Title

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Chapter 5

Isolation of Extracellular RNA from Serum/Plasma

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Abstract

Extracellular RNAs are initiating increased interest due to their potentials in serving as novel biomarkers, mediators of intercellular communication, and therapeutic applications. As a newly emerging field, one of the main obstacles is the lack of standardized protocols for RNA isolations. Here we describe protocols for commercially available kits that have been modified to yield consistent results for isolation of extracellular RNA from both whole serum/plasma and extracellular vesicle-enriched serum/plasma samples.

Key words Cell biology, Extracellular RNA, Extracellular Vesicle, Nucleic acids, Molecular Biology, Isolation, Purification and separation

1 Introduction

Extracellular nucleic acids were first identified in human biofluids in 1948 [1]. Extracellular RNAs (exRNAs) play an important role in intercellular communication in the body. Various reports suggest that RNAs released from one tissue can alter expression in other cells [2]. Most of these exRNAs are noncoding RNAs of various types, many of which have no known function. These types of exRNAs include microRNAs (miRNAs), small interfering RNA (siRNA), piwi-interacting RNA (piRNA), long noncoding RNA (lncRNA), messenger RNA (mRNA) fragments, and transfer RNA (tRNA). It is widely thought that although "free" exRNA can be released from cells without associated macromolecules or a plasma membrane envelope, the ribonucleases present in the extracellular space would rapidly degrade free RNAs. Evidence of exRNA in biofluids suggests that they are protected from the environment through association

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with extracellular vesicles such as exosomes, apoptotic bodies, microvesicles, lipoproteins, and ribonucleoproteins [3].

This chapter describes various RNA isolation methods that can be used to extract RNA from serum and/or plasma. exRNA association with extracellular particles necessitates specific techniques to enrich for each compartment. exRNA can be extracted either from whole serum/plasma or from vesicle-enriched serum/plasma. Vesicle enrichment can be done using one of the four methods, namely: precipitation, membrane filtration, affinity purification, and differential centrifugation.

Precipitation of extracellular vesicles can be done using the ExoQuick (System Biosciences) kit, which is based on the polyethylene glycol (PEG)/sodium chloride (NaCl) method for precipitating macromolecules. This method can precipitate extracellular vesicles as well as lipoprotein and ribonucleoprotein complexes [4]. Amicon ultracentrifugal filters (Millipore) can also be used to enrich for extracellular particles. These are based on size exclusion and hence will enrich all types of extracellular particles smaller than the pore size. This technique can be used to exclude larger particles such as microvesicles or apoptotic bodies. ExoRNeasy kit (Qiagen) uses membrane affinity spin columns for binding all types of extracellular vesicles [5]. New England Peptide's Vn96 peptide binds to heat-shock proteins on the exterior surface of exosomes and other extracellular vesicles and precipitates them through a series of centrifugation steps. Finally, ultracentrifugation can be used to enrich for extracellular vesicles by virtue of their density.

Following this, exRNA can be isolated either from the enriched biofluid or the total biofluid using multiple methods. Some of the methods described in this chapter are miRNeasy micro kit (Qiagen), Circulating RNA isolation kit (Norgen Biotek), miRCURY RNA isolation kit (Exiqon), and SeraMir exosome RNA purification kit (System Biosciences).

2 Materials

2.1 ExoQuick

1. ExoQuick Plasma Prep and Exosome Precipitation kit (System Biosciences EXOQ5TM-1).
2. Phosphate-buffered saline (PBS).

2.2 miRcury Exosome Plus

1. miRcury Biofluids kit (Exiqon—300112/300113).
2. miRcuryTM Exosome Isolation Kit—Serum and plasma (Exiqon—300101).
3. RNase-free water (Ambion—AM9937).

2.3 Millipore

Prepare all solutions using RNase-free water.

1. Qiagen miRNeasy micro kit (217084): Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of 100% ethanol, as indicated on the bottle to obtain a working solution.
2. Chloroform.
3. 200 proof ethanol: 100% solution and freshly prepared 70% solution in water.
4. Phosphate-buffered saline (PBS).
5. AU-0.5 filter, 0.5 mL, 10 kDa MWCO (EMD Millipore—UFC501024).

2.4 ExoRNeasy

Prepare all solutions using RNase-free water.

