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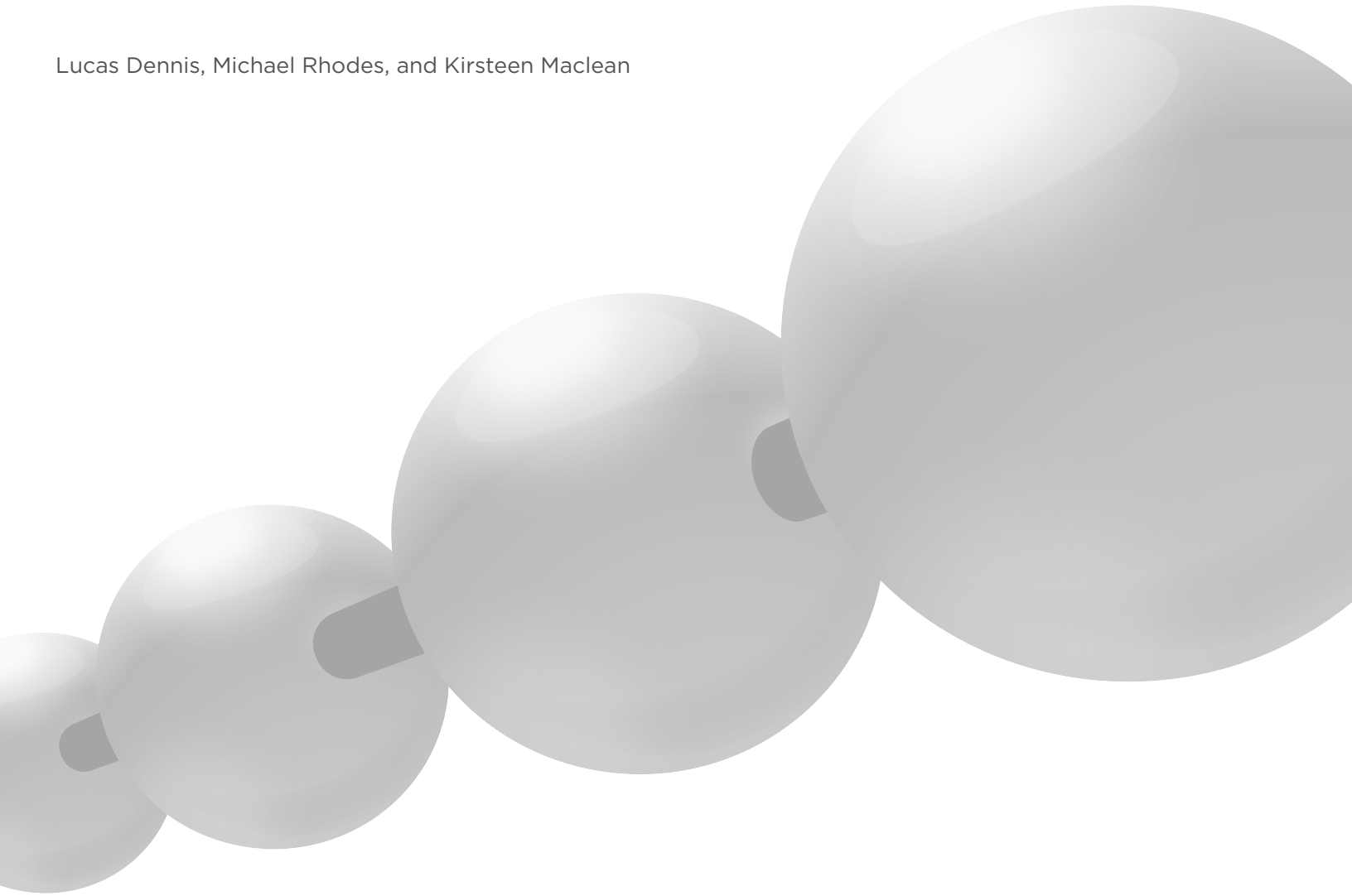
Targeted miRNA Discovery and Validation

Using the nCounter® Platform

Lucas Dennis, Michael Rhodes, and Kirsteen Maclean



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AUGUST 2015 LBL-10112-02

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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]. Much recent work has focused on investigating the promise of miRNA expression signatures as prognostic indicators of disease states[8-10].

