TECH NOTE
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 blood plasma and serum samples. This Tech Note defines the current challenges associated with miRNA studies in plasma and serum samples, points out steps in the processing of collected blood which can have an impact on sample quality, and elucidates 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 plasma and serum 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.

Background

There are many considerations that researchers should be aware of prior to undertaking sample collection and isolation of miRNAs for any downstream profiling technology (Wang et al., 2018; Zaporozhchenko et al., 2018; Becker N and 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 cell-free plasma/serum usually have low abundance of total RNAs, which are short and degraded. The quantity of RNA extracted from plasma and serum is often below threshold amounts required for reliable characterization from standard RNA quality control

Consideration	Recommendation for Plasma	Recommendation for Serum
Contaminants*		
Sample Collection Tube	• EDTA tubes recommended • Citrate tubes acceptable	• Gel separation recommended • Gel-free separation acceptable
Hemolysis	Hemolysis when using whole blood samples should be avoided to avoid poor interpretation of results. Inspect each sample for hemolysis prior to RNA extraction as well as the absorbance of free hemoglobin with an optical density at 414 nm (For guidance, refer to Pizzamiglio et al., 2017, Shah et al., 2016, Kirschner et al., 2013). Further, check levels of hemolysis dependent miR451, miR16 and miR92. (Pizzamiglio et al., 2017 and Juzenas et al., 2017). miR-23a is relatively stable in plasma and serum and is not affected by hemolysis; therefore, it is suggested that a ratio of miR-23A to miR-451 of more than seven is an indicator of possible hemolysis (Foye et al., 2017, Atarod et al., 2015).	
Cellular Contamination	Evaluate results to determine if there are elevated levels of cellular miRNAs (miR-451, miR-16, miR-25). Check if the levels of the five house-keeping mRNAs included in the assay are elevated. Minimize cellular contamination by using gel separation tubes	
Low Abundance of miRNA		
Initial sample volume	1 ml or more of sample input is recommended.	
Pre-extraction spike-Ins	Spike-in synthetic targets to monitor 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.	

Table 1 Key considerations and recommendations. *Further review of contaminants in the 'Blood Collection Guidance' section

and quantification methods, such as absorbance (OD) measurements or analysis by an Agilent Bioanalyzer. It is therefore difficult to appropriately QC samples and assure that they 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 is 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 plasma and serum samples is blood collection method, including the specific type of collection tube utilized.

Both plasma and serum can produce robust results with the nCounter miRNA assay using either EDTA (K2 or K3) or citrate as the plasma anticoagulant; EDTA is the most recommended plasma anticoagulant. While the overall pattern of expression for most miRNAs will be consistent for similar collection methods, small discrepancies in relative expression can be seen for some miRNAs when using collection tube types that are theoretically very similar, a

phenomenon that has been observed previously (Kroh EM, et al., 2010). NanoString strongly recommends data generated from different collection tubes should not be compared directly. (Figure 1)

