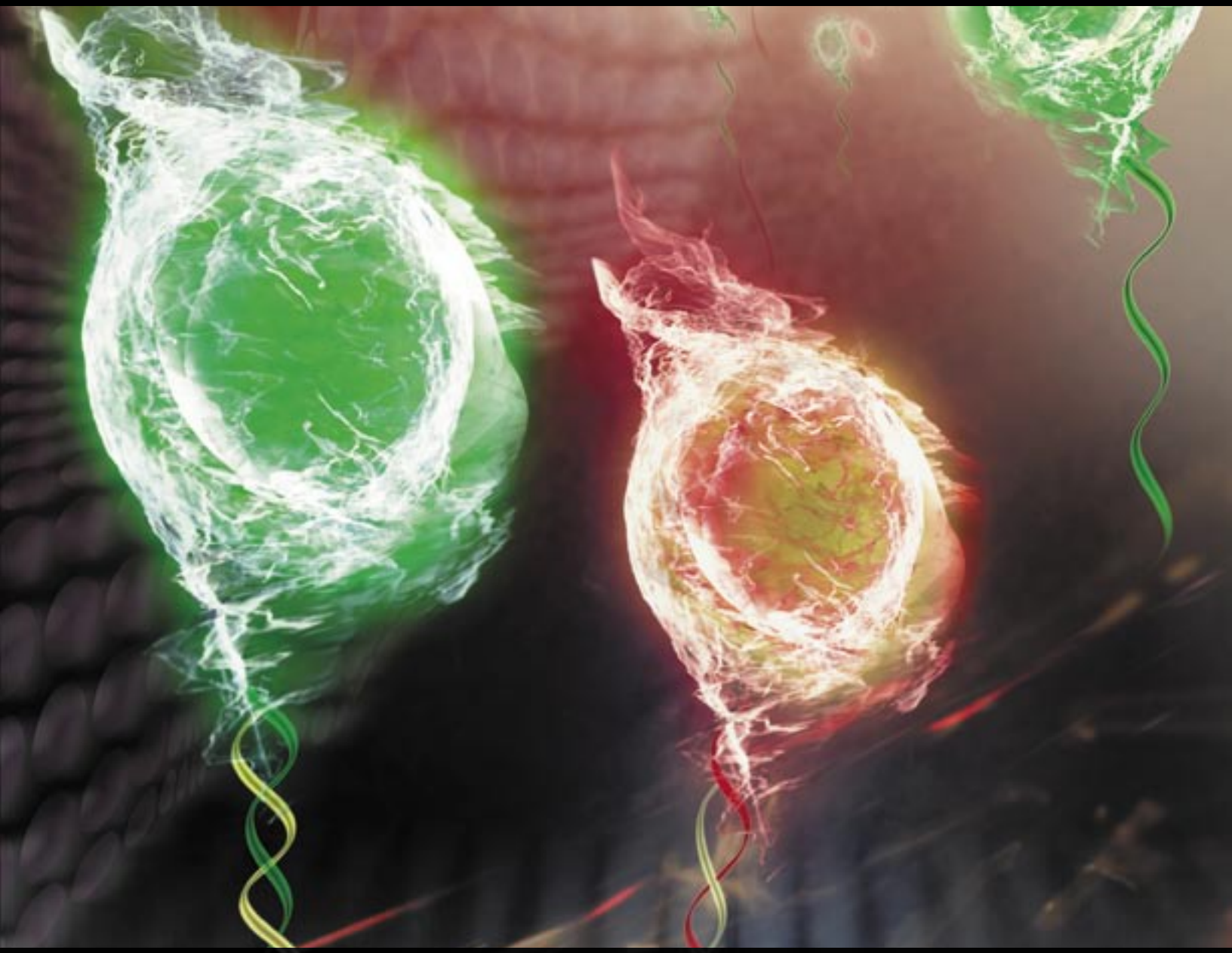


Sensitive, reproducible miRNA profiling

Complete solutions for profiling miRNA expression patterns

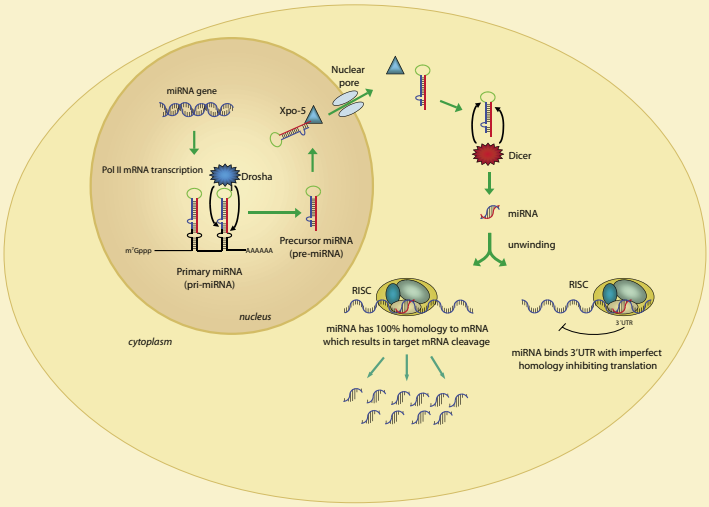
- Complete, optimized platform for miRNA profiling
- Quick and efficient miRNA expression analysis
- Superior sensitivity of miRNA detection
- Excellent reproducibility

miRNA-background

The transcriptional output for the vast majority of complex genomes, including that of human, consists of non-protein-coding RNA (1). These non-coding RNAs appear to be involved in a variety of cellular roles ranging from simple housekeeping to complex regulatory functions. Of the various subclasses of non-coding RNAs, microRNAs (miRNAs) are the most thoroughly characterized. These single-stranded RNAs are typically 19–25 nucleotides in length and are thought to regulate gene expression post-transcriptionally by binding to the 3′ untranslated regions (UTRs) of target mRNAs, inhibiting their translation (2). Recent experimental evidence suggests that the number of unique miRNAs in humans could exceed 800 (3), though several groups have hypothesized that there may be up to 20,000 (4,5) non-coding RNAs that contribute to eukaryotic complexity.

RNA polymerase II transcribes miRNA genes, generating long primary transcripts, pri-miRNAs, that are processed by the RNase III-type enzyme, Drosha, yielding hairpin structures (pre-miRNAs). Pre-miRNA hairpins are exported to the cytoplasm where they are further processed into unstable miRNA duplexes by the RNase III protein Dicer. The less stable of the two strands in the duplex is incorporated into a multiple-protein nuclease complex called the RNA-induced silencing complex (RISC), which regulates protein expression. These RISCs, guided by the miRNA, interact with the 3′ UTR of target mRNAs at regions exhibiting imperfect sequence homology, thus inhibiting protein synthesis by a mechanism that has yet to be fully elucidated. In contrast, RISCs in plants typically interact with their mRNA targets in regions that exhibit perfect sequence homology, leading to mRNA degradation rather than translational inhibition (Figure 1).

