Label IT® µArray® Labeling Kit, Cy™3 **or** Cy™5

Product Name	Quantity	Product No.
Label IT µArray Cy3 Labeling Kit	10 *Label* IT Cy3 reactions (1 µg each)	MIR 8710
	50 *Label* IT Cy3 reactions (1 µg each)	MIR 8750
Label IT µArray Cy5 Labeling Kit	10 *Label* IT Cy5 reactions (1 µg each)	MIR 8810
	50 *Label* IT Cy5 reactions (1 µg each)	MIR 8850

The *Label* IT µArray Labeling Kits, Cy3 or Cy5 are supplied with sufficient reagents to perform 10 or 50 Cy3 or Cy5 labeling reactions (1 µg of nucleic acid each) for microarray hybridization applications. The kit enables direct covalent labeling of mRNA, cDNA, or cRNA for expression profiling analyses and provides specific labeling protocols for each sample type. The kit does not contain sample preparation reagents, purification columns or hybridization components. Please see Section 1.2 for a detailed list of kit components.

Please read the entire protocol carefully and proceed with the section(s) appropriate for the desired experiment.

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1.0 DESCRIPTION

1.1 General Information

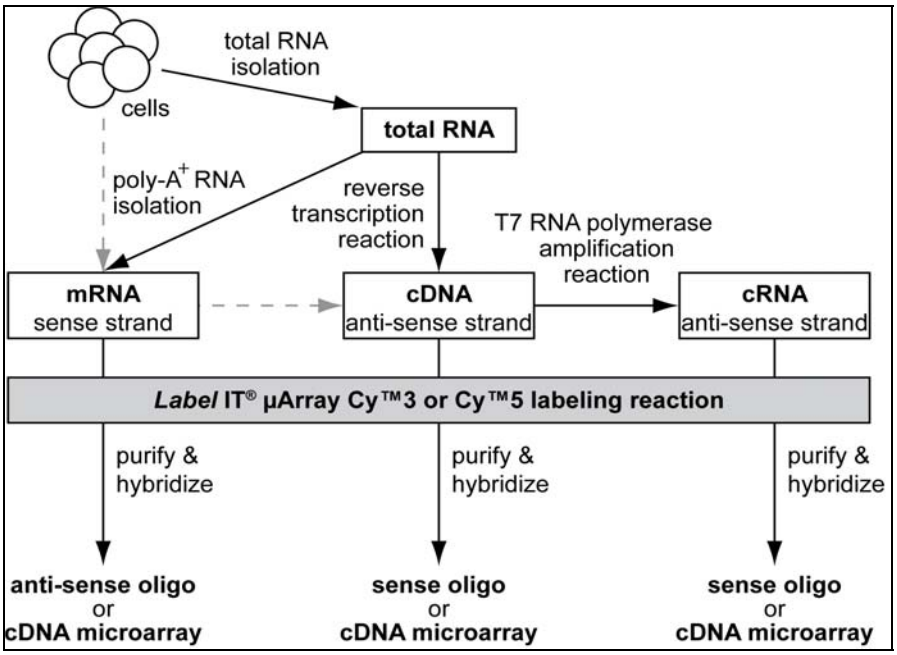
Microarrays represent an established genomics technology that allows the simultaneous hybridization of multiple target molecules on a solid support (i.e. glass slide). Expression profiling analysis, a prominent microarray application, measures the presence and relative amount of specific RNA transcripts by quantifying fluorescent signal from the microarray hybridization. For expression profiling microarray applications, samples derived from total or messenger RNA (mRNA) must be highly labeled with a marker molecule for detection. Mirus Bio' *Label IT* technology is designed to covalently attach marker molecules to nucleic acids in a simple one-step chemical reaction. The reagents directly label nucleic acid bases within the DNA or RNA and do not impact hybridization performance. The ability to label DNA or RNA simply, reproducibly, and uniformly with a detectable marker represents a large technological step forward in nucleic acid labeling technology. Here, the *Label IT* μArray Cy3 and *Label* IT μArray Cy5 Kits have been optimized for the preparation of labeled nucleic acid samples for use in expression profiling hybridizations.

In traditional expression profiling applications, RNA is extracted, labeled independently with distinct reporter molecules and hybridized to a microarray. Target gene expression is quantified from the relative signal derived from the labeled sample following hybridization. Typically, the RNA samples are labeled by enzymatic replication (to either cDNA or cRNA) in the presence of labeled nucleotides. Since *Label* IT Reagents allow the direct chemical modification of nucleic acids, enzymatic replication and incorporation of labeled nucleotides can be eliminated from the labeling process. The *Label* IT μArray Cy3 or Cy5 Labeling Kits allow any type of nucleic acid samples to be directly labeled– mRNA, cDNA, or cRNA (see Figure 1)– depending on experimental design.

The *Label* IT μArray Cy3 or Cy5 Labeling Kits generate high quality microarray hybridization data, with low background and high signal to noise ratios. Furthermore, mRNA samples that are labeled directly with the *Label* IT μArray Cy3 or Cy5 Labeling Kits do not require enzymatic replication step(s), result in sensitive hybridizations, and represent the original sample without any enzymatic replication or incorporation biases. Direct labeling of mRNA allows the detection of low copy number transcripts (less than 10 copies per cell) with the *Labe*l IT μArray Cy3 or Cy5 Labeling Kits. Samples labeled using these kits are compatible with hybridization on a variety of microarray surfaces, facilitating substitution into standard protocols.

