

Label IT[®] μ Array[™] Dual Labeling Kit

Product Name	Quantity	Product No.
Label IT [®] μ Array [™] Dual Labeling Kit	10 labeling reactions: 5 Label IT [®] μ Array [™] Biotin reactions (1 μ g each) 5 Label IT [®] μ Array [™] Fluorescein reactions (1 μ g each)	MIR 8105
	50 labeling reactions: 25 Label IT [®] μ Array [™] Biotin reactions (1 μ g each) 25 Label IT [®] μ Array [™] Fluorescein reactions (1 μ g each)	MIR 8125

The Label IT[®] μ Array[™] Dual Labeling Kit is supplied with sufficient reagents to perform 5 (or 25) biotin and 5 (or 25) fluorescein labeling reactions (1 μ g 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 reagents or biotin/fluorescein detection reagents. A recommended simultaneous detection protocol using Cy[™] 3-conjugated anti-fluorescein antibody and Cy[™] 5-conjugated anti-biotin antibody is provided.

Table of Contents:
Page #

1.0 DESCRIPTION	2
1.1 General Information	2
1.2 Materials Supplied	4
1.3 Materials Required but Not Supplied	4
1.4 Storage and Stability	4
1.5 Abbreviations	4
2.0 RECONSTITUTION PROCEDURE	5
3.0 mRNA LABELING PROCEDURE	5
3.1 mRNA Isolation	6
3.2 Biotin and Fluorescein Labeling of mRNA	6
3.3 Purification of Biotin- and Fluorescein-labeled mRNA	7
4.0 cDNA LABELING PROCEDURE	8
4.1 First Strand cDNA Synthesis	8
4.2 cDNA Purification and Quantification	9
4.3 Biotin and Fluorescein Labeling of cDNA	9
4.4 Purification of Biotin- and Fluorescein-labeled cDNA	10
5.0 cRNA LABELING PROCEDURE	11
5.1 Double Stranded (ds) cDNA Synthesis	11
5.2 ds cDNA Purification	12
5.3 cRNA Synthesis	12
5.4 Biotin and Fluorescein Labeling of cRNA	12
5.5 Purification of Biotin- and Fluorescein-labeled cRNA	13
6.0 HYBRIDIZATION PROCEDURE	14
7.0 DETECTION PROCEDURE	15
8.0 APPLICATION NOTES	16
9.0 TROUBLESHOOTING GUIDE	17
10.0 APPENDIX	18
10.1 Preparation of Buffers and Solutions	18
10.2 General Internet Resources	19
10.3 Related Products	19

1.0 DESCRIPTION

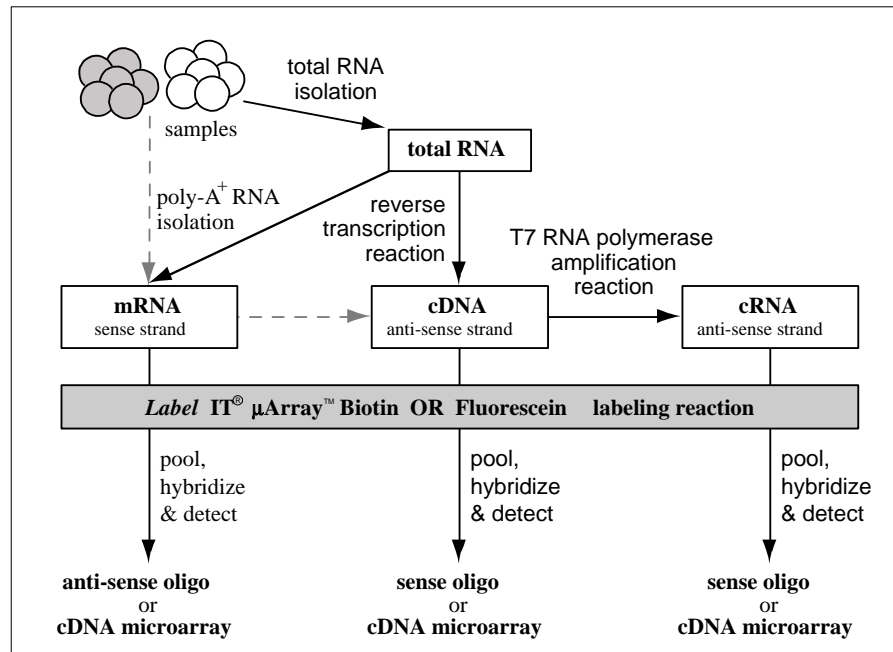
1.1 General Information

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' *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*[®] μ Array[™] Biotin and *Label IT*[®] μ Array[™] Fluorescein Reagents have been optimized for the preparation of labeled nucleic acid samples for use in dual color expression profiling hybridizations.