1. ExoRNeasy Midi kit (Qiagen, 77044): Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of 100% ethanol, as indicated on the bottle to obtain a working solution.
2. RNeasy MinElute Columns (Qiagen, Part of miRNeasy micro kit—217084).
3. Chloroform.
4. 200 proof ethanol: 100% solution and freshly prepared 70% solution in water.

2.5 ME Kit

1. New England Peptide ME™ kit—ME-010-kit.
2. Phosphate-buffered saline (PBS), pH 7.4.
3. Protease Inhibitor Cocktail, Set III EDTA-free (EMD)—539134.
4. Qiagen miRNeasy Micro kit—217084.
5. Chloroform (Sigma-Aldrich, 319988).
6. 100% ethanol (Koptec, V1016).
7. 70% ethanol.
8. RNase-free water (Ambion —AM9937).

2.6 Ultracentrifugation

1. Ultracentrifuge.
2. Swinging bucket rotor (TLA110/MLA5).
3. Qiagen miRNeasy micro kit (217084).
4. Chloroform (Sigma-Aldrich—319988).
5. 100% ethanol (Koptec—V1016).
6. 70% ethanol.
7. RNase-free water (Ambion—AM9937).
8. Phosphate Buffer Solution (PBS).

2.7 miRNeasy Micro Kit

1. Qiagen miRNeasy Micro kit (217084): Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of 100% ethanol, as indicated on the bottle, to obtain the working solutions.
2. Chloroform.
3. 200 proof ethanol: 100% solution and freshly prepared 70% solution in water.

2.8 Norgen

1. Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit (Norgen BioTek—51000).
2. 2-Mercaptoethanol, 99% Extra Pure (Acros Organics—125472500).
3. 100% ethanol (Koptec—V1016).
4. RNase-free water (Ambion—AM9937).
5. Optional: Amicon Ultra 0.5 mL Centrifugal filter devices—3K (Amicon—UFC500396).

2.9 miRcury Biofluids Kit

1. miRcury Biofluids kit (Exiqon—300112/300113)
2. Isopropanol (Sigma-Aldrich—I9516).
3. 100% ethanol (Koptec—V1016).
4. RNase-free water (Ambion—AM9937).

2.10 SeraMir

1. SeraMir Exosome RNA Purification Column Kit (System Biosciences—RA808A-1).
2. 100% ethanol (Koptec—V1016).
3. RNase-free water (Ambion—AM9937).

2.11 Equipment

1. Microfuge for centrifugation at 4 °C and at room temperature.
2. 1.5 mL Microfuge tubes.
3. 15 mL Centrifuge tubes.
4. Vortexer.
5. Phase lock gel tubes, 2 mL (VWR—10847-802).

3 Methods

3.1 ExoQuick

1. Transfer 500 μL of serum/plasma into a 1.5 mL microfuge tube.
   (a) For plasma, add 5 μL thrombin (500 U/mL) to a final concentration of 5 U/mL.
   (b) Incubate at room temperature for 5 min while mixing (gently flicking tube).

(c) Centrifuge at 10,000 × *g* for 5 min. There should be a visible fibrin pellet at the bottom of the tube.

(d) Transfer supernatant to new microfuge tube.

2. Add 125 μL of ExoQuick Exosome Precipitation Solution to the serum and incubate for 30 min at 4 °C.
3. Centrifuge ExoQuick mixture at 1500 × *g* for 30 min at room temperature.
4. Aspirate supernatant.
5. Spin down residual ExoQuick solution by centrifugation at 1500 × *g* for 5 min at room temperature.
6. Remove all traces of fluid by aspiration, taking great care not to disturb the pellet.
7. Resuspend the pellet in 50 μL sterile PBS and proceed with RNA isolation using SeraMir Exosome RNA Purification Column Kit.

3.2 miRCURY Exosome Plus Kit

1. Add 200 μL of Precipitation Buffer A and vortex for 5 s.
2. Incubate for 60 min at 4 °C.
3. Spin for 30 min at 1500 × *g* at room temperature.
4. Remove supernatant and discard (or save for comparative analysis).
5. Resuspend the pellet by vortexing in 270 μL of Resuspension Buffer ending up with ~300 μL of volume and proceed with the miRCURY™ RNA isolation kit—Biofluids
6. Mix 300 μL of resuspended exosomes with 90 μL of Lysis Buffer solution BF.
7. Vortex for 5 s.
8. Incubate for 10 min at room temperature.
9. Add 30 μL of Protein Precipitation Solution BF.
10. Vortex for 5 s.
11. Incubate for 1 min at room temperature.
12. Centrifuge for 3 min at 11,000 × *g*.
13. Transfer the clear supernatant into a new collection tube (2 mL, with lid).
14. Add 400 μL of isopropanol.
15. Vortex for 5 s.
16. Assemble the microRNA Spin Column BF and load sample onto column.
17. Incubate 2 min at room temperature.
18. Spin for 30 s at 11,000 × *g*. Discard flow-through.
19. Add 100 μL Wash Solution 1 BF.

