



# Reach beyond the helix

miRNA analysis products

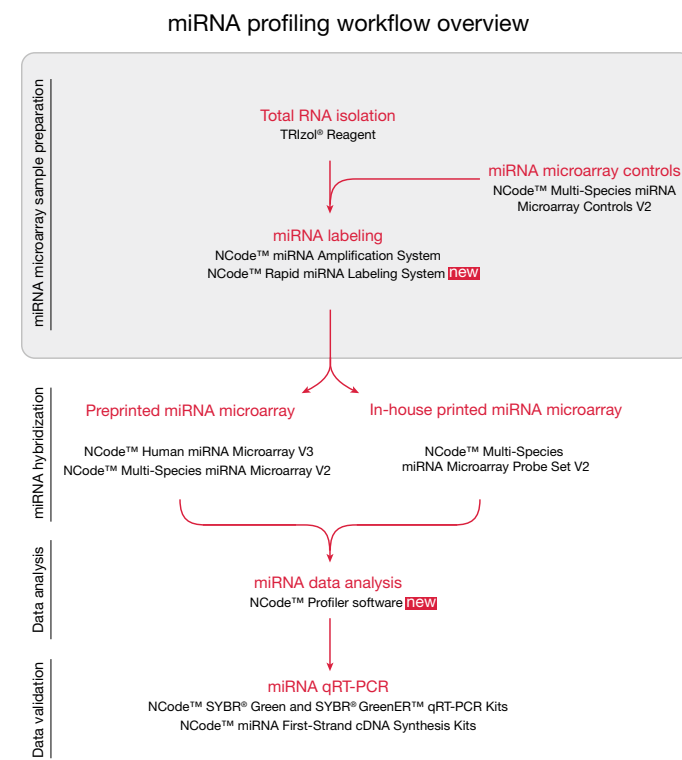
# Complete solutions for miRNA profiling

## NCode™ miRNA analysis products

- Complete, optimized platform for miRNA profiling
- Superior sensitivity of miRNA detection
- Excellent reproducibility
- Experiments completed in as little as one day

Invitrogen offers a broad range of trusted technologies for examining aspects of gene expression arising through coded and epigenetic mechanisms. In particular, noncoding RNAs involved in gene regulation have attracted the interest of a growing number of researchers. Of the various subclasses of regulatory noncoding RNAs, miRNAs are the most thoroughly characterized; however, much remains to be understood about their precise cellular function and role in development and disease. miRNAs are challenging to study, due to their small size, lack of polyadenylated (poly(A)) tails, and mRNA target binding characteristics. NCode™ miRNA analysis products—a key part of Invitrogen’s epigenetics technology platform—have been optimized to address these challenges, providing streamlined solutions for the enrichment, amplification, and profiling of miRNA expression with superb sensitivity and reproducibility. Invitrogen has made a commitment to advancing the field of epigenetics research, and in particular the study of noncoding RNAs. The recently launched NCode™ Rapid miRNA Labeling System (with an improved workflow; Figure 1) and NCode™ Human miRNA Microarray V3 are evidence that Invitrogen will continually update the tools, web resources, and reference information needed to facilitate this research.

Publications citing the NCode™ miRNA analysis platform can be found at [www.invitrogen.com/ncode](http://www.invitrogen.com/ncode).



**Figure 1—miRNA expression profiling with NCode™ miRNA analysis products.** NCode™ miRNA products use simple protocols that enable sensitive and reproducible profiling of miRNA expression patterns.

## Experimental planning

miRNA expression profiling came into prominence in part because of the expectation that a highly expressed miRNA for a given tissue or cell type (or a developmental stage) is likely to play a regulatory role. Since the first published article to report on miRNA profiling using an oligonucleotide microarray in 2004, microarray analysis has become the preferred tool for profiling miRNA expression patterns to gain insight into their relevance in development and disease.

The NCode™ miRNA analysis products are a leading platform for miRNA expression profiling. The workflow, including sample preparation and microarray steps, takes approximately 8–16 hr to complete. During sample preparation, total RNA is isolated and quantified. Amplification of small RNAs may be necessary if there is insufficient starting material. Next, a direct labeling method polyadenylates small RNAs and ligates a fluorescently labeled capture sequence to the tailed RNA. The tagged and tailed miRNAs are subsequently hybridized to the array. Bound miRNAs are detected via the hybridization of branched DNA structures containing Alexa Fluor® dye molecules. The miRNA expression profile is calculated from relative signal intensity detected by a microarray scanner for each spot on the array. Data can be validated by quantitative RT-PCR (qRT-PCR) using the NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit. A qRT-PCR workflow can also be used to easily screen larger numbers of samples for a smaller subset of key miRNAs after they have been identified.