Figure 1—miRNA biogenesis and function



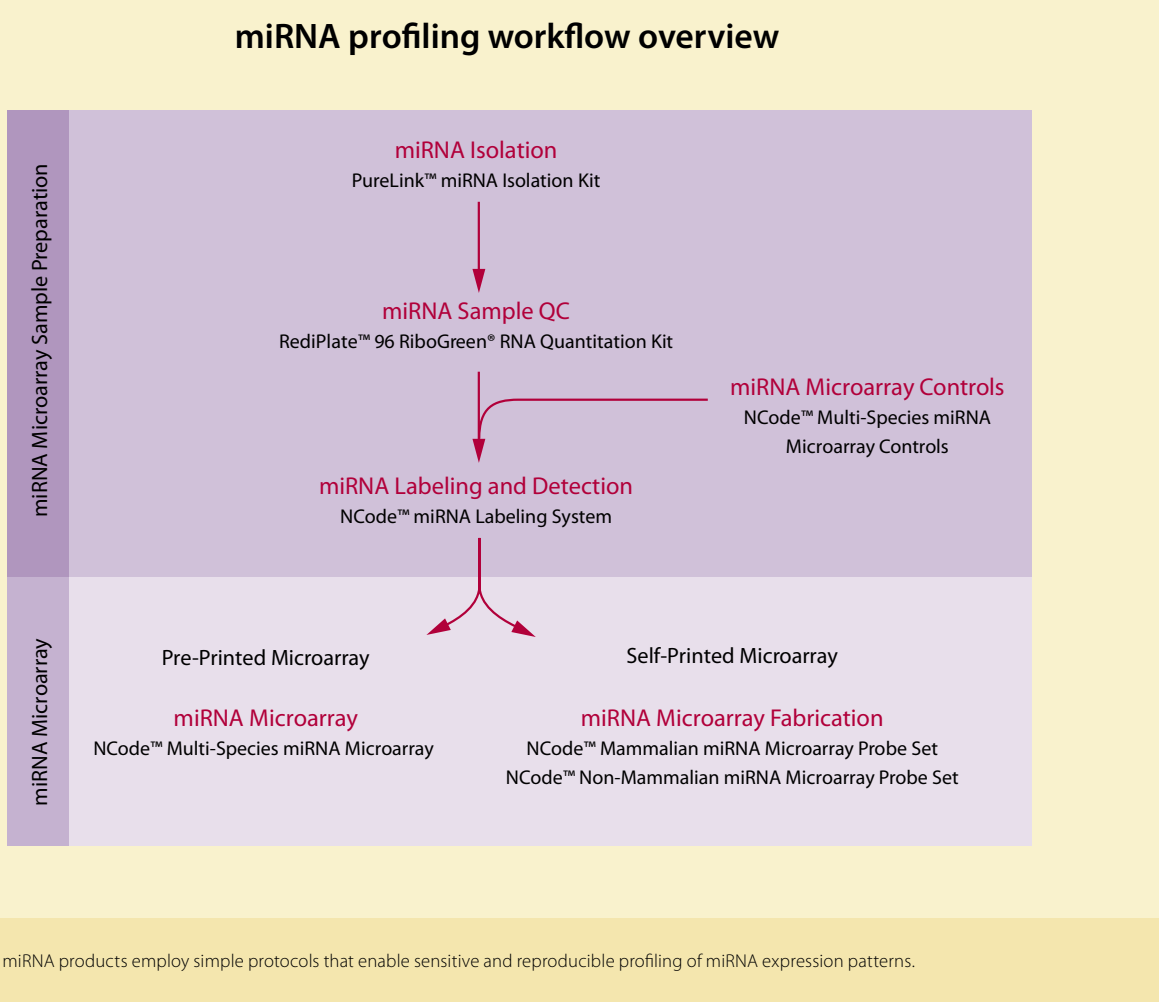
miRNA transcripts, generated by RNA Polymerase II, are processed by RNase III enzymes Drosha (nucleus) and Dicer (cytoplasm) yielding a 19-25 nt miRNA duplex. The less stable strand of the duplex is incorporated into the RISC complex which regulates protein expression.

Profile miRNA with the NCode™ miRNA platform

Though hundreds of miRNAs have been discovered in a variety of organisms, little is known about their cellular function. Several unique physical attributes of miRNAs, including their small size, lack of poly-adenylated tails, and tendency to bind their mRNA targets with imperfect sequence homology, have made them elusive and challenging to study.

NCode™ miRNA Analysis products have been optimized to purify, label, and detect miRNA expression on a miRNA microarray, enabling simple and efficient profiling of miRNA expression patterns in various types of tissue, disease, and developmental states, providing insight into their role in gene regulation (Figure 2).

Figure 2—miRNA expression profiling with NCode™ miRNA analysis products



NCode™ miRNA products employ simple protocols that enable sensitive and reproducible profiling of miRNA expression patterns.

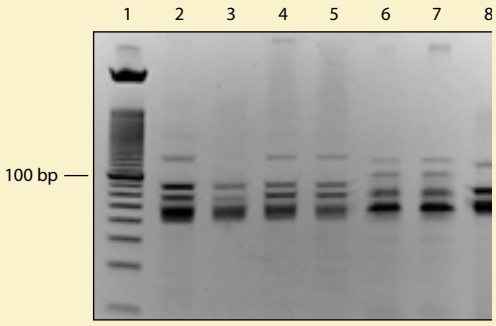
Efficient miRNA purification and enrichment with PureLink™ technology

Successful miRNA profiling experiments begin with the efficient purification of small RNAs. Traditional column-based RNA isolation products are optimized to purify higher molecular weight RNA, resulting in minimal small RNA enrichment, while gel-based extraction methods are laborious and inefficient. Using total RNA to profile miRNA expression can limit sensitivity, as the relative abundance of small RNAs in a total RNA sample is low.

