

EXTRAClean Plasma/Serum RNA Purification Midi Kit

Product # 73710

Product Insert

Cell-free circulating RNA, including exosomal RNA in plasma or serum, has the potential to provide biomarkers for certain cancers and disease states, and includes tumor-specific extracellular RNA in the blood. Exosomes are 40 - 100 nm membrane vesicles, which are secreted by most cell types. Exosomes can be found in saliva, blood, urine, amniotic fluid and malignant ascitic fluids, among other biological fluids. Evidence has been accumulating recently that these vesicles act as cellular messengers, conveying information to distant cells and tissues within the body. The exosomes contain cell-specific proteins, lipids and RNAs, which are transported to other cells, where they can alter function and/or physiology. These exosomes may play a functional role in mediating adaptive immune responses to infectious agents and tumours, tissue repair, neural communication and transfer of pathogenic proteins. Recent work has demonstrated the presence of distinct subsets of microRNAs within exosomes which depend upon the tumour cell type from which they are secreted. For this reason, exosomal RNAs may serve as biomarkers for various diseases including cancer. As the RNA molecules encapsulated within exosomes are protected from degradation by RNAses, they can be efficiently recovered from biological fluids, such as plasma or serum.

Norgen's Purification Technology

Norgen's EXTRAClean Plasma/Serum RNA Purification Midi Kit provides a fast, reliable, reproducible and simple procedure for isolating circulating RNA and exosomal RNA from small plasma/serum inputs ranging from 250 μ L and up to 1.5 mL, with various kit formats addressing different plasma/serum input volumes. Purification is based on spin column chromatography that uses Norgen's proprietary resin separation matrix. The EXTRAClean columns undergo stringent processing and rigorous quality control measures to minimize contamination traces, ensuring optimal results for sensitive applications such as NGS. The kit is designed to isolate all sizes of circulating RNA, including microRNA, as well as all sizes of exosomal RNA. Norgen's EXTRAClean Plasma/Serum RNA Purification Midi Kit provides a clear advantage over other available kits in that they do not require phenol/chloroform or any protease treatments. RNA can be isolated from either fresh or frozen samples using these kits. Moreover, the kits allow the user to elute into a flexible elution volume ranging from 50 μ L to 100 μ L. Typical yields of free-circulating and exosomal RNA vary depending on the input sample, as the amount of RNA present in plasma and serum will depend upon the health status of the individual. Normally, the RNA yield from plasma or serum RNA is highly variable (ranging from 1 to 100 ng/mL). Variability is also observed between samples collected from the same donor at different times during the day. This kit is suitable for the isolation of RNA from fresh or frozen serum or plasma prepared from blood collected on either EDTA or Citrate. Plasma samples prepared from blood collected on heparin should not be used as heparin can significantly interfere with many downstream applications such as RT-PCR.

Advantages

- Versatile plasma/serum input ranges.
- No phenol extractions.
- Fast and easy processing of samples for the purification and the isolation of RNA.
- No time-consuming ultracentrifugation.
- More effective than any other method.
- EXTRAClean resin separation matrix provides highly purified RNA.

Kit Components

Component	Midi Kit Cat# 73710
Number of Preps	20 preps
Lysis Buffer A	100 mL
Wash Solution A	1 x 38 mL* 1 x 18 mL*
Elution Solution A	6 mL
Elution Buffer F	15 mL
EXTRAClean Mini Spin Columns	20
EXTRAClean Midi Spin Columns	20
Collection Tubes	20
Elution tubes (1.7 mL)	20
Product Insert	1

* For the preparation of working solutions, please see Important Notes (Notes prior to use).