## Efficient isolation of total RNA using TRIZOL® Reagents

Isolating high-quality total RNA from your sample is a crucial step for the success of your research. TRIZOL® Reagents purify all RNAs, including those in the 10–200 nucleotide range, and are thus recommended for isolating total RNA for studying miRNAs. Extraction procedures involve ready-to-use monophasic solutions of phenol and guanidine isothiocyanate, and are based upon improvements to the single-step RNA isolation method developed by Chomczynski and Sacchi.<sup>1</sup> Using TRIZOL® Reagents, you can expect:

- More effective purification of total RNA—without depletion of small RNAs—than with column-based purification systems
- A simple, flexible protocol, completed in less than 1 hr
- High-purity RNA from a wide range of animal tissues and cells

## Simplified miRNA fluorescent labeling and sensitive detection

The NCode™ Rapid miRNA Labeling System is a fast and reproducible way to directly label endogenous miRNAs with fluorescent tags. Using this system, miRNAs from total RNA samples are labeled with fluorescent Alexa Fluor® dyes and hybridized to microarrays printed with species-specific antisense miRNA probes. This system has been optimized to ensure sensitive and accurate profiling of miRNA expression patterns from minimal RNA input.

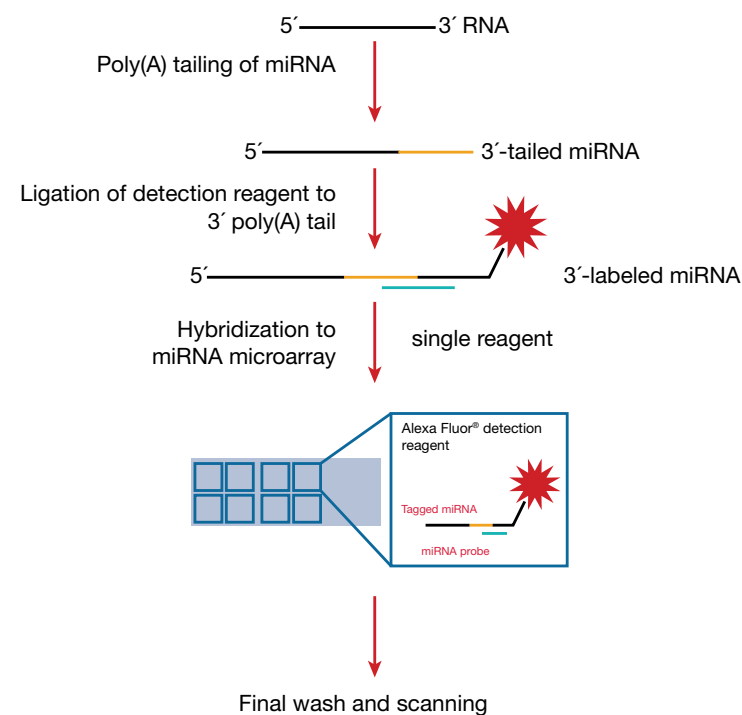
Starting with a 250 ng to 5 µg total RNA sample, miRNAs are tagged directly in a quick and easy protocol with the NCode™ Rapid miRNA Labeling System (Figure 2). Lower starting amounts (100 ng) may be used with similar sensitivities, depending on the scanner type used and the ability to utilize dynamic hybridization. The protocol takes approximately 1 hr to complete and consists of just two steps prior to hybridization: poly(A) tailing, and ligation of a fluorescent dendrimer. After an 8–16 hr hybridization, the microarrays are ready to scan and analyze.

The fluorescent dendrimer—a branched structure of single- and double-stranded DNA conjugated with an Alexa

Fluor® dye—delivers approximately 15 fluorophores to the sample. The high sensitivity achieved with this method is due to the signal-amplification effect of the labeled dendrimer, ensuring maximum signal-to-background ratios and strong signal correlation for increased sensitivity.