20. Spin for 30 s at 11,000 × *g*. Discard flow-through.
21. Add 700 µL Wash Solution 2 BF.
22. Spin for 30 s at 11,000 × *g*. Discard flow-through.
23. Add 250 µl Wash Solution 2 BF.
24. Spin for 2 min at 11,000 × *g*. Transfer spin column to fresh microfuge tube.
25. Add 30 µL water directly onto the membrane of the spin column.
26. Incubate for 1 min at room temperature.
27. Spin for 1 min at 100 × *g* followed by 1 min at 11,000 × *g*.

3.3 Millipore

Carry out all procedures at room temperature unless otherwise specified.

3.3.1 Exosome Isolation

1. Add 500 µL PBS to the AU-0.5 filter, cap, and centrifuge for 10 min at 14,000 × *g*.
2. Reverse the device and centrifuge for 2 min at 1000 × *g* to remove the residual PBS from the filter.
3. Aspirate PBS from the collection tube.
4. Transfer 500 µL serum/plasma to the AU-0.5 filter and cap the device.
5. Centrifuge for 30 min at 14,000 × *g*. There should be ~15 µL sample remaining in the upper chamber.
6. Remove device from the centrifuge and empty the collection tube.
7. Add 0.5 mL PBS to the filter device and gently pipette sample multiple times to mix.
8. Centrifuge for 30 min at 14,000 × *g*.
9. Place filter upside down in a fresh microcentrifuge tube.
10. Centrifuge for 2 min at 2000 × *g* (*see* **Note 1**) to transfer the sample to the collection tube. Discard filter.

3.3.2 RNA Isolation

Add 700 µL Qiazol sample in the collection tube and isolate RNA using the miRNeasy micro kit (starting from **step 3**).

3.4 ExoRNeasy

Carry out all procedures at room temperature unless otherwise specified.

3.4.1 Exosome Isolation

1. Transfer 500 µL of serum/plasma equilibrated to room temperature into a 1.5 mL microfuge tube.
2. Add 500 µL XBP and mix well immediately by gently inverting the tube five times.

3. Add 800 µL of sample/XBP mixture onto the exoEasy spin column and centrifuge for 1 min at 500 × *g*.
4. Repeat **step 3** until all mixture is added onto the column.
5. Discard flow-through.
6. Add 800 µL XWP to the exoEasy spin column.
7. Centrifuge for 5 min at 5000 × *g*. Discard the flow-through together with the collection tube.
8. Transfer the spin column to a fresh collection tube.

3.4.2 RNA Isolation

1. Add 700 µL Qiazol to the membrane of the spin column.
2. Centrifuge for 5 min at 5000 × *g*.
3. Transfer the flow-through, which is the lysate, to a PLG tube (*see* **Note 2**).
4. Vortex for 5 s.
5. Incubate at room temperature for 5 min.
6. Add 90 µL chloroform.
7. Shake vigorously for 15 s.
8. Incubate for 3 min at room temperature.
9. Centrifuge sample for 5 min at 12,000 × *g* at 4 °C.
10. Transfer the upper aqueous phase to a new microcentrifuge tube.
11. Carefully measure the aqueous phase and add 2 volumes of 100% ethanol.
12. Mix gently and thoroughly. Do not centrifuge and do not delay moving on to the next step.
13. Assemble a MinElute spin column in a new collection tube.
14. Load up to 700 µL of the mixture from **step 12**, including any precipitate that may have formed, onto the column.
15. Centrifuge for 15 s at 1000 × *g* (*see* **Note 3**) at room temperature (*see* **Note 4**).
16. Discard flow-through.
17. Repeat **steps 14–16** until entire sample has been loaded.
18. Make sure that ethanol has been added to RWT and RPE buffers.
19. Add 700 µL Buffer RWT to the RNeasy MinElute spin column.
20. Centrifuge for 15 s at ≥8000 × *g* at room temperature to wash the column.
21. Discard the flow-through.
22. Pipet 500 µL Buffer RPE onto the RNeasy MinElute spin column.

