NORGEN BIOTEK CORPORATION

3430 Schmon Parkway
Thorold, ON, Canada L2V 4Y6
Phone: 866-667-4362 • (905) 227-8848
Fax: (905) 227-1061
Email: techsupport@norgenbiotek.com

Plasma/Serum Total cfc-Nucleic Acid Advanced Purification Kit
Product Insert

Product # 68100

Introduction

Cell-free circulating RNA (cfc-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 cfc-RNA, including exosomal RNA, 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.

Cell-Free Circulating DNA (cfc-DNA) resulting from apoptosis, and circulating tumour DNA (ct-DNA) released due to the apoptosis and/or the necrosis of tumour cells, has been found in blood plasma/serum and other bodily fluids. Analyzing cfc-DNA and/or ct-DNA from plasma has now become an important tool in the oncology field for the analysis of the molecular changes taking place during the development or the progress of cancerous cells. Cfc-DNA/ct-DNA has the potential to provide biomarkers for certain cancers and disease states as well as fetal DNA in maternal blood. Corresponding to the size of chromatosome (core histones + linker), cfc-DNA is mostly found in plasma around the size of 167 bp, whereas ct-DNA is usually found around the size of 145 bp. Ct-DNA, in the plasma acquired from cancer patents, accounts for ~10% of the total cfc-DNA which is normally present very low concentrations. Most current plasma/serum cfc-DNA/ct-DNA isolation methods isolate total DNA from plasma or serum with high genomic DNA (gDNA) contamination that usually results due to WBC lysis during blood collection, plasma/serum preparation and/or storage/preservation/transport of blood samples. This gDNA contamination will mask cfc-DNA/ct-DNA normally present in low concentration leading to unreliable results. In addition, cell-free fetal DNA has been widely used as a non-invasive method for prenatal diagnosis including early identification of fetal sex, genetic studies for families at high risk for inherited genetic disorders, screening for Rhesus factor, screening for aneuploidy and identification of preeclampsia.

Norgen's Plasma/Serum Total cfc-Nucleic Acid Advanced Purification Kit provides fast, reliable and simple procedures for isolating the highest quality and quantity of all sizes of circulating DNA and circulating RNA from various amounts of plasma/serum ranging from 1 mL up to 6 mL combined into one elution. Purification is based on using Norgen's proprietary resin separation matrix. The kits are designed to isolate total cfc-nucleic acid from either fresh, preserved or frozen plasma/serum samples. Moreover, these kits allow the user to elute the purified cfc-RNA into a flexible elution volume ranging from 25 µL to 50 µL. The purified plasma/serum total cfc-nucleic acid is eluted in an Elution Solution that is compatible with all downstream applications including PCR, qPCR, methylation-sensitive PCR and Southern Blot analysis, microarrays and NGS.

The kit is compatible with the isolation of total cfc-Nucleic Acid from fresh, preserved or frozen serum/plasma prepared from blood collected on either Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950, 63960), 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 Appendix A for Average Plasma/Serum Yields and Common Total Nucleic acid Quantification Methods.

Component	Product # 68100 (50 preps)
Binding Buffer A	20 mL
Proteinase K	6.5 mL
Slurry E	12.5 mL
Lysis Buffer A	130 mL
Wash Solution A	38 mL
Elution Buffer C	2 x 8 mL
Elution Buffer F	15 mL
Elution Solution A	20 mL
Micro Spin Columns	50
Mini Filter Spin Column	50
Collection Tubes	100
Elution tubes (1.7 mL)	100
Product Insert	1

Customer-Supplied Reagents and Equipment for Manual Isolation

- Benchtop microcentrifuge
- Swinging bucket centrifuge
- Micropipettors
- 15 mL tubes
- 96 – 100% ethanol

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. The kit contains a ready-to-use Proteinase K, which is dissolved in a specially prepared storage buffer. The buffered Proteinase K is stable for up to 2 years after the date of shipment when stored at room temperature.

Quality Control

In accordance with Norgen's Quality Management System, each lot of Norgen's Plasma/Serum Total cfc-Nucleic Acid Advanced Purification Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's Plasma/Serum Total CFC-Nucleic Acid Advanced Purification Kit is designed for research purposes only. It is not intended for diagnostic use.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Safety Information

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 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.

Plasma or serum of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with plasma or serum.

