo Research, D6030)

B. EQUIPMENT AND SUPPLIES (Midi KIt)

1. Centrifuge capable of speeds of ≥8,000 x g (e.g, ThermoFisher RC6), and with rotors capable of holding 15ml tubes (ThermoFisher Scientific, 36-101-0816, or equivalent)
2. Adjustable Calibrated Micropipettors (0.2 – 1000 µl), dedicated for RNA work only.
3. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
4. Hype-Wipe Disinfecting Towelettes (Fisher Scientific 14-412-56 or equivalent)
5. DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450) OR DNase/RNase-free microcentrifuge tubes 2.0 mL, non-stick, low retention, siliconized (Life Technologies AM12475)
6. 5 ml low bind centrifuge tubes (Eppendorf 0030122348)
7. 5 ml Stripettes (Fisher Scientific 07-200-9, or equivalent)
8. Pipet Boy ACU (Integra Biosciences 155000, or equivalent)
9. 15 ml conical tubes (Fisher Scientific 14-959-49D, or equivalent)
10. Qiagen RNeasy Midi Kit (Qiagen 75144)
11. OneStep™ PCR Inhibitor Removal Kit (Zymo Research, D6030)

C. REAGENTS

Guanidine Isothiocyanate **Thermo**

1. **Fisher Catalog #15535016** , or equivalent

Nuclease-Free Water **Thermo Fisher**

2. **Scientific Catalog #AM9937** , or equivalent

Ethanol, 200 proof **Sigma**

3. **Aldrich Catalog #E7023** , or equivalent

Tris (1 M) pH 8.0 RNase-free **Thermo Fisher**

4. **Scientific Catalog #AM9856** , or equivalent

EDTA (0.5 M) pH 8.0 RNase-free **Thermo Fisher**

5. **Scientific Catalog #AM9261** , or equivalent

Heat Primer TE at **70 °C** for at least **00:10:00** prior to use.

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Primer TE (10mM Tris, 0.1mM EDTA, pH8.0)
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PREVIEW RUN

This document contains two separate protocols.

If RNA concentrations are expected to be low, the **RNA Mini** column should be used (follow **Section 1**). If RNA concentrations are expected to be high, the **RNA Midi** column is recommended (follow **Section 2**).

RNeasy Mini 43m 15s

1 Obtain one virus concentrate (if concentrate is frozen, allow thawing) from Virus Concentration from Wastewater Using PEG Precipitation and Ultracentrifugation (protocols.io)

2 Add **500 µL** 6M GITC.

6M GITC
by Jessica Jones,
US Food and Drug Administration

PREVIEW RUN

.

2.1 Aseptically mix **7.09 g** Guanidine Isothiocyanate **Thermo Fisher Catalog #15535016** in sterile distilled or ultrapure water to **4.5 mL**

2.2 Bring total volume up to **10 mL** with additional sterile distilled or ultrapure water

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2.3 Store at **Room temperature** in the dark (or in light occluding tube)

Solution is only stable for 1 month after preparation.

3 Vortex **00:01:15 +/- 15 sec** to dissolve concentrate. 1m 15s

4 Add **700 µL** of 50% EtOH and invert twice.

5 Pipette **700 µL** of sample onto a RNeasy mini spin column.

6 Centrifuge **10000 x g, 00:01:00** . 1m

7 Place column in new collection tube and discard flow through.

8 Add remaining sample from Step 4 to column.

9 Centrifuge **10000 x g, 00:01:00** . 1m

10 Place column in new collection tube and discard flow through.

11 Add **700 µL** RW1 buffer to spin column and incubate for **00:15:00** at room temperature. 15m

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12 Centrifuge **10000 x g, 00:01:00** . 1m

13 Place column in new collection tube and discard flow through.

14 Add **500 µL** RPE to spin column and incubate for **00:15:00** . 15m

15 Centrifuge **10000 x g, 00:01:00** . 1m

16 Add an additional **500 µL** of RPE to Mini spin column.

Incubation is not required at this step.

17 Centrifuge at maximum speed **>16000 x g, 00:02:00** . 2m

Maximum speed should be ≥16,000 x *g.*

18 Transfer column to new collection tube.

19 Centrifuge **16000 x g, 00:01:00** . 1m

20 Carefully transfer column to 1.5 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

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21 Pipette **50 µL** pre-heated (70°C) Primer TE onto silica-gel membrane of column.