23. Centrifuge for 15 s at ≥8000 × *g* to wash the column.
24. Discard the flow-through.
25. Pipet 500 µL Buffer RPE onto the RNeasy MinElute spin column.
26. Centrifuge for 2 min at ≥8000 × *g* (≥10,000 rpm) at room temperature to wash the column.
27. Discard the collection tube with the flow-through (*see* **Note 5**).
28. Transfer the RNeasy MinElute spin column into a new 2 mL collection tube (supplied).
29. Open the lid of the spin column, and centrifuge at full speed (14,000 × *g*) for 5 min to dry the membrane (*see* **Note 6**).
30. Discard the collection tube with the flow-through.
31. Transfer the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14–30 µL RNase-free water directly to the center of the spin column membrane (*see* **Note 7**).
32. Centrifuge for 1 min at full speed to elute the RNA (Optional: Centrifuge for 1 min at 100 × *g* followed by 1 min at full speed) (*see* **Note 8**).

3.5 ME

1. Reconstitute the Vn96 peptide to 2.5 µg/µL by adding 200 µL ME-buffer. Reconstitute the negative control Vn96-Scr peptide by adding 40 µL ME-buffer. Store at 4°°C.
2. Transfer 500 µL of serum/plasma into a 1.5 mL microfuge tube.
3. Add 500 µL PBS.
4. Mix thoroughly.
5. Add 5 µL protease inhibitor cocktail.
6. Centrifuge for 7 min at 10,000 × *g* at room temperature to remove debris.
7. Transfer supernatant to fresh tube, avoiding any pelleted material.
8. Add 20 µL reconstituted Vn96 peptide (use same amount of Vn96-Scr as a negative control).
9. Invert tube ten times.
10. Incubate at room temperature for 30 min on a rotator (*see* **Note 9**).
11. Centrifuge for 7 min at 10,000 × *g* at room temperature to pellet extracellular vesicles.
12. Carefully remove and discard supernatant.
13. Wash by adding 1 mL PBS + 5 µL protease inhibitor cocktail, inverting ten times, centrifuging for 7 min at 10,000 × *g* at

room temperature, and carefully removing and discarding supernatant.

14. Repeat **step 13**.
15. Resuspend pellet in 700 µL Qiazol and isolate RNA using miRNeasy micro kit.

3.6 Ultracentrifugation

3.6.1 Exosome Isolation

1. Start with 500 µL serum/plasma.
2. Bring up volume to fill ultracentrifuge tube (2.3–2.5 mL) with PBS.
3. Centrifuge for 70 min at 100,000 × *g* at 4 °C.
4. Discard supernatant, and resuspend pellet in PBS to fill ultracentrifuge tube.
5. Centrifuge for 70 min at 100,000 × *g* at 4 °C.
6. Discard supernatant.
7. Resuspend pellet in 50 µl PBS and store at −80 °C, or
8. Proceed to miRNeasy RNA isolation by adding 700 µL Qiazol to pellet.

3.7 miRNeasy Micro Kit

1. Transfer 500 µL of serum/plasma into a 15 mL centrifuge tube.
2. Add 2500 µL (5× volumes) of QIAzol Lysis Reagent (*see* **Note 10**).
3. Vortex 5 s.
4. *Optional:* Transfer to PLG tubes. You may need to divide a sample into several PLG tubes depending on the volume (*see* **Note 2**).
5. Incubate for 5 min at room temperature.
6. Add 500 µL chloroform.
7. Shake vigorously for 15 s.
8. Incubate for 3 min at room temperature.
9. Centrifuge sample for 15 min at 12,000 × *g* at **4 °C**.
10. Transfer the upper aqueous phase to a new 15 mL centrifuge tube (*see* **Note 11**). Avoid the white interphase.
11. Carefully measure the aqueous phase and add 1.5× volumes of 100% ethanol.
12. Mix gently and thoroughly. Do not centrifuge and do not delay moving on to the next step.
13. Assemble a MinElute spin column in a new collection tube.
14. Load up to 700 µL of the mixture from **step 12**, including any precipitate that may have formed, onto the column.
15. Spin for 15 s at 1000 × *g* (*see* **Note 3**) at room temperature (*see* **Note 4**).

