Extraction of Vesivirus RNA

PrepSEQ™ Nucleic Acid Extraction Kit is used. The protocol will extract viral RNA from 100 µL of sample. Necessity to remove cells before extraction depends on the total cell number in the sample as described in the procedure.

1. PrepSEQ Nucleic Acid Extraction Kit

1.1 Contents and Storage of Kit

Reagent	Quantity	Description
Lysis Buffer	50 mL	2 bottles Store at ambient.
Binding Solution	1 Empty Bottle	One empty bottle. Fill with isopropanol before use. Store at Ambient after filling with isopropanol.
Wash Buffer Concentrate	26 mL/bottle	2 bottles. Add 74 mL of ethanol to each bottle before use. Store at Ambient.
Elution Buffer	50 mL	1 bottle. Store at Ambient.
Magnetic Particles	1.5 mL	2 tubes Shipped at 2° to 8°C. Store at 2° to 8° C.
Proteinase K Buffer (not needed in this study)	50 mL	1 bottle Shipped at ambient. Store at Ambient.
Proteinase K	1.25 mL	1 tube. Shipped at -15° to -25° C Store at -15° to -25° C

1.2 PrepSEQ: Required Equipment and Additional Materials

Consumable	Description
Magnetic Stand P/N AM10055	Ambion 6-Tube Magnetic Stand
Vortex (VWR) 14216-188 or 14216-186	Vortex-Genie 2T Timed Mixer

Vortex Adapter P/N AM10014	The Vortex Adapter-60 enables hands-free mixing for up to 60 microfuge tubes
1.5 mL tubes P/N AM12450	Ambion Non-Stick 1.5 mL microcentrifuge tubes
2 mL tubes (VWR) 62111-754	2 mL Safe-Lock microcentrifuge tubes with rounded bottoms
Pipettes	MLS*
Aerosol-resistant micropipette tips	MLS*
Isopropanol 100%	MLS*
Ethanol 95%**	MLS*
Bench-top microfuge for 1.5 & 2 mL tubes	MLS*
Heating blocks	MLS*

*MLS: Major Laboratory Supplier
** Do not use denatured ethanol, as it contains components not compatible with the protocol

1.3 PrepSEQ: Reagent preparation

Prior to using the PrepSEQ kit, prepare the following solutions.

- PrepSEQ Binding Solution
  Add 30 mL of 100% Isopropanol to the Binding Solution Bottle. Mark the label to indicate that isopropanol was added, then store at ambient.

- PrepSEQ Wash Buffer Concentrate
  Add 74 mL of 95% Ethanol to the bottle labeled PrepSEQ Wash Solution Concentrate and mix well. Mark the label to indicate that ethanol was added, then store at ambient.

- Resuspension of Magnetic Particles
Magnetic particles may form precipitates with salt during storage at 4^{o}C. It is very important to completely re-suspend the particles. Before each sample preparation, incubate the magnetic particles at 37^{o}C for 10 min followed by vortex until complete suspension.

2. Sample Preparation Procedure

2.1 Removal of cells

Samples with ≤ 10^6 cells/mL

Directly starts from Section 2.2 of Preparation of sample lysate.

Samples with > 10^6 cells/mL

For samples with > 10^6 cells/mL, total lysis of sample creates tight aggregate of magnetic particles with total nucleic acid from cells and reduces recovery rate.

1. Place 200 µl of samples into a 1.5 mL of microcentrifuge tube.

2. Centrifuge at 500x g for 2 minute.

3. Transfer 100 µl to a 2 mL lock-safe microcentrifuge tube.

4. Continue at Step 2 of Section 2.2 (Preparation of sample lysate).

2.2 Preparation of sample lysate

1. Place 100 µl of sample that contains up to total of 10^5 cells into a 2 mL lock-safe microcentrifuge tube.

2. Add 500 µl of Lysis Buffer and vortex for 15 seconds.

3. Incubate at 45^0C for 10 minutes.

4. Vortex tube at maximum speed for 10 seconds.

2.3 Extraction of viral RNA

1. (Important!) Incubate the Magnetic Particles at 37°C for 10 min. Vortex Magnetic Particles until complete resuspension.

