TECHNICAL MANUAL

Wizard® Enviro Total Nucleic Acid Kit

Instructions for Use of Product
A2991

Revised 1/23
TM662

Promega

Wizard® Enviro Total Nucleic Acid Kit

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description 2
2. Product Components and Storage Conditions 2
3. Before You Begin 3
4. Standard Protocol for Capture, Concentration and Clean-Up 4
   - 4.A. Capture and Concentration 5
   - 4.B. Total Nucleic Acid Extraction and Clean-Up 8
5. Supplemental Protocols 10
   - 5.A. Extracting Nucleic Acid from Pelleted Solids 10
   - 5.B. Extracting Nucleic Acid from Sludge and Solids 10
   - 5.C. Scaling the Sample Volume 10
6. Considerations for Processing and Analysis 12
   - 6.A. Quantitating Genome Units in the Sample 12
   - 6.B. Calculating Viral Genetic Material 12
7. Appendix 13
   - 7.A. Troubleshooting 13
   - 7.B. Frequently Asked Questions (FAQ) 13
   - 7.C. Reference 14
   - 7.D. Related Products 14
8. Summary of Changes 15

1. Description

The process of detecting genetic signatures in wastewater samples involves collection of water, either as a grab sample or as a 24-hour composite sample. This is sometimes followed by optional heat pasteurization and sample concentration. Viral or other microbial matter and/or its genetic signature may be present at a low concentration in water samples, making sample concentration a prerequisite for sensitive detection. Concentration of microbial matter can be performed using a variety of methods, such as charged membrane filtration, centrifugal ultrafiltration and flocculation/precipitation using skim milk or polyethylene glycol (PEG)/NaCl. Most of the concentration methods were originally developed to concentrate live matter with the objective of culturing for detection of intact particles, though they have also been used for PCR-based detection. These methods have proven to be inconsistent, labor intensive and time consuming.

To address these issues, we have developed a convenient method to directly capture and concentrate total nucleic acids (TNA) from a large volume of water using PureYield™ columns. The method uses a short protocol that minimizes the need for specialized laboratory equipment. In a first step total nucleic acid from a large volume sample (e.g., 40ml of wastewater) is captured on a PureYield™ Binding Column and then eluted in 1ml. In a second step, the material is further purified and concentrated using the PureYield™ Minicolumn. This method achieves consistent recovery rates and significant reduction in PCR inhibitors (1).

The total nucleic acid extracted using this kit can be analyzed for SARS-CoV-2 targets using a SARS-CoV-2 RT-qPCR kit for wastewater. Please visit the Promega website for more information on these products: **www.promega.com/applications/infectious-diseases/covid19-wastewater-sars-cov-2-detection/**

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Wizard® Enviro Total Nucleic Acid Kit	**1 each**	**A2991**

Contains sufficient materials and reagents for 25 samples. Includes:

- 1 × 320ml Binding Buffer 1 (BBD)
- 1 × 30ml Binding Buffer 2 (BBE)
- 1 × 13ml Protease Solution
- 1 × 85.3ml Column Wash 1(CWE)
- 1 × 206ml Column Wash 2 (RWA)
- 1 × 150ml Nuclease-Free Water
- 5 × 5 each PureYield™ Binding Columns
- 1 × 25 each PureYield™ Minicolumns
- 1 × 25 each PureYield™ Collection Tubes
- 1 × 50 each Elution Tubes
- 1 × 25 each Reservoir Extension Funnel

Storage Conditions: Store all components at +15°C to +30°C.

3. Before You Begin

Materials to Be Supplied by the User

- isopropanol
- ethanol, 95%
- tabletop centrifuge (capable of 3,000 × *g*)
- swinging bucket rotor (that accommodates 50ml tubes)
- 50ml disposable plastic snap-cap tubes (e.g., Eppendorf SnapTec® 50, Cat.# 0030118677) or screw-cap tubes (e.g., Corning® or Falcon® brand)
- 1.5ml microcentrifuge tubes
- heat block (capable of reaching 60°C)
- high-speed microcentrifuge (for tubes, capable of at least 10,000rpm)
- vacuum manifold (e.g., Vac-Man® Laboratory Vacuum Manifold, Cat.# A7231)
- Eluator™ Vacuum Elution Device (Cat.# A1071)
- vacuum pump, single- or double-stage, producing pressure of approximately 650mm Hg (25.6 inches Hg, 12.57psi, 86.7kPa).