The PureLink™ miRNA Isolation Kit is designed to purify small RNA molecules (< 200 nt), including tRNA, 5S rRNA, 5.8S rRNA, and other regulatory RNA molecules such as microRNA (miRNA) and short interfering RNA (siRNA) (Figures 3). The PureLink™ miRNA Isolation Kit utilizes a silica-based two-column system to enrich small RNA species from various sample sources. From sample lysates, RNAs greater than ~200 nt are bound to the first column, allowing the small RNA molecules to flow through. This flow-through fraction, enriched with small RNA molecules, is mixed with a higher concentration of ethanol to increase its binding affinity to the glass fiber membranes in the second column. Bound, small RNAs are then eluted away from the larger RNA molecules such as 28S rRNA, 18S rRNA, and mRNA that can interfere with expression analysis of the small RNAs. Purified RNA is suitable for most downstream applications including microarray analysis, northern blotting, and RT-PCR. The PureLink™ miRNA Isolation Kit provides:

- Fast and easy-to-use protocol—less time at the bench
- Effective purification of RNA species less than 200 nt—enhances downstream analysis

Figure 3— Small RNA molecule purification from various samples using the PureLink™ miRNA Kit



A variety of small RNA molecules were efficiently purified with the PureLink™ miRNA Kit and run on a NuPAGE® Novex 12% Bis-Tris Gel. Lane 1: 10 bp Ladder; Lane 2: 2 x 10^6 HeLa cells; Lane 3: 2 x 10^6 293F cells; Lane 4: 5.5 mg Rat spleen; Lane 5: 5 mg Mouse liver; Lane 6: 300 mg Spinach; Lane 7: 1 x 10^7 Yeast cells; Lane 8: 2 x 10^9 Bacteria cells.

Fast and reproducible miRNA labeling with the NCode™ miRNA Labeling System

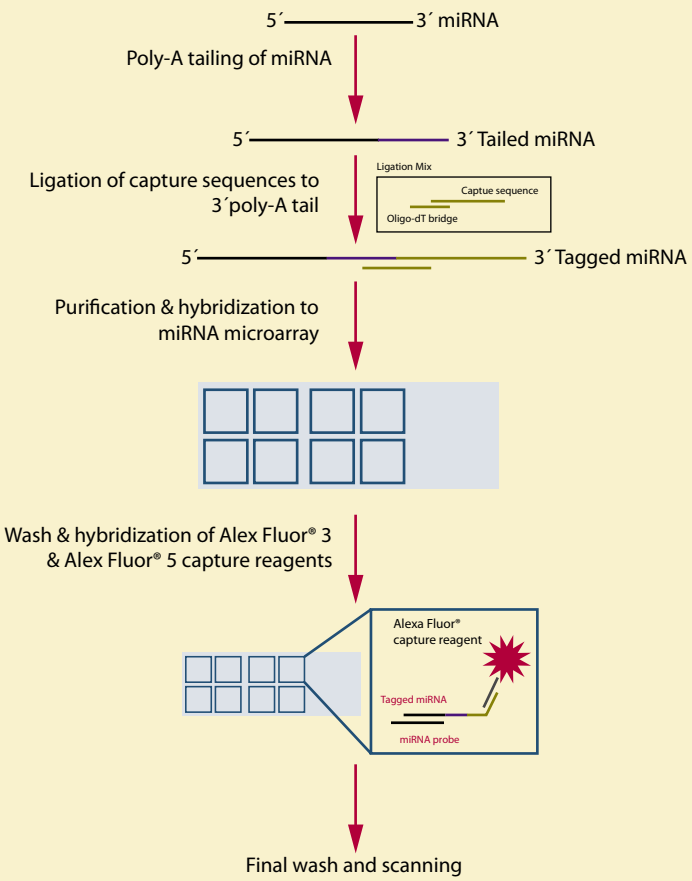
Given their size (18–25 nt) and lack of polyA tails, miRNAs are not effectively labeled by traditional mRNA labeling procedures such as random-primed or oligo(dT)-primed cDNA synthesis. The NCode™ miRNA Labeling System is a fast and reproducible labeling system that contains all the necessary components to directly label endogenous miRNAs with fluorescent tags. It has been optimized to ensure sensitive and accurate profiling of miRNA expression patterns from minimal RNA input.