The use of the *Label* IT μArray Cy3 or *Label* IT μArray Cy5 Reagents allow the direct labeling of nucleic acid samples with the popular Cy3 or Cy5 fluorophores. These fluorophores can be detected by standard microarray scanners with no additional detection reagents or steps required. The *Label* IT μArray Cy3 or Cy 5 Labeling Kits provide the optimal labeling density for microarray applications, characterized by high fluorescent signal with minimal quenching.

Figure 1. Guide to Nucleic Acid Labeling for Expression Profiling Microarray Applications



PolyA$^+$ RNA (or mRNA) isolated directly from cells or from total RNA preparations can be labeled directly with Cy3 or Cy5 using the *Label* IT µArray Cy3 or Cy5 Labeling Kits, then hybridized to a cDNA or anti-sense oligo microarray. RNA (either total or polyA$^+$ RNA) can also be reverse transcribed into first-strand cDNA samples and then labeled with Cy3 or Cy5 using the *Label* IT µArray Cy3 or Cy5 Labeling Kits. If the amount of RNA is limited, some applications may require sample amplification to generate cRNA, which can then be labeled with Cy3 or Cy5 using the *Label* IT µArray Cy3 or Cy5 Labeling Kits.

Table 1. Selecting a Sample Type for the *Label* IT µArray Cy3 or Cy5 Labeling Kits

Sample Type	Criteria/Features
mRNA	Allows direct hybridization of biological material No enzymatic replication bias No enzymatic incorporation bias Compatible with cDNA and anti-sense* oligo microarrays
cDNA	No enzymatic incorporation bias Compatible with cDNA and sense oligo microarrays
cRNA	Use when amplification of limited starting material is required No enzymatic incorporation bias Compatible with cDNA and sense oligo microarrays

* Currently, most oligo arrays use sense-strand capture sequences, and are therefore not compatible with hybridization of labeled sense-strand RNA. Verify the design of oligo arrays before selecting a sample type to label with the *Label* IT µArray Cy3 or Cy5 Labeling Kits.

1.2 Materials Supplied

Table 2. Kit Components

Component*	MIR 8710 10 reactions	MIR 8750 50 reactions	MIR 8810 10 reactions	MIR 8850 50 reactions	Reagent Cap Color
Label IT Cy3 Reagent	dried pellets	dried pellets	--	--	Lilac
Label IT Cy5 Reagent	--	--	dried pellets	dried pellets	Blue
Reconstitution Solution	100 µl	200 µl	100 µl	200 µl	Brown
10X Labeling Buffer M	100 µl	500 µl	100 µl	500 µl	Purple
0.5 M EDTA	75 µl	375 µl	75 µl	375 µl	Green
Reagent D1	150 µl	500 µl	150 µl	500 µl	Blue
Neutralization Buffer N1	200 µl	500 µl	200 µl	500 µl	White
10X STOP Reagent	100 µl	500 µl	100 µl	500 µl	Red
5X Fragmentation Buffer	250 µl	1.25 ml	250 µl	1.25 ml	Orange

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

NOTE: A standard Cy3 or Cy5 labeling reaction is defined for 1 µg nucleic acid sample. The *Label* IT µArray Cy3 or the *Label* IT µArray Cy5 labeling reactions can be scaled up or down to label different amounts of DNA or RNA as required for alternate microarray hybridization conditions. **There are distinct labeling protocols for different nucleic acid sample types; please refer to the appropriate labeling procedure in this protocol.**

1.3 Materials Required but Not Supplied

General materials

- MB-grade water (DNase- and RNase-free)
- Low-retention microcentrifuge tubes
- RNA sample (starting material)
- Purification kit/reagents (see specific section of protocol for recommendations)
- Non-powdered gloves

See specific section of protocol for additional reagents recommended or required for sample preparation and microarray hybridization.

1.4 Storage and Stability

Store the *Label* IT µArray Cy3 or Cy5 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 µArray Reagents are stable for 6 months after reconstitution. Unreconstituted *Label* IT µArray Reagents and all other reagents are stable for up to 1 year from the date of purchase.

1.5 Abbreviations

BSA – bovine serum albumin
SDS – sodium dodecyl sulfate (lauryl sulfate sodium salt)
SSC – sodium chloride + sodium citrate buffer (see Appendix, Section 9.1 for preparation)
RT – room temperature
MB – grade (molecular biology grade) – DNase- and RNase-free
ds – double strand

2.0 RECONSTITUTION PROCEDURE

1. Warm the vials of *Label* IT µArray Cy3 Reagent or *Label* IT µArray Cy5 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 the 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 3. Reconstitution of *Label* IT µArray Reagents