Specifications

Kit Specifications	
Sample Type	Plasma/Serum
Anti-coagulant (for Plasma) †	EDTA or Citrate
Sample Volume Range	250 µL to 1.5 mL
Minimum Elution Volume	50 µL
Maximum Elution Volume	100 µL
Time to Complete 10 Purifications	35 - 40 minutes
Size of RNA Purified	All sizes, including miRNA and small RNA (<200 nt)
Average Yields ‡	Variable depending on specimen

† This kit is suitable for the isolation of RNA from fresh or frozen serum or plasma prepared from blood collected on either EDTA or Citrate. Plasma samples prepared from blood collected on heparin should not be used as heparin can significantly interfere with many downstream applications such as RT-PCR

‡ Please check page 6 for Average Plasma/Serum Yields and Common RNA Quantification Methods

Storage Conditions and Product Stability

All solutions should be kept tightly sealed and stored at room temperature. This kit is stable for 2 years after the date of shipment. It is recommended to warm Lysis Buffer A for 20 minutes at 60°C if any salt precipitation is observed.

Precautions and Disclaimers

This kit is designed for research purposes only. It is not intended for human or diagnostic use. Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at www.norgenbiotek.com.

Lysis Buffer A contains guanidine thiocyanate, and should be handled with care. Guanidine thiocyanate forms highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of this solution.

If liquid containing these buffers is spilled, clean with suitable laboratory detergent and water. If the spilled liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Caution: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Customer-Supplied Reagents and Equipments

- Benchtop microcentrifuge
- Swinging bucket centrifuges
- Vortexer
- Micropipettors
- 96 – 100% ethanol
- 100% Isopropanol
- Optional: β - Mercaptoethanol

Procedure

All centrifugation steps are carried out in a benchtop microcentrifuge. Various speeds are required for different steps, so please check your microcentrifuge specifications to ensure that it is capable of the proper speeds. All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) (r)}}$$

where RCF = required gravitational acceleration (relative centrifugal force in units of g); r = radius of the rotor in cm; and RPM = the number of revolutions per minute required to achieve the necessary g -force.

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware are not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defence against these enzymes.

- The RNA area should be located away from microbiological work stations
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water
- Clean all surfaces with commercially available RNase decontamination solutions
- When working with purified RNA, ensure that they remain on ice during downstream applications

Notes Prior to Use

- All centrifugation steps are performed at room temperature.
- Ensure that centrifuge tubes used are capable of withstanding the centrifugal forces required.
- The provided spin columns are optimized to be used with a benchtop centrifuge and not to be used on a vacuum apparatus
- Most standard benchtop microcentrifuges will accommodate Norgen's EXTRAClean Mini Spin Columns and EXTRAClean Midi Spin Columns.

- Most standard swinging bucket centrifuges will accommodate Norgen's EXTRAClean Midi. Do not use a fixed-angle rotor.
- Norgen's EXTRAClean Midi Spin Columns are centrifuged in a 15 mL centrifuge tube.
- Centrifuging Norgen's EXTRAClean Spin Columns at a speed higher than recommended may affect RNA yield.
- Centrifuging Norgen's EXTRAClean Spin Columns at a speed lower than recommended will not affect RNA yield. However, centrifugation at a lower speed may require longer time for the solutions to pass through the spin column
- When placing Norgen's EXTRAClean Midi Spin Columns into the swinging bucket centrifuge make sure that lids of the tubes are not tightly closed. Tightly closed lids may cause back pressure which may cause column clogging or disintegration.
- Ensure that all solutions are at room temperature prior to use.
- It is highly recommended to warm up **Lysis Buffer A** at 60°C for 20 minutes and mix well until the solutions become clear again if precipitates are present.
- Prepare a working concentration of the **Wash Solution A**:
 - Add **42 mL** of 96 - 100% ethanol (provided by the user) to the supplied bottle containing **18 mL** of the concentrated Wash Solution A. This will give a final volume of **60 mL**.
 - Add **90 mL** of 96 - 100% ethanol (provided by the user) to the supplied bottle containing **38 mL** from the concentrated Wash Solution A. This will give a final volume of **128 mL**.
 - The labels on the bottles have a box that may be checked to indicate that the ethanol has been added.
- The use of β -mercaptoethanol in lysis is highly recommended to isolate RNA for sensitive downstream applications. Add 10 μ L of β -mercaptoethanol (provided by the user) to each 1 mL of **Lysis Buffer A**.
- Ensure that samples have not undergone more than one freeze-thaw cycle, as this may lead to RNA degradation.
- It is recommended to not work with samples that were hemolyzed as this will affect the RNA profile outcome.
- This kit is suitable for the isolation of RNA from serum or plasma prepared from blood collected on either EDTA or citrate. Plasma samples prepared from blood collected on heparin should not be used as heparin can significantly interfere with many downstream applications such as RT-PCR.
- Frozen plasma or serum samples should be centrifuged for 2 minutes at 400 x g (~2,000 RPM) before processing. Only clear supernatant should be processed, as column clogging may be encountered if frozen samples are directly processed.
- If any of the solutions do not go through the EXTRAClean Spin Columns within the specified centrifugation time, spin for an additional 1-2 minutes until the solution completely passes through the column. Do NOT exceed the centrifugation speed as this may affect RNA yield.