In traditional expression profiling applications, RNA is extracted from both test and reference samples, labeled independently with distinct reporter molecules, pooled, and hybridized simultaneously to a single microarray. Target gene expression is quantified from the relative signal derived from each 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*[®] μ Array[™] Dual Labeling Kit allows any type of nucleic acid samples to be directly labeled— mRNA, cDNA, or cRNA (see Figure 1)— depending on experimental design.

The *Label IT*[®] μ Array[™] Dual Labeling Kit generates 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*[®] μ Array[™] Dual Labeling Kit do not require the traditional 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 *Label IT*[®] μ Array[™] Dual Labeling Kit. Samples labeled using this kit are compatible with hybridization on a variety of microarray surfaces, facilitating substitution into standard protocols.

The use of biotin and fluorescein as marker molecules for hybridization require a post-hybridization secondary detection procedure with different fluorophor-labeled conjugates. As such, biotin- and fluorescein-labeled hybridization samples provide flexibility and compatibility with a variety of established dual color secondary detection strategies. A simple method of Cy[™]3-conjugated anti-fluorescein and Cy[™]5-conjugated anti-biotin antibody detection is provided in this protocol, allowing use of standard microarray scanners.

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 biotin or fluorescein using the *Label IT[®] μArray™* Dual Labeling Kit, then pooled and simultaneously 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 biotin or fluorescein using the *Label IT[®] μArray™* Dual Labeling Kit. If the amount of RNA is limited, some applications may require amplification of the samples. The RNA samples can also be used to generate cRNA samples, which can then be labeled with biotin or fluorescein using the *Label IT[®] μArray™* Dual Labeling Kit.

Table 1. Selecting a Sample Type for the *Label IT[®] μArray™* Dual Labeling Kit

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™* Dual Labeling Kit.

1.2 Table 2. Materials Supplied

Component*	Relevant Labeling Procedure	MIR 8105 2 x 5 reactions	MIR 8125 2 x 25 reactions	Reagent Cap Color
<i>Label IT</i> [®] μ Array [™] Biotin Reagent	mRNA, cDNA, cRNA	dried pellet	dried pellet	Brown
<i>Label IT</i> [®] μ Array [™] Fluorescein Reagent	mRNA, cDNA, cRNA	dried pellet	dried pellet	Yellow
Reconstitution Solution	mRNA, cDNA, cRNA	44 μ l	220 μ l	Brown
10X Labeling Buffer M	mRNA, cDNA, cRNA	100 μ l	500 μ l	Purple
0.5 M EDTA	cDNA, cRNA	75 μ l	375 μ l	Green
Reagent D1	cDNA, cRNA	150 μ l	750 μ l	Blue
Neutralization Buffer N1	cDNA, cRNA	190 μ l	950 μ l	White
10X STOP Reagent	mRNA, cRNA	100 μ l	500 μ l	Red
5X Fragmentation Buffer	cRNA	250 μ l	1.25 ml	Orange

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

NOTE: A standard biotin or fluorescein labeling reaction is defined for 1 μ g nucleic acid sample. For the two nucleic acid samples being compared in a microarray hybridization, label one sample with biotin and the second sample with fluorescein. It may be beneficial, in subsequent analyses, to interchange the marker molecules used for the two samples. The *Label IT*[®] μ Array[™] Biotin and the *Label IT*[®] μ Array[™] Fluorescein 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 (siliconized) microcentrifugation 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, purification, microarray hybridization, and secondary detection.