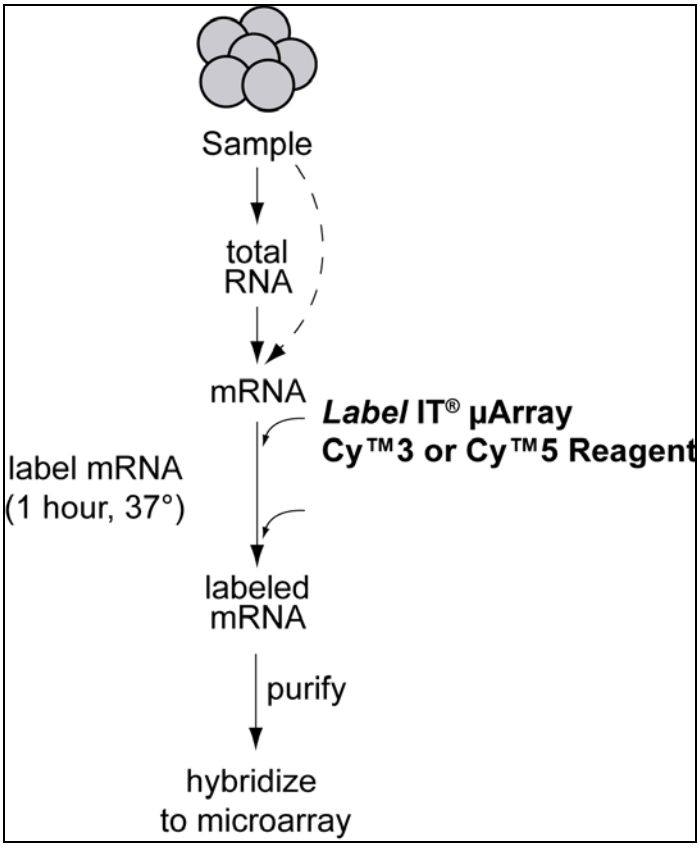
Labeling Kit	Labeling Reagent	Volume of Reconstitution Solution
MIR 8710	*Label* IT µArray **Cy3**	22 µl
MIR 8750	*Label* IT µArray **Cy3**	110 µl
MIR 8810	*Label* IT µArray **Cy5**	22 µl
MIR 8850	*Label* IT µArray **Cy5**	110 µl

3. Store unused, reconstituted *Label* IT µArray Reagents tightly capped at -20°C, protected from exposure to light. For subsequent use, warm the vial to RT and spin briefly before opening.

3.0 mRNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use MB-grade reagents, water and plasticware. Use non-powdered gloves during all steps of sample labeling, microarray hybridization, washing and scanning. PolyA$^+$ RNA isolation reagents and post-labeling purification reagents are required but not supplied.

Figure 2. Guide to mRNA Sample Preparation and Labeling



3.1 mRNA Isolation

NOTE: This section applies to analysis of eukaryotic mRNA. Some microarray applications may not require polyA$^+$ isolation but may require isolation of alternate RNA populations, such as small RNA samples (microRNA) or bacterial non-ribosomal mRNA. Isolate the desired RNA population before proceeding with the labeling protocol. See Application Notes, Section 7.0, Part C.

1. Isolate polyA$^+$ enriched RNA from the samples intended for microarray hybridization. Direct isolation of mRNA from tissue/cells and mRNA isolation from a total RNA sample are both suitable. Mirus Bio has successfully tested a variety of commercially available mRNA isolation kits, including PolyATract® mRNA Isolation Systems (Promega Corp., www.promega.com), Poly(A)Purist™ mRNA Purification Kit (Ambion, Inc., www.ambion.com), and Oligotex® mRNA Kits (Qiagen Inc., www.qiagen.com). **The mRNA must be eluted in water or dilute buffer for optimal labeling with the *Label* IT µArray Cy3 or Cy5 Labeling Kits.** The standard elution reagents provided with the kits listed above are appropriate. Use the purification kits as recommended by the manufacturer. Prepare the amount of mRNA from each sample that is needed for the intended hybridization(s).
   NOTE: Consult the manufacturer's literature accompanying the polyA$^+$ isolation kit for expected yields of polyA$^+$ RNA. Generally, eukaryotic total RNA consists of 1 to 4% mRNA.
2. Using a clean RNase-free (50 µl) microcell cuvette, determine the absorbance of the mRNA samples at 260 nm. Use the elution buffer as the blank. Recover the RNA samples from the microcell cuvette. Quantify the mRNA samples using 40 µg/ml for 1 $0D_{260}$.

3.2 Cy3 or Cy5 Labeling of mRNA Samples

1. Reconstitute *Label* IT µArray Reagent according to Section 2.0. If the reagent has already been reconstituted, warm to RT and quick spin before use.
2. Perform independent labeling reactions according to the examples shown. Mark the reaction tubes accordingly. **Add the *Label* IT µArray Reagent last.**

Table 4. Labeling Reaction Setup
For standard 100 µl labeling reactions with 1 µg mRNA each:

Labeling Reaction	
Purified mRNA Sample (1 µg)	up to 86 µl
10X Labeling Buffer M	10 µl
MB-grade water	bring volume to 96 µl
Label IT µArray Cy3 or Cy5 Reagent	4 µl
Total Volume	100 µl

NOTE: This example labels 1 µg of mRNA at a 4:1 (v:w) ratio of *Label* IT Reagent to mRNA. If there is a need to increase or decrease the density of labels in the final product, simply modify the ratio of labeling reagent to mRNA during the labeling reaction or adjust the incubation time of the labeling reaction. In addition, the labeling reaction may be scaled up or down, depending on the amount/volume of mRNA to be labeled. If the mRNA sample is more dilute, simply increase the reaction volume. Alternatively, concentrate the mRNA sample (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction. When scaling the labeling reaction, the amount of *Label* IT Reagent should never constitute more than 20% of the total reaction volume. Ensure that the final concentration of Labeling Buffer M is 1X.

