WHITEPAPER

MicroRNA Biomarker Discovery and Validation

Using the nCounter® Platform

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Introduction

MicroRNAs (miRNAs) are a class of small, non-coding RNA that regulate the gene expression of target mRNAs via post-transcriptional gene silencing[1]. These short RNAs have been implicated in the widespread control of critical biological processes such as proliferation, differentiation, and apoptosis[2-4]. Due to their central role in developmental processes, perturbations in miRNA expression patterns have been implicated in many diseases, including cancer, Alzheimer's disease, heart disease, and diabetes[5-7]. Recent work has focused on the promise of developing miRNA expression signatures as prognostic biomarkers of disease [8-11].

The nCounter miRNA expression essay enables users to rapidly and efficiently profile the top 800 highly-curated human miRNAs from miRBase 22 in a single tube. This product provides a sensitive, reproducible, and highly-multiplexed method for detecting specific miRNAs within purified RNA isolated from any source, including biological fluids, formalin-fixed paraffin-embedded (FFPE) samples, and fresh or frozen cells and tissues[12] (**FIGURE 1**). Refer to the Tech Note for nCounter miRNA Expression Analysis in Plasma and Serum Samples for additional details. A significant advantage of the assay is that it uses NanoString's nCounter platform to offer direct digital counts of each miRNA without the use of reverse transcription or amplification, making it highly specific. In addition to high performance on FFPE and fresh/frozen tissue, the nCounter miRNA expression assay is highly reproducible (**FIGURE 2**) with replicate count correlations (R^2) greater than 0.99. It is a unique biomarker discovery and signature development tool that enables collection of expression data (**FIGURE 3**) in a short amount of time with minimal hands-on manipulation.

nCounter technology is based on a novel method of direct molecular barcoding and digital detection of target molecules through the use of color-coded probe pairs[13]. The nCounter miRNA Sample Preparation Kit provides reagents for ligating unique oligonucleotide tags (miRtags) onto the 3' end of target miRNAs such that short RNA targets can be detected by nCounter probes (**FIGURE 4**). Sample preparation involves multiplexed ligation of the specific tags to their target miRNA and an enzymatic purification to remove nonligated tags. nCounter miRNA expression assays are highly specific, and can distinguish between miRNAs that differ by just a few base pairs, as shown in cross-hybridization data with the let-7 family, a family of highly homologous miRNAs which can differ in sequence by as little as a single nucelotide (**FIGURE 5**). Sequence specificity of the ligation reaction is ensured by the use of T_m-optimized bridging oligos that are complementary to a portion of both the target miRNA and miRNA-specific tag along with careful, stepwise control of annealing and ligation temperatures.

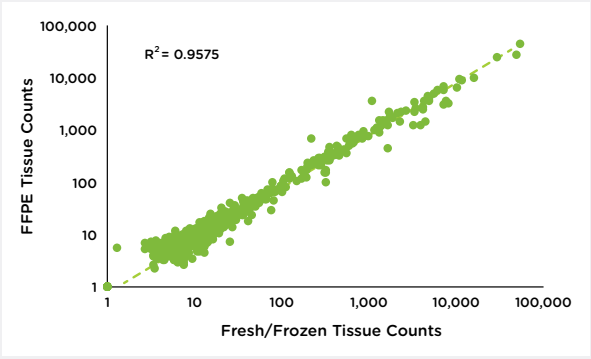

FIGURE 1: Expression correlation is preserved when profiling matched FFPE and fresh/frozen tissues. Sections of tissue from a single human liver were formalin-fixed and paraffin-embedded (FFPE) or frozen. 100 ng of total RNA was isolated from the matched FFPE and frozen tissue and profiled using the nCounter miRNA Expression Assay. Count correlations between the samples were high ($R^2 > 0.95$) indicating that generation and handling of FFPE samples has no significant impact on performance of the nCounter assay.