The nCounter miRNA Expression Assay enables users to rapidly and efficiently profile 800 highly curated human miRNAs. This product provides a sensitive, reproducible, and highly multiplexed method for detecting specific miRNAs within purified RNA isolated from any source, including fresh or frozen cells and tissues, formalin-fixed paraffin-embedded (FFPE) samples (**FIGURE 1**), and biological fluids[11] (refer to the ***nCounter miRNA Analysis in Plasma and Serum Samples*** Tech Note for additional details). The assay utilizes NanoString's nCounter platform to offer direct, digital counts of each miRNA without the use of reverse transcription or amplification. It is a unique, targeted discovery and validation tool that enables collection of expression data 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[12]. The nCounter miRNA Sample Preparation Kit provides reagents for ligating unique oligonucleotide tags (miRtags) onto the 3' end of target miRNAs so that short RNA targets can be detected by nCounter probes (**FIGURE 2**). Sample preparation involves multiplexed ligation of the specific tags to their target miRNA and an enzymatic purification to remove non-ligated tags. 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.

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 3**). 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/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 cell signaling and cancer biology[13,14], neuroscience[15], stem cell research[16-17], autophagy[18], infectious disease[19,20], immunology[21,22], and many others[23,24].

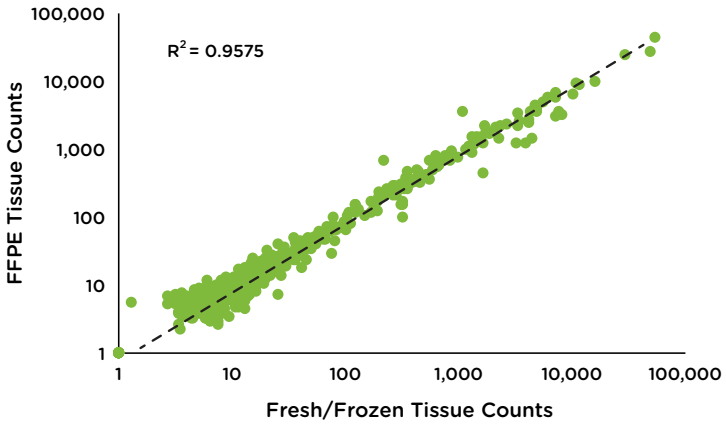

FIGURE 1 Expression correlation is preserved when profiling matched FFPE and fresh/frozen tissues. Sections of tissue from a single human liver were formalin-fixed and paraffin-embedded (FFPE) or frozen. 100 ng of total RNA was isolated from the matched FFPE and frozen tissue and profiled using the nCounter 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.

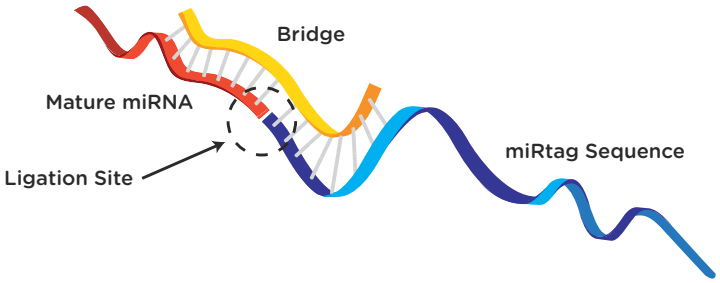

FIGURE 2 Specific capture of miRNA targets via ligation. miRNAs (red) are specifically ligated (circled region) to unique tags (blue) for downstream detection via hybridization with an nCounter CodeSet. Ligation specificity is ensured by T_m-optimization and T_m-balancing of the bridge (yellow) sequence that is complementary to each mature miRNA and cognate miRtag coupled with careful thermal control of the ligation reaction.