Some collection tubes contain 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 culturing blood), and heparin tubes. Treating samples with heparinase 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 circulating cell types in blood have their own unique miRNA profile and given the low concentration of extracellular miRNAs in plasma or serum, miRNA from a small number of lysed cells can disproportionately represent a large amount of the miRNAs detected in the assay. Hemolysis of whole blood samples should be avoided to prevent poor interpretation of results. Each sample should be visually inspected for the presence of a red/pink hue indicative of hemolysis prior to the RNA extraction step as well as checked for the
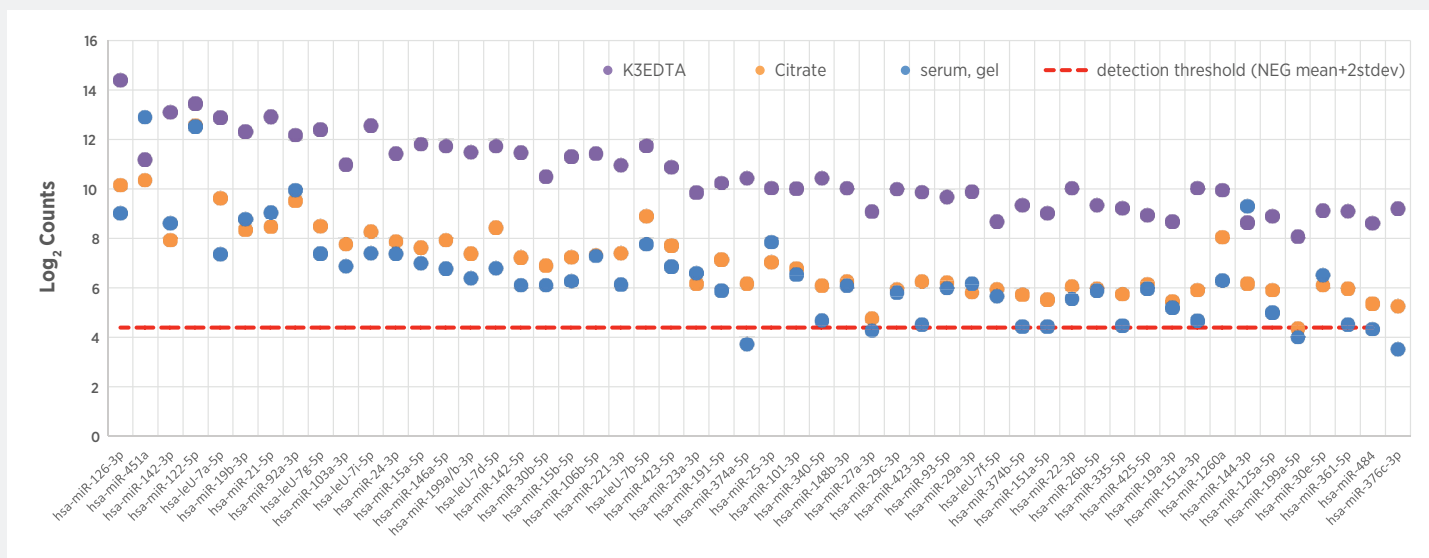

Figure 1 Log2 counts for 52 individual miRNAs collected from a common donor are displayed for five sample collection tube types: K2 EDTA plasma (gray), K3 EDTA plasma (yellow), citrate plasma (green), serum with gel separator (blue), and serum without gel separator (orange). miRNAs are listed across the x-axis and Log2 counts are plotted along the y-axis. In general, while blood collected in all three tube types displays similar miRNA profiles, EDTA plasma yields more detectable miRNAs than citrate plasma or serum.

absorbance of free hemoglobin at 414 nm, as shown in Figure 2 (For guidance, refer to Pizzamiglio et al., 2017, Shah et al., 2016, Kirschner et al., 2011). Furthermore, levels of hemolysis-dependent miRNAs such as miR451, miR16 and miR92 should be investigated (Pizzamiglio et al., 2017 and Juzenas et al., 2017). Given that miR-23a is relatively stable in plasma and serum and is not affected by hemolysis, it is suggested that a ratio of miR-23a to miR-451 of more than seven is an indicator of possible hemolysis (Foye et al., 2017, Atarod et al., 2015, Kirschner et al., 2013).

Cellular miRNA contamination due to lysis can vary from sample to sample if the blood is not collected and processed promptly and carefully, and can 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 the serum, snap freeze, and store at -80°C (NOTE: A gel-based separation can help to minimize cellular RNA contamination). At this point, as indicated above, it is important to note the color of the serum. Cellular mRNA contamination can also confound results and skew interpretation of the miRNA profile in serum or plasma. The NanoString miRNA panels contain probes for five mRNA house-keeping genes to monitor the presence of cellular contamination during the collection of serum or plasma, so be certain to check if the counts of all five of the house-keeping mRNAs included in the assay are elevated.

Addition of Synthetic Spike-Ins for Monitoring Extraction Efficiency (Optional)

To monitor the efficiency of RNA recovery rates during purification, one can utilize a synthetic spike-in control RNA target. If adopting this approach, we recommend the use of a single spike-in probe (osa-miR-414). Data from this spike-in may also be optionally used during normalization (see below) if other normalization methods are not robust. This synthetic spike-in RNA (unmodified, HPLC or gel purified) can be ordered from IDT Technologies or another vendor. The sequence for this spike in target is: UCAUCCUCAUCAUCAUCGUCC (MIMAT0001330). NanoString miRNA assays include probes for three to five such synthetic spike-in miRNA targets. Contact **support@nanostring.com** if you wish to use more than one.