1.4 Storage and Stability

Store the *Label IT*[®] μ Array[™] Biotin and the *Label IT*[®] μ Array[™] Fluorescein 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*[®] μ Array[™] Reagents are stable for 6 months after reconstitution. Unreconstituted *Label IT*[®] μ Array[™] 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 10.1 for preparation)
- SSPE – sodium chloride + sodium phosphate + EDTA buffer (see Appendix, Section 10.1 for preparation)
- SSPE-T – sodium chloride + sodium phosphate + EDTA + Triton X-100 buffer (see Appendix, Section 10.1 for preparation)
- RT – room temperature
- MB – molecular biology

2.0 RECONSTITUTION PROCEDURE

1. Warm the vials of *Label IT*[®] μ Array[™] Biotin Reagent and *Label IT*[®] μ Array[™] Fluorescein Reagent to room temperature and quick spin before opening.
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*[®] μ Array[™] Reagents

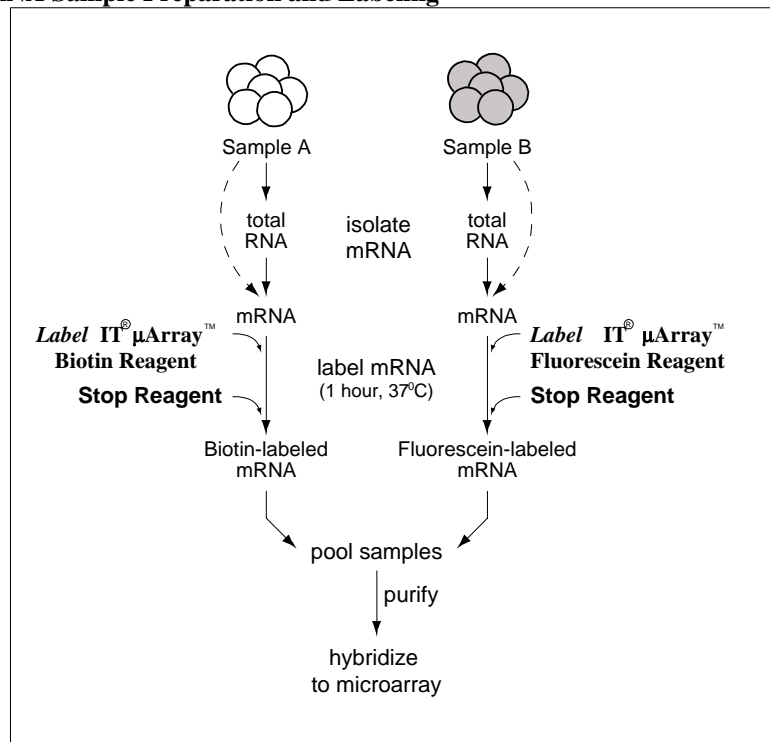
<i>Label IT</i> [®] μ Array [™] Dual Labeling Kit	Labeling Reagent	Volume of Reconstitution Solution
MIR 8105	<i>Label IT</i> [®] μ Array [™] Biotin	22 μ l
	<i>Label IT</i> [®] μ Array [™] Fluorescein	22 μ l
MIR 8125	<i>Label IT</i> [®] μ Array [™] Biotin	110 μ l
	<i>Label IT</i> [®] μ Array [™] Fluorescein	110 μ l

3. Store unused, reconstituted *Label IT*[®] μ Array[™] Reagents tightly capped at -20°C. 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 RNase- and DNase-free reagents, water, and plasticware. Use non-powdered gloves during all steps of sample labeling, microarray hybridization, washing, detection, 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 analyses of eukaryotic mRNA. Some microarray applications may not require polyA⁺ isolation but may require isolation of alternate RNA populations, such as bacterial non-ribosomal mRNA. Isolate the desired RNA population before proceeding with the labeling protocol. See Application Notes, Section 8.0, Part C.

1. Isolate polyA⁺ enriched RNA from both samples intended for each microarray hybridization. Direct isolation of mRNA from tissue/cells and mRNA isolation from a total RNA sample are both suitable. Mirus 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[™] Dual Labeling Kit.** 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 OD₂₆₀.