Designed for Targeted Discovery and Validation

nCounter miRNA panel content is based on miRBase, a bioinformatics repository for small RNA sequence and annotation information[25,26]. These panels are periodically revised to account for updates to miRBase and to ensure that they include the most biologically relevant miRNAs and controls. For a comparison of NanoString's Human v2 and v3 miRNA panel content, refer to **TEXT BOX 1**.

The miRNAs included in the nCounter Human v3 miRNA panel account for greater than 95% of all observed sequencing reads in miRBase 21 (released on June 26, 2014). Since the release of miRBase version 20, a confidence level has been assigned to each miRNA hairpin based on a set of quantitative guidelines associated with the mapping of sequencing reads[27]. Version 3 of the Human miRNA Expression Panel contains a probe for all of the miRNA hairpins denoted in miRBase 21 as "high confidence" (**FIGURE 4**). Additionally, NanoString uses proprietary metrics such as observed read ratios and expression analytics in order to screen potential content for inclusion in the panel. Together, these methods help ensure that the miRNA panel 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 panels[28,29]. This metric is important as not all potentially clinically relevant miRNA biomarkers are classified as "high confidence" by miRBase. NanoString's panel design philosophy uses a holistic set of selection criteria to ensure that each miRNA panel contains a comprehensive collection of miRNAs enriched for biological activity and ideal for both targeted discovery and validation experiments.

A large number of control probes are included in each nCounter miRNA panel to help ensure robust performance. miRNA panel 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 panels.

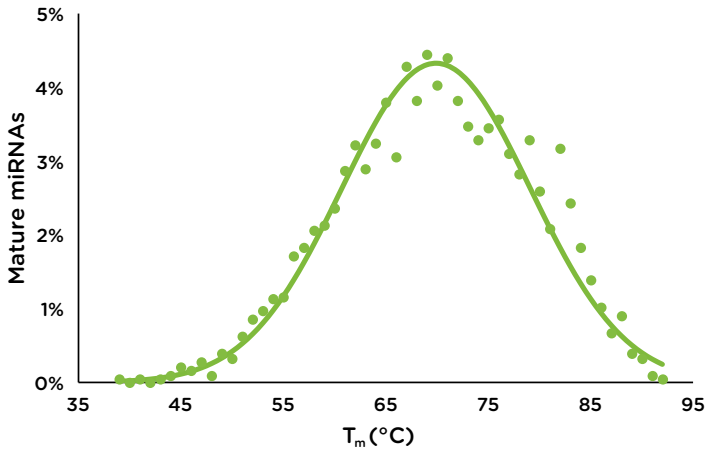

FIGURE 3 Calculated T_m distribution of human mature miRNA sequences. Percentage G/C content of mature miRNAs is highly variable, causing a broad T_m distribution. The nCounter miRNA sample preparation method normalizes this T_m distribution while preserving miRNA specificity via miRtag ligation in order to generate ideal targets for downstream detection via hybridization with nCounter Capture and Reporter probes.

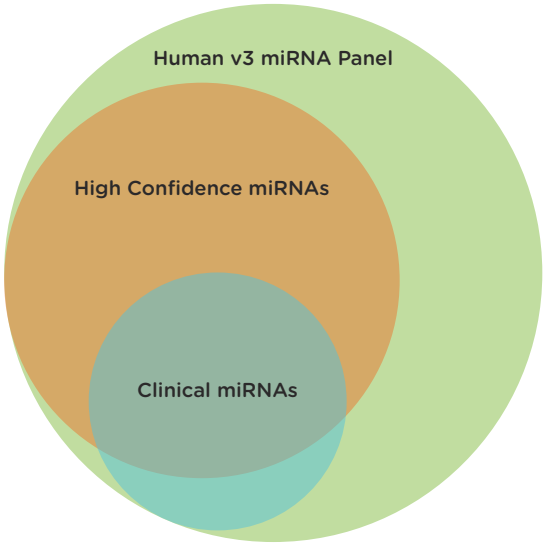

FIGURE 4 Overlap of high confidence and clinical miRNAs in the Human v3 miRNA panel. NanoString's Human v3 miRNA Panel provides 100% coverage of the miRBase high confidence and published clinical miRNAs.

