

Label IT[®] miRNA Labeling Kit, Cy[™]3/Cy[™]5

Product Name	Quantity	Product No.
<i>Label IT</i> miRNA Labeling Kit, Cy [™] 3/Cy [™] 5	10 labeling reactions: 5 <i>Label IT</i> Cy [™] 3 reactions (1 µg each) 5 <i>Label IT</i> Cy [™] 5 reactions (1 µg each)	MIR 8305
	50 labeling reactions: 25 <i>Label IT</i> Cy [™] 3 reactions (1 µg each) 25 <i>Label IT</i> Cy [™] 5 reactions (1 µg each)	MIR 8325

The *Label IT* miRNA Labeling Kit is supplied with sufficient reagents to perform 5 (or 25) Cy[™]3 and 5 (or 25) Cy[™]5 labeling reactions (1 µg of nucleic acid each) for microarray hybridization applications. This kit enables direct, covalent labeling and hybridization of microRNA (miRNA), and a simple post-labeling purification protocol to maximize recovery of valuable small RNA. Please see Section 1.3 for a detailed list of kit components.

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1.0 DESCRIPTION

1.1 miRNA Microarray Technology

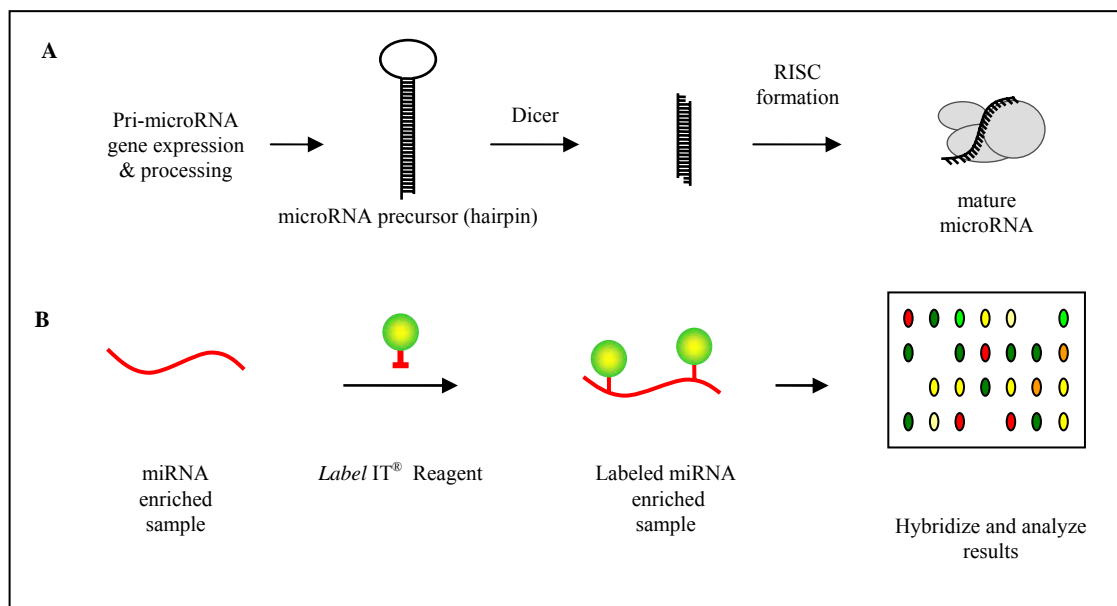
Microarrays represent an established genomics technology that allows the simultaneous hybridization of multiple target molecules on a solid support (i.e. glass slide). Expression profiling analysis, a prominent microarray application, measures the presence and relative amount of specific miRNA transcripts by quantifying fluorescent signal from the microarray hybridization. Mirus Bio's *Label IT* technology is designed to covalently attach marker molecules to nucleic acids in a simple one-step chemical reaction. The *Label IT* Reagents label nucleic acid bases within the RNA and the labels do not impact hybridization performance. The ability to label RNA simply, reproducibly, and uniformly with a detectable marker represents a large technological step forward in nucleic acid labeling technology. Samples that are labeled directly with the *Label IT* Reagents do not require enzymatic replication steps, result in sensitive hybridizations, and represent the original sample without any enzymatic replication or incorporation biases.