Simple labeling

After enriching for miRNAs with the PureLink™ miRNA Purification Kit, miRNAs are tagged directly in a quick and easy-to-use protocol with the NCode™ miRNA Labeling System (Figure 4). The protocol consists of just three steps prior to hybridization:

1. Poly-A tailing (15 min)
2. Ligation of unique capture sequences using a bridging oligo (30 min)
3. Purification of tagged miRNA (10 min)

Figure 4—NCode™ miRNA Labeling System overview



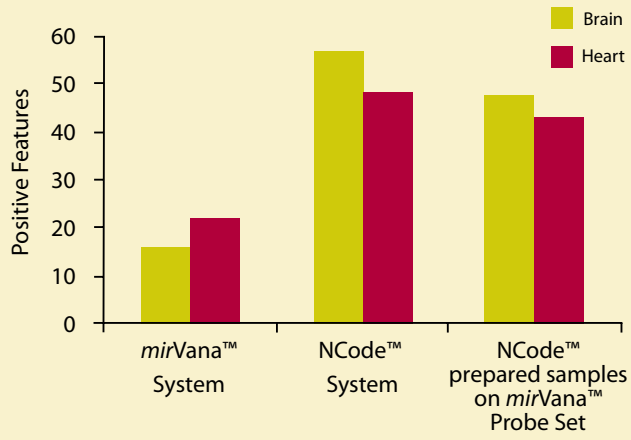
The NCode™ miRNA Labeling System protocol consists of only three quick steps before hybridization, minimizing hands-on time.

Sensitive detection and reproducible results

After an overnight hybridization, the microarrays are incubated with signal amplifying Alexa Fluor® 3 and Alexa Fluor® 5 capture reagents.* The capture reagents are comprised of DNA polymers each containing 900 Alexa Fluor® molecules and sequences complementary to the ligated tags on the hybridized miRNAs (Figure 4). Each tagged miRNA will bind only one highly fluorescent DNA polymer, ensuring maximum signal-to-background ratios, and strong signal correlation for increased sensitivity. Using the NCode™ miRNA Labeling System, you can:

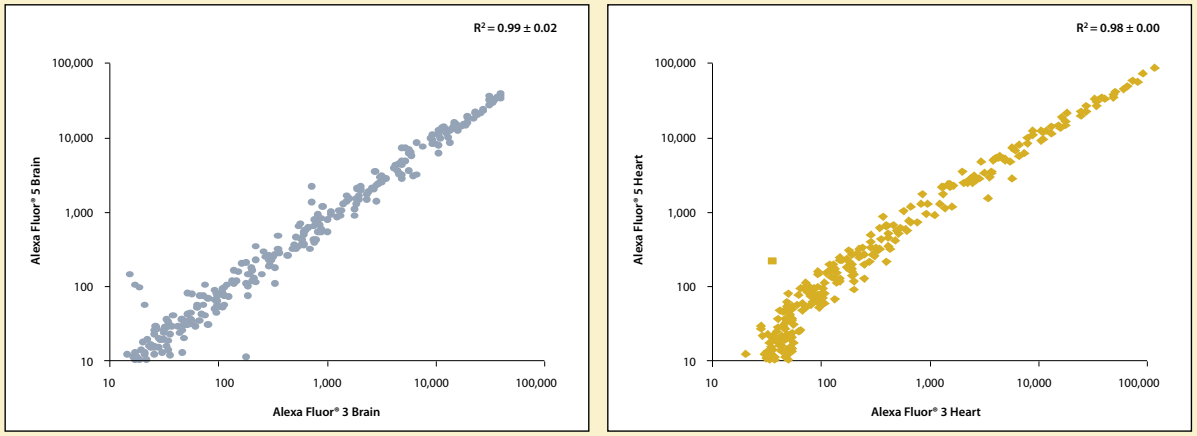
- Prepare your miRNA samples in less than one hour (Figure 4)
- Profile miRNAs with less total RNA (Figure 5)
- Detect miRNAs expressed at low levels
- Obtain reproducible results (Figure 6)