Note: Start-up bundles include hardware (Eluator™ Vacuum Elution Device, Vac-Man® Laboratory Vacuum Manifold, Vac-Man® Jr Laboratory Vac Manifold #10, Polypropylene Vacuum Flask, and vacuum-grade tubing) and a vacuum pump. The vacuum pump is available by region. See Section 7.C, Related Products.

Prepare the following solutions prior to beginning nucleic acid extraction in Section 4:

Column Wash 1 (CWE): Add 57ml of isopropanol to the Column Wash 1 (CWE) bottle and mark on the bottle “plus isopropanol”. This reagent is stable at +15°C to +30°C when tightly capped.

Column Wash 2 (RWA): Add 350ml of 95% ethanol the Column Wash 2 (RWA) bottle and mark the bottle “plus ethanol”. This reagent is stable at +15°C to +30°C when tightly capped.

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4. Standard Protocol for Capture, Concentration and Clean-Up

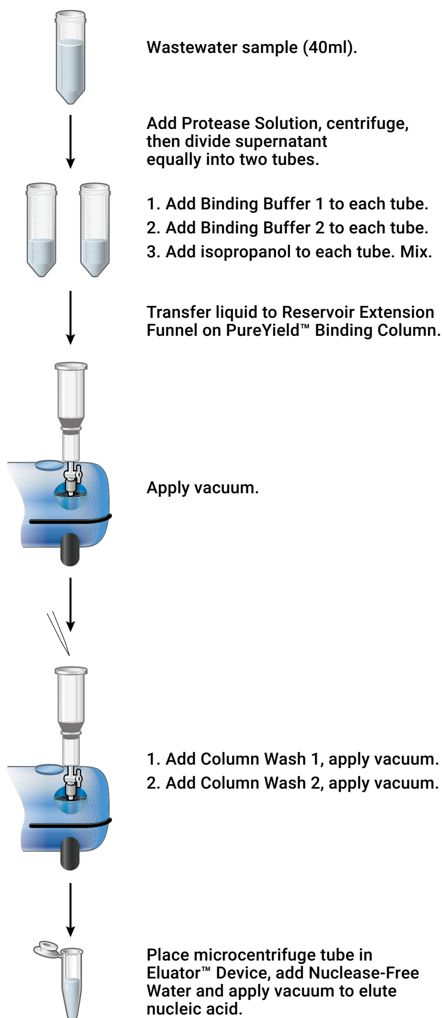

Figure 1. Schematic for direct capture of nucleic acid from wastewater using a Reservoir Extension Funnel on a PureYield™ Binding Column.

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4.A. Capture and Concentration

1. Dispense 40ml of wastewater into a 50ml conical tube.

Note: Either pasteurized or unpasteurized wastewater samples can be processed in this protocol. To pasteurize wastewater, incubate at 60°C for 1 hour. Please follow your institution's biosafety guidelines.

2. Preheat 1.2ml of Nuclease-Free Water, per sample, to 60°C for 2–5 minutes.
3. Add 0.5ml of Protease Solution to each wastewater sample. Mix well by inversion and incubate for 30 minutes at ambient temperature.
4. Centrifuge at 3,000 × *g* for 10 minutes to remove solids.

   Note: It is important to remove solids to avoid clogging the PureYield™ Binding Column.
5. Carefully decant 20ml of the supernatant into each of two clean 50ml conical tubes. Discard the 50ml conical tube containing the pellet into an appropriate biohazard waste container.