Using the NCode™ Rapid miRNA Labeling System, you can expect:

- Less time at the bench—hybridize in one step, with no miRNA enrichment required
- Increased sensitivity—reliably detect attomolar levels of miRNA (approximately 2–10 copies per cell)
- Rapid turnaround—complete your experiments in a single day using the 8 hr hybridization protocol with dynamic hybridization



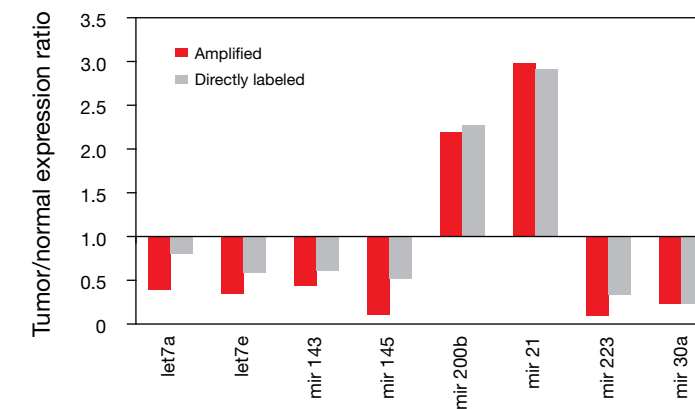
**Figure 2—NCode™ Rapid miRNA Labeling System titration.** The NCode™ Rapid miRNA Labeling System is recommended when starting with 250 ng to 5 µg total RNA. Lower amounts can be used, but miRNAs expressed at extremely low levels may be missed. When starting with less than 50 ng of total RNA, we recommend using the NCode™ miRNA Amplification System to increase the quantity of miRNA that can be detected.

## Ultrasensitive miRNA amplification

In many instances, RNA samples derived from laser capture microdissection (LCM), flow-sorted (FACS) samples, or needle biopsies do not yield sufficient starting amounts of RNA for standard profiling methods or for the experimental replicates needed for statistical reliability. In such cases, it is useful to apply linear miRNA amplification methods to the enriched miRNA population, thereby increasing the relative target abundance levels.

The NCode™ miRNA Amplification System, based on mRNA amplification methods, enables robust and efficient linear amplification and microarray profiling of small amounts of RNA species, such as miRNA, from as little as 50 ng of total RNA or the enriched equivalent. The NCode™ miRNA Amplification System delivers:

- Increased sensitivity, with consistent 2,000- to 5,000-fold amplification of miRNA
- Amplified product that faithfully represents the initial miRNA sample (Figure 3)
- Reproducible results with precise amplification from reaction to reaction



**Figure 3—Ultrasensitive and accurate miRNA microarray profiling from small samples.** Total RNA from large-cell carcinomas and adjacent normal tissue was isolated from multiple patients and enriched for miRNA with the PureLink™ miRNA Isolation Kit. Enriched miRNA from 300 ng of total RNA was amplified using the NCode™ miRNA Amplification System and labeled for array analysis with the NCode™ miRNA Labeling System. Additionally, the miRNA from 10 µg of total RNA from the same source was labeled with the NCode™ miRNA Labeling System without amplification and hybridized to NCode™ Multi-Species miRNA Microarrays for analysis. The ratios of miRNA expression in tumor samples vs. adjacent normal tissues were calculated for all human miRNA.

## Comprehensive miRNA expression profiling

Whether you prefer to profile miRNAs using in-house printed arrays or preprinted arrays, the NCode™ platform allows you to comprehensively screen most known miRNAs for the most studied species. Enjoy the advantages of a leading preprinted miRNA profiling microarray, or gain the flexibility of a probe set and controls for array fabrication in your lab.

The NCode™ microarrays offer:

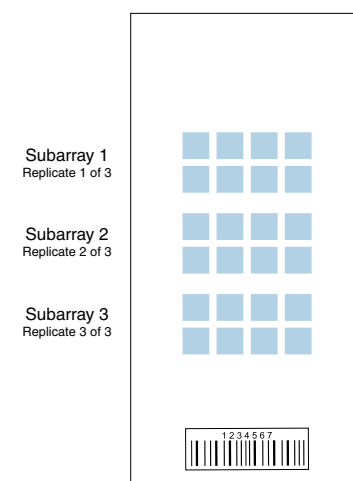
- Arrays spotted in triplicate with positive and negative controls throughout for monitoring hybridization specificity
- Increased sensitivity from maximum hybridization intensities and normalized melting temperatures for uniform hybridization
- Easy normalization of signal intensities during scanning using Alexa Fluor® dye control probes
- Detection and identification of miRNAs that are conserved in other species but have not yet been validated for your model system