3. Incubate the labeling reactions at 37°C for 1 hour.
   NOTE: If condensation appears at the top of the tubes, perform a quick spin after 30 minutes of incubation.
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. Place on ice or store at -80°C until ready to proceed with purification.

Important: The mRNA labeling reactions must NOT be treated with Reagent D1 and Neutralization Buffer N1.

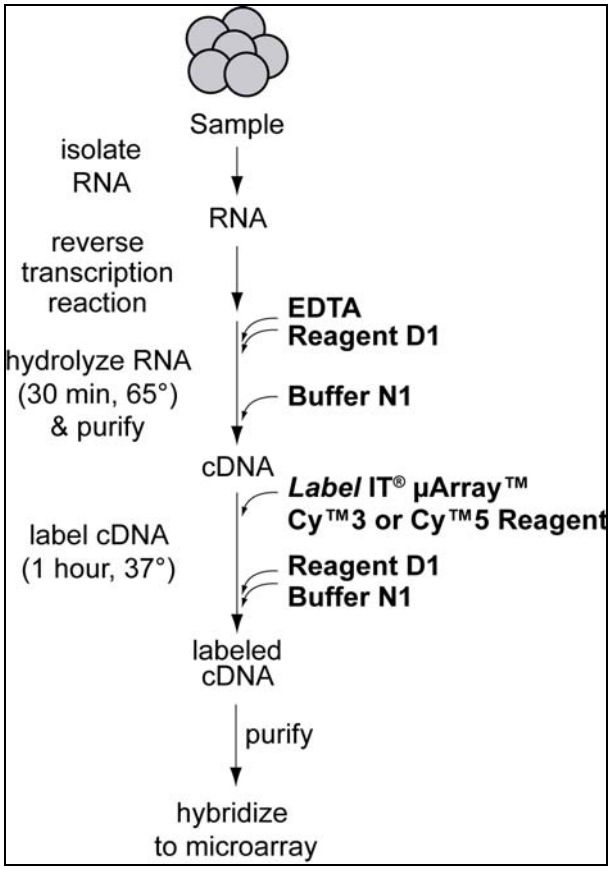
3.3 Purification of Cy3- or Cy5-labeled mRNA Samples

1. Perform one of the following purification procedures; each is compatible with the *Label* IT μArray Cy3or Cy5 Labeling Kits. Post-labeling purification is required for improved signal-to-noise ratios and lower background levels during hybridization.
    a. Silica membrane-based microspin column purification kit (i.e., Rapid Total RNA Purification System (Marligen Biosceinces Inc., www.marligen.com), MEGAClear™ Purification Kit (Ambion, Inc.), or Microarray Target Purification Kit (Roche Diagnostics Corp., www.roche-applied-science.com).
    b. Solid Phase Reversible Immobilization (SPRI) purification (RNAClean™, Agencourt Bioscience Corp., www.agencourt.com).
    c. Microcon Centrifugal Filter Unit (Millipore Corp., www.millipore.com) purification, using size YM-30, according to the manufacturer's directions for desalting samples.
    d. Gel Filtration (G50) microspin column purification, according to the manufacturer's recommendations.
2. Store the labeled and purified mRNA samples at –80°C, or proceed directly with the microarray hybridization. See Section 6.0 for recommendations.

4.0 cDNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use MB-grade reagents, water, and plasticware. Use non-powdered gloves during all steps of cDNA synthesis, sample labeling, microarray hybridization, washing and scanning. Reverse transcription reagents and sample purification reagents are required but not supplied.

Figure 3. Guide to cDNA Sample Preparation and Labeling



4.1 First Strand cDNA Synthesis

1. Perform reverse transcription reactions, using either total RNA or polyA$^+$ enriched RNA starting material, according to established protocols or enzyme manufacturer's recommendations. For example, Mirus Bio recommends using 1 µg cDNA per hybridization.
2. Since the cDNA samples will be directly labeled post-synthesis, **the RNA templates must be removed** after reverse transcription. To hydrolyze the RNA, add to each of the completed reverse transcription reactions: 0.05 volume of 0.5 M EDTA (for example, add 2 µl 0.5 M EDTA to a 40 µl reaction) and 0.1 volume of Reagent D1 (for example, add 4 µl Reagent D1 to the same 40 µl reaction).
3. Incubate at 65°C for 30 minutes and then allow the samples to slowly cool to RT.
4. Neutralize the samples by adding 0.125 original reaction volume of Neutralization Buffer N1 (for example, add 5 µl Neutralization Buffer N1 per 40 µl original reverse transcription reaction). Mix by pipetting. Place on ice until ready to proceed with purification.

4.2 cDNA Purification and Quantification

1. Purify the cDNA samples using a silica membrane-based column. Mirus Bio recommends the Microarray Target Purification kit (Roche Diagnostics Corp.).
2. Using a clean DNase-free (50 µl) microcell cuvette, determine the absorbance of the cDNA samples at 260 nm. Use the elution buffer as the blank. Recover the cDNA samples from the microcell cuvette. Quantify the cDNA samples using 37 µg/ml for 1 $0D_{260}$. Store the samples at -20°C or colder.