Following the lysis procedures specified by the RNA extraction protocol, we recommend adding 5 μL of a 200 pM solution of the spike-ins, assuming a final elution volume of 15-25 μl. The spike-in solution should be added at least five minutes after the addition of lysis buffer to the sample. Please consult with **support@nanostring.com** for appropriate guidance.
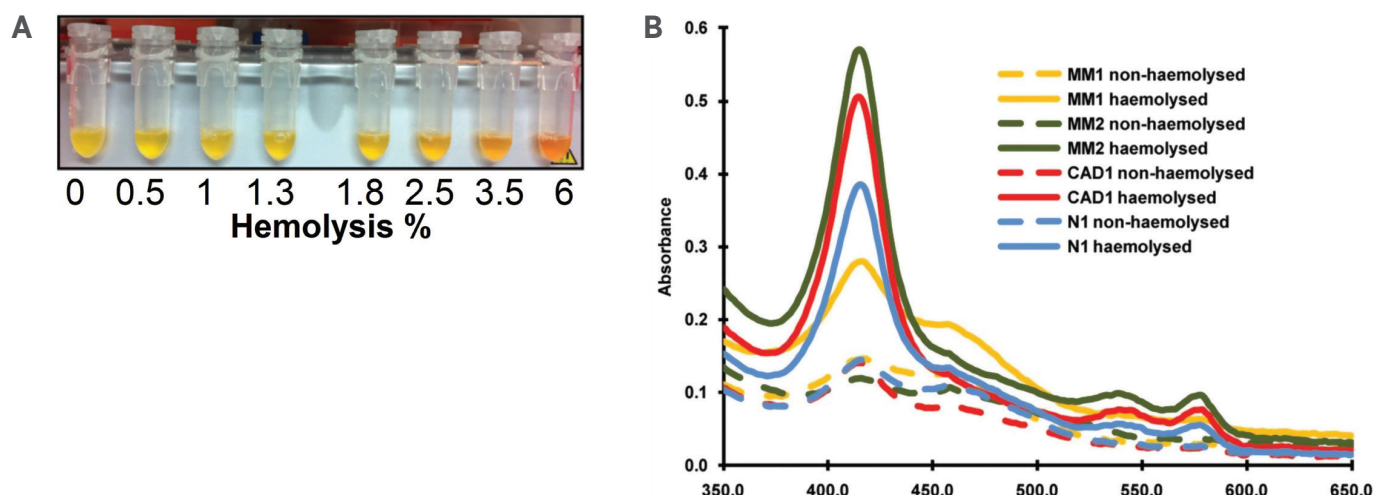

Figure 2 A. Visual inspection of serum samples deliberately spiked with different volumes of lysed erythrocytes (from 0 to 6% v/v) indicate an increase in color to a darker hue of red/pink as the number of lysed erythrocytes increases. Adapted from Marzi MJ et al. 2016. **B.** Adsorption spectra of matching hemolyzed and non-hemolyzed plasma samples from four different patients collected in different EDTA tubes show the characteristic Soret band adsorption of free hemoglobin at 414, 541 and 576 nm. Adapted from Kirschner MB et al., 2011.

Total RNA Extraction and Isolation

nCounter miRNA assays are compatible with miRNA extracted by a variety of extraction methods/kits. Comparison of a limited number of miRNA purification kits show that the Norgen slurry kit or midi/maxi plasma serum kit (see below for product numbers) extract a higher number of detectable miRNA compared to others tested. We recommend using the Norgen kits indicated below and starting with a minimum of 1 ml of plasma or serum to produce significant counts, but robust detection of some miRNAs may require larger volumes.

As stated previously, circulating miRNAs are relatively low in concentration, and extraction yields frequently approach the limit of UV/Vis spectrophotometric detection; therefore, typical cautions regarding sample cleanup using sodium acetate precipitation or mini-columns (see nCounter miRNA Assay Manual) are of even greater importance in producing reliably 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. Therefore, we strongly recommend the use of a secondary cleanup process (precipitation- or column-based, as outlined below) following purification with any commercial kit.

As with the choice of blood collection tube, an important factor to consider for collecting comparable data is consistency in the choice of the RNA extraction kit. Different extraction kits yield varying efficiencies at extracting different miRNAs, often resulting in variances in miRNA expression profiles (Ramon-Nunez et al., 2017; Ban et al., 2017; Meerson et al., 2016; Pritchard et al., 2012). NanoString has observed that counts measured for individual miRNAs can differ between extraction kits; therefore, we recommend utilizing a single extraction kit and buffer throughout an entire study if you intend to directly compare miRNA counts from different experiments.