TEXT BOX 1 Content Comparison

NanoString's Human v3 miRNA Panel contains 799 probes that represent >95% of all human miRBase reads. 675 probes are shared between the v3 and v2 panels, representing an 85% content overlap. Panel content was updated to maximize coverage of miRBase high confidence annotated miRNAs, remove coverage of miRBase "dead entries" (sequences mapping to confirmed non-miRNAs), and ensure coverage of clinically relevant content. The table below shows a direct comparison of the miRNA content between the v3 and v2 panels. For greater detail about the Human v3 miRNA Panel content, refer to the target list ("gene list") available on NanoString's website at **www.nanostring.com/products/miRNA**.

	Version 2	Version 3
Total Endogenous Probes	800	799
Number of Probes Shared by v2 and v3	675	675
Percentage of Panel Overlap	85%	85%

	Version 2	Version 3
Number of Probes Corresponding to High Confidence miRNA	293	407
Percent Coverage by Panel	72%	100%

TABLE 1 Description of the controls in nCounter miRNA panels.

Control Type	Number	Description	Use
Positive Controls	6	Probes that recognize synthetic mRNA targets included in the CodeSet 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 CodeSet (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.

miRNA Panel Performance and Application Data

nCounter miRNA Expression assays have been demonstrated to be a highly reproducible and sensitive means of profiling large numbers of miRNAs[31]. NanoString's unique miRNA profiling method is capable of single nucleotide mismatch discrimination (**FIGURE 5**), enabling measurement of expression differences between highly homologous miRNAs[31]. Combined with excellent assay-to-assay precision (**FIGURE 6**), nCounter miRNA assays are capable of accurately quantifying changes in miRNA expression (**FIGURE 7**), a critical attribute for the discovery and validation of robust biomarkers.

The amount of research being conducted using NanoString's miRNA panels is growing, and the paragraphs below outline several fascinating applications of the miRNA panels in a variety of different areas of research, including cancer biology and progression, as well as hematopoiesis.

Since the discovery that miRNA deregulation was linked to chronic lymphocytic leukemia, aberrant miRNA expression patterns were found to have a profound influence on cancer-related signaling pathways[5]. nCounter miRNA panels have been successfully utilized to identify miRNA biomarkers that differentiate between normal and disease states in cancer as well as heart disease, neurodegeneration, and colitis[6,32-35]. These panels have been used to identify biomarkers that can stratify and help predict patient outcomes[36-38] and to identify miRNAs that directly affect specific targets known to be involved in cancer progression[39].

In order to identify patterns of differential expression specific for each cell lineage, Teruel-Montoya *et al.* used the nCounter Human miRNA Expression panel to profile the miRNA content of highly purified normal hematopoietic cells from the same individuals[17]. Using Trizol to extract 100 ng RNA, the group estimated the contribution of each hematopoietic cell type to miRNA content in blood volume. The authors suggest that such data can 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.

A

miRNA	Sequence
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGUGUGGUU
Let-7c	UGAGGUAGUAGGUUGUAUGGUU
Let-7d	AGAGGUAGUAGGUUGCAUAGUU
Let-7e	UGAGGUAGGAGGUUGUAUAGUU
Let-7f	UGAGGUAGUAGAUUGUAUAGUU
Let-7g	UGAGGUAGUAGUUUGUAUAGUU
Let-7i	UGAGGUAGUAGGUUGUGCUGUU

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).