Figure 5—Sensitivity of the NCode™ miRNA Labeling System



Detectable positives for mouse self-self hybridizations. Triplicate homotypic hybridizations for mouse brain and mouse heart miRNA prepared with the mirVana™ miRNA Isolation and Labeling Kits (Ambion) or the PureLink™ miRNA Enrichment Kit and NCode™ miRNA Labeling System. Samples prepared with the mirVana™ System were hybridized in Ambion's recommended hybridization solution/conditions and detected on the mirVana™ Probe Set. NCode™ labeled samples were hybridized as described in the protocol and detected on both the NCode™ miRNA Mouse Probe Set and the mirVana™ probes. The number of positive features detected for the samples labeled with the NCode™ miRNA Labeling System is 50% greater than for samples labeled with Ambion's mirVana™ Labeling Kit on either probe set. For an miRNA to be scored positive, both channels must be greater than the lower limit of detection (~300-500 RFUs) for at least four of the six replicate features across the three replicate arrays.

Figure 6—Reproducibility of NCode™ miRNA Labeling System



Linear correlation of mouse brain and heart homotypic hybridizations. Data points represent mean normalized data of triplicate arrays. Mouse heart and brain miRNA were enriched using the PureLink™ miRNA Isolation Kit and labeled with the NCode™ miRNA Labeling System. Mouse miRNAs were detected on the NCode™ miRNA array. The mean R^2 value is ≥0.98 for both data sets, showing a high correlation between channels with minimal variability across arrays.

* Capture reagents contain 3DNA™ reagent manufactured under license from Genisphere, Inc.

Comprehensive miRNA profiling with the NCode™ Multi-Species miRNA Microarray

Microarrays have become instrumental for profiling miRNA expression patterns. The NCode™ Multi-Species miRNA Microarray contains probes for profiling miRNAs from a variety of species, allowing you to comprehensively screen most known miRNA molecules for the most studied species. There are several key factors that must be addressed when using microarrays to successfully profile miRNAs.

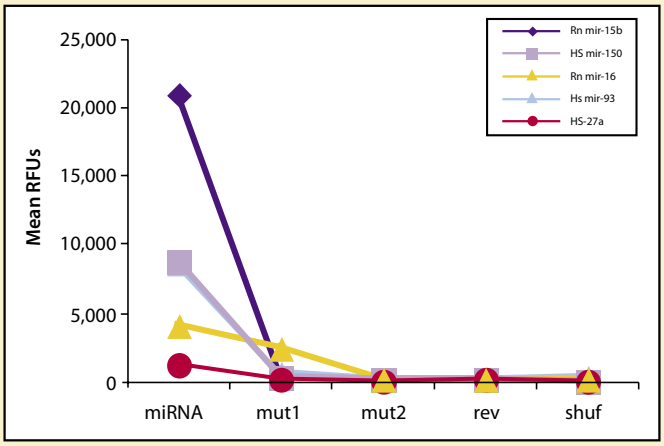
Validated probe design

Careful consideration must be taken when designing probes for analyzing miRNAs to maximize specificity and sensitivity.