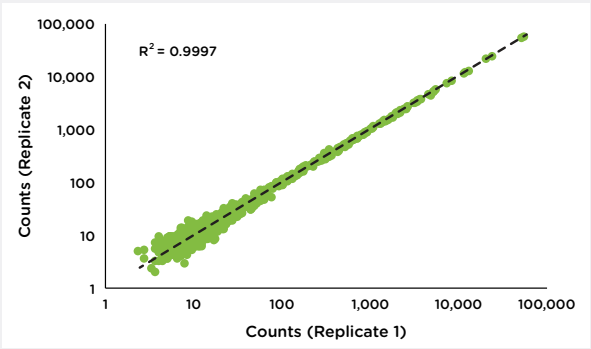

FIGURE 2: nCounter Human v3 miRNA Expression assay is highly reproducible. Count correlations between the preparation of the same RNA sample (100 ng) were extremely high (R2 > 0.99) demonstrating the precision of the nCounter Human v3 miRNA assay.

FIGURE 3: Differential miRNA expression analysis on the nCounter platform. nCounter miRNA assays are capable of accurately quantifying changes in miRNA expression. Human Reference RNA (100 ng per assay) and Brain Reference RNA (100 ng per assay) were profiled in triplicate using the nCounter Human v3 miRNA assay. Hundreds of miRNAs are significantly differentially expressed between samples.

This approach specifically captures all mature miRNAs in a sample despite the large overall sequence diversity and associated broad T_m range between miRNAs (**FIGURE 6**). Control RNA included in the Sample Preparation Kit is used to monitor ligation efficiency and specificity through each step of the reaction (**TABLE 1**). All nCounter assays are run using an nCounter instrument. miRNA expression data can be easily imported into the free nSolver™ Analysis Software (www.nanostring.com/products/analysis-software/nsolver) for data normalization and visualization, including heat maps, histograms, and violin plots. The nCounter platform has been used to profile miRNAs from a variety of sample types and in a wide range of biological areas of research, including biomarker discovery[14], signature development[15], immuno-oncology[16], oncology[17], neuroscience[18], stem cell research[19], autophagy[20], infectious disease[21], immunology[22], and many others[23,24].

Designed for Biomarker Discovery and Validation

nCounter miRNA assay content is based on miRBase, a bioinformatics repository for small RNA sequence and annotation information[25,26]. These assays are periodically revised to account for updates to miRBase and to ensure that they include the most biologically-relevant miRNAs and controls. The miRNAs included in the nCounter Human v3 miRNA assay account for 98% of all observed sequencing reads in miRBase 22 (released on 12 March 2018). Additionally, NanoString uses proprietary metrics such as observed read ratios and expression analytics in order to screen potential content for inclusion in the assay. Together, these methods help ensure that the miRNA assay content is heavily weighted toward biologically-significant miRNAs. In addition to this data-driven selection, NanoString also performs literature reviews to ensure that actionable and clinically relevant miRNAs are included in our nCounter miRNA assays[28,29]. This metric is important as not all potentially clinically relevant miRNA biomarkers are classified as "high confidence" by miRBase. NanoString's assay design philosophy uses a holistic set of selection criteria to ensure that each miRNA assay contains a comprehensive collection of miRNAs enriched for biological activity and ideal for both targeted discovery and validation experiments. For greater detail about the Human v3 miRNA expression assay content, refer to the target list (gene list) available on NanoString's website at www.NanoString.com/products/miRNA

A large number of control probes are included in each nCounter miRNA assay to help ensure robust performance. miRNA assay controls have been updated to include probes for both hybridization and ligation based on sequences generated by the External RNA Controls Consortium (ERCC), an independent consortium of academic, public, and private organizations sponsored by the National Institute of Standards and Technology (NIST)[30]. ERCC sequences were developed to enable generation of reproducible research control probes for gene expression analysis and related biological questions via the use of synthetically-derived sequences that do not occur in most genomes. **TABLE 1** outlines each type of control included in nCounter miRNA expression assays.