Several studies have focused on the identification of novel miRNAs in the progression of colorectal cancer[14,40]. 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. A comprehensive study by Hur *et al.* identified a metastasis-specific miRNA signature in patient colorectal cancers providing evidence that miRNAs may be clinically applicable to predict prognosis and distant metastasis in colorectal cancer. Adoption of the nCounter miRNA profiling platform enabled identification and evaluation of differentially expressed miRNA tissue and serum based biomarkers.

In addition to the studies mentioned here, NanoString maintains a comprehensive and frequently updated list online of all of the publications that use NanoString assays, including miRNA, other small RNA species, gene expression (mRNA), and CNV. For more references and information, visit NanoString's website at **www.nanostring.com/community/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 panels are a set of unique and powerful targeted discovery and validation tools, providing excellent specificity, low false-positive rates[31], and a direct digital readout. With a simple workflow, these panels enable investigators to generate comprehensive expression data sets in short amounts of time with minimal hands-on manipulation.

This white paper highlighted many of the diverse applications for NanoString miRNA panels, 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 panel designs that ensure each panel contains biologically relevant content and appropriate controls.

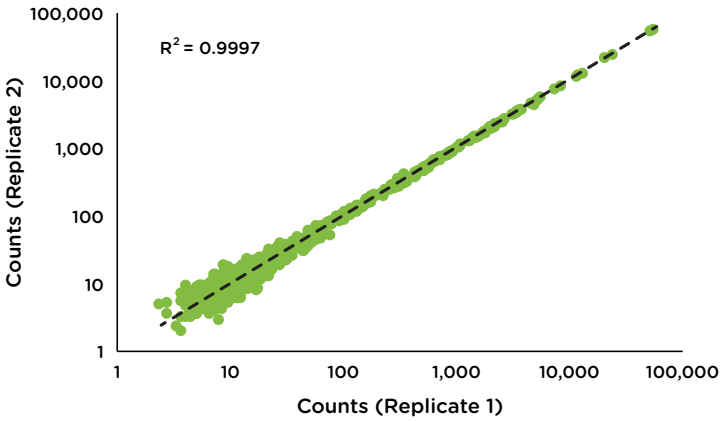

FIGURE 6 nCounter Human v3 miRNA Expression assay is highly reproducible. Count correlations between the preparation of the same RNA sample (100 ng) were extremely high (R^2 > 0.99) demonstrating the precision of the nCounter Human v3 miRNA assay.

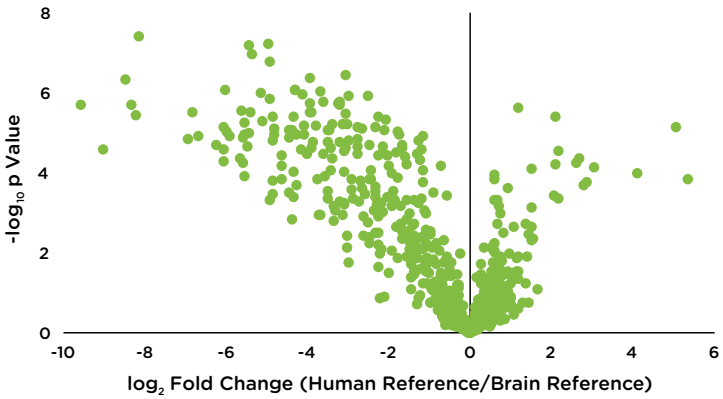

FIGURE 7 Differential miRNA expression on the nCounter platform. nCounter miRNA assays are capable of accurately quantifying changes in miRNA expression. Human Reference RNA (100 ng per assay) and Brain Reference RNA (100 ng per assay) were profiled in triplicate using the nCounter Human v3 miRNA assay. Hundreds of miRNAs are significantly differentially expressed between samples.

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AUGUST 2015 LBL-10112-02