1.2 Overview of miRNA Labeling

miRNAs are derived from endogenous genes and undergo a series of processing steps to participate in natural RNA interference (RNAi) phenomena. In the final steps, a hairpin miRNA precursor is cleaved by Dicer to form a double-stranded ~21mer miRNA. As shown in Figure 1A, one strand, the mature miRNA, is preferentially retained and incorporated into RNA-Induced Silencing Complex (RISC). The short length of mature miRNAs, and the lack of a universal feature such as a poly(A) tail, make them unfavorable candidates for standard microarray labeling protocols that rely on enzymatic replication. Enzymatic end-labeling strategies have known variability in efficiency and may result in the addition of non-specific sequence to the labeled miRNA.

The *Label IT* CyTM3 and CyTM5 Reagents allow one-step, direct labeling of samples (containing miRNAs) with no enzymatic steps or alterations to sequence or length (See Figure 1B). The *Label IT* miRNA Labeling Kit, CyTM3/CyTM5 provides the optimal labeling density for microarray applications using the popular CyTM3 and CyTM5 fluorophores, characterized by high fluorescent signal with minimal quenching. These fluorophores can be detected by standard microarray scanners with no additional detection reagents or steps required. Following purification, labeled samples are ready for hybridization.

Figure 1. One Step Direct Labeling of miRNA Enriched Samples



1.3 Materials Supplied

Table 1. Kit Components

Component*	MIR 8305 2 x 5 reactions	MIR 8325 2 x 25 reactions	Reagent Cap Color
<i>Label IT</i> Cy TM 3 Reagent	dried pellet	dried pellet	Purple
<i>Label IT</i> Cy TM 5 Reagent	dried pellet	dried pellet	Blue
Reconstitution Solution	44 µl	220 µl	Brown
10X Labeling Buffer M	100 µl	500 µl	Purple
10X STOP Reagent	100 µl	500 µl	Red
Precipitation Enhancer Solution	20 µl	100 µl	Gray
1X Hybridization Solution	225 µl	1125 µl	White

* Extra volume of each component is supplied to allow for slight variations in pipetting.

NOTE: A standard Cy[™]3 or Cy[™]5 labeling reaction is defined for 1 µg nucleic acid sample. For the two nucleic acid samples compared in a microarray hybridization, label one sample with Cy[™]3 and the second sample with Cy[™]5. The *Label IT* Cy[™]3 and Cy[™]5 labeling reactions can be scaled up or down to label different amounts of sample as required for alternate microarray hybridization conditions.

1.4 Materials Required but Not Supplied

- MB-grade water (DNase- and RNase-free)
- Low-retention microcentrifuge tubes
- miRNA enriched sample (starting material)
- miRNA specific microarray slides
- Non-powdered gloves
- See specific section of protocol for additional reagents recommended or required for sample preparation and microarray hybridization.

1.5 Storage and Stability

Store the *Label IT* Cy[™]3 and Cy[™]5 Reagents at –20°C both as dried pellets and after reconstitution. Store all other supplied reagents at 4°C or –20°C. The *Label IT* Reagents are stable for 6 months after reconstitution. Unreconstituted *Label IT* Reagents and all other reagents are stable for up to 1 year from the date of purchase.

1.6 Abbreviations

- BSA – bovine serum albumin
- SDS – sodium dodecyl sulfate (lauryl sulfate sodium salt)
- SSC – sodium chloride + sodium citrate buffer (see Section 7.0)
- RT – room temperature
- MB – grade (molecular biology grade) – DNase- and RNase-free
- nt – nucleotide

2.0 RECONSTITUTION PROCEDURE

1. Warm the vials of *Label IT* Cy[™]3 Reagent and Cy[™]5 Reagent to RT and quick spin to collect the contents before opening. The Reconstitution Solution freezes at 4°C; ensure it is completely thawed before use in order to obtain required volume.
2. Add the indicated amount of Reconstitution Solution to the dried pellet (it may not be visible). To ensure reconstitution of the pellet, mix well by gently pipetting up and down, and then quick spin to collect volume.

