

INTRODUCTION

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

SPECIFICATIONS

Storage	Store the <i>Label IT</i> Reagent at -20°C in both its dried pellet and reconstituted forms. Store the 2X Hybridization Solution at -20°C . All other materials can be stored at room temperature or at 4°C .
Product Guarantee	The unreconstituted <i>Label IT</i> Reagent is stable for 1 year from date of purchase, and reconstituted <i>Label IT</i> Reagent is stable for 6 months from date of reconstitution when properly stored and handled. All other components are stable for 1 year from date of purchase when properly stored and handled.
Kit Size	Kits provide material to complete 10 or 50 reactions (1 μg for each reaction).



Cap the *Label IT* Reagent tightly and avoid exposure to moisture and light.

For Research Use Only.

MATERIALS

Materials Supplied

Label IT miRNA Labeling Kits are supplied in *one* of the following formats.

Kit Component	Product MIR No.				Reagent Cap Color
	9305	9410 9510 9610	9325	9450 9550 9650	
Label IT Reagent	dried pellet	dried pellet	dried pellet	dried pellet	varies with reagent
Reconstitution Solution	44 µl	44 µl	220 µl	220 µl	brown
10X Labeling Buffer M	100 µl	100 µl	500 µl	500 µl	purple
10X STOP Reagent	100 µl	100 µl	500 µl	500 µl	red
Purification Columns (with Collection Tubes)	5	10	25	50	N/A
Column Binding Buffer	100 µl	200 µl	500 µl	1 ml	teal
Column Wash Buffer	1 ml	2 ml	5 ml	10 ml	N/A
Column Elution Solution	150 µl	300 µl	750 µl	1.5 ml	gold
2X Hybridization Solution	150 µl	300 µl	750 µl	1.5 ml	white
Elution Tubes	5	10	25	50	N/A

Labels available in Label IT miRNA Labeling Kits.

Product MIR No.	Label
MIR 9305, 9325	Cy TM 3/Cy TM 5
MIR 9410, 9450	Biotin
MIR 9510, 9550	Cy TM 3
MIR 9610, 9650	Cy TM 5

Materials required, but not supplied

- DNase-free and RNase-free water
- Microcentrifuge tubes
- Ethanol (96 – 100%)
- miRNA-containing sample (starting material)
- miRNA specific microarray slides
- Microarray scanning equipment

BEFORE YOU START:

Important Tips for Optimal Plasmid DNA Transfection

The suggestions below generally yield strong labeling with minimal background and will maximize performance with most microarrays.

- **Reagent preparation.** Prior to first use, warm the vial containing the *Label IT* Reagent to room temperature and centrifuge briefly (pulse) to collect the dried pellet. For subsequent uses, warm the vial of reconstituted *Label IT* Reagent to room temperature before opening.
- **Reaction scalability.** The *Label IT* miRNA labeling reactions can be scaled up or down to label different amounts of sample as required for alternate microarray hybridization conditions.
- **Reaction volumes.** When adjusting reactions volumes, 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.
- **DNase and RNase-free materials.** Wear gloves at all times when working with RNA. Use DNase-free and RNase-free reagents and plastic ware. Use powder-free gloves during all steps of sample labeling, microarray hybridization, washing and scanning.
- **Addition of *Label IT* Reagent.** Add the *Label IT* Reagent to the labeling reaction last.
- **RNA preparation:** Mirus Bio recommends the mirVana™ miRNA Isolation Kit (Ambion) for isolation of either total or low molecular weight enriched RNA. Both total and miRNA-enriched samples are compatible with this labeling kit. However, please note that not all miRNA microarray platforms are optimized for use with total RNA. Capture sequence design, hybridization temperature and other factors can greatly influence the specificity of hybridization.
- **miRNA concentration.** Determine the concentration of the miRNA-containing samples (use 40 µg/ml for one absorbance unit at 260 nm).
- **Hybridization considerations.** There are a large variety of hybridization applications and formats available, and only general recommendations are provided in this protocol. Variability in microarray slides, surface chemistries, capture sequences, and manufacturing processes, we recommend optimizing the hybridization specifically for the microarray being used. This protocol provides general recommendations for RNA input amount that are applicable for most arrays tested (see table below). The microarray manufacturer may provide ranges for RNA input amounts that fall outside the ranges recommended here. In this case, please follow the recommended range provided by the manufacturer.



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

Labeled Sample	Recommended Mass per Array
miRNA-containing sample	LMW enriched RNA 100 – 1000 ng
	Total RNA 500 ng – 5 µg

- Do not allow the slide to dry during the hybridization procedure. Protect the slide from exposure to light during and after the hybridization procedure.
- To verify expression profiling results, it may be useful to perform additional hybridizations with the same miRNA samples labeled with the opposite Cy™ dye (perform a ‘dye swap’ experiment).
- Microarray expression profiling is a useful method for assessing relative expression differences, but those differences should be validated using another method such as qRT-PCR or quantitative sequencing.