Primer TE (10mM Tris, 0.1mM EDTA, pH8.0)
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US Food and Drug Administration

PREVIEW RUN

21.1 Mix components together.

21.1.1 **100 µL**

Tris (1 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #AM9856**

, or equivalent.

21.1.2 **20 µL**

EDTA (0.5 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #AM9261**

, or equivalent.

21.1.3 **9.88 mL**

Nuclease-Free Water **Thermo Fisher Scientific Catalog #AM9937**

, or equivalent.

21.2 Store at **Room temperature** .

22 Centrifuge **10000 x g, 00:01:00** . 1m

Material that passed through column contains the viral RNA being isolated and is in the 1.5

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ml low-retention/siliconized RNase/DNase free microcentrifuge tube in which the column was placed.

23 Pipette an additional **50 µL** pre-heated (70°C) Primer TE onto silica-gel membrane of column.

24 Pipette the eluted RNA (from Step 22) **50 µL** back onto column.

25 Centrifuge **10000 x g, Room temperature, 00:01:00** . 1m

26 Discard column and place tube with RNA on **On ice** to prepare Zymo column.

27 Prepare Zymo column per manufacturer's instructions.

27.1 Insert column into a collection tube.

27.2 Open the cap and add **600 µL** of Prep-solution.

27.3 Centrifuge at **8000 x g, Room temperature, 00:03:00** . 3m

28 Transfer Zymo column into a clean 1.5 or 2.0 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

29 Transfer RNA from Step 26 to prepared Zymo One Step RT-PCR inhibitor remover column.

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30 Centrifuge **8000 x g, 00:03:00** . 3m

31 Discard column and save RNA in the 1.5 or 2.0 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

32 Proceed with RT-qPCR or store RNA at **-70 °C** .

RNeasy Midi 5m 45s

33 Obtain two virus concentrates (if concentrate is frozen, allow thawing) from Virus Concentration from Wastewater Using PEG Precipitation and Ultracentrifugation (protocols.io)

Total volume should be approximately **400 µL** .

34

Optional chloroform clean-up.

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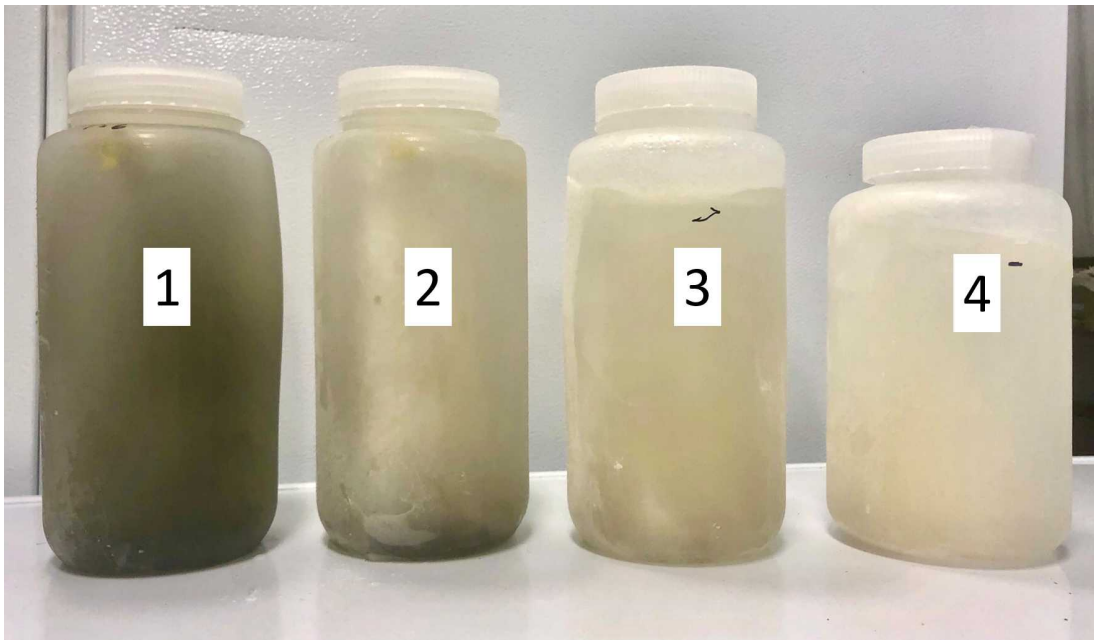

If high amounts of solids were present in the original wastewater sample (i.e., bottles 1 and 2), the chloroform clean-up is recommended prior to proceeding with the Midi column extraction. If few solids were present (i.e., bottles 3 and 4), this step can be omitted and the sample can be extracted starting with Step 35.