The NCode™ Multi-Species miRNA Microarray uses a validated probe design algorithm that generates probes with:

- Maximum hybridization intensities for increased sensitivity (Figure 8)
- Maximum specificity for discerning between closely related miRNAs (Figures 7 and 8)
- Normalized melting temperatures for uniform hybridization

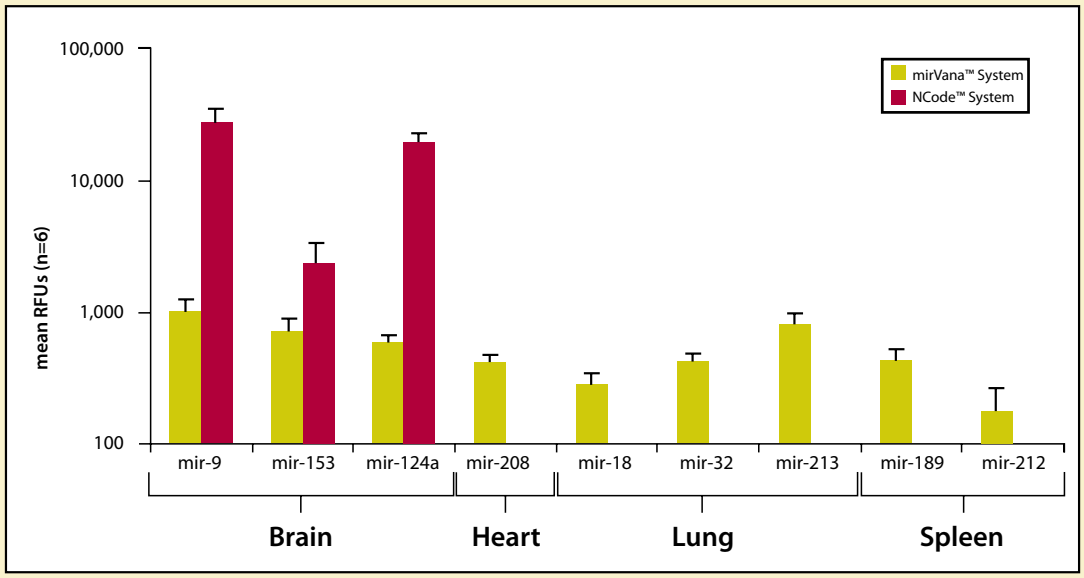
Figure 7—Probe specificity of NCode™ Multi-Species Microarray



Discrimination between perfect match and mismatch probes. Data points represent the mean RFUs (n=6) from NCode™ miRNA Arrays hybridized with mouse heart miRNA labeled with the NCode™ miRNA Labeling System. For a subset of miRNAs, probes were designed to evaluate the specificity of the NCode™ miRNA Detection System. These include probes with a single (mut1) or double (mut2) mismatch, the reverse complement (rev), and a probe in which the sequence was randomly shuffled (shuf). The data illustrate that the system is consistently able to discriminate between sequences with a double mismatch, and often between the perfect match and probes with a single mismatch, across a broad dynamic range of perfect match intensities. The reverse and shuffle probes are always negative.

Comprehensive miRNA profiling with the NCode™ Multi-Species miRNA Microarray, continued

Figure 8—Improved sensitivity and specificity with NCode™ miRNA analysis products



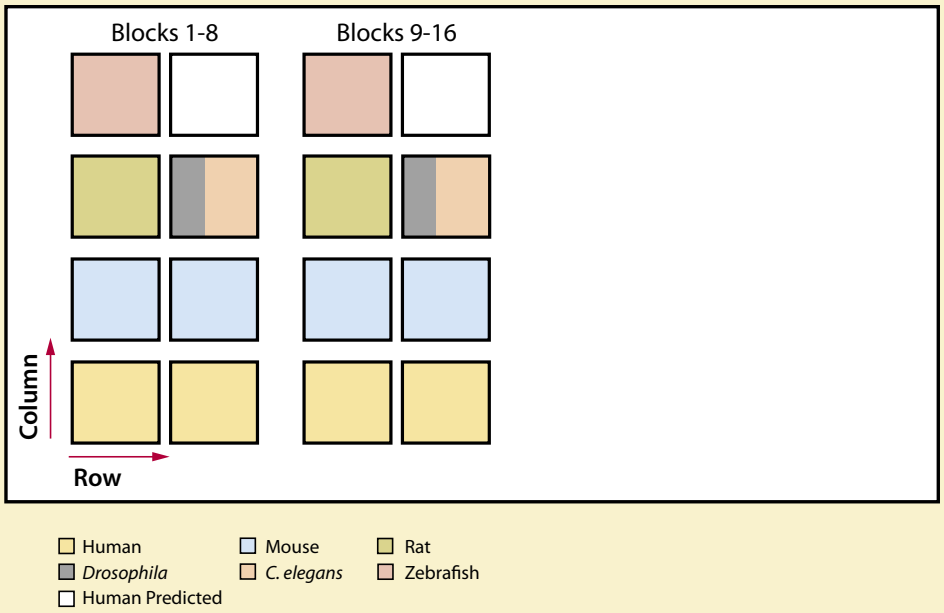
Detection of validated tissue-specific miRNAs. The graph shows the mean Alexa Fluor® 3 signal, using the NCode™ miRNA Labeling System or the Cy™3 signal obtained using the *mir*Vana™ Labeling System (n=6) from mouse brain miRNA for nine tissue-specific miRNAs; three brain, one heart, three lung, and two spleen (6). The NCode™ miRNA Detection System shows more sensitive and specific detection of the validated, tissue-specific miRNAs than the Ambion system.