### NCode™ Human miRNA Microarray

The NCode™ Human miRNA Microarray V3 consists of Corning® epoxide-coated glass slides printed with optimized probe sequences targeting nearly all of the known human miRNAs in the Sanger miRBase Sequence Database, Release 10.0, plus 373 novel putative human miRNAs (Table 1). Each microarray slide comes fully blocked and ready to use. The novel putative human miRNA sequences were discovered through deep sequencing and are biologically validated, allowing a dramatically improved detection rate over predicted miRNA sequences.

### NCode™ Multi-Species miRNA Microarray

The NCode™ Multi-Species miRNA Microarray V2 consists of optimized probe sequences targeting nearly all of the known and predicted mature miRNAs in the Sanger miRBase Sequence Database, Release 9.0, for human, mouse, rat, *D. melanogaster*, *C. elegans*, and zebrafish sequences (Figure 4 and Table 1). These species-specific, unmodified oligonucleotides are 34–44 bases in length, and each microarray slide comes fully blocked and ready to use.



**Figure 4—NCode™ miRNA microarray content.** Each epoxide slide contains probes for profiling designated miRNA sequences, spotted in triplicate. NCode™ probes for the NCode™ miRNA Microarray Control are printed throughout the array to facilitate analysis, as are mismatch and shuffled controls for monitoring hybridization specificity.

### Controls

The NCode™ Multi-Species miRNA Microarray Control V2 is a synthetic 22-nucleotide miRNA positive control that has been screened for no detectable cross-hybridization or interference with endogenous miRNAs from model organisms. Use this control to assess the efficiency of array labeling and hybridization. The NCode™ Multi-Species miRNA Microarrays and NCode™ miRNA Microarray Probe Sets include oligonucleotide probes that are complementary to this control.

### Probes

For researchers planning to spot their own miRNA microarrays, Invitrogen has probes from the NCode™ Human miRNA Microarray V3 and NCode™ Multi-Species miRNA Microarray V2 available in 384-well plates. Each well contains 500 pmol of lyophilized oligonucleotide probe, ready for resuspension and printing.

**Table 1—Number of probe sequences in the NCode™ miRNA microarrays.**

| Probe content                                     | Human miRNA array V3 probes | Multi-species miRNA array V2 probes |
|---|-----------------------------|-------------------------------------|
| Based on Sanger miRBase Sequence Database release | Version 10.0                | Version 9.0                         |
| Human   | 710 plus 373 putative       | 553                                 |
| Mouse   |                             | 427                                 |
| Rat   |                             | 261                                 |
| <i>C. elegans</i>                                 |                             | 115                                 |
| <i>D. melanogaster</i>                            |                             | 85                                  |
| Zebrafish   |                             | 371                                 |
| Small nucleolar RNAs                              | 29                          | 10                                  |
| Mismatch controls                                 | 76                          | 76                                  |
| NCode™ positive control                           | 1 (72)*                     | 1 (72)*                             |
| NCode™ dye normalization controls                 | 5                           | 5                                   |

\* The NCode™ positive control is spotted throughout three subarrays (72 wells).

## Simplified data analysis using the NCode™ Profiler

miRNA researchers now have a tailored tool to identify differentially expressed miRNA markers on microarrays. NCode™ Profiler software is an advanced experimental design and analysis solution designed for two-dye expression profiling microarray experiments. The software eliminates inherent challenges with miRNA array design and data analysis, providing:

- Simplified experimental design steps
- Confidence when interpreting results using proven statistical analysis with dye swap or loop design normalization models
- Reliable rankings of miRNA markers for selected tissues
- Easy export of normalized data to visualization software for clustering (tree) and heat map analysis

### Experimental design

The NCode™ Profiler software enables simple design of array experiments (Figure 5); it applies a loop design and dye swap/latin squares normalization methods<sup>2,3</sup> to your experimental criteria for samples, species, and controls. The loop design and latin squares analysis support sample-to-sample comparison within the experiment without the need for indirect comparison to a reference sample. The recommended experiments are designed with statistical considerations to reduce or eliminate any general miRNA effect, array effect, dye effect, overall tissue effect, array-miRNA interaction, or dye-miRNA interaction from the data.