LABELING PROTOCOL

The procedure below describes how to perform a standard miRNA labeling reaction.

A. Prepare *Label IT* Reagent

1. Before the first use, warm the vial containing the *Label IT* Reagent to room temperature and centrifuge briefly (pulse) to collect the dried pellet.
2. Warm the Reconstitution Solution to room temperature. The Reconstitution Solution remains frozen at 4° C, so please be sure that it is completely thawed before use.
3. For the first use only, add the pre-warmed Reconstitution Solution to each *Label IT* pellet according to the table:

Product MIR No.	Reconstitution Solution to Add
9305, 9410, 9510, 9610	22 µl to each <i>Label IT</i> pellet
9325, 9450, 9550, 9650	110 µl to each <i>Label IT</i> pellet

4. To ensure complete reconstitution of the pellet, mix well by vortexing and then centrifuge briefly (pulse) to collect the solution.

B. Label Nucleic Acid Sample

1. Set up the labeling reaction. The table below demonstrates a typical reaction that will label 1 µg of miRNA containing sample. Add the reagents in the order listed, and be sure to add the *Label IT* Reagent last.

Standard miRNA Labeling Reaction	
DNase, RNase-free water	Bring to volume
10X Labeling Buffer M	10 µl
Purified miRNA containing sample (1 µg)	Up to 86 µl
Label IT Reagent	4 µl
Total Volume	100 µl

2. Incubate the reaction at 37°C for one hour.
3. Stop the labeling reaction by adding 0.1 volumes of 10X STOP Reagent to the labeling reaction. For example, add 10 µl 10X STOP Reagent to 100 µl sample reaction. Vortex gently to mix
4. If performing dual color hybridization, pool the CyTM3 and CyTM5 samples together before proceeding to the column purification steps.
5. Place the reactions on ice or store at ≤ -20°C until you are ready to proceed with the purification steps.



The labeling reaction can be adjusted to label more or less RNA as needed. See the **APPENDIX Part C** for help with adjusting labeling reactions.



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

C. Purify Labeled miRNA with the Purification Column

Prepare the labeled sample for the column

1. Add 0.1 volume of Column Binding Buffer to the labeled sample (e.g. add 10 µl Column Binding Buffer to a 100 µl sample).
2. Add 2.5 volumes of ethanol (96 – 100%) to the labeled sample (e.g. add 250 µl ethanol to a 100 µl sample). Mix well by pipetting.

Apply the labeled sample to the column

1. Apply the labeled sample to the Purification Column and collection tube provided. **Do not** apply more than 720 µl to the column for any centrifugation. If the sample volume is greater than 720 µl, repeat steps 1-3 in the same column while removing the eluate until all the labeled sample has passed through the column.
2. Centrifuge for 30 seconds at 11,000 x g.
3. Discard the flow-through from the collection tube. Use the same collection tube for all wash steps.

Wash the column

1. If not done previously, add 4 volumes of 96 – 100% ethanol to the bottle containing Column Wash Buffer according to the table.

Product MIR No.	Ethanol to Add
9305	4 ml
9410, 9510, 9610	8 ml
9325	20 ml
9450, 9550, 9650	40 ml

2. Apply 600 µl of Column Wash Buffer to the column.
3. Centrifuge the column for 30 seconds at 11,000 x g. Discard the flow-through in the collection tube.
4. Apply 200 µl of Column Wash Buffer to the column.
5. Centrifuge for 2 minutes at 11,000 x g. Discard the flow-through in the collection tube.
6. Transfer the column to a 1.5 ml elution tube.
7. Apply 28.5 µl Elution Buffer to the column (when using a 22 x 60 mm standard LifterSlip[™]).
8. Centrifuge for 1 minute at 11,000 x g.
9. Remove the column from the elution tube. The purified labeled sample is now ready to use.



When using the Cy[™]3/Cy[™]5 dual channel kits, we recommend pooling the appropriate labeled samples for competitive hybridizations prior to purification.



Ensure that 4 volumes of 96 – 100% ethanol have been added to the Column Wash Buffer before proceeding to the next step.