Data Analysis and Normalization

All data analysis and normalization can be performed using the nSolver™ Analysis Software (available by complimentary download from the NanoString Technologies website). Analyses within nSolver can be customized to suit the specific needs of individual experiments, and any miRNA data set will default to our recommended method for cell- or tissue-derived miRNA data (the Top 100 method). However, we generally do not recommend the use of this method for

plasma and serum samples as these sample types usually do not contain 100 miRNA targets above background. For such data sets, we recommend normalizing in nSolver using one of the methods below, in order of decreasing preference. For guidance on the general use of nSolver to implement the methods listed below, refer to the nSolver User Manual and nSolver support page (https://www.nanostring.com/support/data-analysis/nsolver-data-analysis-support).

1. Housekeeping Method (Recommended). Normalize to the geometric mean of at least three stably expressed miRNA targets. We recommend identifying such stably expressed targets using a data driven approach similar to that described by Gouin et al., 2018) (See Box 2).

2. Total miRNA Method. Normalize to the geometric mean of all the miRNAs that are expressed robustly above the background (greater than 50 average raw counts). This method may be less robust if relatively few targets are expressed above background.

3. Spike-In Method. If the spike in QC probe was used during RNA purification, normalize to this probe. Counts of the spike-in probe(s) will reflect variance in RNA purification recovery as well as variance in ligation efficiencies across samples, which are two of the major sources of variance for plasma and serum samples. However, this method will not normalize variation from sample input differences, and as such is not recommended when there is variation in biofluid sample volumes from which RNA was purified.

4. Ligation Method. Normalize data using the geometric mean of ligation positive control probes. This will only normalize data to the variation in ligation efficiency from lane to lane and is not recommended except to address specific experimental questions, or when the above methods have all yielded unsatisfactory results.

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

Identification of Stably Expressed (Housekeeper) miRNA targets

Gouin et al., 2018 recently outlined a method for the identification and confirmation of stably expressed miRNA targets suitable for use as normalizing housekeeping targets from extracellular vesicle samples. In brief, the method describes a consensus approach which utilizes the results from three published algorithms (GEnorm, NormFinder, and BestKeeper) to generate a single

Recommended purification kit and other materials:

• Norgen Plasma/Serum Circulating exosomal RNA Purification Kit, Slurry Format (Norgen, Cat #42800)

• Norgen Plasma/Serum RNA Purification Midi or Maxi kit (Norgen, Cat #56100 and 56200).

Protocol

1. Use up to 5 ml of plasma/serum, with a recommended minimum of 1 ml. Lower inputs are expected to result in fewer detected targets.

2. Follow the extraction protocol outlined for the selected extraction kit by elution from the column.

3. Elute in 100 µl of elution buffer, or as per kit protocol. Do not concentrate the sample by eluting in a lower volume. This both lowers the efficiency of the elution from the column and serves to concentrate any carryover extraction reagents.

4. Concentrate and clean the sample using either sodium precipitation or using a size-exclusion filter with a small pore size (~3 kDa MW cutoff), see below.

RNA clean-up and concentration (Choose A or B):

A. Column clean-up and concentration method:

Required Materials

• AMICON ULTRA 0.5ML - 3KDa cutoff (UFC500324)

a. Add 320 µl of RNase-free water and load onto an Amicon Ultra YM-3 column.

b. Centrifuge at room temperature at 14,000g for exactly 90 minutes; this is an important step to get to the correct concentration/volume.

c. Invert the column and collect in a fresh tube by centrifuging at 8,000g for 2 minutes.

d. Transfer to a fresh RNase-free tube and determine the volume collected.

e. Dilute to 15-20 µl (should be just lower than this volume). Use 3 µl of this final solution in the NanoString miRNA ligation reaction.

B. Ethanol/Sodium acetate precipitation method:

Required Materials

• Linear acrylamide (Thermo Fisher, Cat # AM9520)
• Sodium Acetate (3M) (Thermo Fisher, Cat # AM9740
• Ethanol, 200 proof [100%]

a. Combine 0.1 vols of 3M sodium acetate, 3 volumes of ice cold 100% ethanol, 3 µl 5 mg/ml linear acrylamide and mix well by pipetting up and down. Do not vortex as it may shear the RNA.

b. Precipitate for 1 hour at -20°C or -80°C overnight.

