Tech Note for nCounter® miRNA Expression Analysis in Plasma and Serum Samples

Introduction

nCounter® miRNA Expression Assays allow users to rapidly and efficiently profile hundreds of miRNAs simultaneously. When appropriate sample handling and data analysis guidelines are implemented, nCounter miRNA assays can generate reliable data from plasma and serum samples. This Tech Note will define the current challenges associated with miRNA studies in blood plasma and serum samples, point out steps in the processing of collected blood which can have an impact on sample quality, and elucidate the ways in which variables in sample preparation can be controlled to produce reliable data using nCounter miRNA Assays. There are two broad categories of considerations that must be accounted for when performing miRNA analysis of serum and plasma samples: 1) sample contaminants that can confound results and 2) the generally low abundance of miRNA in plasma and serum. TABLE 1 provides a brief synopsis of key considerations and recommendations which are described in detail throughout this tech note.

TABLE 1. Key considerations and recommendations.

Consideration	Guidance for Plasma	Guidance for Serum
Contaminants		
Sample Collection Tube	• EDTA tubes recommended • Citrate tubes acceptable	• Gel separation recommended • Gel-free separation acceptable
Hemolysis	Minimize hemolysis, inspect each sample for hemolysis prior to RNA extraction	
Cellular Contamination	Evaluate results to determine if elevated levels of cellular miRNAs (mir-451, miR-16, miR-25, miR-106, miR-7g) or the housekeeping gene, B2M, is elevated.	
	Minimize cellular contamination by using gel separation tubes	
Low miRNA Abundance		
Initial sample volume	Add a 400 µL equivalent* of initial sample to the hybridization.	
Pre-extraction Spike-Ins	Spike-in synthetic targets to control for variability in RNA extraction efficiency	
Post-Extraction Enrichment	Concentrate eluted miRNA with a size exclusion filter (~3 kDa MW cutoff). This step also minimizes contamination.	

**In this Tech Note, an "equivalent" is defined as the volume of final, purified RNA that would correspond to an initial volume of plasma or serum. For example, if an RNA extraction was performed on 500 µL of plasma via an extraction method with 80% yield and half of the eluted material were used in the hybridization, the input to the hyb would be a 200 µL equivalent (500 µL x 0.8 x 0.5 = 200 µL).*

Background

There are many considerations that researchers should be aware of prior to undertaking sample collection and isolation of miRNAs for any downstream miRNA profiling technology (Becker N & Lockwood CM, 2013). Whole blood is a complex tissue containing red blood cells, white blood cells, platelets, macrophages, and trace numbers of displaced cells, such as circulating tumor cells. Plasma and serum are the cell-free portions of blood and therefore contain very little nucleic acid material. Indeed, NanoString's 100 ng total RNA recommendation for standard miRNA profiling of tissues and cells assumes an RNA profile similar to that observed in most tissues, *i.e.,* about 90% of the RNA is ribosomal, 9–10% is mRNA and less than 1% is small RNA. Samples such as serum/plasma should be devoid of rRNA and mRNA, thereby reducing the overall amount of total RNA. The quantity of RNA extracted from plasma and serum is often below threshold amounts required for reliable information from standard RNA quality control methods and quantitation methods, such as absorbance (OD) measurements or the Agilent Bioanalyzer. It is therefore difficult to appropriately QC and thus assure that samples meet recommended purity guidelines and are free of chaotropic salts/phenol that can inhibit the ligation reaction. Furthermore, plasma and serum samples contain a high concentration of nucleases. These nucleases ensure that very little RNA will be present in cell-free blood samples, making extraction of measurable volumes of miRNA from plasma and serum technically challenging. Herein, we provide some guidance on overcoming the challenges of low sample input and enzyme inhibition when utilizing the nCounter system.