### Data analysis

Once an experiment has been conducted, the raw data can be imported and analyzed using the advanced statistical methods compatible with NCode™ microarrays. For each sample on the array, pairwise differential expression is determined and the test statistics, fold change, and P-values are generated. Additionally,

the ranking of each miRNA marker with respect to the other markers within the sample is given. Visit the Invitrogen website at [www.invitrogen.com/ncode](http://www.invitrogen.com/ncode) for a free download. NCode™ Profiler software runs on Microsoft Windows® XP and Windows Vista® operating systems.

## Sensitive and specific miRNA qRT-PCR

qRT-PCR is the standard for validating microarray data and quantitating the exact copy number of miRNAs. It is an invaluable tool for highly sensitive and accurate profiling of miRNA population subsets. Some commercially available miRNA qRT-PCR systems utilize proprietary, predesigned miRNA-specific primers for reverse transcription. Unfortunately, such primers require that the miRNA sequence be publicly available, and that a commercial qRT-PCR assay has been developed for that specific sequence. This limits the availability of qRT-PCR assays for many model organisms, recently discovered miRNAs, and proprietary miRNA sequences.

The NCode™ SYBR® Green and NCode™ SYBR® GreenER™ miRNA qRT-PCR kits overcome these limitations by combining a carefully optimized polyadenylation reaction with the market-leading

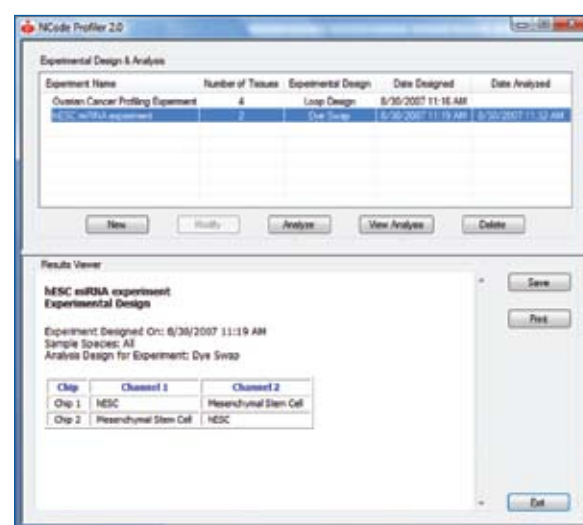


Figure 5—Experimental Design Module in NCode™ Profiler software.

reverse transcriptase, SuperScript® III RT, in a “universal” first-strand cDNA synthesis reaction (Figure 6). The miRNA-specific amplification occurs during the PCR reaction, in which the sequence of the miRNA of interest is used as the target-specific PCR primer. Use NCode™ SYBR® Green and NCode™ SYBR® GreenER™ miRNA qRT-PCR kits to:

- Quantitate almost any miRNA or small noncoding RNA, as soon as its sequence is discovered or predicted, using a flexible and inexpensive assay
- Obtain excellent sensitivity over a broad dynamic range of miRNA abundance (Figure 7)
- Profile closely related miRNAs with single-nucleotide discrimination (Figure 8)
- Archive cDNA for later validation—the use of the same total RNA sample for experiments over time will ensure more reliable comparison

Recommended primers for amplification of known and novel miRNAs with the NCode™ SYBR® Green and NCode™ SYBR® GreenER™ miRNA qRT-PCR kits are available at our website. For help with primer analysis, visit [www.invitrogen.com/ncode](http://www.invitrogen.com/ncode) and follow the link for the NCode™ miRNA database.