4.3 Cy3 or Cy5 Labeling of cDNA Samples

1. Reconstitute *Label* IT µArray Reagent according to Section 2.0. If the reagent has already been reconstituted, warm to RT and quick spin before use.
2. Perform independent labeling reactions according to the examples shown. Mark the tubes accordingly. **Add the *Label* IT µArray Reagent last.**

Table 5. Labeling Reaction Setup
For standard 100 µl labeling reactions with 1 µg cDNA each:

Labeling Reaction	
Purified cDNA Sample (1 µg)	up to 86 µl
10X Labeling Buffer M	10 µl
MB-grade water	bring volume to 96 µl
Label IT µArray Cy3 or Cy5 Reagent	4 µl
Total Volume	100 µl

NOTE: This example labels 1 µg of cDNA at a 4:1 (v:w) ratio of *Label* IT Reagent to cDNA. If there is a need to increase or decrease the density of labels in the final product, simply modify the ratio of labeling reagent to cDNA during the labeling reaction or adjust the incubation time of the labeling reaction. In addition, the labeling reaction may be scaled up or down, depending on the amount/volume of cDNA to be labeled. If the cDNA sample is more dilute, simply increase the reaction volume. Alternatively, concentrate the cDNA sample (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction. When scaling the labeling reaction, the amount of *Label* IT Reagent should never constitute more than 20% of the total reaction volume. Ensure that the final concentration of Labeling Buffer M is 1X.

3. Incubate the labeling reactions at 37°C for 1 hour.

   NOTE: If condensation appears at the top of the tubes, perform a quick spin after 30 minutes of incubation.

4. **Important: The cDNA labeling reactions must be treated immediately with Reagent D1 and Buffer N1.** Add 0.1 volume of Reagent D1 (for example, add 10 µl to a 100 µl labeling reaction), mix well, and incubate for 5

minutes at RT. Immediately add 0.1 volume of Neutralization Buffer N1 (for example, add 10 µl to the same 100 µl labeling reaction), mix well, and incubate on ice for at least 5 minutes.

NOTE: It is not necessary to stop the cDNA labeling reactions with the 10X STOP Reagent because treatment with Reagent D1 and Buffer N1 is sufficient to terminate the labeling reaction.

4.4 Purification of Cy3- or Cy5-labeled cDNA Samples

1. Perform one of the following purification procedures; each is compatible with the *Label* IT µArray Cy3 or Cy5 Labeling Kits. Post-labeling purification is required for improved signal-to-noise ratios and lower background levels during hybridization.
   a. Silica membrane-based microspin column purification kit (for example, Rapid PCR Purification System (Marligen Biosciences Inc.), or Microarray Target Purification Kit (Roche Diagnostics Corp).
   b. Microcon Centrifugal Filter Unit (Millipore Corp.) purification, size YM-30, according to the manufacturer's directions for desalting samples.
   c. Gel Filtration (G50) microspin column purification, according to the manufacturer's recommendations.
3. Store the labeled and purified cDNA samples at –20°C, or proceed directly with the microarray hybridization. See Section 6.0 for recommendations.

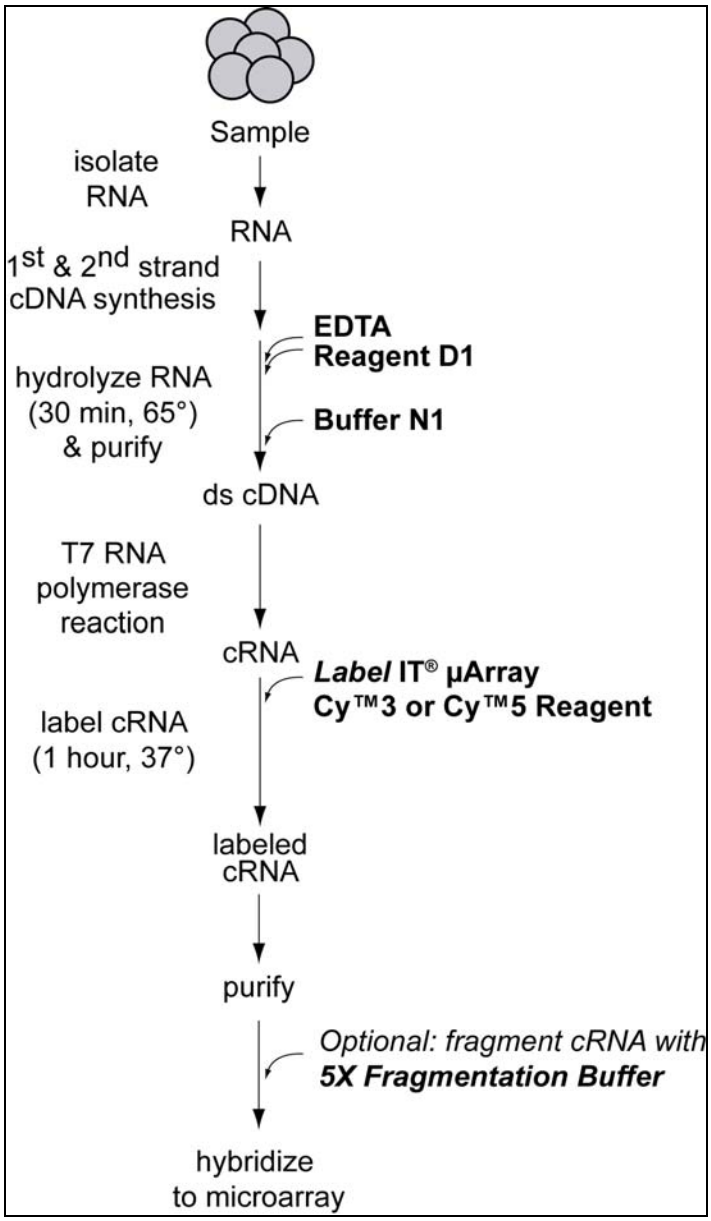
5.0 cRNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use MB-grade reagents, water and plasticware. Use non-powdered gloves during all steps of cRNA synthesis, sample labeling, microarray hybridization, washing and scanning.