   Note: If you wish to process the pelleted solids to collect additional total nucleic acid, see Section 5.A.
6. To each tube containing 20ml of the clarified supernatant, add 6ml of Binding Buffer 1 (BBD) followed by 0.5ml of Binding Buffer 2 (BBE).
7. Mix well by inversion.
8. Add 24ml of isopropanol to each tube.
9. Mix well by inversion.
10. Prepare the vacuum manifold assembly. Please refer to the Q*uick Start Guide for Assembly of a Vacuum Apparatus with the Welch® Vacuum Pump* #TB355 for details. Note: For optimal results, we recommend confirming that the vacuum pressure is ≥60 kPa. Lower vacuum pressure may result in slow sample flowthrough.
11. Remove the vacuum port cap. Attach a Reservoir Extension Funnel to the PureYield™ Binding Column, then connect the column to the vacuum manifold by pressing the nozzle gently into the vacuum port (Figure 2). Using the Reservoir Extension Funnel allows up to 100ml of sample mixture to be added to the PureYield™ Binding Column at one time.

Figure 2. The Reservoir Extension Funnel and PureYield™ Binding Column attached to a Vac-Man® Vacuum Manifold port. See *Quick Start Guide for Assembly of a Vacuum Apparatus with the Welch Vacuum Pump Technical Bulletin* #TB355 for setup details.

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4.A. Capture and Concentration (continued)

12. Pour the mixture from each tube from Step 8 into the Reservoir Extension Funnel on the PureYield™ Binding Column (combine both tubes of the same sample if applicable), turn on the pump and apply vacuum to capture TNA on the column.

    Notes:

    a. To ensure even vacuum pressure is applied to samples, close all Luer-Lok® Stopcocks at unused positions on the vacuum manifold before turning on the vacuum pump.

    b. Empty the liquid waste collected in the blue Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231). Dispose of the alcohol-containing waste following your institutional policies.

    c. If the manifold flow rate is <2ml/minute or the pressure reading on the vacuum pump is lower than 10 inches (250mm) of Hg or >60kPa, check that all of the valves on the unused ports of the vacuum manifold are closed. If flow rate is still lower than recommended (or desired), there may be a leak in the tubing connections, the Luer-Lok® Stopcocks or the vacuum manifold.

13. Add 5ml of Column Wash 1 (CWE) and apply a vacuum to pull the liquid through the PureYield™ Binding Column.

14. Add 20ml of Column Wash 2 (RWA) and apply a vacuum to pull the liquid through the PureYield™ Binding Column. Continue to draw a vacuum for an additional 30 seconds after all visible liquid has passed through the membrane.

15. Release the vacuum by turning off the vacuum pump and opening ports at unused positions or unseating the stopper of the sidearm flask. Remove the column from the vacuum manifold.

16. Assemble the elution device by placing a 1.5ml microcentrifuge tube into the base of the Eluator™ Vacuum Elution Device (Cat.# A1071) and securing the tube cap in the open position, as shown (Figure 3, Panel A). Insert the PureYield™ Binding Column into the top of the Eluator™ Device, making sure the column is fully seated on the collar as shown in Figure 3, Panel B.

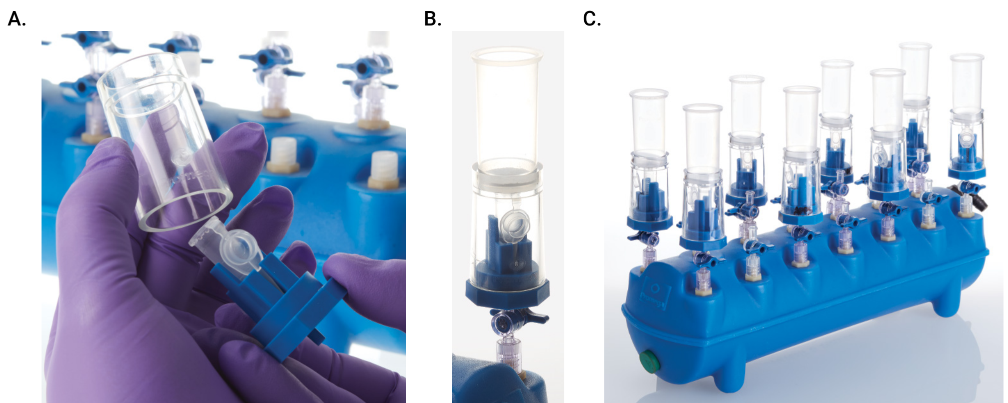

Figure 3. Elution by vacuum. Panel A. A 1.5ml microcentrifuge tube is placed in the base of the Eluator™ Vacuum Elution Device with the tube cap locked as shown. **Panel B.** The final Eluator™ Vacuum Elution Device assembly, including the binding column, ready for use on a vacuum manifold. **Panel C.** Example of eight Eluator™ Vacuum Elution Devices with binding columns assembled onto a Vac-Man® Laboratory Vacuum Manifold.