Tip: Orient the hinge of the micro-centrifuge tube outward to assist in locating the nucleic acid pellet, which will then be located on the same side of the hinge.

c. Centrifuge at max speed (13,000 rpm) in a cold microcentrifuge for 30 minutes. A white pellet should be visible. Slowly remove the supernatant by decanting and use absorbent paper to remove the excess supernatant.

d. Wash the pellet with 1 ml ice cold 75% ethanol without disturbing the pellet, and centrifuge for 10 minutes at maximum speed.

Tip: RNA at this step can be more easily dislodged than in previous steps. Extra care should be taken when discarding the supernatant.

e. Repeat the wash in step (d), remove the supernatant, and use absorbent paper to remove the excess supernatant.

f. Allow the pellet to air-dry at room temperature. This takes about 5-10 minutes. Leave your tubes propped upside down at a 45 degree angle on a clean tissue paper, this will facilitate drying.

Note: Ethanol carryover will inhibit the assay (pipette out any leftover droplets). Do not over dry, it will decrease solubility.

g. Resuspend the pellet in 8-15 µl RNase-free water. The pellet is not visible after the pellet is dry. It is important to pipette up and down 15-20 times to completely dissolve the pellet. Be sure to wash RNA off the walls of the tube. Use 3 µl of this final solution in the NanoString miRNA ligation reaction.

consolidated list of housekeepers which are strongly supported based on their favorable stability scores within all three algorithms. Because each of the three algorithms will not generate lists of completely overlapping genes, the rationale here is that agreement between the three disparate mathematical approaches will yield a more robust set of putative housekeeping targets.

Although we would encourage use of the above approach as the most robust method, in our own experience the published algorithms may not always be freely available, nor is learning the use of three separate tools an insignificant time commitment. We therefore outline here a simplified approach which should still yield results largely consistent with those of Gouin et al. Briefly, we use one of the above algorithms to identify putative housekeeping miRNAs (available as a free download, NormFinder), and confirm those identified targets using a built-in variation metric from within the nSolver software.

First, import raw data (RCC files) into nSolver and remove any samples which failed QC. Build an experiment and set background subtraction to the Mean +1 SD of the NEG control probes. This will set most low expression probes to one count. Importantly, keep the normalization options turned off. Export data from the finished experiment into an excel file derived from the normalized data table.

NormFinder is readily obtainable as an Excel plug-in or R script (https://moma.dk/normfinder-software), and we briefly describe the workflow for using the Excel plug-in here.

Open the Excel file containing the NormFinder macro ("Normfinder_0953.xlsx") and choose the "Enable" option for the plug-in. Without closing Excel, open the file containing the background corrected miRNA data exported from nSolver.

Format the Excel data file to meet the expected configuration of NormFinder. The column immediately to the left of the first sample should contain the miRNA probe name (in some nSolver export formats this column will instead be a less recognizable miRNA ID number), and the row of data immediately above the raw gene counts can optionally be used to identify sample groups. Samples with the same pathology or treatment should be labeled with the same identifier in this row. If not already done, sort background subtracted data by average counts across all samples, and delete all miRNAs expressed below 50 mean counts when averaged across all samples.

Run NormFinder, which can be found in the Add-In tab.

Select the remaining miRNA data, being sure to include the gene ID column and Group ID row if relevant. NormFinder will create a new worksheet in the current excel file which lists all the genes and a stability value for each. Identify five potential housekeepers with the best (smallest) stability value.

Confirmation of Stable Expression in nSolver

Open nSolver and copy the previous background subtraction experiment to create a new one. In this version of the experiment if background correction is still preferred, we recommend changing the method to background threshold (mean +1 SD can still be used). For normalization, keep the POS control normalization option check box off, but turn on the CodeSet Content (Housekeeping Gene) normalization, and select the top five candidates from the NormFinder output above. Any putative housekeeping targets with an unusually high %CV may be removed from normalization. The absolute value of the %CV is not important here, but rather how this value compares to the %CV of other miRNA targets present above background. Putative housekeepers that have an outlier %CV value, or a %CV value that is more than 2x as high as the %CV of other putative housekeepers should be removed from normalization. We recommend using at least three such targets in the final normalization.

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.

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FEB 2019 TN_MK1342