

EXTRAClean Urine Cell-Free Circulating RNA Purification Midi Kit
Product Insert

Product # 73910

Introduction

Recent evidence indicates that cell-free circulating RNA (cfc-RNA), including exosomal RNA in urine, contains valuable information for the discovery of biomarkers that can help with early detection of certain cancer types and for monitoring the disease status. The advantage for using urine as a source for cancer biomarkers is that it can be acquired in large quantities without using invasive procedures. In addition, repeated sampling from the same individual is applicable, which facilitates longitudinal studies. There are many advantages favouring the use of urinary nucleic acids for cancer biomarker discovery over blood, tissue samples or other bodily fluids, including: (1) urine is non-infectious for HIV and less infectious for many other pathogens; (2) the profile of urinary nucleic acids is similar to that in plasma or serum; (3) nucleic acid purification from urine is technically much easier because of its low protein concentration (1000-fold lower than blood).

Norgen's Purification Technology

Norgen's EXTRAClean Urine Cell-Free Circulating RNA Purification Midi Kit provide a fast, reliable reproducible and simple procedure for isolating circulating RNA and exosomal RNA from various urine inputs ranging from 2 mL and up to 10 mL, with various kit formats addressing different urine 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 Urine Cell-Free Circulating RNA Purification Midi Kit provides a clear advantage over other available kits in that they do not require phenol/chloroform or any protease treatments. Moreover, the kit allows the user to elute into a flexible elution volume ranging from 50 µL to 100 µL. The purified RNA is of the highest integrity, and can be used in a number of downstream applications including real time PCR, reverse transcription PCR, Northern blotting, RNase protection and primer extension, and expression array assays.

Advantages

- Versatile urine input ranges
- No phenol extractions
- No carrier RNA
- Bind and elute all RNA irrespective of size or GC content, without bias
- Concentrate circulating RNA and exosomal RNA into flexible elution volumes
- EXTRAClean resin separation matrix provides highly purified RNA

Kit Components

Component	Midi Kit (Cat. #73910)
Number of Preps	20 preps
Binding Solution K	75 mL
Lysis Buffer A	20 mL
Wash Solution A	18 mL
Elution Solution A	6 mL
EXTRAClean Mini Spin Columns	20
EXTRAClean Midi Spin Column	20
Collection Tubes	20
Elution tubes (1.7 mL)	20
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These kits are suitable for the isolation of cfc-RNA from fresh urine samples, frozen urine samples or urine samples collected on most urine preservative.

It has been noticed that urine samples stored at -70°C, -20°C or at 4°C will develop some precipitation due to the aggregation of some of the highly abundant proteins in urine. Eliminating these precipitates using centrifugation or filtration may cause the loss of some of the cfc protein-bound RNA. Furthermore, these precipitates may affect the quality of the purified nucleic acid. We recommend the use of Norgen's Urine Preservative when collecting urine samples. Norgen's Urine Preservative is designed for the preservation of nucleic acids and proteins in fresh urine samples at ambient temperatures, therefore no protein precipitation will occur and the purified nucleic acids will be of a higher quality. The components of the Urine Preservative allow samples to be stored for over 2 years at room temperature with no detected degradation of urine DNA, RNA or proteins. Norgen's Urine Preservative is available as a liquid format in Norgen's Urine Preservative Single Dose Ampules, as well as in a dried format in Norgen's Urine Collection and Preservation Tubes

Specifications

Kit Specifications	
Sample Type	Fresh or frozen urine
Sample volume Range	2 to 10 mL
Minimum Elution Volume	50 µL
Maximum Elution Volume	100 µL
Time to Complete 10 Purifications	40 - 45 minutes
Size of RNA Purified	All sizes, including miRNA and small RNA (< 200 nt)
Average Yields ¥	Variable depending on specimen

¥ Please check page 6 for Average Urine Yields and Common RNA Quantification Methods

Storage Conditions and Product Stability

All buffers should be kept tightly sealed and stored at room temperature. These kits are 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 and Binding Solution K 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 Equipment

- 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 is 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.
- Most standard swinging bucket centrifuges will accommodate Norgen's EXTRAClean Midi Spin Columns. Do not use a fixed-angle rotor
- Norgen's EXTRAClean Midi Spin Columns are centrifuged in 15 mL and 50 mL centrifuge tubes, respectively.
- 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 by adding 42 mL of 96 - 100% ethanol (provided by the user) to the supplied bottle containing 18mL of concentrated Wash Solution A. This will give a final volume of 60 mL. The label on the bottle has 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.
- 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.