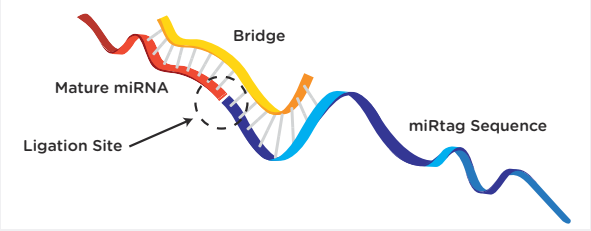

FIGURE 4: Specific capture of miRNA targets via ligation. miRNAs (red) are specifically ligated (circled region) to unique tags (blue) for downstream detection via hybridization with the nCounter miRNA expression assay. Ligation specificity is ensured by T_m-optimization and T_m- balancing of the bridge (yellow) sequence that is complementary to each mature miRNA and cognate miRtag coupled with careful thermal control of the ligation reaction.

A

miRNA	Sequence
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGU**G**U**G**GUU
Let-7c	UGAGGUAGUAGGUUGUAU**G**GUU
Let-7d	**A**GAGGUAGUAGGUUG**C**AUAGUU
Let-7e	UGAGGUAG**G**AGGUUGUAUAGUU
Let-7f	UGAGGUAGUAG**A**UUGUAUAGUU
Let-7g	UGAGGUAGUAG**U**UUGUAUAGUU
Let-7i	UGAGGUAGUAG**G**UUGU**GCU**GUU

B

	Let-7a	Let-7b	Let-7c	Let-7d	Let-7e	Let-7f	Let-7g	Let-7i
Let-7a	100%	1%	5%	4%	17%	4%	0%	0%
Let-7b	0%	100%	0%	0%	0%	0%	0%	0%
Let-7c	0%	11%	100%	0%	0%	0%	0%	0%
Let-7d	0%	0%	0%	100%	0%	0%	0%	0%
Let-7e	0%	0%	0%	0%	100%	0%	0%	0%
Let-7f	1%	0%	0%	0%	0%	100%	0%	0%
Let-7g	0%	0%	0%	0%	0%	0%	100%	0%
Let-7i	0%	0%	0%	0%	0%	0%	0%	100%

FIGURE 5: nCounter miRNA Expression assays are highly specific. (A) Single-nucleotide mismatch cross-hybridization rates were empirically determined for the let-7 family, a family of highly homologous miRNAs which can differ in sequence by as little as a single nucleotide. (B) Each let-7 miRNA target was assayed alone and in the presence of all let-7 probes. Cross-hybridization was calculated as: (off-target counts/on-target counts)..

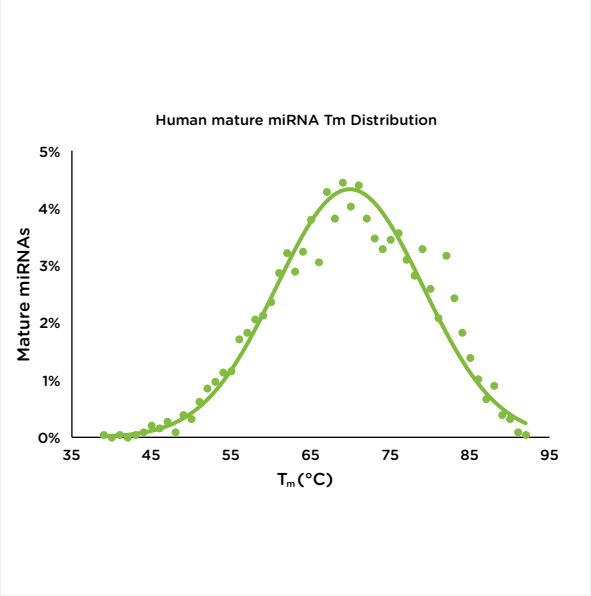

FIGURE 6: Calculated Tm distribution of human mature miRNA sequences. Percentage G/C content of mature miRNAs is highly variable, causing a broad Tm distribution. The nCounter miRNA sample preparation method normalizes this Tm distribution while preserving miRNA specificity via miRtag ligation in order to generate ideal targets for downstream detection via hybridization with nCounter Capture and Reporter probes.