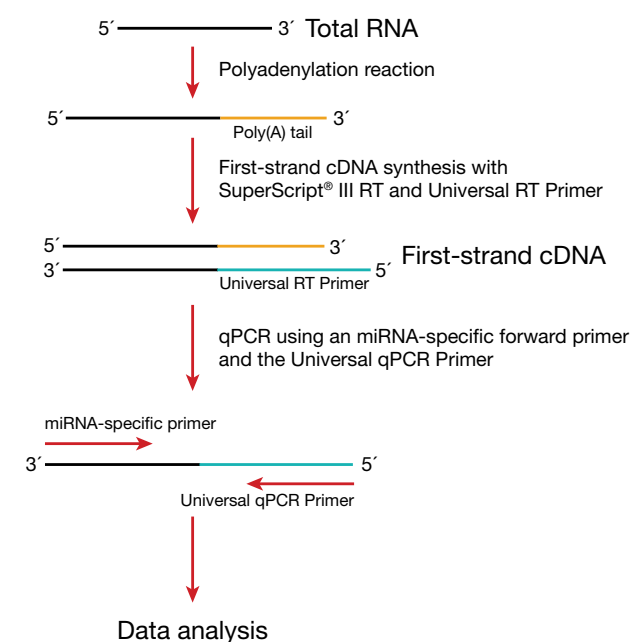


Figure 6—The NCode™ SYBR® Green and SYBR® GreenER™ miRNA qRT-PCR Universal workflow. miRNAs are polyadenylated using poly(A) polymerase and ATP. Following polyadenylation, SuperScript® III RT and a specially designed Universal RT Primer are used to synthesize cDNA from the tailed miRNA population. Subsequently, the first-strand cDNA is analyzed in qPCR using SYBR® Green or SYBR® GreenER™ detection reagent, the Universal qPCR Primer provided in the kits, and a user-designed forward primer that targets the specific miRNA sequence of interest.

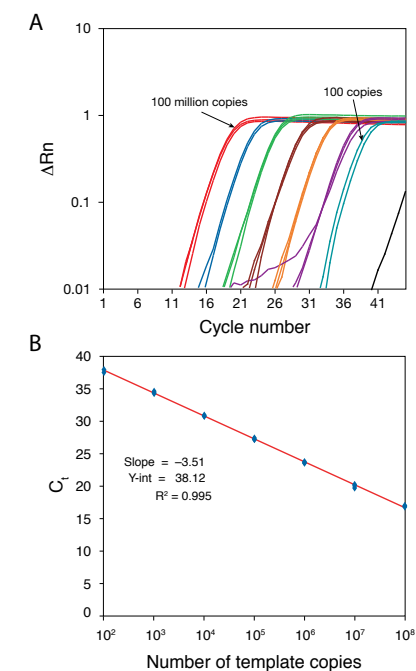
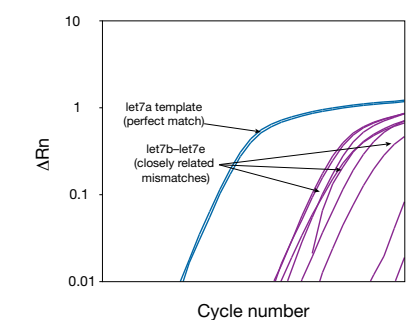


Figure 7—Accurate quantitation over a broad dynamic range of samples. A synthetic miRNA was amplified with the NCode™ SYBR® Green miRNA qRT-PCR Kit. **A.** The assay has a dynamic range of 6–7 orders of magnitude (10<sup>8</sup> to 100 copies). **B.** The standard curve shows excellent linear correlation between copy number and threshold cycle.



| Primer | Template | C <sub>t</sub> |
|--------|----------|----------------|
| let7a  | let7a    | 26.82          |
| let7a  | let7b    | 38.21          |
| let7a  | let7c    | 37.22          |
| let7a  | let7d    | 38.60          |
| let7a  | let7e    | 41.55          |
| let7a  | NTC      | ND             |

Figure 8—Profile highly homologous miRNA with excellent specificity. The NCode™ SYBR® Green miRNA qRT-PCR Kit was used to profile synthetic let7 family miRNA templates from 100,000 copies by using the let7a sequence as the primer. At an annealing temperature of 65°C, the assay clearly discriminates the closely related family members. NTC: no-template control. ND: not detectable.

## Complete service solutions for miRNA profiling

Several unique physical attributes of miRNAs, including their small size, lack of poly(A) tails, and tendency to bind their mRNA targets with imperfect sequence homology, have made them elusive and challenging to study. Invitrogen has leveraged its expertise in this field to develop an advanced miRNA profiling service for research organizations. The combination of leading products and technical expertise in the field of miRNA places Invitrogen Custom Services at the forefront. Invitrogen's NCode™ miRNA Analysis Services offers:

- State-of-the-art technologies, equipment, and high-throughput capabilities provided by expert scientific staff with extensive miRNA expertise
- Dynamic hybridization used to further increase sensitivity
- Fast, reliable results at a competitive price (typical turn-around time ~2–3 weeks)
- Confidential consultation and service support