16. Discard flow-through.
17. Repeat **steps 14–16** until entire sample has been loaded.
18. Make sure that ethanol has been added to RWT and RPE buffers as instructed in the manufacturer's manual.
19. Add 700 µL Buffer RWT to the RNeasy MinElute spin column.
20. Centrifuge for 15 s at 8000 × *g* at room temperature to wash the column.
21. Discard the flow-through.
22. Pipet 500 µL Buffer RPE onto the RNeasy MinElute spin column.
23. Centrifuge for 15 s at 8000 × *g* to wash the column.
24. Discard the flow-through.
25. Pipet 500 µL 80% ethanol (*see* **Note 12**) onto the RNeasy MinElute spin column.
26. Centrifuge for 2 min at ≥8000 × *g* (≥10,000 rpm) at room temperature to wash the column.
27. Discard the collection tube with the flow-through (*see* **Note 5**).
28. Transfer the RNeasy MinElute spin column into a new 2 mL collection tube (supplied).
29. Open the lid of the spin column, and centrifuge at full speed for 5 min to dry the membrane (*see* **Note 6**).
30. Discard the collection tube with the flow-through.
31. Transfer the RNeasy MinElute spin column into a new 1.5 mL collection tube (supplied).
32. Add 14 or 30 µL RNase-free water directly to the center of the spin column membrane (*see* **Note 7**).
33. Centrifuge for 1 min at full speed to elute the RNA. (Optional: Centrifuge for 1 min at 100 × *g* followed by 1 min at full speed.) (*see* **Note 8**).

3.8 Norgen

1. Set Incubator to 60 °C and warm PS Solution A, PS Solution B, and PS Solution C for 20 min to ensure no precipitates are present.
2. Add 50 mL of 96–100% ethanol to Wash Solution and check off box on bottle noting addition of ethanol.
3. Add 10 µL 2-Mercaptoethanol per 1 mL PS Solution B. (*see* **Note 13**).
4. Transfer 500 µL serum/plasma into a microfuge tube.
5. Add 100 µL PS Solution A (*see* **Note 14**) and 900 µL PS Solution B (containing 2-Mercaptoethanol).

6. Vortex for 15 s.
7. Incubate for 10 min at 60 °C.
8. Add 1.5 mL 100% Ethanol.
9. Vortex for 15 s.
10. Centrifuge for 30 s at 100 × *g* at room temperature. Carefully decant supernatant, discarding it. DO NOT disrupt pellet.
11. To the pellet add 750 µL PS Solution C.
12. Vortex for 15 s.
13. Incubate for 10 min at 60 °C.
14. Add 750 µL 100% Ethanol.
15. Vortex for 15 s.
16. Assemble a mini filter Spin Column with a collection tube.
17. Transfer 650 µL of the mixture to the Filter Column.
18. Centrifuge for 1 min at 16,000 × *g* at room temperature.
19. Discard flow-through.
20. Repeat **steps 17–19** until all of the mixture has been added to the Filter Column.
21. Add 400 µL Wash Solution to the column.
22. Centrifuge for 1 min at 16,000 × *g* at room temperature.
23. Discard flow-through.
24. Repeat **steps 21–23** two more times for a total of three washes.
25. Open the cap of the column and centrifuge for 3 min at 16,000 × *g* at room temperature to dry the column. Discard collection tube.
26. Transfer the spin column to a fresh 1.7 mL elution tube.
27. Apply 30 µL water to the column (*see* **Note 15**).
28. Centrifuge for 2 min at 300 × *g* at room temperature to load the solution onto the column.
29. Centrifuge for 3 min at 16,000 × *g* at room temperature to elute the RNA.
30. *Optional*: Cleanup by Amicon filtration
    (a) Dilute the eluted RNA with 320 µL DNA/RNA-free water.
    (b) Place the Amicon column into a collection tube. Load sample onto column.
    (c) Spin for 8 min at 14,000 × *g* at room temperature until the volume of the sample is ~20 µL.
    (d) Discard the flow-through.
    (e) Invert the column and place it into a new tube.
    (f) Centrifuge for 2 min at 8000 × *g* at room temperature.
    (g) The final volume should be ~20 µL. The sample can be further concentrated using a speed-vac.