miRNA assay Performance and Application Data

One of the benefits of the direct digital counting methodology afforded by the NanoString nCounter platform is that it is perfectly positioned for translational research needs, enabling the utility of very poor quality RNA, particularly from highly-degraded clinical FFPE samples to address novel treatment options in cancer[31]. Profiling of miRNAs from FFPE and their potential as novel biomarkers in a variety of tumor types has therefore gained much traction. Recent examples have focused on the identification of novel miRNAs in the progression of colorectal cancer[32,33]. Using both fresh frozen and FFPE human tissue as well as mouse models of colorectal cancer, Valeri *et al.* performed nCounter miRNA profiling on extracted RNA and characterized the up-regulation of miR-135b as a potential biomarker for colorectal cancer[32]. Furthermore, a comprehensive study by Hur *et al.* identified a metastatic-specific miRNA signature in patient colorectal cancers, providing evidence that miRNAs may be clinically applicable to predict prognosis and distant metastasis in colorectal cancer[33]. To date, triple-negative breast cancer (TNBC) has been one of the most challenging breast tumor subtypes to treat, not least because only a small proportion of patients respond to neoadjuvant therapy regimes[34]. miRNAs have therefore emerged as attractive candidates for novel molecular biomarkers in the progression of breast cancer. Cascione *et al.* wished to stratify TNBC using NanoString miRNA expression profiling by investigating the profiles in tumor, adjacent, non-tumor,

TABLE 1.
Description of the controls in nCounter miRNA Expression assays.

Control Type	Number	Description	Use
Positive Controls	6	Probes that recognize synthetic mRNA targets included in the assay at specified concentrations (targets do not require ligation).	Positive controls used by the QC metrics in nSolver to confirm linear response to input amounts, and confirm that low input signal is above background.
Negative Controls	8	Probes that recognize synthetic mRNA targets not included in the assay (targets do not require ligation).	Negative controls used by the QC metrics in nSolver to determine background signal independent of ligation success. This can then be used to set threshold for defining expression of miRNA.
Ligation Positive Controls	3	Probes that recognize synthetic miRNA targets included in the Sample Preparation Kit (Sample Preparation Kit includes targets, bridges, and miRtags).	Ligation positive controls monitor ligation efficiency, independent of the miRNAs in the sample.
Ligation Negative Controls	3	Probes that recognize synthetic miRNA targets not included in the Sample Preparation Kit (no miRNA target).	Ligation negative controls monitor non-specific ligation.
mRNA Reference Controls	5	Probes that recognize endogenous mRNA targets commonly expressed in tissues.	Measurement of mRNA can be used to determine if there is cellular contamination in cell free samples. When miRNA is extracted from cells, even if ligation fails, the mRNA will be seen. It is not recommended that these mRNA be used as reference genes to normalize samples.
Spike-in Controls	5	Probes that recognize exogenous miRNA targets not included in the Sample Preparation Kit to monitor RNA isolation/purification steps upstream of the assay (optional, added by user if desired).	If user adds the appropriate small molecules to samples, they can be used to monitor steps of sample processing prior to the NanoString protocol.

and lymph node metastatic lesions from over 170 women. Their overall results helped define specific miRNA expression signatures that further characterize the complexity and diversity of TNBC as well as its metastasis[35]. Other clinical utility with the adoption of the NanoString nCounter platform lies in examining the hematopoeitic cell of origin of blood based miRNAs as well as the ability to explore expression profiling from individual blood cell types. Teruel-Montoya *et al*. used the nCounter Human miRNA expression assay to profile the miRNA content from RNA generated from platelets, T cells, B cells, granulocytes, and other hematological cell types. The authors suggested that such data could be used as a basis for interpretation of miRNA-disease association studies. For example, a miRNA elevated in acute myelogenous leukemia (AML), but absent or very low in normal granulocytes (as the authors demonstrated), might participate in the pathogenesis of AML. Further insight into the differential expression of miRNAs by blood cell type therefore is also relevant to understanding the systemic effects of blood cell delivered miRNAs[36].

