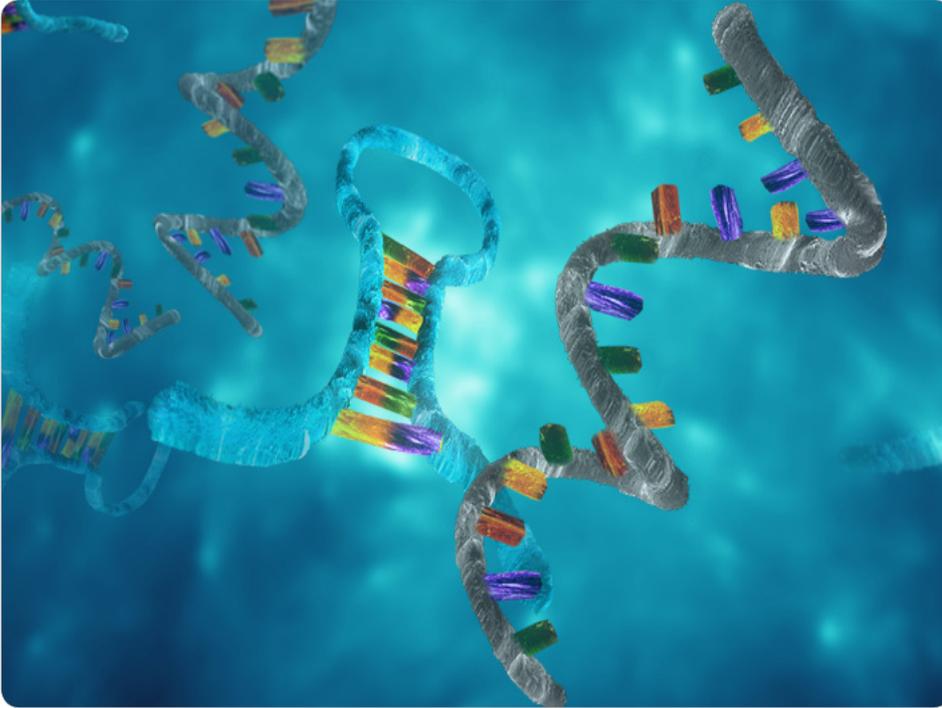




nCounter[®] miRGE[™] Assay CodeSet



Product Highlights

- **Two assays in a single reaction**
- **Highly multiplexed**, simultaneous measurement of subsets of miRNAs and mRNAs in a single tube reaction
- **No reverse transcription, no amplification and fewer pipetting steps** than other assays
- Truly **digital data acquisition** delivers sensitive and **highly reproducible** results
- Compatible with total RNA isolated from any source, **including FFPE samples**

nCounter[®] miRGE[™] Assay Overview

The **nCounter miRGE Assay** is designed to provide a single-tube, sensitive, reproducible and highly multiplexed method for detecting subsets of both mRNAs and miRNAs across all biological levels of expression. After processing with the nCounter miRNA Sample Preparation Kit, messenger RNA and tagged miRNAs in total RNA are quantified without the use of reverse transcription or amplification. Instead, the individual sequences are counted with sequence-specific fluorescent barcodes. The assay can be run on total RNA isolated from any source, including Formalin-Fixed Paraffin Embedded (FFPE) samples.

MicroRNAs (miRNAs) are small non-coding RNAs that modify gene expression patterns via posttranscriptional regulation of mRNA targets. A single miRNA has the potential to regulate the activity of many of downstream mRNA targets; conversely, multiple miRNAs can target a single mRNA. This remarkable

regulatory potential means that aberrant expression of small numbers of miRNAs can have a wide impact on global gene expression. Many of these gene expression changes are related to cellular proliferation, differentiation, metabolism, and apoptosis. It is therefore important to understand the interactions between miRNAs and their mRNA targets. The NanoString nCounter miRGE Assay allows researchers the ability to simultaneously investigate expression levels of both miRNAs and the mRNAs they potentially regulate in a single reaction.

The NanoString nCounter miRGE Assay delivers accurate and sensitive profiling of 100 to 200 mRNA targets and between 5 and 30 miRNA targets. The system provides exceptional ease-of-use for the combined analysis of miRNA and mRNA expression.

Molecules That Count[®]

nCounter miRNA Sample Preparation Kit

The nCounter miRNA Sample Preparation Kit provides reagents for ligating unique oligonucleotide tags onto miRNAs that are in a total RNA sample, allowing these short RNAs to be counted with great specificity. Since the tagging reactions only affect miRNAs in the sample, the mRNA transcripts are unaltered, allowing them to be quantified in a downstream hybridization.