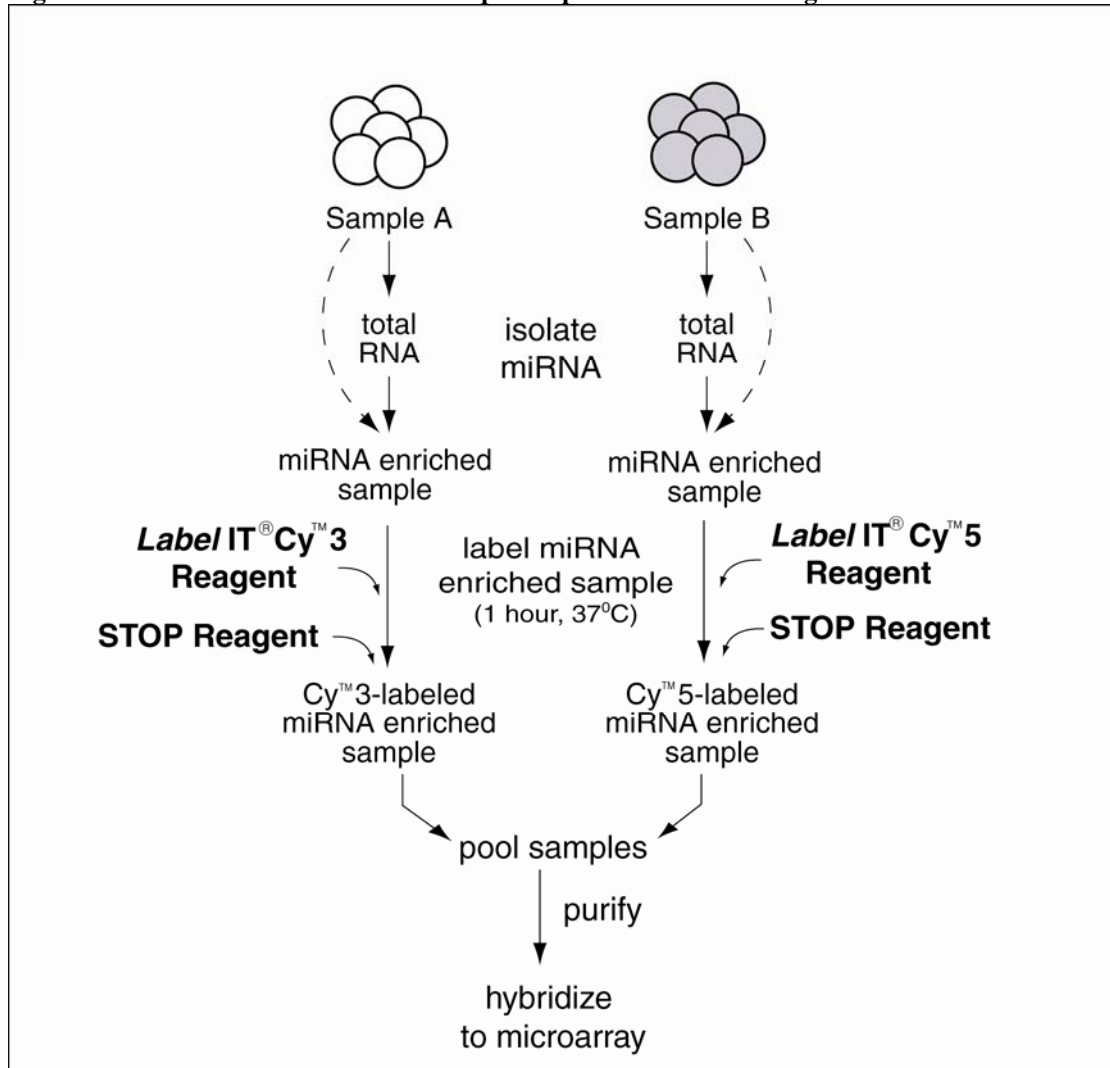
Table 2. Reconstitution of *Label IT* Reagents

Labeling Kit	Labeling Reagent	Volume of Reconstitution Solution
MIR 8305	<i>Label IT</i> Cy [™] 3	22 µl
	<i>Label IT</i> Cy [™] 5	22 µl
MIR 8325	<i>Label IT</i> Cy [™] 3	110 µl
	<i>Label IT</i> Cy [™] 5	110 µl

3.0 LABELING PROCEDURE

Wear gloves at all times when working with RNA. Use MB-grade reagents, and plasticware. Use non-powdered gloves during all steps of sample labeling, microarray hybridization, washing and scanning.

Figure 2. Guide to miRNA Enriched Sample Preparation and Labeling



3.1 Isolation of miRNA Enriched Sample

Recommended: isolate miRNA enriched samples from tissue or total RNA. Mirus Bio recommends the *mirVana*[™] miRNA Isolation Kit (Ambion) using the enrichment procedure for small RNAs. Alternative enrichment procedures include the PureLink[™] miRNA Isolation Kit (Invitrogen), gel purification of desired miRNA enriched sample, or size-selection with a Microcon YM-100 column (Millipore Corp).