The comprehensive nCounter miRNA assays are also being used to discover biomarkers and develop expression signatures from biofluids including serum and plasma[37-39]. The discovery of these relatively stable, extracellular circulating miRNAs has generated a great deal of interest over conventional biomarkers, particularly their likelihood as candidates for non-invasive early disease monitoring and prognostic biomarkers of disease progression or resolution. To this end, Permuth-Wey J. *et al*. recently discovered novel miRNA biomarkers in plasma samples from patients with intraductal papillary mucinous neoplasms of the pancreas, a precursor to pancreatic ductal adenocarcinoma (PDAC), one of the most fatal cancers worldwide. These exciting developments using nCounter miRNA expression assays represent a potential new technology and methodology for a disease which, to date, lacks early detection or intervention at an operable stage[40]. Similarly, Armstrong *et al*. profiled miRNAs derived from matched FFPE tumors and plasma and urine-based biofluids from patients with bladder cancer using the nCounter miRNA expression assay. Bladder cancer remains one of the most expensive cancers to treat due to the high rate of local recurrence and the requirement for frequent long-term follow-up with invasive and uncomfortable cystoscopic evaluations.

There is a clear unmet need therefore for additional, improved urine- (or other bio-specimen) based alternatives to cystoscopy for the screening, initial evaluation, and follow-up of bladder cancer; miRNA profiling may afford such a novel methodology[12]. In other examples, Stylii *et al*. profiled miRNAs from cerebrospinal fluid (CSF) samples in patients with aneurysmal subarachnoid hemorrhage (SAH), prognosis of which is very poor relating to a high mortality rate. The findings indicated that temporal miRNA profiling using NanoString can detect differences between CSF from aneurysmal SAH and non-SAH patients. Moreover, the miRNA profile of CSF samples from patients who develop cerebral vasospasm; a common complication from patients suffering from SAH, may be distinguishable from those who do not[41]. Further, in a study of miRNA expression profiling in extracellular vesicles from serum samples, Rodosthenis *et al*. sought to uncover whether air pollution from particulate matter could be linked to cardiovascular disease and mortality through a deregulation of miRNAs. The group identified several miRNAs following exposure to particulate matter and interestingly conducted further studies and pathway analysis mapping to demonstrate the role of these identified miRNAs in cardiovascular disease related pathways[42]. The breadth of biological applications accommodated using the NanoString miRNA expression assays continues to expand. Recently, Wang *et al*. investigated the role of differentially-expressed miRNAs following human enterovirus 71 (EV71) infection, the causative agent of hand, foot, and mouth disease. The group adopted NanoString miRNA profiling to characterize the responses of serum miRNA profiles in normal samples as well as in samples from EV71 infected patients suffering symptoms ranging from mild to severe. 44 miRNAs were observed in patients with infections and were found to regulate a number of key genes involved in known signaling pathways associated with the diagnosis of infection[43].

These are just a few examples of the nCounter miRNA profiling platform enabling identification and evaluation of differentially-expressed miRNA tissue and biofluid-based biomarkers.

In addition to the studies mentioned herein, NanoString maintains a comprehensive and frequently-updated online list of all of the publications that utilize NanoString assays, including miRNA, gene expression (mRNA), gene fusions, single cell and CNV. For more references and information, see NanoString's Publications page, https://www.nanostring.com/scientific-content/publications.

Summary and Conclusion

Many diverse platforms have been employed to investigate miRNA profiles and signatures, from broad screening to targeted platforms and methods. NanoString's miRNA expression assays are a set of unique and powerful biomarker discovery and signature development tools, providing excellent specificity, low false-positive rates[31], and a direct digital readout. With a simple workflow, these assays enable investigators to generate comprehensive expression data sets quickly with minimal hands-on manipulation. This white paper highlighted many of the diverse applications for NanoString miRNA assays, including cancer and hematopoiesis. nCounter miRNA profiling has enabled the identification of individual or multiple miRNA biomarkers that reflect changes in disease states or effects of therapeutic agents, demonstrating the versatility of the nCounter platform to both identify and validate differentially expressed miRNAs. Such studies are possible due to carefully curated assay designs that ensure each assay contains biologically-relevant content and appropriate controls.

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