Note: The procedure outlined below is for processing 250 μ L to 1.5 mL inputs of Plasma/Serum. If the sample volume is lower than 1.5 mL Plasma/Serum, simply bring the volume of your sample up to 1.5 mL using Nuclease-free water and proceed as outlined below.

1. Place 1.5 mL of plasma/serum sample in a 15 mL tube (provided by the user) and add 4.5 mL of Lysis Buffer A. Mix well by vortexing for 10 seconds.
2. Add 3 mL of 100% Isopropanol (provided by the user). Mix well by vortexing for 10 seconds.
3. Transfer 4.5 mL of the mixture from Step 2 into an EXTRAClean Midi Spin Column assembled with one of the provided collection tubes. Centrifuge for 3 minutes at 1,000 x g (~2,200 RPM). Discard the flowthrough and reassemble the spin column with its collection tube. (Note: Make sure that lid of the tubes is not tightly closed during centrifugation).
4. Repeat Step 3 one more time until all the mixture from Step 2 has been transferred to the EXTRAClean Midi Spin Column.

5. Apply 3 mL of Wash Solution A to the column and centrifuge for 3 minutes at 1,000 x g (~2,200 RPM). Discard the flowthrough and reassemble the spin column with its collection tube. (Note: Make sure that lid of the tubes is not tightly closed during centrifugation).
 6. Repeat step 5 one more time, for a total of two washes.
 7. Spin the column, empty, for 3 minutes at 2,000 x g (~3,000 RPM). Discard the collection tube.
 8. Transfer the EXTRAClean Midi Spin column to a fresh 15 mL tube (not provided). Apply 400 µL of Elution Buffer F to the column and let stand at room temperature for 2 minutes. Centrifuge for 2 minutes at 500 x g (~1,600 RPM).
 9. Reload the eluted RNA from Step 8 back to the EXTRAClean Midi Spin Column and let stand at room temperature for 2 minutes. Centrifuge for 2 minutes at 500 x g (~1,600 RPM).
 10. To the elution from Step 9, add 300 µL of Lysis Buffer A and mix well by vortexing for 10 seconds.
 11. Add 400 µL of 96-100% Ethanol (provided by the user). Mix well by vortexing for 10 seconds.
 12. Transfer 700 µL of the mixture from Step 11 into an EXTRAClean Mini Spin Column assembled with one of the provided collection tubes. Centrifuge for 2 minutes at 3,300 x g (~6,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
 13. Repeat Step 12 one more time to transfer the remaining mixture into the EXTRAClean Mini Spin Column.
- Optional Step: An optional On-Column DNA Removal Protocol is provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol.
14. Apply 400µL of Wash Solution A to the column and centrifuge for 1 minute at 3,300 x g (~6,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
 15. Repeat Step 14 two more times, for a total of three washes.
 16. Spin the column, empty, for 2 minutes at 13,000 x g (~14,000 RPM). Discard the collection tube.
 17. Transfer the spin column to a fresh 1.7 mL Elution tube. Apply 50 µL of Elution Solution A to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 400 x g (~2,000 RPM), followed by 2 minutes at 5,800 x g (~8,000 RPM).
 18. For maximum recovery, transfer the eluted buffer back to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 400 x g (~2,000 RPM), followed by 2 minutes at 5,800 x g (~8,000 RPM).
 - Plasma/Serum RNA is ready for the downstream application of your choice. For an explanation of expected yields and recommendations for quantification of the RNA, please refer to Appendix B.