NOTE: Standard RNA isolation protocols may not retain small RNA species, including miRNAs.

3.2 CyTM3 and CyTM5 Labeling

1. Reconstitute *Label IT* Reagents according to Section 2.0. If the reagents have already been reconstituted, warm to RT and quick spin before use.
2. Perform independent labeling reactions according to the examples shown (see Table 3 below). If necessary, determine the concentration of the miRNA enriched samples (use 33 µg/ml for one absorbance unit at 260 nm). For the two miRNA enriched samples compared in a microarray hybridization, label one sample with CyTM3 and the second sample with CyTM5 in separate reactions. Mark the reaction tubes accordingly.

NOTE: Add the *Label IT* Reagent last.

3. Incubate the labeling reactions at 37°C for 1 hour.
4. Stop the labeling reactions with the addition of 0.1 volumes of the 10X STOP Reagent (for example, add 10 µl to a 100 µl labeling reaction). Vortex gently to mix.
5. In preparation for post-labeling purification via ethanol precipitation, add 2 µl of Precipitation Enhancer Solution per 100 µl labeling reaction.

Note: Blocker nucleic acids for subsequent hybridization can also be added at this step, if desired.

6. Place on ice or store at -80°C until ready to proceed with purification.

Table 3. Labeling Reaction Setup

CyTM3 Labeling Reaction	
Purified miRNA Enriched Sample A (1 µg)	up to 86 µl
10X Labeling Buffer M	10 µl
MB-grade Water	bring volume to 96 µl
<i>Label IT</i> Cy TM 3 Reagent	4 µl
Total Volume	100 µl

CyTM5 Labeling Reaction	
Purified miRNA Enriched Sample B (1 µg)	up to 86 µl
10X Labeling Buffer M	10 µl
MB-grade Water	bring volume to 96 µl
<i>Label IT</i> Cy TM 5 Reagent	4 µl
Total Volume	100 µl

NOTE: When adjusting reactions, maintain a 1X final concentration of Buffer M and ensure that the *Label IT* Reagent does NOT constitute greater than 20% of total reaction volume.

A. To label a different amount (mass) of miRNA enriched sample: Use 4 µl of *Label IT* Reagent per 1 µg of sample (for example, use 8 µl of *Label IT* Reagent to label 2 µg of sample).

B. To increase (or decrease) the final labeling density: Increase (or decrease) the ratio of labeling reagent to miRNA enriched sample in the reaction, or adjust the incubation time of the labeling reaction.

3.3 Purification of Labeled Sample using Ethanol Precipitation

1. **Recommended:** For dual-channel applications, pool the appropriate labeled samples for competitive hybridizations prior to purification.
2. Add 0.1 volume of 5 M sodium chloride, then 2.5 volumes of ice cold 100% ethanol to the labeling reaction. Mix and place at -20°C (or colder) for at least 30 minutes.
3. Centrifuge at full speed in a microcentrifuge for at least 30 minutes to pellet the labeled miRNA enriched sample. Orient the tubes in the microcentrifuge in such a way that it is known where the pellet forms. Remove the ethanol, being careful not to disturb the pellet.
4. To thoroughly wash the pellet, add 500 µl RNase- and DNase-free 70% ethanol (RT), and vortex briefly.
5. After an additional centrifugation (4°C) at full speed for 20 minutes, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become difficult to resuspend.
6. Resuspend the labeled sample(s) in the appropriate volume of 1X Hybridization Solution (pre-warmed to hybridization temperature) and proceed with hybridization.

NOTE: Ensure the hybridization solution is pre-warmed and completely in solution before each use.

4.0 HYBRIDIZATION PROCEDURE

This section includes recommendations and suggestions for miRNA microarray hybridizations. These conditions were optimized by Mirus Bio scientists using arrays fabricated in-house on a variety of slide substrates, and validated using the miRMAX™ version 1 microarrays (Bionomics Research & Technology Center). Because of the variability in microarray slides, surface chemistries, capture sequences, and manufacturing processes, the hybridization process should be optimized specifically for the microarray being used. Be sure that the microarray contains verified capture sequences that are complementary to directly labeled miRNA species. Conditions and buffers other than those mentioned here are also likely to be compatible with the labeled samples using this protocol.