3.2 Biotin and Fluorescein Labeling of mRNA Samples

1. Reconstitute Label IT[®] μArray[™] Reagents according to procedure in Section 2.0.
2. Perform independent labeling reactions according to the examples shown. For the two mRNA samples being compared in a microarray hybridization, label one sample with biotin and the second sample with fluorescein. Mark the reaction tubes accordingly. **Add the Label IT[®] μArray[™] Reagent last.**

Table 4. Labeling Reaction Setup

Biotin Labeling Reaction	
Purified mRNA Sample A (1 μg)	up to 86 μl
10X Labeling Buffer M	10 μl
MB-grade water	bring volume to 96 μl
Label IT [®] μArray [™] Biotin Reagent	4 μl
Total Volume	100 μl

Fluorescein Labeling Reaction	
Purified mRNA Sample B (1 μg)	up to 86 μl
10X Labeling Buffer M	10 μl
MB-grade water	bring volume to 96 μl
Label IT [®] μArray [™] Fluorescein 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 A is 1X.

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

NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of each reaction.

4. Stop the labeling reactions with the addition of 0.1 volume of the 10X STOP Reagent (10 μ l to a 100 μ l labeling reaction). Vortex gently to mix. 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.

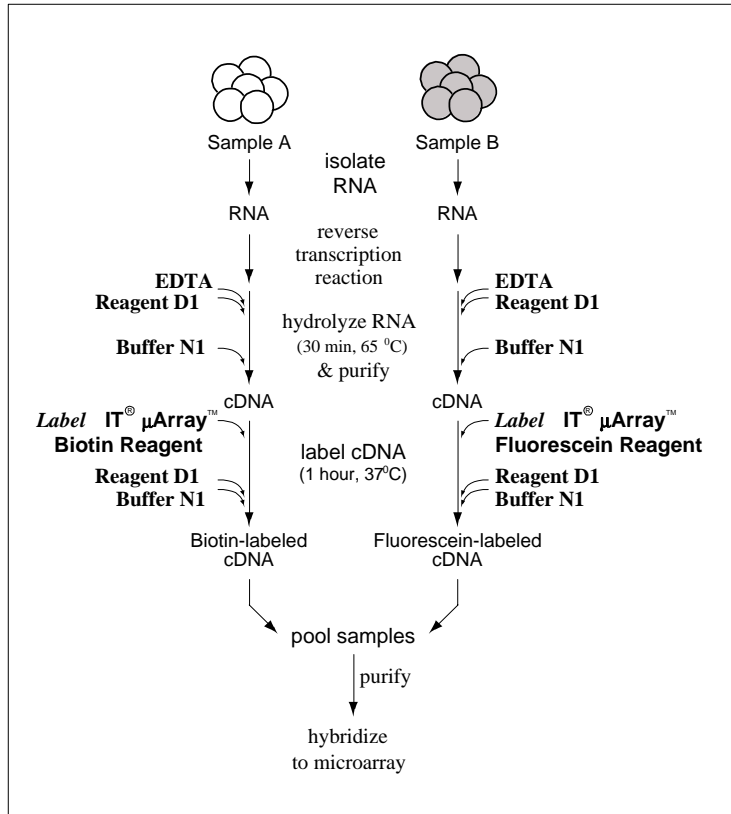
3.3 Purification of Biotin- and Fluorescein-labeled mRNA