Appendix A

Protocol for Optional On-Column DNA Removal

Norgen's EXTRAClean Plasma/Serum RNA Purification Midi Kit isolates RNA with minimal amounts of genomic DNA contamination. However, an optional protocol is provided below for maximum removal of residual DNA that may affect sensitive downstream applications. It is recommended that Norgen's RNase-Free DNase I Kit (Product # 25710) be used for this step.

1. For every on-column reaction to be performed, prepare a mix of 15 µL of DNase I and 100 µL of Enzyme Incubation Buffer using Norgen's RNase-Free DNase I Kit (Product # 25710). Mix gently by inverting the tube a few times. DO NOT VORTEX.
 Note: If using an alternative DNase I, prepare a working stock of 0.25 Kunitz unit/µL RNase-free DNase I solution according to the manufacturer's instructions. A 100 µL aliquot is required for each column to be treated.
2. Perform the procedure up to Step 13.

3. Apply 400 μL of Wash Solution A to the column and centrifuge for 30 seconds at 6,000 RPM. Discard the flowthrough and reassemble the spin column with its collection tube.
4. Apply 100 μL of the RNase-free DNase I solution prepared in Step 1 to the column and centrifuge at 8,000 x g (~10,000 RPM) for 1 minute.
 Note: Ensure that the entire DNase I solution passes through the column. If needed, spin at 13,000 x g (~14,000 RPM) for an additional minute.
5. After the centrifugation in Step 4, pipette the flowthrough that is present in the collection tube back onto the top of the column.
 Note: Ensure Step 5 is performed in order to ensure maximum DNase activity and to obtain maximum yields of RNA, in particular for small RNA species.
6. Incubate the column assembly at 25 - 30°C for 15 minutes.
7. Without any further centrifugation, proceed directly to the second wash step in Step 15.

Appendix B

Cell-Free Circulating RNA Yield

Plasma/Serum RNA, like RNA in other cell-free bodily fluids, is normally found in very low amounts (1 - 100 pg/ μL), therefore measuring cell-free RNA concentration using common quantification methods is very difficult and challenging. Typical yields of plasma/serum RNA vary significantly from sample to sample. Variability is also observed between samples collected from the same donor at different times during the day and therefore there is no absolute yield for RNA purified from bodily fluids including plasma or serum. Cell-free circulating RNA yield varies depending on a number of factors including age, sex, diet, exercise and most importantly the health status of the donor.

Below is a list of the most common RNA quantification methods, as well as the limit of detection for each of these methods. **Unfortunately, none of these methods can be used reliably for measuring the concentration of RNA purified from plasma or serum unless large plasma/serum volumes have been processed.** This would only be applicable if plasma/serum contains the maximum amount of RNA that can fit within the specification range of these quantification tools. It should be noted that the specifications outlined below are based on measuring a pure RNA, which will not be the case for the RNA purified from plasma or serum. Plasma/Serum RNA is short fragmented RNA which is usually present in less than 1000 bp. Purified plasma/serum RNA usually contains traces of proteins which will interfere with most quantification methods, leading to the overestimation of the purified RNA concentration. Therefore, purified RNA contaminated with more proteins will be presented at a higher concentration as compared to RNA purified with less protein contaminants, which in this case will depend on the method used for plasma/serum RNA purification. **The only reliable method that can assess the quality and the relative quantity of the purified plasma/serum RNA is RT-qPCR amplification of a standard RNA using a small RNA amplicon such as the 5S rRNA housekeeping gene.**