Blood Collection Guidance

One of the major factors to consider prior to embarking on any miRNA profiling study using serum and plasma samples is the blood collection method of choice, including the specific type of collection tube utilized. Generally, blood samples are collected as serum or plasma. Serum, in which the blood is allowed to coagulate before centrifugal separation, is essentially blood with all of the cells and clotting factors removed. Plasma is treated with an anticoagulant additive and whole cells are centrifugally separated, leaving the clotting factors present, but inhibited (reversibly or irreversibly, depending on the anticoagulant). Laboratory anticoagulants, such as EDTA, citrate, and oxalate act by chelating calcium. Another common anticoagulant, heparin, works via antithrombin activation.

Both plasma and serum can produce robust results in the nCounter miRNA Assay, and using either EDTA (K2 or K3) or citrate as the plasma anticoagulant is acceptable. While the overall pattern of expression for most miRNAs is the same, small discrepancies in relative expression levels can be seen with theoretically very similar tube types for some miRNAs, a phenomenon that has been previously observed (Kroh EM, *et al*., 2010). It should be noted that citrate plasma tubes in general produce a lower number of overall miRNA counts compared to EDTA. NanoString recommends that data generated from different collection tubes not be compared directly (TABLE 2); this is true not just for citrate plasma, but also serum and EDTA plasma (see FIGURE 1).

FIGURE 1. Log_2 counts for 52 individual miRNAs are displayed for three sample collection tube types, K2 EDTA plasma (orange), K3 EDTA plasma (gray), citrate plasma (red). When citrate counts are normalized to align with K2 EDTA counts (pink), there is significant overlap of data generated by the three types of tubes. miRNAs are listed across the x-axis and Log_2 counts are plotted along the y-axis. RNA was prepared from fractionated plasma. In general, Citrate plasma tubes produce lower counts overall than other collection methods.

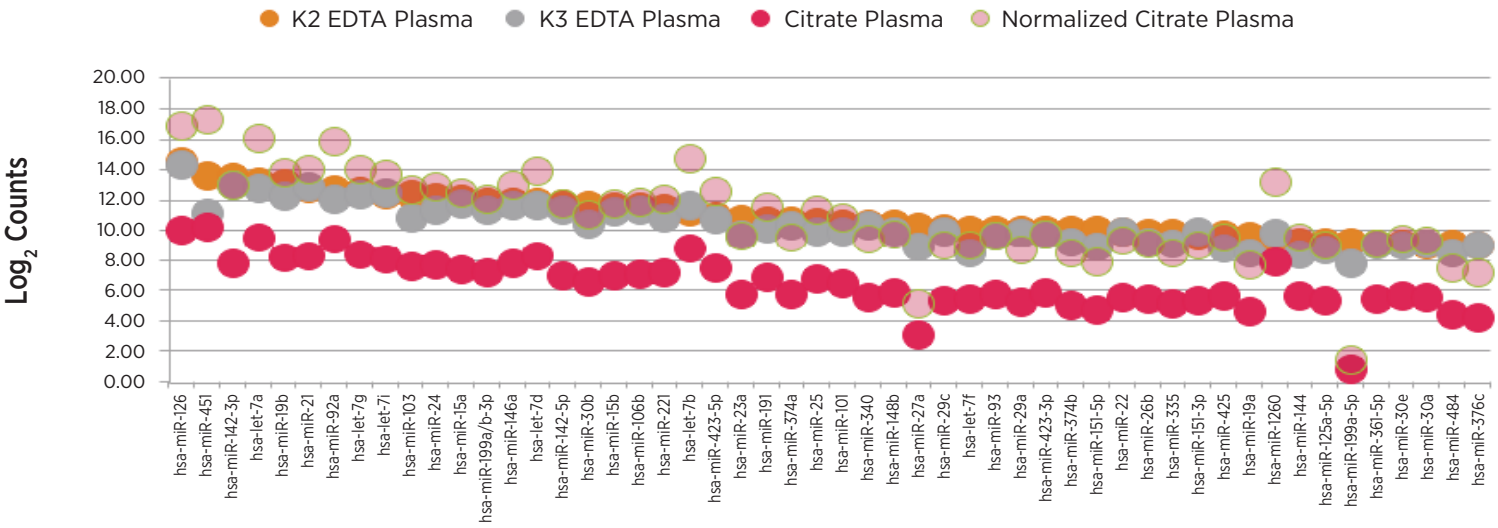

TABLE 2. The number of miRNAs detected above background and average counts for a variety of serum and plasma collection tubes.