Reverse transcription, T7 RNA polymerase amplification, and sample purification reagents are required but not supplied.

NOTE: This protocol involves an enzymatic amplification of the sample and is recommended only when starting materials may be limiting.

Figure 4. Guide to cRNA Sample Preparation and Labeling



5.1 Double Stranded (ds) cDNA Synthesis

1. Generate ds cDNA samples that incorporate the T7 promoter sequence according to established protocols (see Section 9.2). Prepare ds cDNA samples from both RNA samples intended for each microarray hybridization.
2. Since the final cRNA samples will be directly labeled post-synthesis, **the RNA template must be removed from the ds cDNA samples.** Mirus Bio recommends that this hydrolysis step be performed before the purification of the ds cDNA samples. To hydrolyze the original RNA, add to the completed second-strand cDNA reactions: 0.05 volume of 0.5 M EDTA (for example, add 7.5 µl 0.5 M EDTA to a 150 µl reaction) and 0.1 volume of Reagent D1 (for example, add 15 µl Reagent D1 to the same 150 µl reaction).

3. Incubate at 65°C for 30 minutes and then allow the samples to slowly cool to RT.
4. Neutralize the samples by adding 0.125 volume of Neutralization Buffer N1 (for example, add 18.75 µl Neutralization Buffer N1 to the same 150 µl reaction).

5.2 ds cDNA Purification

1. Many protocols recommend phenol:chloroform extraction and ethanol precipitation to purify ds cDNA. Although this method is acceptable, Mirus Bio recommends the Microarray Target Purification Kit (Roche Diagnostics Corp.) which is a silica membrane-based microspin column.
2. Quantify the purified ds cDNA samples by A260 absorbance, if desired. Concentrate the ds cDNA by speed vac or lyophilization for the RNA amplification step, if necessary.

5.3 cRNA Synthesis

1. Use the purified ds cDNA samples as templates in T7 RNA polymerase reactions, according to established protocols. Mirus Bio routinely uses the MEGAscript™ High Yield Transcription Kit (Ambion, Inc.). Purify the cRNA using a silica membrane-based column such as the Microarray Target Purification Kit (Roche Diagnostics Corp.).
2. Using a clean (RNase-free) 50 µl microcell cuvette, determine the absorbance of the cRNA samples at 260 nm. Use the elution buffer as the blank. Quantify the cRNA samples using 40 µg/ml for 1 $0D_{260}$.

5.4 Cy3 or Cy5 Labeling of cRNA Samples

1. Reconstitute *Label* IT µArray Reagent according to Section 2.0. If the reagent has already been reconstituted, warm to RT and quick spin before use.
2. Perform independent labeling reactions according to the examples shown. Mark the tubes accordingly. **Add the *Label* IT µArray Reagent last.**

Table 6. Labeling Reaction Setup
For standard 100 µl labeling reactions with 1 µg cRNA each:

Labeling Reaction	
Purified cRNA Sample (1 µg)	up to 86 µl
10X Labeling Buffer M	10 µl
MB-grade water	bring volume to 96 µl
Label IT µArray Cy3 or Cy5 Reagent	4 µl
Total Volume	100 µl

NOTE: This example labels 1 µg of cRNA at a 4:1 (v:w) ratio of *Label* IT Reagent to cRNA. If there is a need to increase or decrease the density of labels in the final product, simply modify the ratio of labeling reagent to cRNA during the labeling reaction or adjust the incubation time of the labeling reaction. In addition, the labeling reaction may be scaled up or down, depending on the amount/volume of cRNA to be labeled. If the cRNA sample is more dilute, simply increase the reaction volume. Alternatively, concentrate the cRNA sample (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction. When scaling the labeling reaction, the amount of *Label* IT Reagent should never constitute more than 20% of the total reaction volume. Ensure that the final concentration of Labeling Buffer M is 1X.

3. Incubate the labeling reactions at 37°C for 1 hour, protected from light.
NOTE: If condensation appears at the top of the tubes, perform a quick spin after 30 minutes of incubation.

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). Place on ice or at -80°C until ready to proceed with purification.
Important: The cRNA labeling reaction must NOT be treated with Reagent D1 and Neutralization Buffer N1.