Preparation of Cell-free Urine Sample

1. Collect and transfer 15-50 mL of the urine into a conical tube and centrifuge at 200 x g (~1,000 RPM) for 10 minutes to remove urine exfoliated cells and debris. Decant cell-free urine into new 15-50 mL conical tube.
2. Centrifuge the cell-free urine at 1,800 x g (~3,000 RPM) for 10 minutes to remove any residual debris or bacterial cells.
3. Transfer cell-free urine into a fresh 15-50 mL conical tube.
 - Cell-Free Urine is now ready for the purification of cell-free RNA

Note: The procedure outlined below is for 10 mL inputs of urine. If processing a sample volume lower than 10 mL urine, simply bring the volume of your samples up to 10 mL using Nuclease-free water and proceed as outlined below.

1. Place 10 mL of cell-free urine in a 50 mL tube (provided by the user). Add 3 mL Binding Solution K and mix well by vortexing for 10 seconds.
2. Transfer 4.5 mL of the mixture from Step 1 into a 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.
3. Repeat Step 2 two more times to transfer the remaining mixture into the EXTRAClean Midi Spin Column.
4. Apply 400 μ L of Lysis Buffer A to the column and centrifuge for 3 minutes at 500 x g (~1,600 RPM). Do Not Discard the flowthrough.
5. Apply an additional 400 μ L of Lysis Buffer A to the column and centrifuge for 3 minutes at 500 x g (~1,600 RPM). Do Not Discard the flowthrough.
6. Reload the 800 μ L flowthrough from Step 5 back to the column and let stand at room temperature for 20 minutes. Centrifuge for 3 minutes at 1,000 x g (~2,200 RPM). Do Not Discard the flowthrough.
7. To the flowthrough from Step 6 add 400 μ L 100% Isopropanol and mix well by pipetting.
8. Transfer 600 μ L of the mixture from Step 7 to an EXTRAClean Mini Spin column assembled with one of the provided collection tubes. Centrifuge for 2 minutes at 3,300 x g (~7,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.

9. Repeat Step 8 one more time to transfer the remaining mixture into the EXTRAClean Mini Spin Column.
10. Apply 600 μ L of Wash Solution A to the column and centrifuge for 1 minute at 3,300 x g (~7,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
11. Repeat Step 10 one more time, for a total of two washes.
12. Spin the column, empty, for 2 minutes at 14,000 x g (~14,000 RPM). Discard the collection tube.
13. Transfer the spin column to a fresh 1.7 mL Elution tube. Apply 50 μ L of Elution Solution A to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 200 x g (~2,000 RPM), followed by 2 minutes at 5,200 x g (~8,000 RPM).
14. 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).
 - Urine cfc-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 A.

Appendix A

Cell-Free Circulating RNA Yield

Urine cfc-RNA, like RNA in other cell-free bodily fluids, is normally found in very low amounts (1 - 100 pg/ μ L), therefore measuring cell-free RNA concentration using common quantification methods is very difficult and challenging. Typical yields of urine cfc-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 urine. 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 urine unless large urine volumes have been processed.** This would only be applicable if urine 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 urine. Urine RNA is short fragmented RNA which is usually present in less than 1000 bp. Purified urine 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 urine RNA purification. **The only reliable method that can assess the quality and the relative quantity of the purified urine 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/ μ L	25 - 250 ng/ μ L	----	----	50-2000 pg/ μ L
Qualitative range	5 - 500 ng/ μ L	5 - 250 ng/ μ L	50 - 5000 pg/ μ L	250 - 5000 pg/ μ L	50-2000 pg/ μ L
Quantitation accuracy	20% CV	20% CV	30% CV	----	-----

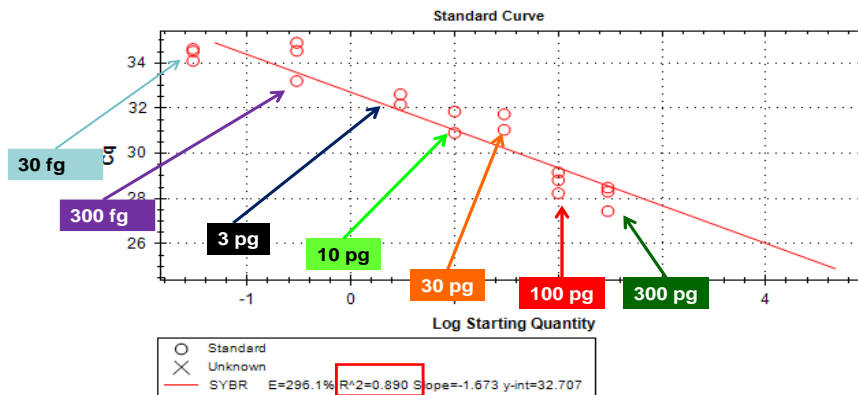
2) NanoDrop 2000

- Detection Limit: 2 ng/ μ l (dsDNA)

3) Quant-iT™ RiboGreen® RNA Assay Kit

- Quantitation Range: 1-200 ng

4) qPCR 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 RNA will be contaminated with the Wash Solution A. This may reduce the quality of your purified RNA 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?

- Urine samples contain very little RNA. This varies from individual to individual based on numerous variables. In order to increase the yield, the amount of urine 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 Urine 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.

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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