Collection Tube	miRNAs above background	Avg Counts for miRNAs above background
K2EDTA	144	2,531
K3EDTA	142	2,281
Citrate	59	585
Serum, no gel	35	398
Serum, gel	54	441

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There are some anticoagulants and other additives which have been found to inhibit the ligation step in the nCounter miRNA Assay. These include citrate tubes containing SPS (sodium polyanethol sulfonate, used for blood culturing), and heparin tubes. Heparinase treatment of samples to reduce endogenous heparin can improve results, however, whenever possible, NanoString does not recommend the use of heparin tubes. Tubes with sodium fluoride and oxalate can improve yield of some miRNAs, but are also expected to decrease performance of the nCounter miRNA Assay.

Hemolysis of plasma and serum samples during collection can significantly alter the overall miRNA content and therefore should be taken into consideration. All of the circulating cell types in blood have their own unique miRNA profiles and given the low concentration of miRNAs in plasma or serum, RNA from a small number of lysed cells can represent a disproportionately large proportion of the miRNAs detected. Cellular miRNA contamination due to lysis can vary from sample to sample if the blood is not collected and processed promptly and carefully, and confound downstream data analysis (Kim DJ *et al.*, 2012).

To minimize cellular contamination after serum collection, allow blood to coagulate for an hour at room temperature (RT). Spin at 10,000g for 10 min at RT, collect serum, snap freeze, and store at -80°C (**NOTE:** A gel-based separation can help to minimize cellular RNA contamination). At this point, it is important to note the color of the serum. If hemolysis has occurred, indicated by a red/pink hue, signal levels for cellular miRNAs such as miR-451, miR-16, miR-25, miR-106, and miR-7g are likely to be elevated (Kirschner MB, *et al.*, 2011).

Addition of Synthetic Spike-Ins

To control for variances in the starting material as well as the efficiency of the downstream total RNA extraction step, NanoString recommends the use of synthetic control RNA spike-ins. All NanoString miRNA CodeSets are "spike-in ready" in that they contain probes for a number of recommended spike-in miRNAs (for example, the Human v2 miRNA Panel contains 5 spike-in probes: ath-miR-159a, cel-miR-248, cel-miR-254, osa-miR-414, osa-miR-442). Spike-in oligos can be ordered from IDT Technologies or other oligo vendors (unmodified, HPLC, or gel purified), aliquoted and stored at -20°C at 1–5 pg/µL.

Following the lysis procedures specified by the RNA extraction protocol, we recommend adding 5 µL spike-in oligo mixture containing an optimized amount of synthetic RNA oligos (**BOX 1**). Please consult with **support@nanostring.com** for appropriate guidance.

BOX 1. Optimal Spike-in Concentration

NanoString recommends the use of small RNA spike-ins as means of assessing miRNA extraction efficiency from biofluids. Adding a known amount of a measurable target molecule allows for downstream normalization of data to account for any differences in purification efficiency observed from sample to sample. In order to serve as a monitor of this process, small RNA spike-ins must be added to a sample after lysis, but prior to extraction/purification.

NanoString recommends that 15–30 attomoles of a spike-in molecule be present in the final hybridization reaction with the nCounter miRNA CodeSet. As nCounter miRNA detection does not involve amplification, the amount of spike-in oligo needed to be added to a lysed sample must be carefully controlled and take into account the following information:

1. The elution volume of the purified sample
2. The fraction of the eluted sample profiled with the nCounter miRNA Expression Assay

For example:

If 500 µL of plasma is expected to be lysed for the extraction protocol and the protocol results in a final elution volume of 15 µL, of which 3 µL will be assayed in a nCounter miRNA Expression Assay, then the number of spike-in molecules that should be added to the sample is 1000 attomoles and can be derived by the following formula:

Elution Volume ÷ Amount of sample added to nCounter miRNA Sample Preparation x Post-ligation dilution factor* x Target number of molecules in a hybridization

15 µL/3 µL x 10 x 20 attomoles = 1000 attomoles

This means that 1000 attomoles (or 5 µL of a 200 pM solution) should be added to the 500 µL of plasma to be extracted after addition of a lysis buffer. NanoString does not recommend direct addition of a spike-in to a sample prior to addition of lysis buffer as degradation of the spike-in may occur.