D. Perform miRNA microarray hybridization

This section includes recommendations and suggestions for miRNA microarray hybridizations. These conditions were optimized by Mirus Bio scientists using various commercially available miRNA microarrays, and validated using the NCode[™] Multi-Species miRNA Microarray Kit V2 (Invitrogen Inc), miRCURY[™] LNA miRNA array (Exiqon), and mirVana[™] miRNA Bioarrays, Version 2 (Ambion). Due to variability in microarray slides, surface chemistries, capture sequences, and manufacturing processes, the hybridization process should be optimized specifically for the microarray being used. Ensure that the microarray contains verified capture sequences that are complementary to directly labeled miRNA species. MicroRNAs labeled using *Label IT* are also likely to work with many other conditions and buffers.



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

Prepare for hybridization

1. Add 28.5 µl of 2X Hybridization Solution and mix well before hybridization.
2. Denature the labeled sample in 1X Hybridization Solution at 65°C for 3 minutes. Centrifuge the sample at maximum speed for 1 minute to pellet any particulates. Gently remove the supernatant to apply to the microarray.



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

Hybridize the labeled miRNA to the array

Note: Use a 22 x 60 mm standard LifterSlip[™] with 57 µl Hybridization Solution. For other microarray formats, scale the hybridization volumes and masses as appropriate. Ensure that Hybridization Solution is at a 1X final concentration.

1. Hybridize labeled RNA samples according to the following recommendations:

LMW Enriched RNA	Total RNA
100 – 1000 ng	500 ng – 5 µg

2. Hybridize at ~ 37°C for ~ 16 hours (overnight)

Wash the Array

Note: Perform post-hybridization washes with ample volume of pre-warmed buffers and moderate agitation. You should be able to completely submerge the microarray slide in the buffer.

1. Wash 2 x 5 minutes with 1X SSC, 0.1% SDS at the hybridization temperature.
2. Wash 1 x 5 minutes with 1X SSC at the hybridization temperature.
3. Wash 2 x 1 minute with 0.1X SSC at room temperature.
4. Treat the arrays as follows:



Hybridization temperature and duration may vary based on T_m of the capture sequences on the microarray used.

For Biotin Labeled miRNA	For Cy [™] 3 and/or Cy [™] 5 Labeled miRNA
DO NOT DRY. Proceed to a biotin detection procedure. See Appendix for methods.	Dry and proceed with detection and analysis.

APPENDIX

A. Detect biotin labeled, hybridized miRNA

Biotin detection reagent(s) such as fluoro-conjugated streptavidin, BSA (ultrapure), Triton X-100, and 20X SSPE are required but not supplied. There are a variety of streptavidin/avidin and anti-biotin antibody fluorescent conjugates that can be used to detect hybridized biotin-labeled samples on glass slides. As an example, this section provides a general protocol using Cy3[™]-conjugated streptavidin that is compatible with a majority of microarray scanners.

Note: See Section B for required solution

1. Prepare 6X SSPE-T Solution and Biotin Detection Solution as directed in Part B.
2. After the post-hybridization washes, incubate the slide(s) in 6X SSPE-T for 5 minutes at room temperature.
3. One at a time, remove a slide from the 6X SSPE-T wash and briefly blot the edge of the slide on a paper towel to wick off any excess buffer (do not allow the slide to dry).
4. Immediately overlay ~200 µl Biotin Detection Solution on the array. Apply a large coverslip to distribute the Biotin Detection Solution evenly over the slide. Incubate under humidified conditions at 37°C for 20 minutes
5. Remove the Biotin Detection Solution and coverslip by immersing the slide in 6X SSPE-T. Wash three times for 5 minutes in 6X SSPE-T at room temperature with gentle shaking
6. Dry the slide by centrifuging briefly, or blow dry using compressed air.
7. Scan the slide (532 nm laser excitation for Cy3[™] detection).
8. Store the slide at room temperature, protected from light.



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

B. Preparation of Commonly Used Buffers

20X SSC	
3M NaCl, 0.3M Sodium Citrate, pH 8.0	
NaCl	175.3 g
Sodium Citrate	88.2 g
Water	800 ml
Mix well and adjust to pH 8.0 with a few drops of 10N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclaving.	
Total Volume	1000 ml

20X SSPE	
NaH ₂ PO ₄ – H ₂ O	27.6 g
NaCl	175.3 g
EDTA	7.4 g
Water	800 ml
Mix well and adjust to pH 7.4 with 10N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclaving.	
Total Volume	1000 ml



Use RNase and DNase-free components.

B. Preparation of commonly used buffers

2X SSC, 0.1% SDS	
20X SSC	100 ml
10% SDS	10 ml
Water	890 ml
Mix well and adjust to pH 8.0 with a few drops of 10N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclave	
Total Volume	1000 ml

1X SSC	
20X SSC	50 ml
Water	950 ml
Total Volume	1000 ml

0.1 X SSC	
20X SSC	5 ml
Water	995 ml
Total Volume	1000 ml

6X SSPE-T	
20X SSPE	300 ml
Triton X-100	0.05 ml
Water	700 ml
Total Volume	1000 ml

Biotin Detection Solution	
6X SSPE-T	200 µl
BSA (25 mg/ml)†	0.8 µl
Streptavidin-Cy3™ (0.8 mg/ml)*	0.5 µl
Total Volume	1000 ml
Use 200 µl per slide. Make fresh, immediately before use.	