1. Recommended: Pool the *Label IT*[®] μ Array[™] Biotin and *Label IT*[®] μ Array[™] Fluorescein mRNA samples to be compared on each microarray.
2. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] μ Array[™] Dual Labeling Kit. Post-labeling purification is recommended for improved signal-to-noise ratios and lower background levels during hybridization.
 - a. Ethanol precipitation
 - i. **Recommended:** Add blocker/suppressor nucleic acids (e.g. species-specific Cot-1 DNA, sheared salmon DNA) required for the microarray hybridization. This will increase the recovery of the labeled mRNA.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2.5 volumes of ice-cold 100% ethanol. Mix and store at -20°C (or colder) for at least 30 minutes. Recommended: store precipitated sample at -20°C (or colder) until immediately before hybridization to a microarray.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C for at least 30 minutes to pellet the labeled mRNA (plus blockers). Aspirate the ethanol, being careful not to disturb the pellet. **NOTE:** Orient the tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small RNA quantities can be invisible to the naked eye.
 - iv. Gently wash the pellet once with 500 μ l MB-grade 70% ethanol. Centrifuge at full speed for at least 15 minutes at 4°C and remove 70% ethanol wash. Pulse-spin and 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.
 - v. Resuspend the labeled mRNA in hybridization buffer (see Section 6.0 for recommendations), or buffer of choice.
 - b. Microcon Centrifugal Filter Unit (Millipore Corp., www.millipore.com) purification, using 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.
 - d. RNeasy[®] Mini Kit (Qiagen Inc.), according to the manufacturer's protocol for RNA cleanup, eluting the mRNA in water.
2. Store the labeled, pooled 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 RNase- and DNase-free reagents, water, and plasticware. Use non-powdered gloves during all steps of cDNA synthesis, sample labeling, microarray hybridization, washing, detection, 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. Prepare the appropriate amount of cDNA from both RNA samples intended for each microarray 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., www.roche-applied-science.com).
2. Using a clean RNase-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 OD₂₆₀. Store the samples at -20°C or colder.

4.3 Biotin and Fluorescein Labeling of cDNA Samples

1. Reconstitute *Label IT*[®] μ Array[™] Reagents according to procedure in Section 2.0.
2. Perform independent labeling reactions according to the examples shown. For the two cDNA samples being compared in a microarray hybridization, label one sample with biotin and the second sample with fluorescein. Mark the tubes accordingly. **Add the *Label IT*[®] μ Array[™] Reagent last.**

Table 5. Labeling Reaction Setup

 For standard 100 μ l labeling reactions with 1 μ g cDNA each:

Biotin Labeling Reaction	
Purified cDNA Sample A (1 μ g)	up to 86 μ l
10X Labeling Buffer M	10 μ l
MB-grade water	bring volume to 96 μ l
<i>Label IT</i> [®] μ Array [™] Biotin Reagent	4 μ l
Total Volume	100 μ l

Fluorescein Labeling Reaction	
Purified cDNA Sample B (1 μ g)	up to 86 μ l
10X Labeling Buffer M	10 μ l
MB-grade water	bring volume to 96 μ l
<i>Label IT</i> [®] μ Array [™] Fluorescein Reagent	4 μ l
Total Volume	100 μ l

NOTE: This example labels 1 μ g of mRNA 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 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 cDNA to be labeled. If the cDNA 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 A is 1X.

3. Incubate the labeling reactions at 37°C for 1 hour.
NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of each reaction.
4. **Important: The cDNA labeling reactions must be treated immediately with Reagent D1 and Buffer N1.** Add 0.1 volume of Reagent D1 (10 μ l to a 100 μ l labeling reaction), mix well, and incubate for 5 minutes at RT. Immediately add 0.1 volume of Neutralization Buffer N1 (10 μ l to the same 100 μ l 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 Biotin- and Fluorescein-labeled cDNA Samples