17. Place the Eluator™ Device assembly onto a vacuum manifold (Figure 3, Panel B). Add 500µl of preheated (60°C) Nuclease-Free Water to the PureYield™ Binding Column. Check that the vacuum manifold is properly assembled again (e.g., unused ports are closed, sidearm flask stopper is secured) and then apply maximum vacuum for 1 minute or until all liquid has passed through the column. Repeat the process by adding another 500µl of preheated Nuclease-Free Water to the PureYield™ Binding Column to elute a total of 1ml of TNA solution.

    Note: You can reuse the Eluator™ Vacuum Elution Device after cleaning with 70% ethanol or other standard laboratory disinfectants.

4.B. Total Nucleic Acid Extraction and Clean-Up

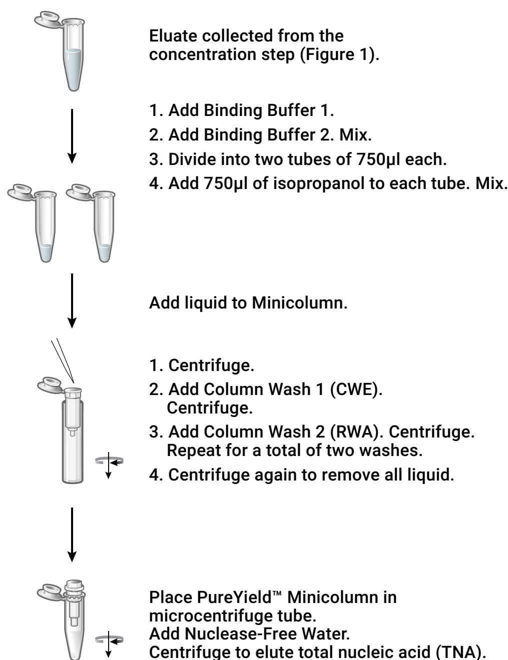

Figure 4. Protocol for clean-up and concentration of TNA using PureYield™ Minicolumns.

1. Add 400µl of Binding Buffer 1(BBD) and 100µl of Binding Buffer 2 (BBE) to 1ml of liquid eluted in Section 4.A, Step 15.
2. Mix well by inversion and divide the contents into two 1.5ml tubes containing 750µl each.
3. Add 750µl of isopropanol to each tube and mix well.
4. Place the PureYield™ Minicolumn into a PureYield™ Collection Tube. Pass the entire volume of the mixture through the column, 750µl at a time, using a microcentrifuge set at 10,000rpm for 1 minute.
5. Add 300µl of Column Wash 1 (CWE) and pull through the PureYield™ Minicolumn by centrifugation. Discard the flowthrough.

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4.B. Total Nucleic Acid Extraction and Clean-Up (continued)

6. Add 500µl of Column Wash 2 (RWA) and pull through the PureYield™ Minicolumn by centrifugation. Repeat this wash one time. Discard the flowthrough.
7. Centrifuge for 30 seconds to remove any residual wash solution.
8. Preheat 50µl of Nuclease-Free Water per sample to 60°C for 2–5 minutes.
9. Transfer the PureYield™ Minicolumn to a new 1.5ml microcentrifuge tube and add 20µl of preheated (60°C) Nuclease-Free Water to the column. Let the water soak into the column filter for approximately 1 minute.
10. Centrifuge at 10,000rpm for 1 minute to elute. Repeat elution with another 20µl of preheated Nuclease-Free Water, for a total of 40µl.
11. Store sample at or below –20°C until further analysis. TNA purified using this method can be directly used for RT-qPCR.