The nCounter miRGE sample preparation procedure involves a multiplexed annealing of the specific tags to their target miRNAs, a ligation reaction, and an enzymatic purification to remove the excess unligated tags. Sequence specificity between each miRNA and its appropriate tag is ensured by careful, stepwise control of annealing and ligation temperatures. Controls included in the nCounter miRNA Sample Preparation Kit allow the user to monitor the ligation efficiency and specificity, hybridization efficiency, and background levels through the entire process. This unique sample preparation method has a total hands-on time of approximately 1 hour.

nCounter Analysis System

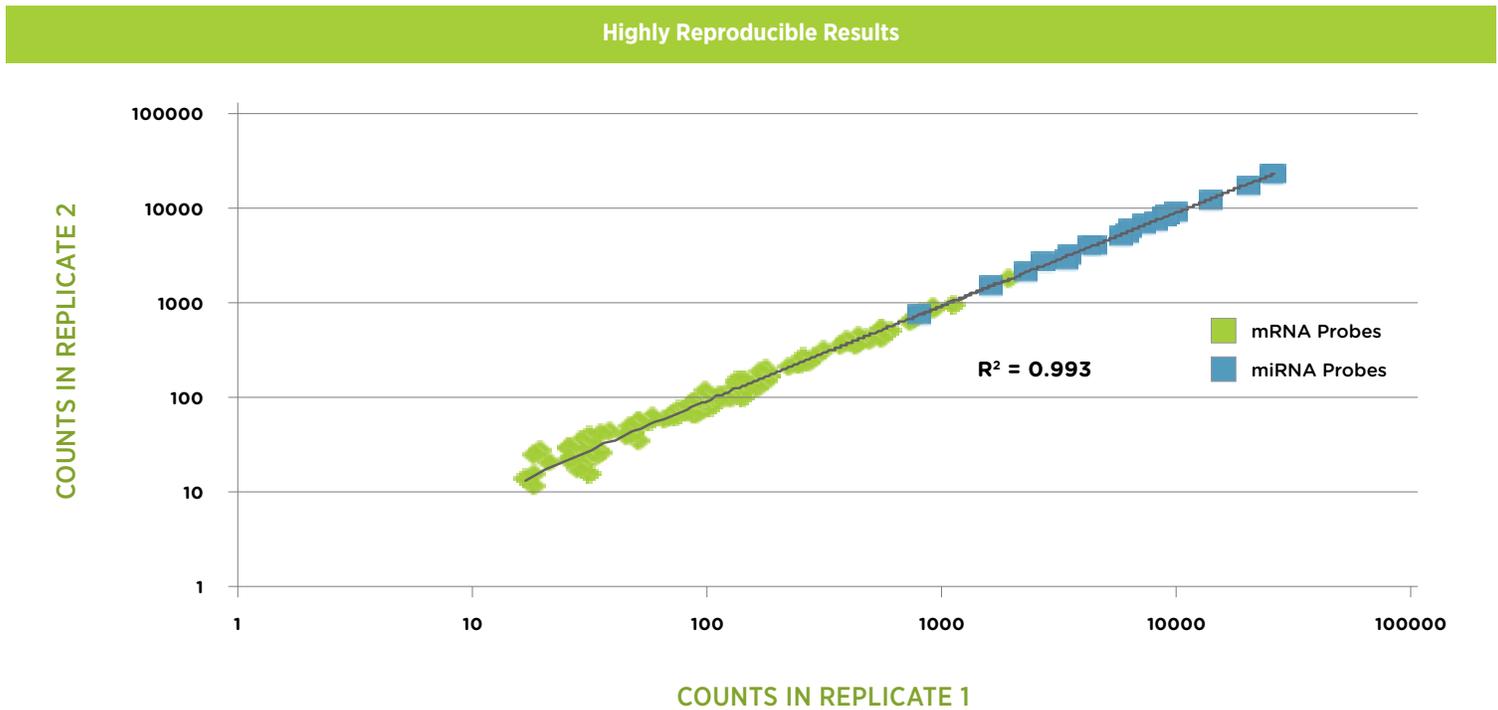
After sample preparation, the nCounter Analysis System is used to obtain quantitative expression data. The nCounter Analysis System delivers direct multiplexed measurements of tagged miRNA and mRNA expression, providing digital readouts of the relative abundance of hundreds of expression targets simultaneously. The nCounter platform is based on two target-specific probes that bind to their cognate sequence in a solution-based hybridization reaction. A Reporter Probe carries the fluorescent barcode and a Capture Probe allows the complex to be immobilized for data collection. The protocol does not include any amplification steps that might introduce bias into the results.