34.1 Chloroform **Sigma**
Add **800 µL** of **Aldrich Catalog #288306** .

34.2 Vortex **00:00:45 +/- 15 sec** . 45s

34.3 Centrifuge **3000 x g, Room temperature, 00:05:00** . 5m

34.4 Transfer aqueous layer to 5 ml Eppendorf low bind centrifuge tube.

35 Add **2 mL** 6M GITC.

6M GITC
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35.1 Aseptically mix **7.09 g** Guanidine Isothiocyanate **Thermo Fisher Catalog #15535016** in sterile distilled or ultrapure water to **4.5 mL**

35.2 Bring total volume up to **10 mL** with additional sterile distilled or ultrapure water

35.3 Store at **Room temperature** in the dark (or in light occluding tube)

Solution is only stable for 1 month after preparation.

36 Vortex **00:01:15 +/- 15 sec** . 1m 15s

37 Add **2.8 mL** of 50% EtOH and invert twice.

38 Pipette **4 mL** of sample onto a RNeasy Midi spin column.

39 Centrifuge **5000 x g, Room temperature, 00:05:00** . 5m

40 Discard flow through and return Midi column to tube.

41 Add remaining sample (from Step 40) to Midi column.

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42 Centrifuge **5000 x g, Room temperature, 00:05:00** . 5m

43 Place column in new collection tube (sterile, nuclease-free 15 ml conical tube) and discard flow through.

44 Add **4 mL** RW1 buffer to Midi spin column.

45 Incubate for **00:15:00** at **Room temperature** . 15m

46 Centrifuge **5000 x g, Room temperature, 00:05:00** . 5m

47 Place column in new collection tube (sterile, nuclease-free 15 ml conical tube) and discard flow through.

48 Add **4 mL** RPE to Midi spin column and incubate for **00:15:00** at **Room temperature** . 15m

49 Centrifuge **5000 x g, Room temperature, 00:05:00** . 5m

50 Transfer column to new collection tube and add an additional **4 mL** of RPE to the Midi spin column.

Incubation not required at this step.

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51 Centrifuge **5000 x g, Room temperature, 00:10:00** . 10m

52 Carefully transfer column to a sterile, nuclease-free 15 ml conical tube.

53 Pipette **150 µL** heated (**70 °C**) Primer TE onto silica-gel membrane of Midi column.

54 Incubate for **00:01:00** at **Room temperature** . 1m

55 Centrifuge **5000 x g, Room temperature, 00:03:00** . 3m

Material that passed through column contains the viral RNA being isolated and is in the sterile, nuclease-free 15 ml conical tube in which the column was placed.

56 Add an additional **150 µL** of heated Primer TE onto the Midi column.

57 Pipette the eluted RNA (Step 55) back onto column (~ **150 µL**).

58 Centrifuge **5000 x g, Room temperature, 00:03:00** . 3m

59 Discard column and place tube with RNA **On ice** to prepare Zymo column.

60 Prepare two (2) Zymo One Step RT-PCR inhibitor remover columns per manufacturer's instructions.

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60.1 Insert Zymo column into a collection tube.

60.2 Open the cap and add **600 µL** of Prep-solution.

60.3 Centrifuge at **8000 x g, Room temperature, 00:03:00** . 3m

61 Transfer Zymo column into a clean 1.5 or 2.0 ml low-retention/siliconized RNase/DNase free micriocentrifuge tube.

62 Transfer RNA from Step 59 to prepared Zymo One Step RT-PCR inhibitor remover columns (~ **150 µL** each) .

63 Centrifuge **8000 x g, Room temperature, 00:03:00** . 3m

64 Combine RNA from columns into one fresh low-retention/siliconized RNase/DNase free micriocentrifuge tube.

65 Proceed with RT-qPCR (RT-qPCR Detection of Process Controls (Murine noroviurs and crAssphage) from Wastewater (protocols.io) and RT-qPCR Detection of SARS-CoV-2 from Wastewater Using the AB 7500 (protocols.io)) or store at **-70 °C** .

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