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

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

Important Notes

- All centrifugation steps are performed at room temperature.
- Ensure that centrifuge tubes used are capable of withstanding the centrifugal forces required.
- Most standard benchtop microcentrifuges will accommodate Norgen's Micro and Mini Spin Columns.
- Centrifuging Norgen's spin columns at a speed lower than recommended in the procedure will not affect nucleic acid yield. However, centrifugation at a lower speed may require longer time for the solutions to pass through the spin column
- 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.
- Ensure that all solutions are at room temperature prior to use, and that no precipitates have formed. If necessary, warm the solutions and mix well until the solutions become clear again.
- Prepare a working concentration of the **Wash Solution A** by adding **90 mL of 96 - 100% ethanol** (provided by the user) to the supplied bottle containing the **38 mL** concentrated **Wash Solution A**. This will give a final volume of 128 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added
- Ensure that samples have not undergone more than one freeze-thaw cycle, as this may lead to RNA loss.
- Always **vortex** the **Proteinase K** before use.
- It is highly recommended to warm **Lysis Buffer A** at 60ºC for 20 minutes and mix well until the solutions become clear again if precipitates (crystals) are present.
- ***The kit is compatible with the isolation of cfc-RNA from fresh, preserved or frozen serum/plasma prepared from blood collected on either Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950, 63960), 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.***
- ***If any of the solutions do not go through the Spin Columns within the specified centrifugation time, spin for an additional 1-2 minutes at maximum speed until the solution completely passes through the column.***
- ***Frozen plasma (from Blood collected on EDTA or Citrate Tubes) 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.***

- ***VERY IMPORTANT! Frozen plasma recovered from Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950, 63960) may contain some precipitates upon thawing. DO NOT discard any precipitates before cfc-RNA purification. Briefly vortex the plasma and proceed immediately for cfc-RNA purification. Discarding any precipitates may significantly lower cfc-DNA yield.***

Total cfc-Nucleic Acid Purification from 0.5 mL up to 6 mL Plasma/Serum

Important Notes:

- This procedure as written is for processing 1 mL Plasma/Serum samples. To process a different Plasma/Serum volume higher than 1 mL, please check Table 1 for the appropriate volumes of Binding Buffer A, Elution Buffer C and Proteinase K.
- To process a different Plasma/Serum volume lower than 1 mL, please bring up the volume of your sample up to 1 mL using 1X PBS (pH 7.4) then follow the procedure outline below.
- Please refer to the notes in the Important Notes section for the pre-treatment of frozen plasma/serum samples

1. Place 1 mL of plasma/serum sample in a 15 mL tube (provided by the user) followed by the addition of 50 µL **Binding Buffer A**. Mix well by vortexing for 20 seconds. Incubate for 5 minutes at room temperature
2. After incubation, centrifuge for **1 minute** at **50 x g (~500 RPM)**. Discard completely the supernatant.
3. Add 34 µL **Elution Buffer C to** the pellet resulting from **Step 2.** Mix well by vortexing for 30 seconds. **(*Note: The pellet resulting from Step 2 must be completely resuspended in Elution Buffer C*)**
4. Add 17 µL of **Proteinase K** to the resuspended pellet**.** Mix well by vortexing for 10 seconds then incubate at **room temperature for 30 minutes** with shaking slowly end-over-end.
5. After incubation, add 200 µL **Slurry E** followed by the addition 1.5 mL of **Lysis Buffer A,** and mix well by vortexing for 10 seconds. ***(Note*: *Slurry E contains resin and must be mixed well before every pipeting)***. Incubate for 2 minutes at room temperature.
6. After incubation add 2 mL **96-100% Ethanol** and mix well by vortexing for 10 seconds.
7. Incubate for 2 minutes at room temperature then mix well by vortexing for 10 seconds.
8. Transfer 700 µL of the mixture from **Step 7** into a Mini Spin column assembled with one of the provided collection tubes. Centrifuge for **30 seconds at 13,000 x g (~14,000 RPM)**. Discard the flowthrough and reassemble the spin column with its collection tube.
9. Repeat **Step 8** until the entire mixture from **Step 7** is transferred into the Mini Filter Spin column.
10. Apply 600 µL of **Wash Solution A** to the column and centrifuge for **30 seconds at 13,000 x g (~14,000 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 13,000 *x g* (~14,000 RPM)**. Discard the collection tube.
13. Transfer the spin column to a fresh 1.7 mL Elution tube. Apply 250 µL of **Elution Solution F** 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)**.
14. Add 500 µL of **Lysis Buffer A** to the eluted RNA**,** and mix well by vortexing for 10 seconds
15. Add 750 µL **96-100% Ethanol** and mix well by vortexing for 10 seconds.
16. Transfer 500 µL of the mixture into a Micro Spin column assembled with one of the provided collection tubes. Centrifuge for **1 minute at 3,300 *x g* (~6,000 RPM)**. Discard the flowthrough and reassemble the spin column with its collection tube.
17. Repeat **Step 16** one more time to transfer the remaining mixture from **Step 15**.
18. Apply 600 µL 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.
19. Repeat **Step 18** one more time, for a total of two washes.
20. Spin the column, empty, for **2 minutes at 13,000 *x g* (~14,000 RPM)**. Discard the collection tube.
21. Transfer the spin column to a fresh 1.7 mL Elution tube. Apply 25 - 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)**.