Mirus Bio recommends performing a sodium borohydride-based pre-soak step and/or BSA-based pre-hybridization step to minimize irregular background and non-specific fluorescence. Necessary non-specific blocker nucleic acid (for example, sheared salmon sperm DNA) for the hybridization can be added to the labeled sample prior to purification. Please see Section 7 for preparation of recommended buffers.

Important: Do not allow the slide to dry during the hybridization procedure. Protect the slide from exposure to light during and following the hybridization procedure.

1. Pre-soak the required microarray slide(s) for 30 min at the hybridization temperature using Pre-soak Buffer (not supplied, pre-warmed, see recipe in Section 7) with mild agitation.
2. Rinse slides 2-3 times in 2X SSC to remove excess Pre-soak Buffer. Transfer immediately to Pre-hybridization Buffer that has been pre-warmed to hybridization temperature (not supplied, see recipe in Section 7).
3. Pre-hybridize the required microarray slide(s) for at least 45 minutes at the hybridization temperature with moderate agitation.
4. Dip slide in dH₂O and quickly dry (centrifuge slide or use compressed air). Microarray slide is now ready for hybridization.
5. Denature labeled sample in 1X Hybridization Solution at 65°C for 3 minutes. Spin the sample at maximum speed for 1 minute to pellet any particulates.
6. Perform the microarray hybridization using the protocol outlined in Table 4.

Table 4. Hybridization Conditions for miRNA Microarrays

Labeled Sample	Recommended Mass per Array ^a	Hybridization Temp./Duration ^b	Post-Hybridization Washes ^c
miRNA enriched sample	≤ 500 ng from each labeled sample	30-45°C ~16 hours (overnight)	<ol style="list-style-type: none"> 1. 2X SSC, 0.1% SDS at hybridization temperature, 2 x 5 min. each 2. 1X SSC at hybridization temperature, 2 x 5 min. each 3. 0.1X SSC at RT, 2 x 1 min. each 4. Briefly dip slide in dH₂O and dry

NOTES:

^a**Recommended Mass per Array:** : Using 22 x 40 mm coverslip area (we recommend LifterSlip™ coverslips from Erie Scientific Co.) with 30 µl Hybridization Solution. Hybridization volumes and masses should be scaled as appropriate for other microarray formats.

^b**Hybridization Temperature/Duration:** Hybridization temperature may vary, based on T_m of capture sequences of microarray used. For example, miRMAX™ version 1 microarray hybridizations were optimized at 30°C using the Label IT miRNA Labeling Kit.

^c**Post-Hybridization Washes:** Perform post-hybridization washes with ample volume of pre-warmed buffers and moderate agitation.

5.0 APPLICATION NOTES

A. Hybridization

Due to the variety of hybridization applications and formats available, general recommendations have been provided in this protocol. Hybridization performance may require empirical optimization depending on the particular application.

B. Adjusting the Density of CyTM 3 and CyTM 5 Labels

The labeling protocol in Section 3.2 has been optimized for microarray hybridization performance. If required, the labeling density can be adjusted by increasing or decreasing the ratio of labeling reagent to nucleic acid in the labeling reaction. Also, the labeling density can be controlled by adjusting the incubation time (≤ 3 hours).

C. Dye Swap Experiments

To verify expression profiling results, it may be advantageous to perform additional hybridizations with the same nucleic acid samples labeled with the opposite CyTM dye (perform a ‘dye swap’ experiment).

6.0 TROUBLESHOOTING GUIDE

Poor Hybridization Signal

Problem	Solution
Suboptimal amount of sample applied to microarray	- label and hybridize more sample to microarray - label and pool equal amounts of the two nucleic acid samples
Poor quality RNA samples	- ensure the use of high quality miRNA enriched samples - ensure that the size range of miRNA enriched sample is appropriate - use proper laboratory techniques when handling RNA samples - use the labeled sample promptly, avoid prolonged storage, and multiple freeze/thaws
Poorly enriched sample	- ensure that the size range of miRNA enriched sample is appropriate
Signal lost by exposure to light, environmental conditions	- minimize exposure of the labeling reagents, labeled samples and hybridized microarray(s) to light throughout the entire procedure - avoid unnecessary scanning (duration, power) of the microarray
Poor quality microarray	- use verified capture sequences - use verified strand sequence (ensure that slide capture sequences are complementary to directly labeled mature miRNAs) - optimize microarray production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted microarrays
Weak Cy TM 3 or Cy TM 5 signal	- ensure that the samples have been purified and quantified properly before labeling - ensure that the kit components have been stored properly - increase the ratio of labeling reagent to nucleic acid - increase the duration of labeling reactions
Hybridization signal ‘stripped’ from microarray	- decrease stringency of hybridization incubation or post-hybridization washes by increasing salt concentration and/or decreasing temperature
Suboptimal hybridization time	- extend duration of hybridization