** This accounts for a 1:10 dilution that is part of the nCounter miRNA analysis protocol*

The spike-in controls will help normalize for extraction efficiency by correcting for the variance between samples due to slight variations in the extraction of miRNA following addition of lysis buffer to the sample, but will not normalize for variation due to input amounts of miRNA which can vary due to differences in starting amount or biological differences.

FIGURE 3 below displays the improved coefficient of variation (CV) between replicate samples when a spike-in normalization step is performed. Un-normalized samples exhibit a CV of 20%, normalization of the samples improves this measure to 5%.

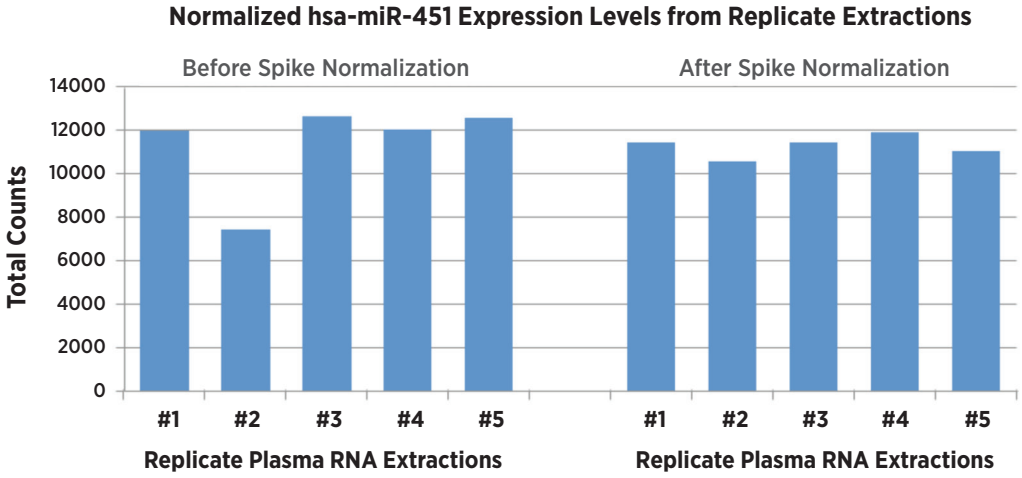


FIGURE 3. Spike Normalization Reduces Percent CV from 20% to 5%. Use of miRNA spike-ins for normalization improves Coefficient of Variation between replicate samples. Samples 1–5 are EDTA plasma spiked with synthetic miR-10b and miR-122, split into 5 aliquots and extracted in parallel. The x-axis represents the different samples. Total counts for hsa-miR-451 are plotted on the y-axis.

Total RNA Extraction and Isolation

The nCounter miRNA Assays are compatible with miRNA extracted from a variety of extraction methods/kits. Generally, 400 µL of plasma or serum should be enough to produce significant counts, but robust detection of some miRNAs may require larger volumes. There may be a diminishing return with increased sample volume when samples are prepared on commercial miRNA extraction columns, likely due to finite binding capacity of the column matrix. Contact your miRNA extraction kit manufacturer for column-specific binding capacity and sample volume guidelines.