Probes specific for a set of mRNAs and miRNAs are combined with a series of internal controls to form the miRGE Assay Codeset. After hybridization of the Codeset with the tagged preparation, samples are transferred to the nCounter Prep Station where excess probes are removed and probe/target complexes are aligned and immobilized in the nCounter Cartridge. Cartridges are then placed into the nCounter Digital Analyzer for data collection. Each miRNA and mRNA of interest is identified by the “color code” generated by six ordered fluorescent spots present on the Reporter Probe. The Reporter Probes on the surface of the cartridge are then counted and tabulated for each mRNA and miRNA species.

miRGE Assay Performance Data

To demonstrate the reproducibility of data generated using the nCounter miRGE Assay, 100 ng of total RNA was isolated from mouse lung tissue and processed following the nCounter miRGE Assay Manual. Processed samples were hybridized with a miRGE CodeSet interrogating 20 miRNA and 185 mRNA targets. To account for minor differences in hybridization and purification efficiencies, the raw molecular counts were normalized with internal positive spike controls present in each sample. Counts for individual probes in technical replicates were highly correlated between sample preparations ($R^2 > 0.99$, Figure 1).

FIGURE 1: Counts for 20 miRNAs and 185 mRNAs species in mouse lung total RNA were highly reproducible between technical replicates.



Given that many miRNAs share similar nucleotide sequences at their 5' end ("seed" regions), the ability of the miRGE Assay to specifically identify individual miRNAs among highly homologous family members was examined. Individual synthetic Let7 family miRNAs (Table 1) for both human and mouse were prepared and analyzed following the nCounter miRGE Assay protocol and the counts for specific family members were examined. Counts for these specific Let7 probes in a single target assay were then expressed as a percentage relative to the perfect match probe in that assay (Table 2). The majority of probes exhibited less than 1 percent cross-hybridization, indicating that the nCounter miRGE Assay can accurately distinguish between highly similar miRNAs with great sequence specificity.

TABLE 1: Sequences for miRNAs in the Let7 family are highly similar.

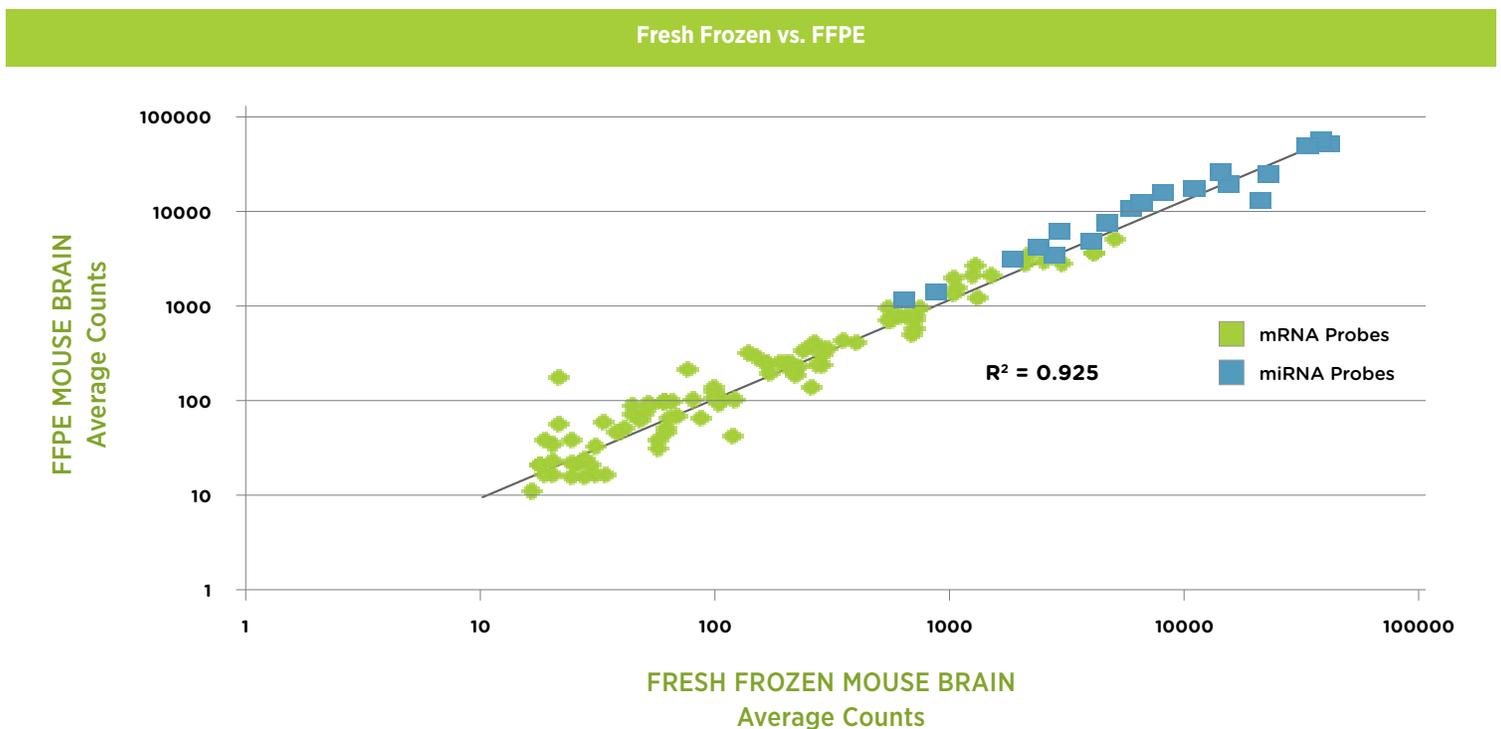
miRNA	Sequence
Let7e	ugagguaggagguuguauaguu
Let7f	ugagguaguagauuguauaguu
Let7i	ugagguaguaguuugugcuguu