Common RNA Quantification Methods

1) Bioanalyzer RNA Quantification Kits

	RNA 6000 Nano Kit		RNA 6000 Pico Kit		Small RNA kit
	Total RNA	mRNA	Total RNA	mRNA	Total RNA
Quantitative range	25 - 500 ng/ μL	25 - 250 ng/ μL	----	----	50-2000 pg/ μL
Qualitative range	5 - 500 ng/ μL	5 - 250 ng/ μL	50 - 5000 pg/ μL	250 - 5000 pg/ μL	50-2000 pg/ μL
Quantitation accuracy	20% CV	20% CV	30% CV	----	-----

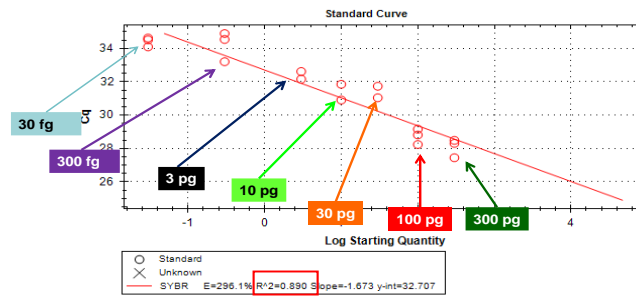
2) NanoDrop 2000

- Detection Limit: 2 ng/μL (dsDNA)

3) Quant-iT™ RiboGreen® RNA Assay Kit

- Quantitation Range: 1-200 ng

4) qPCR DNA Standard Curve (generated by Norgen)



Frequently Asked Questions

1. What if a variable speed centrifuge is not available?

- A fixed speed centrifuge can be used, however reduced yields may be observed.

2. At what temperature should I centrifuge my samples?

- All centrifugation steps are performed at room temperature. Centrifugation at 4°C will not adversely affect kit performance.

3. What if I added more or less of the specified reagents' volume?

- Adding more or less than the specified volumes may reduce both the quality and the quantity of the purified RNA. Eluting your RNA in high volumes will increase the yield but will lower the concentration. Eluting in small volumes will increase the concentration but will lower the overall yield.

4. What if I forgot to do a dry spin before my final elution step?

- Your purified DNA will be contaminated with the Wash Solution A. This may reduce the quality of your purified DNA and will interfere with your downstream applications.

5. Can I perform a second elution?

- Yes, but it is recommended that the 2nd elution be in a smaller volume (50% of 1st Elution). It is also recommended to perform the 2nd elution into a separate elution tube to avoid diluting the 1st elution.

6. Why do my samples show low RNA yield?

- Plasma/Serum samples contain very little RNA. This varies from individual to individual based on numerous variables. In order to increase the yield, the amount of Plasma/Serum input could be increased.

7. Why do the A260:280 ratio of the purified RNA is lower than 2.0?

- Most of the Free-Circulating Plasma/Serum RNA is short RNA fragments. The A260:280 ratio is normally between 1 – 1.6. This low A260:280 ratio will not affect any downstream application.

8. Why does my isolated RNA not perform well in downstream applications?

- If a different Elution Buffer was used other than the one provided in the kit, the buffer should be checked for any components that may interfere with the application. Common components that are known to interfere are high salts (including EDTA), detergents and other denaturants. Check the compatibility of your elution buffer with the intended use.

9. Do I need to do a DNase treatment for my RNA Elution?

- You may need to do a DNase treatment to your isolated Plasma/Serum miRNA. It is recommended to use Norgen's RNase-Free DNase I Kit (Cat# 25710). Also please refer to the protocol for optional on-column DNA removal outlined on **Page 6**.

Technical Support

Contact our Technical Support Team between the hours of 9:00 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at support@norgenbiotek.com.

Norgen's purification technology is patented and/or patent pending. See www.norgenbiotek.com/patents

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