Highest probe content

The NCode™ Multi-Species Microarray contains more DNA probe content than any other commercially available microarray (Figure 9). In addition to probes targeting all the miRNAs in Sanger mirBase 7.0 for human, mouse, rat, *Drosophila, C.elegans*, and zebrafish, the NCode™ Array contains probes for 144 predicted human miRNAs that have been generated using comparative regulatory motif analyses (7). Each species is separated on the microarray to simplify visualization of array images. This combination of probe content enables you to:

- Compare cross-species miRNA expression
- Validate existence of predicted miRNA species

Figure 9—NCode™ Multi-Species miRNA Microarray



Each epoxide slide contains probes for profiling human, mouse, rat, *Drosophila, C. elegans,* and zebrafish, spotted in duplicate. Probes are separated by species as shown in image. NCode™ Probes for the NCode™ miRNA Controls are printed throughout the array to facilitate analysis, as are mismatch and shuffled controls for monitoring hybridization specificity. The actual probe layout in Gene List files may vary. See manual for instructions for downloading the appropriate Gene List file for your microarray product.

Probe Content	Number of Probes
Human	329
Human predicted*	144
Mouse	250
Rat	201
Drosophila	93
C. elegans	130
Zebrafish	163
NCode™ Controls	20
Mismatch controls	24

* Predicted miRNAs not yet validated

Monitor performance with NCode™ Multi-Species miRNA Microarray Controls

As with any microarray experiment, controls are necessary to minimize and monitor experimental variability. The NCode™ Multi-Species miRNA Microarray Controls include a set of 10 synthetic, exogenous controls that are spiked into the microarray labeling reaction. These probes do not cross-react with any probes to endogenous miRNAs on the microarray. They are spotted throughout the NCode™ Multi-Species miRNA Microarray and are available with the NCode™ Mammalian and Non-Mammalian miRNA Microarray Probe Sets. By using the NCode™ Control Set, you will be able to:

- Assess the performance of the labeling reaction
- Evaluate hybridization efficiency
- Determine signal linearity
- Normalize data

Perform quick and efficient miRNA analysis with the NCode™ miRNA analysis products

NCode™ miRNA analysis products will enable you to profile miRNA expression patterns sensitively and reproducibly with the convenience of an integrated solution. Visit **www.invitrogen.com/ncode** and see how the NCode™ platform will facilitate your miRNA studies.

Ordering information

Product	Quantity	Cat. no.
miRNA purification		
PureLink™ miRNA Isolation Kit	25 rxns	K1570-01
miRNA Quantitation		
RediPlate™ 96 RiboGreen® RNA Quantitation Kit	1 Kit	R-32700
miRNA Fluorescent Labeling		
NCode™ miRNA Labeling System	20 rxns	MIRLS-20
miRNA Microarrays		
NCode™ Multi-Species miRNA Microarray	5 arrays	MIRA-05
miRNA Microarray Controls		
NCode™ Multi-Species miRNA Microarray Controls	100 rxns	MIRAC-01
miRNA Microarray Probe Sets		
NCode™ Mammalian miRNA Microarray Probe Set	500 pmol	MIRMPS-01
NCode™ Non-Mammalian miRNA Microarray Probe Set	500 pmol	MIRNPS-01

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