Use RNase and DNase-free components.

* Final solution concentration of 2 µg/ml streptavidin-Cy[™]3 (Jackson ImmunoResearch Labs Inc., www.jacksonimmuno.com, or Zymed Laboratories Inc., www.zymed.com)

† Final solution concentration of 0.1 mg/ml BSA (Sigma-Aldrich, St. Louis, MO; Cat. #A9418)

C. Adjusting the Labeling Reaction

The *Label IT* miRNA labeling reaction (see **Labeling Protocol Part B**) may be adjusted for labeling more or less miRNA.

The following table provides some example reaction conditions for using RNA input amounts that are different from the standard reaction provided in the **Labeling Protocol Part B**.

Reagent	250 ng Input RNA	1 µg Input RNA	2 µg Input RNA
DNase, RNase-free water	88.5 µl	84 µl	78 µl
10X Labeling Buffer M	10 µl	10 µl	10 µl
Purified miRNA containing sample (500 ng per µl)	0.5 µl	2 µl	4 µl
Label IT Reagent	1 µl	4 µl	8 µl
Total Volume	100 µl	100 µl	100 µl

TROUBLESHOOTING GUIDE

Problem	Solution
Poor Hybridization Signal	
Suboptimal amount of sample applied to microarray	Label and hybridize more sample to the microarray.
	Label and pool equal amounts of the two nucleic acid samples (for dual labeling).
Poor quality RNA samples	Use high quality miRNA-containing samples.
	Use proper laboratory techniques when handling RNA samples.
	Use the labeled sample promptly. Avoid prolonged storage and multiple freeze/thaws.
Poorly extracted sample	Ensure that sample contains miRNA (~22 nt size range).
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 [™] 3 or Cy [™] 5 signal	Use samples that have been properly purified and quantified before labeling.
	Ensure that the kit components have been stored properly.
	Increase the incubation time of the labeling reactions.
Hybridization signal 'stripped' from microarray	Decrease the stringency of the hybridization incubation or post-hybridization washes by increasing salt concentration and/or decreasing temperature.
Suboptimal hybridization time	Extend the duration of the hybridization.

TROUBLESHOOTING GUIDE continued

Problem	Solution
High Background	
Excess sample applied to microarray	Quantify the amount of labeled sample and use less in hybridization.
Labeled samples not purified efficiently	Repeat the purification step.
Ink or marker used to identify microarray	Avoid using markers or stickers to identify a slide; use a diamond scribe pen, if necessary.
Low stringency hybridization or wash conditions	Increase the hybridization temperature.
	Increase the stringency of post-hybridization washes by decreasing salt concentration and/or increasing temperature.
Salt from wash buffer remaining on microarray	Dip the slide rapidly in water before drying.
Array dried during hybridization steps or between wash 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	Centrifuge the hybridization sample prior to applying it to the microarray. See PROCEDURE PART D.

RELATED PRODUCTS

- *Label IT* Fluorescence *In Situ* Hybridization Kits
- *Label IT* μ Array Labeling Kits
- *Label IT* Tracker Intracellular Localization Kits
- *Label IT* Tracker siRNA Intracellular Localization Kits

REFERENCES - RESOURCES

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- Ambros V, Chen X. The regulation of genes and genomes by small RNAs. *Development* 2007:134(9):1635
- The miRNA Registry <http://microrna.sanger.ac.uk>

For details on our products, visit www.mirusbio.com or www.TheTransfectionExperts.com.

Contact Mirus Bio for additional information.



Mirus Bio LLC
545 Science Drive
Madison, WI 53711
Toll-free: 888.530.0801
Direct: 608.441.2852
Fax: 608.441.2849

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Use of Mirus Bio *TransIT*[®] polyamine transfection reagents are covered by U.S. Patent No. 5,744,335, No. 6,180,784, No. 7,101,995, No. 7,601,367 and patents pending. Mirus Bio *Label IT*[®] nucleic acid labeling and modifying reagents are covered by U.S. Patent No. 6,262,252, No. 6,593,465, No. 7,049,142, No. 7,326,780 and No. 7,491,538. CyTM3 and CyTM5 products or portions thereof are manufactured under license from Carnegie Mellon University and are covered by U.S. Patent No. 5,268,486.

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