### Your partner in discovery

NCode™ miRNA Analysis Services uses optimized procedures to label and profile miRNA expression patterns with superior sensitivity and specificity. Each NCode™ miRNA analysis service is customizable to fit specific scientific goals, and our expert staff will work with you to design service projects to achieve your needs. We understand the need for a flexible environment in performing custom services and therefore offer many ways to arrange service agreements. To request more information or to discuss your miRNA profiling experiments, please contact Invitrogen Custom Services ([custom.services@invitrogen.com](mailto:custom.services@invitrogen.com)).

### miRNA analysis services available

Invitrogen offers services for each step of the miRNA analysis workflow. These services include:

- Total RNA isolation from cells or tissue
- miRNA expression profiling with any of the NCode™ miRNA microarrays
- miRNA quantitation using qRT-PCR analysis
- Additional assistance with complex or unusual data analysis

### Reach beyond the helix

Invitrogen's epigenetics technologies comprise products and services that enable researchers to reach beyond the helix, providing deeper understanding of the nature of gene expression. Our trusted and proven tools help researchers probe the mechanisms of epigenetics with clarity, and offer workflow advantages that accelerate the pace of discovery.

Stay up-to-date on the latest products and services for epigenetics research—visit [www.invitrogen.com/epi](http://www.invitrogen.com/epi) and [www.invitrogen.com/ncode](http://www.invitrogen.com/ncode) for more information about:

- Product selection, including focus areas and new technologies
- Experimental planning assistance and troubleshooting
- Key publications on top applications

## Ordering information

| Product   | Quantity      | Cat. no.   |
|---|---------------|------------|
| <b>Total RNA isolation</b>  |               |            |
| TRIzol® Reagent   | 100 ml        | 15596026   |
| <b>miRNA enrichment</b>   |               |            |
| PureLink™ miRNA Isolation Kit   | 20 rxns       | K157001    |
| <b>miRNA quantitation</b>   |               |            |
| Quant-IT™ RiboGreen® RNA Assay Kit  | 2,000 assays  | R11490     |
| <b>miRNA labeling and amplification</b>   |               |            |
| NCode™ Rapid miRNA Labeling System  | 20 rxns       | MIRLSRPD20 |
| NCode™ miRNA Amplification System   | 20 rxns       | MIRAS20    |
| <b>miRNA microarray products</b>  |               |            |
| NCode™ Human miRNA Microarray V3  | 5 arrays      | MIRA305    |
| NCode™ Multi-Species miRNA Microarray V2  | 5 arrays      | MIRA205    |
| NCode™ Multi-Species miRNA Microarray Probe Set V2  | 500 pmol      | MIRMPS201  |
| NCode™ Multi-Species miRNA Microarray Control V2  | 10 µl         | MIRAC201   |
| NCode™ Human miRNA Microarray Probe Set V3  | 500 pmol      | MIRHPS3-01 |
| <b>miRNA qRT-PCR</b>  |               |            |
| NCode™ SYBR® Green miRNA qRT-PCR Kit (10 poly(A) rxns, 20 cDNA rxns)  | 100 qPCR rxns | MIRQ100    |
| NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit (10 poly(A) rxns, 20 cDNA rxns)   | 100 qPCR rxns | MIRQER100  |
| NCode™ miRNA First-Strand cDNA Synthesis Kit (10 poly(A) rxns, 20 cDNA rxns)  | 10 rxns       | MIRC10     |
| NCode™ miRNA First-Strand cDNA Synthesis Kit (50 poly(A) rxns, 100 cDNA rxns)   | 50 rxns       | MIRC50     |
| <b>miRNA Profiling Custom Services*</b>   |               |            |
| *Please contact <a href="mailto:custom.services@invitrogen.com">custom.services@invitrogen.com</a> for a project quote. We provide customizable services for total RNA isolation, labeling and hybridization, analysis, and qRT-PCR detection to help you meet your experimental goals. |               |            |

### References

1. Chomczynski, P. and Sacchi, N. (1987) *Anal Biochem* 162:156–159.
2. Dudoit, S. et al. (2002) *Statistica Sinica* 12:111–139.
3. Yang, Y.H. et al. (2002) *Nucleic Acids Res* 30:e15.