High Background

Problem	Solution
Excess sample applied to microarray	- quantify the amount of labeled sample and use less in hybridization
Insufficient blocking of the microarray	- perform the recommended pre-hybridization blocking step
Labeled samples not efficiently purified	- repeat purification
Suboptimal blocker DNA used in the hybridization	- use more or alternate blocker nucleic acids during hybridization
Ink or marker used to identify microarray	- avoid using markers or stickers to identify slide; use a diamond scribe pen, if necessary
Low stringency hybridization or wash conditions	- increase hybridization temperature - increase stringency of post-hybridization washes by decreasing salt concentration and/or increasing temperature
Salt from wash buffer remaining on microarray	- dip slide rapidly in water before drying
Array allowed to dry during hybridization steps	- do not allow the slide to dry until the final step
Poor quality microarray	- optimize microarray production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted microarrays
Punctate background	- ensure the hybridization sample has been spun prior to application to the array as indicated in Section 4.0

7.0 PREPARATION OF BUFFERS

NOTE: Ensure all components are MB-grade.

20X SSC

3 M NaCl, 0.3 M Sodium Citrate, pH 8.0	
NaCl	175.3 g
Sodium Citrate	88.2 g
Water	800 ml
Mix well and adjust pH to 8.0 with a few drops of 10 N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclaving.	
Total Volume	1000 ml
Store at RT	

Pre-Soak Buffer

2X SSC, 0.05% SDS, 0.25% Sodium Borohydride	
20X SSC	10 ml
10% SDS	0.5 ml
NaBH ₄	0.25 g
Water	~ 90 ml
Total Volume	100 ml
Prepare fresh	

Pre-hybridization Buffer

5X SSC, 0.1% SDS, 1% BSA	
20X SSC	25 ml
10% SDS	1 ml
BSA	1 g
Water	~ 74 ml
Total Volume	100 ml
Prepare fresh	

Post-Hybridization Buffers

2X SSC, 0.1% SDS	
20X SSC	100 ml
10% SDS	10 ml
Water	890 ml
Total Volume	1000 ml
Store at RT	

1X SSC	
20X SSC	50 ml
Water	950 ml
Total Volume	1000 ml
Store at RT	

0.1X SSC	
20X SSC	5 ml
Water	995 ml
Total Volume	1000 ml
Store at RT	

8.0 ADDITIONAL RESOURCES

Internet Sites for microarray applications

"Anatomy of a Comparative Gene Expression Study" <http://www.cs.wustl.edu/~jbuhler/research/array/>
Chipping away at the mysteries of science and medicine. <http://www.ncbi.nlm.nih.gov/About/primer/microarrays.html>
DNA Microarray (Genome Chip) web site, by Leming Shi, Ph.D. <http://www.gene-chips.com/>
Y. F. Leung's Functional Genomics - Microarray web site: <http://ihome.cuhk.edu.hk/%7eb400559/array.html>
Microarray protocols at microarrays.org: <http://www.microarrays.org/protocols.html>

Internet Sites for miRNA applications

The miRNA Registry <http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml>

References:

Genomics, Biogenesis, Mechanism and Function. David Bartel. Cell, Vol. 116, 281-297, January, 2004.
Small RNAs With a Big Role in Gene Regulation. Lin He and Gregory J. Hannon. Nature Reviews: Genetics, Volume 5, July 2004.

9.0 RELATED PRODUCTS

Label IT miRNA Labeling Kit, Cy[™]3 or Cy[™]5 (MIR 8510, MIR 8550, MIR 8610, MIR 8650)

Label IT miRNA Labeling Kit, Biotin (MIR 8410, MIR 8450)

Label IT μ Array Biotin Labeling Kit (MIR 8010, MIR 8050)

Label IT μ Array Dual Labeling Kit (Fluorescein and Biotin; MIR 8105, MIR 8125)

Label IT μ Array Cy[™]3/ Cy[™]5 Labeling Kit (MIR 8205, MIR 8225)

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Label IT Reagents are covered by U.S. Patent No. 6,262,252 and No. 6,593,465.

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