5. Supplemental Protocols

Wastewater samples may contain a range of targets and solids. In some cases, processing either the solid fraction or a variable amount of water may be desirable. In the direct capture protocol described in Section 4, suspended solids are removed by centrifugation prior to filtration. In other cases, sludge is allowed to precipitate by gravity and then analyzed. Larger or smaller water volumes are also sometimes prescribed by local sampling protocols. The following section describes how to extract total nucleic acids by processing either solids or a variable amount of wastewater.

5.A. Extracting Nucleic Acid from Pelleted Solids

The pelleted solids collected in Section 4.A can be processed to recover total nucleic acid as described here.

1. Add 5ml of Nuclease-Free Water to the pellet from Section 4.A, Step 4. This is the pellet recovered after treatment with Protease Solution and after decanting the supernatant that contains the soluble suspension into a separate tube.
2. Add 1.5ml of Binding Buffer 1 (BBD) and 125µl of Binding Buffer 2 (BBE).
3. Add 6ml of isopropanol.
4. Mix well by inversion.
5. Centrifuge the mixture at 3,000 × *g* for 10 minutes.
6. The supernatant will contain nucleic acid from the solids. Add the supernatant to the Reservoir Extension Funnel on the PureYield™ Binding Column turn on the pump and apply a vacuum to capture the TNA on the column.
7. Add 5ml of Column Wash 1 (CWE) and apply a vacuum to pull the liquid through the PureYield™ Binding Column.
8. Add 20ml of Column Wash 2 (RWA) and apply a vacuum to pull the liquid through the PureYield™ Binding Column. Continue the vacuum for an additional 30 seconds after all fluid has passed through the membrane.
9. Elute captured nucleic acid using the Eluator™ Vacuum Elution Device (Figure 3) by eluting in 500µl of Nuclease-Free Water, twice, for a total elution volume of 1ml.

10. Add 400µl of Binding Buffer 1 and 100µl of Binding Buffer 2 to the 1ml of liquid eluted in Step 9.
11. Mix well by inversion and divide the contents into two 1.5ml tubes containing 750µl each.
12. Add 750µl of isopropanol to each tube and mix well.
13. Place the PureYield™ Minicolumn into a PureYield™ Collection Tube. Pass the entire volume of the mixture through the column, 750µl at a time, using a microcentrifuge set at 10,000rpm for 1 minute.
14. Add 300µl of Column Wash 1 (CWE) and pull through the PureYield™ Minicolumn by centrifugation. Discard the flowthrough.
15. Add 500µl of Column Wash 2 (RWA) and pull through the PureYield™ Minicolumn by centrifugation. Repeat this wash one time. Discard the flowthrough.
16. Centrifuge for 30 seconds to remove any residual wash solution.
17. Preheat 50µl of Nuclease-Free Water per sample to 60°C for 2–5 minutes.
18. Transfer the PureYield™ Minicolumn to a new 1.5ml microcentrifuge tube and add 20µl of preheated (60°C) Nuclease-Free Water to the column. Let the water soak into the column filter for approximately 1 minute.
19. Centrifuge at 10,000rpm for 1 minute to elute. Repeat elution with another 20µl of preheated Nuclease-Free Water, for a total of 40µl.
20. Store sample at or below –20°C until further analysis. TNA purified using this method can be directly used for RT-qPCR.

5.B. Extracting Nucleic Acid from Sludge and Solids

An additional method for processing waste samples is to extract nucleic acid from settled solids from large volumes of wastewater or from sludge. Processing settled material can increase the sensitivity of assaying for pathogens by allowing material from larger samples to be efficiently processed.