Optional: The Cy3- or Cy5-labeled cRNA sample(s) can be fragmented to less than 200 nucleotides in size, which may improve efficiency of hybridization with oligo-based capture sequences. Add 0.25 volumes of 5X Fragmentation Buffer to the labeled, pooled and purified cRNA sample(s) (i.e., 50 µl to a 200 µl sample) and incubate at 94°C for 15 minutes. Place immediately on ice**.** Purification is recommended, using the *mir*Vana miRNA Isolation Kit (Ambion, Inc.), for improved signal-to-noise ratios and lower background.

5.5 Purification of Cy3- or Cy5-labeled cRNA

1. Perform one of the following purification procedures; each is compatible with the *Label* IT µArray Cy3 or Cy5 Labeling Kits. Post-labeling purification is required for improved signal-to-noise ratios and lower background levels during hybridization.
    a. Silica membrane-based microspin column purification kit (for example, Rapid Total RNA Purification System (Marligen Biosciences Inc.), MEGAClear™ Purification Kit (Ambion, Inc.), or Microarray Target Purification Kit (Roche Diagnostics Corp.)
    b. Solid Phase Reversible Immobilization (SPRI) purification (RNAClean, Agencourt Bioscience Corp.).
    c. Microcon Centrifugal Filter Unit (Millipore Corp.) purification, using size YM-30, according to the manufacturer's directions for desalting samples.
    d. Gel Filtration (G50) microspin column purification, according to the manufacturer's recommendations.

2. Store the labeled, pooled and purified cRNA samples at –80°C, or proceed directly with the microarray hybridization. See Section 6.0 for recommendations.

6.0 HYBRIDIZATION PROCEDURE

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. Before hybridization, concentrate the Cy3- or Cy5-labeled samples, if needed. If required, blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A^+ DNA, sheared salmon DNA, etc.) necessary for the hybridization can be added to the purified labeled sample prior to concentration. Drying down of the sample in a speed vac is not recommended because re-suspension of labeled samples can be difficult.
2. Prehybridize the required microarray slide(s) for at least 45 minutes at the hybridization temperature using a prehybridization buffer containing 5X SSC, 0.1% SDS and 1% BSA (for example, Sigma A9418).
3. Dip slide in dH_2O and quickly dry (centrifuge slide or use compressed air).
4. Dilute or resuspend the Cy3- or Cy5-labeled samples in the desired volume of hybridization buffer (see recommendations below).
5. When using formamide based hybridization buffers, denature labeled sample at 65°C for 1 minute. Spin the sample at maximum speed for 1 minute to pellet particulates.
6. Perform the microarray hybridization using the protocol of choice. Due to the variety of hybridization applications and formats available, general recommendations are provided. These conditions were determined to be optimal by Mirus Bio scientists, using arrays fabricated in-house on a variety of slide substrates. Other conditions are also compatible with samples labeled with the *Label* IT µArray Cy3 or Cy5 Labeling Kits, and should be optimized for the type of sample, microarray, and surface in the particular application. Please see APPENDIX Section 9.1 for the preparation of the recommended buffers.

Table 7. Standard Hybridization Conditions for cDNA Microarrays (printed PCR products):

Labeled Sample	Recommended Mass per Array[a]	Hybridization Buffer	Hybridization Temp. / Duration	Post-Hybridization Washes[b]
mRNA	≥ 2 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	50°C ~16 hours (overnight)	**1.** 1X SSC/0.2% SDS at 50°C 2 x 5 min. each **2.** 0.1X SSC/0.1% SDS at 50°C 1 x 5 min. **3.** 0.1X SSC, RT 1 x 5 min.
cDNA	≥ 2 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	45°C ~16 hours (overnight)	**1.** 1X SSC/0.2% SDS at 45°C 2 x 5 min. each **2.** 0.1X SSC/0.1% SDS at 45°C 1 x 5 min. **3.** 0.1X SSC, RT 1 x 5 min.
cRNA (unfragmented)	≥ 2 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	45°C ~16 hours (overnight)	**1.** 1X SSC/0.2% SDS at 45°C 2 x 5 min. each **2.** 0.1X SSC/0.1% SDS at 45°C 1 x 5 min. **3.** 0.1X SSC, RT 1 x 5 min.

[a] Using 22 x 40 mm coverslip area with 30 µl hybridization buffer. Hybridization volumes and masses should be scaled as appropriate for other microarray formats.
[b] Perform post hybridization washes with ample volume of prewarmed buffers and moderate agitation.

Table 8. Hybridization Conditions for Long Oligo (~50mers) Microarrays:

Labeled Sample	Recommended Mass per Array[a]	Hybridization Buffer	Hybridization Temp. / Duration	Post-Hybridization Washes[b]
mRNA[c]				
cDNA	≥ 1 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	30°C ~16 hours (overnight)	**1.** 1X SSC/0.2% SDS at 45°C 2 x 5 min. each **2.** 0.1X SSC/0.1% SDS at 45°C 1 x 5 min **3.** 0.1X SSC, RT 1 x 5 min.
cRNA (fragmented)	≥ 2 µg[d]	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	30°C ~16 hours (overnight)	**1.** 1X SSC/0.2% SDS at 45°C 2 x 5 min. each **2.** 0.1X SSC/0.1% SDS at 45°C 1 x 5 min. **3.** 0.1X SSC, RT 1 x 5 min.