   Note: This step can be performed while incubation of sample in Lysis Solution at Step 3 of Section 2.2.

2. Add 30µl of Magnetic Particles Suspension to the sample. Pipette up and down several times to mix.

3. Add 330 µl of Binding Solution. Invert tube 4 times to mix.

4. Incubate with shaking at Setting #7 on vortex mixer for 10 minutes to capture RNA.

5. Centrifuge the tube in a regular bench-top microfuge for 15 seconds. This step will spin down solution on the tube wall and inside the cap and pellet most of magnetic particle to the tube wall.

Note: When solution is too viscous, centrifugation will not help to pellet the particles. 4 minutes in the magnet stand will pull the particles to the tube wall. This situation may

happen when there is a lot of cellular nucleic acid (such as 10^6 cells), or a lot of protein (such as serum). Under such situations, the particles aggregate very tightly and solutions are so viscous that particles are difficult to travel through the tube wall. Very often, the eluates could be viscous too. However, it does not seem to hurt recovery of RNA.

6. Place tubes into Magnetic Stand with magnetic particle pellet oriented toward the magnet; complete capture of magnetic particles will happen in about 1 – 2 min. Aspirate supernatant carefully without disturbing the magnetic particles.

7. Add 300 µl of Wash Solution to the tube. Invert tubes back and forth twice and then vortex tube at Setting #7 on vortex mixer for 5 sec.

(Note: Flush the magnetic particles gently with Wash Solution helps to detach the magnetic particles from the tube wall.)

8. Centrifuge the tube in a regular bench-top microfuge for 15 seconds.

9. Place tubes into Magnetic stand with bead pellet oriented toward the magnet; complete capture of magnetic particles will happen in about 1 min. Aspirate supernatant carefully without disturbing the magnetic particles.

10. Add 300 µl of Wash Solution to the tube. Invert tubes back and forth twice and then vortex tube at Setting #7 on vortex mixer for 5 sec.

(Note: Flush the magnetic particles gently with Wash Solution helps to detach the magnetic particles from the tube wall.)

11. Centrifuge the tube in a regular bench-top microfuge for 15 seconds.

12. Place tubes into Magnetic stand with bead pellet oriented toward the magnet; complete capture of magnetic particles will happen in about 1 min. Aspirate supernatant carefully without disturbing the magnetic particles.

13. Use P200 to aspirate residual solution at the bottom of the tube.

14. With lid open, air-dry the Magnetic Particles for 5 minutes at room temperature.

(Note: Ethanol in the wash solution decreases recovery and causes PCR inhibition.)

15. Add 100 µl of Elution buffer.

(Note: If the magnetic particle pellet attaches to the tube wall tightly, place the tubes in the bench top microfuge with the bead pellet oriented toward the center, and spin for 30 seconds to detach the magnetic particles into the elution buffer. If it is difficult to re-suspend the magnetic particles, use P200 to pipette up-and-down several times to re-suspend the particles. Be careful not to let the particle pellet stick inside the tip.)

16. Vortex tube at high speed on vortex mixer for 10 sec.

17. Incubate 5 minutes at 45°C and vortex tubes 2-3 times during this incubation.

18. Centrifuge the tube in a regular bench-top microfuge for 15 seconds.

19. Place tubes into Magnetic stand with bead pellet oriented toward the magnet; complete capture of magnetic particles will happen in about 1 min.

20. Transfer the liquid phase with eluted RNA to a non-stick 1.5-mL microfuge tube.

21. Centrifuge the tube at top speed for 3 min to pellet residual magnetic particles. Place the tube on magnet stand with the magnetic particles towards the magnet. Transfer the liquid phase with eluted RNA to a non-stick 1.5-mL microfuge tube without disturbing the Magnetic particles.

(Note: Magnetic particles can be PCR inhibitory.)

22. Set up RT-PCR of the extracted nucleic acid.