1. To 2ml of solid material (sludge or settled solids) add 8ml of Nuclease-Free Water resulting in a 10ml final volume.
2. Add 200µl Protease Solution, mix well and incubate for 30 minutes.
3. Add 3ml of Binding Buffer 1 (BBD) and 250µl of Binding Buffer 2 (BBE).
4. Add 12ml of isopropanol.
5. Mix well by inversion.
6. Centrifuge the mixture at 3,000 × *g* for 10 minutes.
7. The supernatant will contain nucleic acid from the solids. Add the supernatant to the Reservoir Extension Funnel on the PureYield™ Binding Column turn on the pump and apply a vacuum to capture the TNA on the column.
8. Add 5ml of Column Wash 1 (CWE) and apply a vacuum to pull the liquid through the PureYield™ Binding Column.
9. Add 20ml of Column Wash 2 (RWA) and apply a vacuum to pull the liquid through the PureYield™ Binding Column. Continue the vacuum for an additional 30 seconds after all fluid has passed through the membrane.
10. Elute captured nucleic acid using the Eluator™ Vacuum Elution Device (Figure 3) by eluting in 500µl of Nuclease-Free Water, twice, for a total elution volume of 1ml.
11. Add 400µl of Binding Buffer 1 and 100µl of Binding Buffer 2 to the 1ml of liquid eluted in Step 10
12. Mix well by inversion and divide the contents into two 1.5ml tubes containing 750µl each.
13. Add 750µl of isopropanol to each tube and mix well.
14. Place the PureYield™ Minicolumn into a PureYield™ Collection Tube. Pass the entire volume of the mixture through the column, 750µl at a time, using a microcentrifuge set at 10,000rpm for 1 minute.
15. Add 300µl of Column Wash 1 (CWE) and pull through the PureYield™ Minicolumn by centrifugation. Discard the flowthrough.
16. Add 500µl of Column Wash 2 (RWA) and pull through the PureYield™ Minicolumn by centrifugation. Repeat this wash one time. Discard the flowthrough.
17. Centrifuge for 30 seconds to remove any residual wash solution.
18. Preheat 50µl of Nuclease-Free Water per sample to 60°C for 2–5 minutes.
19. Transfer the PureYield™ Minicolumn to a new 1.5ml microcentrifuge tube and add 20µl of preheated (60°C) Nuclease-Free Water to the column. Let the water soak into the column filter for approximately 1 minute.
20. Centrifuge at 10,000rpm for 1 minute to elute. Repeat elution with another 20µl of preheated Nuclease-Free Water, for a total of 40µl.
21. Store sample at or below –20°C until further analysis. TNA purified using this method can be directly used for RT-qPCR.

5.C. Scaling the Sample Volume

If the input volume of wastewater sample is different than the standard protocol (Section 4), use the reagent volumes listed in Table 1. Buffers are available separately, if you are using input volumes larger than 40ml. Use Table 1 as a guide for buffer volumes. Use appropriately-sized containers to accommodate the final volumes. Since the sensitivity of a downstream PCR or sequencing assay is dependent on the number of copies of the target organism nucleic acid in the sample, larger input volumes can be used. Conversely, as input volume decreases, the ability to detect the presence of lower abundance organisms will be diminished. We recommend a minimum sample volume of 40ml.

Note: The amount of buffer and columns provided with this system is sufficient for a 40ml sample volume. Protease Solution, Binding Buffer 1 and Binding Buffer 2 are available separately (see Section 7.C).

Table 1. Proportional Sample and Reagent Volumes.

Sample Volume (ml)	Protease Solution (ml)	Binding Buffer 1 (ml)	Binding Buffer 2 (ml)	Isopropanol (ml)
5	0.1	1.5	0.125	6
10	0.125	3	0.25	12
20	0.25	6	0.5	24
40	0.5	12	1	48
80	1	24	2	96

6. Considerations for Processing and Analysis

6.A. Quantitating Genome Units in the Sample

The nucleic acid purified using this method can be used for various downstream analysis and detection techniques, such as qPCR, RT-qPCR, ddPCR or sequencing. To quantitate genome units for a particular bacterial or viral target, qPCR or RT-qPCR is a commonly used technique.

6.B. Calculating Viral Genetic Material

Use a SARS-CoV-2 RT-qPCR kit for wastewater to determine the viral copy number in an RT-qPCR assay (molecular assay). Use the following equation to determine the viral genome units/liter in the water.

$$\text{Viral genome (copies/liter)} = \frac{\text{Copies in RT-qPCR} \times 1{,}000}{\text{Volume of nucleic acid extract used in RT-qPCR (ml)}^{*} \times \text{Concentration factor}}$$

*If 5µl of nucleic acid extract is used in RT-qPCR, the value in ml is 0.005.

$$\text{Concentration factor} = \frac{\text{Water sample volume used (ml)}}{\text{Volume of nucleic acid extracted (ml)}}$$

Notes:

- If a sample volume of 40ml is used and total nucleic acid is eluted in 40µl after the extraction and clean-up step, the concentration factor = 1,000.
- If a sludge sample volume of 2ml is used and total nucleic acid is eluted in 100µl of Nuclease-Free Water after the extraction and clean-up step, the concentration factor = 20.
- If a sample volume of 40ml is used and total nucleic acid is eluted in 80µl of Nuclease-Free Water after the extraction and clean-up step, the concentration factor = 500.