Commercial miRNA extraction mini-columns are an easy way to reliably obtain clean miRNA samples from plasma and serum, typically by first removing proteins in an organic phase separation, and then binding and washing nucleic acids on a silica-based column. As stated previously, circulating miRNAs are relatively low in concentration and extraction yields frequently approach the limit of UV/vis spectrophotometric detection, therefore the typical cautions regarding sample cleanup using mini-columns (see *nCounter miRNA Assay Manual*) are of even greater importance in reliably producing clean miRNA. The primary issue with mini-columns is carryover from the extraction process contaminating the samples, which can both complicate spectrophotometric concentration determination and inhibit ligation in the NanoString miRNA assay. Some ways to avoid contamination are:

a. Perform the organic phase separation carefully (if there is one in the kit you choose) and avoid phenol carryover. Phenol has a spectrophotometric profile similar to nucleic acids, making it difficult to determine the concentration of the sample, or whether the extraction was successful at all.

b. Wash the column thoroughly, and then completely remove the wash buffers before eluting the RNA. Guanidinium carryover from the binding buffer will inhibit assay ligation, and can also obfuscate spectrophotometric concentration determinations. Adding 2 extra washes with the last wash buffer or 80% ethanol will reduce this carryover significantly.

c. Elute in a larger volume (*e.g.*, 100–200 µL) and then reduce the volume to 20–25 µL; this can be accomplished by either an ethanol/sodium acetate precipitation or using a size-exclusion filter with a small pore size (~3 kDa MW cutoff). This will affect maximum recovery from the extraction column and then both concentrate the sample to increase count data and wash away the buffer residues that might inhibit detection.

As with blood collection tube choice, an important factor for collecting comparable data is consistency in the choice of RNA extraction kit. Different extraction kits have varying efficiency at extracting different miRNAs, often resulting in variances in miRNA expression profiles (Pritchard CC. *et al.*, 2012, Blondal T *et al.*, 2012, Podolska A *et al.*, 2012, Jacob NK *et al.*, 2013). NanoString has observed that counts measured for individual miRNAs can differ between extraction kits (data not shown). Therefore we recommend utilizing a single extraction kit and buffer throughout a study where direct comparisons of miRNA counts are intended.

Data Analysis and Normalization

All data analysis and normalization can be performed using the nSolver™ Analysis Software (available by complimentary download from NanoString Technologies). There are several options for normalization in nSolver, in which specific miRNA counts are normalized to a selection of stably-expressed miRNAs based on CVs calculated across all experimental samples or with the use of the spike-in controls. Normalization options include:

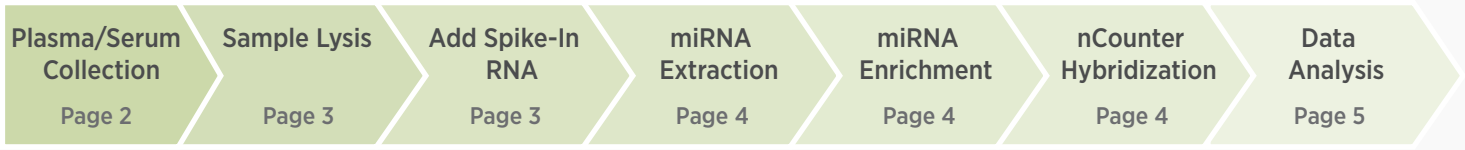
1. Calculate the Geometric mean of the top 100 miRNAs in all samples, effectively normalizing relative to total miRNA present (recommended).
2. Calculate the Geometric mean of user selected miRNA(s) normalizing all samples relative to the selected miRNA(s). Requires miRNA(s) with constant expression. Not widely used due to lack of widely-recognized housekeeping miRNAs.
3. Calculate the Geometric mean of Housekeeping mRNAs that is not ideal but can be used as a surrogate for sample quantity.
4. Calculate the Geometric mean of ligation control probes normalizing for variability in ligation efficiency.

Contact support@nanostring.com for further guidance on data analysis.

Conclusion

The identification of stable circulating miRNAs in various disease states offers the potential for discovery of novel biomarkers and new biological insights. As outlined herein, important considerations regarding the low-abundance of miRNA in plasma/serum and potential contaminants associated with these sample types must be addressed to ensure reliable results. BOX 2 provides an overview of the workflow for analyzing serum and plasma with nCounter miRNA assays and page numbers of this Tech Note to be referenced for key steps. Using the guidelines outlined in this tech note, reproducible and relevant data can be obtained using the nCounter miRNA assays.

BOX 2: Serum/Plasma miRNA Workflow



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