3.9 miRCURY

1. Add 80 mL of 100% ethanol to "Wash Solution 2 BF."
2. Transfer 500 μL of serum/plasma into a 1.5 mL microfuge tube.
3. Add 150 μL "Lysis Solution BF."
4. Vortex for 5 s.
5. Incubate for 10 min at room temperature.
6. Add 50 μL of "Protein Precipitation Solution BF."
7. Vortex for 5 s.
8. Incubate for 1 min at room temperature.
9. Centrifuge sample for 5 min at 12,000 × *g* at room temperature.
10. Transfer the cleared supernatant to a new microcentrifuge tube, avoiding any precipitate.
11. Add 675 μL isopropanol.
12. Vortex for 5 s.
13. Assemble the microRNA Spin Column BF and load sample onto column.
14. Incubate 2 min at room temperature.
15. Spin for 30 s at 11,000 × *g*. Discard flow-through.
16. Add 100 μL Wash Solution 1 BF.
17. Spin for 30 s at 11,000 × *g*. Discard flow-through.
18. Add 700 μL Wash Solution 2 BF.
19. Spin for 30 s at 11,000 × *g*. Discard flow-through.
20. Add 250 μl Wash Solution 2 BF.
21. Spin for 2 min at 11,000 × *g*. Transfer spin column to fresh microfuge tube.
22. Add 30 μL water directly onto the membrane of the spin column.
23. Incubate for 1 min at room temperature.
24. Spin for 1 min at 100 × *g* followed by 1 min at 11,000 × *g*.

3.10 SeraMir

1. Add 350 μL Lysis Buffer to the resuspended exosome pellet and vortex 15 s.
2. Incubate for 5 min at room temperature.
3. Add 200 μL of 100% Ethanol.
4. Vortex 10 s.
5. Assemble spin column and collection tube. Transfer mixture to spin column.
6. Centrifuge for 1 min at 17,000 × *g* rpm at room temperature (check to see that the liquid has all flowed through; if not, spin longer).

7. Discard flow-through.
8. Repeat **steps 6** and 7 until all lysate has been spun.
9. Add 400 μL Wash Buffer.
10. Centrifuge for 1 min at 17,000 × *g* at room temperature (check to see that the liquid has all flowed through; if not, spin longer).
11. Discard flow-through.
12. Repeat **steps 9–11** once again (total of two washes).
13. Centrifuge for 2 min at 17,000 × *g* at room temperature to dry.
14. Transfer spin column to fresh microfuge tube.
15. Add 30 μL Elution Buffer or water directly to membrane in spin column.
16. Centrifuge for 2 min at 400 × *g* at room temperature (loads buffer onto membrane) (*see* **Note 8**).
17. Centrifuge for 1 min at 17,000 × *g* at room temperature to elute RNA.

4 Notes

1. The manufacturer's protocol suggests spinning at 1000 × *g*.
2. PLG makes separation of aqueous phase from the interface easier, and thus is particularly useful for large numbers of samples or less experienced personnel, but it is expensive.
3. The manufacturer's protocol is for 8000 × *g*, but some labs have found that 1000 × *g* for the binding step gives better results.
4. The centrifuge *must* be above 20 °C so that excessive precipitation does not occur.
5. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur and result in lower RNA yields.
6. To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise). It is important to dry the spin column membrane since residual ethanol may reduce RNA yields. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.
7. As little as 10 μL RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be

reduced by approximately 20%. Do not elute with less than 10 μL RNase-free water, as the spin column membrane will not be sufficiently hydrated. The dead volume of the RNeasy MinElute spin column is 2 μL: elution with 14 μL RNase-free water results in a 12 μL eluate.

8. Centrifuging at a low speed first helps the solvent wet the surface of the membrane prior to the full-speed centrifuging step. This results in a better yield/RNA recovery from the membrane.
9. This is different from the manufacturer's protocol, which suggests 15 min at RT or overnight at 4 °C.
10. Adapted from miRNeasy serum kit—added 5× the volume of Qiazol.
11. If there is poor phase separation, the original biofluid can be diluted. To "rescue" the exRNA preparation, additional buffer, Qiazol, and chloroform can be added (maintaining the ratio 1:5:1).
12. 80% ethanol should be prepared with ethanol (96–100%) and RNase-free water.
13. If the kit will be used up within 3 months, the 2-Mercaptoethanol can be added directly to the Solution B bottle.
14. Solution A contains a resin and must be mixed well before addition to samples.
15. The manufacturer's protocol suggests eluting with 100 μL of Elution Solution A. This volume was selected to match that of other kits to enable fair comparisons. Eluting with larger volumes will lead to a better yield, but more dilute samples.

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