7. Appendix

7.A. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Lower than expected A_{260} (yield)	Use more starting material.
	Sample is relatively low in TNA content or degraded.
	Insufficient lysis.
	Check wastewater storage conditions. We recommended processing wastewater within 48 hours of sample collection. Store wastewater at +4°C to +10°C for use within a week.
	Store wastewater samples at –20°C for long-term storage. Frozen samples should not be thawed more than once.
	Check that the correct amount of Binding Buffers 1 and 2 was used to ensure sufficient lysis and binding.
Inhibitors present	Reduce the amount of starting material used per sample.
Sample takes a long time to flow through the PureYield™ Column	Reduce the amount of starting material used per sample.
	Check the pressure in the vacuum pump and vacuum system.
	Consider replacing the stopcocks, tubing or vacuum manifold.

7.B. Frequently Asked Questions (FAQ)

1. I forgot to preheat the Nuclease-Free Water prior to extraction. Is this ok?

   Answer: Total nucleic acid will still be eluted if the Nuclease-Free Water is not preheated.

2. Can nucleic acid extracted from the first step be stored for later use?

   Answer: Store the extracted nucleic acid at –20°C for short-term storage (<6 months) and at –80°C for long-term storage (>6 months).

   Consider eluting total nucleic acid in a TE buffer such as Elution Buffer (Cat.# A8281) for long-term storage. Consider the appropriate downstream application when using a TE buffer for Elution. TE Buffer (10mM Tris [pH 8], 0.1mM EDTA).

3. Can a laboratory vacuum line used for the vacuum process?

   Answer: The laboratory in-line vacuum can be used with the Vac-Man® Manifold (Cat.# A7231), if the pressure is appropriate. However, house vacuum systems can be quite variable and we recommend a dedicated vacuum source for best results.

7.C. Reference

1. Mondal, S. *et al*. (2021) A direct capture method for purification and detection of viral nucleic acid enables epidemiological surveillance of SARS-CoV-2. *Sci Total Environ*. **795**, 148834.

7.D. Related Products

Buffers and Solutions

Product	Size	Cat.#
Binding Buffer 1 (BBD)	320ml	A2981
Binding Buffer 2 (BBE)	30ml	MC1501
Protease Solution	30ml	A1442
Elution Buffer	50ml	A8281
RQ1 RNase-Free DNase	1,000 units	M6101

Start-Up Bundle (includes Kit, Pump, Vacuum Trap, Vac-Man® Manifold, Eluator™ Device, tubing)

Product	Size	Cat.#
Wizard® Enviro Start-Up Kit 110V Pump (Cat.# A2991; NA)	1 each	A3050
Wizard® Enviro Start-Up Kit 220V Pump (Cat.# A2291; EU)	1 each	A3060

Nucleic Acid Purification and Detection

Product	Size	Cat.#
ReliaPrep™ RNA Miniprep System*	50 preps (cells)	Z6010
	50 preps (tissues)	Z6110
ReliaPrep™ RNA Clean-Up and Concentration System*	10 preps	Z1071
Eluator™ Vacuum Elution Device	4 each	A1071
Vac-Man® Laboratory Vacuum Manifold	1 each	A7231
Vac-Man® Jr. Laboratory Vacuum Manifold	1 each	A7660
Luer-Lok® Stopcocks	10 each	A7261

*Additional kit sizes are available.

8. Summary of Changes

The following changes were made to the 1/23 version of this document:

1. Revised Sections 1, 3, 4 and 7.D.
2. Added new Section 7.C.
3. Updated document font.

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