TABLE 2: Low cross-hybridization between miRNAs of the Let7 family demonstrated the superior sensitivity of the nCounter miRGE Assay.

nCounter miRNA Probes		miRNA Target							
		Let7a	Let7b	Let7c	Let7d	Let7e	Let7f	Let7g	Let7i
HUMAN	Let7e	1%	-	-	-	100%	-	-	-
	Let7f	-	-	-	-	-	100%	-	-
MOUSE	Let7e	1%	-	-	-	100%	-	-	-
	Let7i	3%	-	-	-	1%	-	-	100%

To examine the ability of the nCounter miRGE Assay to measure mRNA and miRNA expression levels in Formalin-Fixed Paraffin-Embedded (FFPE) tissues, FFPE-derived purified total RNA was processed and hybridized with a miRGE CodeSet as described in the assay manual. Counts for each mRNA and miRNA probe were then compared to counts generated with matched purified RNA from fresh tissue. These results demonstrate that high quality data, comparable to that obtainable with fresh samples ($R^2 \geq 0.92$), can be achieved from FFPE-derived RNA samples and the nCounter miRGE Assay kit.

FIGURE 2: Molecular counts of miRNAs and mRNAs correlate extremely well in total RNA purified from fresh frozen or FFPE-preserved matched tissue ($R^2 > 0.92$).



System Performance

Description	Specifications
Level of multiplexing	100 – 200 mRNAs 5 – 30 miRNAs
Recommended amount of starting material	100 ng purified total RNA
Sample types supported	Purified total RNA
Sample prep reaction volume	10 µL
Hybridization reaction volume	35 µL
Limit of detection:	
• miRNA	> 2.5 copies per cell copy per cell
• mRNA	1 copy per cell
Fold change sensitivity	> 2-fold change
Hybridization spike correlation	$R^2 \geq 0.95$
Linear dynamic range	2×10^6 total counts
nCounter Prep Station throughput	12 samples < 2.5 hours
nCounter Digital Analyzer throughput	12 samples / 4 hours (up to 72 samples per day unattended running in continuous mode)
Controls	6 positive hybridization controls 8 negative hybridization controls 2 positive ligation controls

Ordering Information

Description	Quantity / Use	Part Number (P/N)
nCounter miRGE Assay Kit	48 assays 96 assays	MIR-P1CC-48 MIR-P1CC-96
nCounter Analysis System (includes the Prep Station and Digital Analyzer)	1	NCT-SYS-120
Additional nCounter Prep Station	1	NCT-PREP-120
Additional nCounter Digital Analyzer	1	NCT-DIGA-120

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