- ***Plasma/Serum Total Nucleic acid is ready for the downstream application of your choice.***

Table 1. Binding Buffer A, Elution Buffer C and Proteinase K to be added to different Plasma/Serum Sample Volumes

Sample Volume (mL)	Binding Buffer A (µL) (Step 1)	Elution Buffer C (µL) (Step 3)	Proteinase K (µL) (Step 4)
1.5	75	50	26
2	100	70	34
2.5	125	85	43
3	150	100	51
3.5	175	120	60
4	200	135	68
4.5	225	155	77
5	250	170	85
5.5	275	185	90
6	300	200	100

Appendix A

Cell-Free Circulating RNA and DNA 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

Plasma/Serum Cell-free circulating DNA (cfc-DNA) is normally found in very low amounts (1 - 100 pg/µL), therefore measuring cfc-DNA concentration using common DNA quantification methods is very difficult and challenging. Typical yields of cfc-DNA 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 cfc-DNA purified from bodily fluids including plasma or serum. Cell-free circulating DNA 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 and DNA 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 and/or DNA 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/DNA 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 and/or DNA. Plasma/Serum RNA is short fragmented RNA which is usually present in less than 1000 bp. Plasma/Serum DNA is short fragmented DNA which is usually present in less than 1000 bp. Purified plasma/serum RNA and/or DNA usually contains traces of proteins which will interfere with most quantification methods, leading to the overestimation of the purified nucleic acid concentration. Therefore purified nucleic acid contaminated with more proteins will be presented at a higher concentration as compared to nucleic acid purified with less protein contaminants, which in this case will depend on the method used for plasma/serum total nucleic acid purification. ***The only reliable method that can assess the quality and the relative quantity of the purified plasma/serum RNA and/or DNA is PCR amplification of a small RNA/DNA amplicon such as the 5S rRNA housekeeping gene***.

Common Nucleic Acid Quantification Methods

1) 2100 Bioanalyzer 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
Qualititative 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	----	------

	DNA 1000 Kit	DNA 7500 Kit	DNA 12000 Kit	High Sensitivity DNA Kit
Size Range	**25–1000 bp**	**100–7500 bp**	**100–12000 bp**	**50-7000 bp**
Quantitation accuracy	**20% CV***	**20% CV***	**25% CV***	**20% CV**
Quantitative range	**0.5-50 ng/µL**	**0.5-50 ng/µL**	**0.5-50 ng/µL**	**5-500 pg/µL**

2) NanoDrop 2000

- Detection Limit: 2 ng/µl (dsDNA)

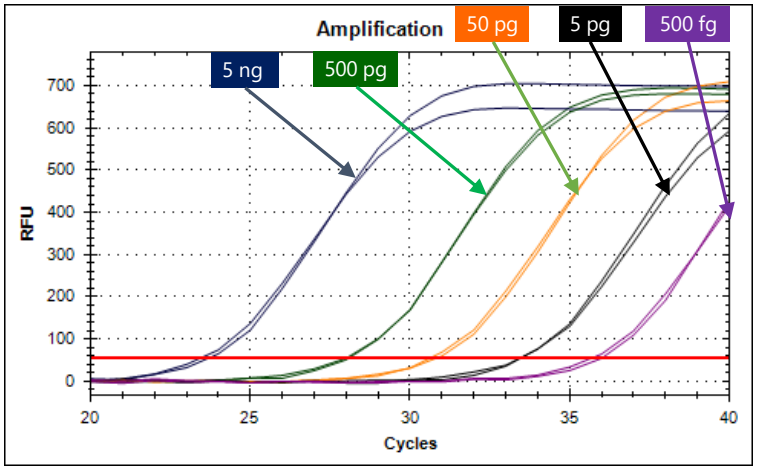
3) Quant-iT™ RiboGreen® RNA Assay Kit

- Quantitation Range: 1-200 ng

4) Quant-iT™ Pico Green® dsDNA Assay Kit

- Quantitation Range: 25 pg/mL

4) qPCR RNA Standard Curve
(Generated using Norgen's Low Abundance RNA Quantification Kit Cat# 58900))
(Generated using Norgen's Low Abundance DNA Quantification Kit Cat# 57200))



Frequently Asked Questions

1. **What if a variable speed centrifuge is not available and the speed differs from the recommended?**
   - 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 Nucleic Acid 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 Nucleic Acid will be contaminated with the Wash Solution A. This may reduce the quality of your purified Nucleic Acid 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. What if my incubation time varied from what is specified in the product manual?

- Varying the incubation time will result in a reduction in your Nucleic Acid yields.

7. Why do my samples show very low RNA yield?

- Plasma/Serum samples contain very little cfc-Nucleic Acid. This varies from individual to individual. In order to increase the yield, the amount of Plasma/Serum input could be increased.

8. Why does my purified cfc- Nucleic Acid not perform well in downstream applications?

- If a different Elution solution 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 Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Plasma/Serum Cell-Free Circulating RNA an DNA Purification Kits or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362. or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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

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