[a] Using 22 x 40 mm coverslip area with 30 µl hybridization buffer. Hybridization volumes and masses should be scaled as appropriate for other microarray formats.
[b] Perform post hybridization washes with ample volume of prewarmed buffers and moderate agitation.
[c] Currently, the majority of commercially available oligo arrays are generated using sense-strand capture sequences, and are therefore not compatible with hybridization of labeled sense-strand RNA. **Anti-sense** oligo arrays must be used to capture labeled sense-strand RNA samples, such as mRNA.
[d] The labeling reactions may need to be scaled up for optimal hybridization performance.

7.0 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. Labeling Total RNA for Gene Expression Analysis
Direct labeling of total RNA for gene expression analysis is another attractive application of the *Label* IT µArray Cy3 or Cy5 Labeling Kits. In some applications, the hybridization of purified labeled total RNA may provide satisfactory hybridization performance. However, Mirus Bio recommends that the sample be enriched for labeled mRNA via polyA$^+$ purification, for better hybridization sensitivity. In Section 3.0, Mirus Bio recommends the isolation of polyA$^+$ RNA prior to labeling with the *Label* IT µArray Cy3 or Cy5 Labeling Kit since this strategy is more economical and provides consistently superior hybridization performance than isolating polyA$^+$ RNA after labeling total RNA.

C. Alternate RNA Samples
For microarray analysis of RNA samples other than eukaryotic mRNA, such as bacterial (or eukaryotic) ribosomal RNA, bacterial mRNA, microRNA, an RNA transcribed *in vitro*, etc., we recommend isolating the RNA material required for the microarray hybridization and then labeling as described in the mRNA labeling protocol (Section 3.0). Label IT miRNA Labeling Kits for miRNA expression profiling applications are listed in Section 9.3 (Related Products).

D. Alternate DNA Samples
For microarray analysis of DNA samples other than cDNA (for example, genomic DNA, PCR products, etc.), we recommend isolating the alternate DNA material required for the microarray hybridization and then labeling as described in the cDNA labeling protocol (Section 4.0).

E. Adjusting the Density of Cy3 or Cy5 Labels
The labeling protocols in Sections 3.0, 4.0 and 5.0 have 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 (≤ 4 hours).

F. Use of Reagent D1 and Buffer N1
Treat labeled RNA (for example, mRNA and cRNA) with the addition of 10X STOP Reagent. Labeled RNA samples should never be treated with Reagent D1 and Buffer N1 (as per mRNA and cRNA labeling protocols). Treat labeled DNA (for example, cDNA) with Reagent D1 and Buffer N1 after labeling (as per cDNA protocol). DNA samples do not need to be treated with the 10X Stop Reagent as Reagents D1 and N1 are sufficient to stop the labeling reaction.

8.0 TROUBLESHOOTING GUIDE

Poor Hybridization Signal

Problem	Solution
Suboptimal amount of sample applied to microarray	- label and hybridize more sample to microarray - - use an alternate purification method
Poor quality RNA samples	- use higher quality RNA samples - prepare new cDNA or cRNA from higher quality RNA samples - use proper laboratory techniques when handling RNA samples - label and hybridize more sample to microarray - use the labeled sample promptly, avoid prolonged storage
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 - optimize microarray production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted arrays
Weak Cy3 or Cy5 signal	- for cDNA and cRNA labeling, ensure that residual starting material has been hydrolyzed before labeling - 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
Improper treatment of Cy3-or Cy5-labeled cDNA	- ensure that the D1/N1 steps are performed as described
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 - use alternate purification method, do not use ethanol precipitation
Suboptimal blocker/suppressor DNA used in the hybridization	- add more or alternate blocker and/or suppressor DNA to hybridization buffer
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 rapidly in water before drying slide
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	- assure the labeled sample has been denatured and spun as indicated in Section 6.5

9.0 APPENDIX

9.1 Preparation of Buffers and Solutions 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	

Prehybridization 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	

Hybridization Buffer

5X SSC, 50% Formamide, 0.1% SDS	
20X SSC	250 µl
100% Formamide	500 µl
10% SDS	10 µl
Water	240 µl
Total Volume	1000 µl
Aliquot and store at -20ºC	

0.1X SSC, 0.1% SDS	
20X SSC	5 ml
10% SDS	10 ml
Water	985 ml
Total Volume	1000 ml
Store at RT	

Post-Hybridization Buffers

1X SSC, 0.2% SDS	
20X SSC	50 ml
10% SDS	20 ml
Water	930 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	

9.2 General Internet Resources

"Anatomy of a Comparative Gene Expression Study"
http://www.cs.wustl.edu/~jbuhler//research/array/
Microarrays: Chipping away at the mysteries of science and medicine, from NCBI: A Science Primer
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

9.3 Related Products

Label IT µArray Biotin Labeling Kit (MIR 8010, MIR 8050)
Label IT µArray Dual Labeling Kit (Fluorescein and Biotin; MIR 8105, MIR 8125)
Label IT µArray Cy3/Cy5 Labeling Kit (MIR 8205, MIR8225)

Label IT miRNA Labeling Kit, Cy3/Cy5 (MIR 8305, MIR 8325)
Label IT miRNA Labeling Kit, Biotin (MIR 8410, MIR 8450)
Label IT miRNA Labeling Kit, Cy3 (MIR 8510, MIR 8550)
Label IT miRNA Labeling Kit, Cy5 (MIR 8610, MIR 8650)