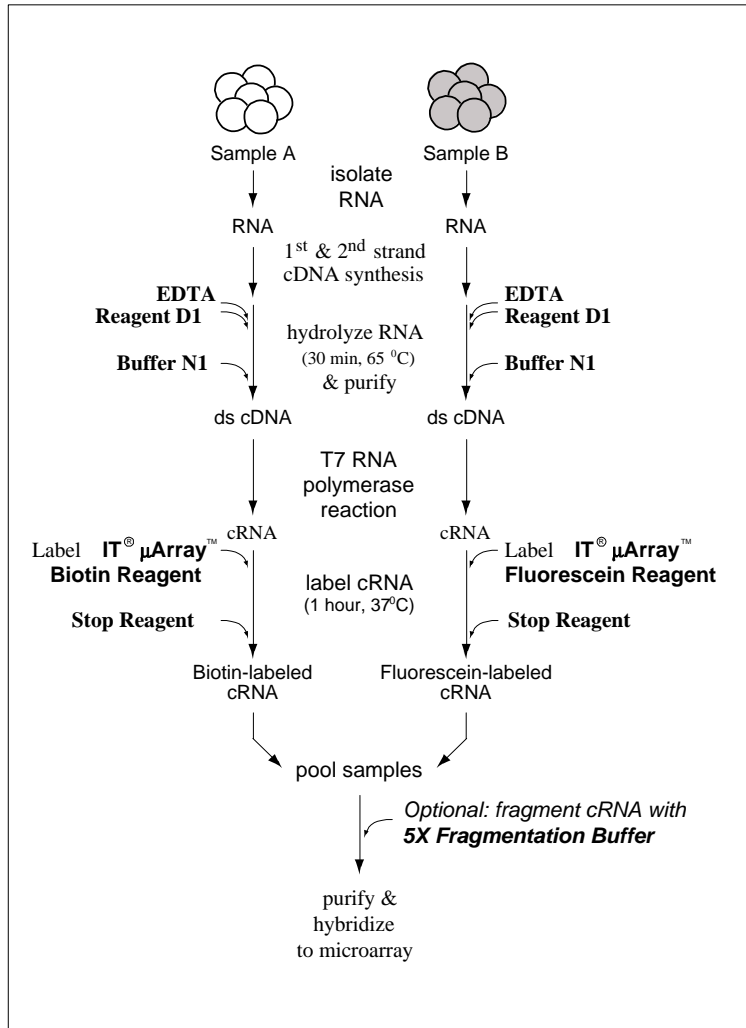
1. **Recommended:** Pool *Label IT*[®] μ Array[™] Biotin and *Label IT*[®] μ Array[™] Fluorescein cDNA labeling reactions.
2. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] μ Array[™] Dual Labeling Kit.
Post-labeling purification is recommended for improved signal-to-noise ratios and lower background levels during hybridization.
 - a. Ethanol precipitation
 - i. Recommended: Add blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon DNA) required for the hybridization. This will increase the recovery of the labeled cDNA.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2 volumes of ice-cold 100% ethanol. Mix and store at –20°C (or colder) for at least 30 minutes. Recommended: store precipitated sample at –20°C (or colder) until immediately before hybridization to a microarray.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C for at least 30 minutes to pellet the labeled cDNA (plus blockers). Aspirate the ethanol, being careful not to disturb the pellet.
NOTE: Orient the tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small DNA quantities can be invisible to the naked eye.
 - iv. Gently wash the pellet once with 500 μ l MB-grade 70% ethanol. Centrifuge at full speed for at least 15 minutes at 4°C and remove 70% ethanol wash. Pulse-spin and 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.
 - v. Resuspend the labeled cDNA pellet in hybridization buffer (see Section 6.0 for recommendations), or buffer of choice.
 - 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.
 - d. QIAquick[®] PCR Purification Kit (Qiagen Inc.), according to the manufacturer's protocol.
3. Store the labeled, pooled 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 RNase- and DNase-free reagents, water and plasticware. Use non-powdered gloves during all steps of cRNA synthesis, sample labeling, microarray hybridization, washing, detection 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 using either total RNA or polyA⁺ enriched RNA starting material, according to established protocols, which incorporate the T7 promoter sequence (see Section 10.2). Prepare 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 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 room temperature.
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). Ensure that the pH is ~7.5 using pH indicator strips.

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 recommends the Microarray Target Purification kit (Roche Diagnostics Corp.) to purify the ds cDNA samples from the hydrolyzed RNA (and salts).
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 routinely uses the MEGAscript™ High Yield Transcription Kit (Ambion, Inc.). Purify the cRNA using the Microarray Target Purification kit (Roche Diagnostics Corp.), according to the manufacturer’s protocol for RNA cleanup, eluting the cRNA in water.
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 OD₂₆₀.

5.4 Biotin and Fluorescein Labeling of cRNA Samples

1. Reconstitute *Label IT*® µArray™ Reagents according to procedure in Section 2.0.
2. Perform independent labeling reactions according to the examples shown. For the two cRNA samples being compared in a microarray hybridization, label one sample with biotin and the second sample with fluorescein. 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:

Biotin Labeling Reaction	
Purified cRNA 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> ® µArray™ Biotin Reagent	4 µl
Total Volume	100 µl

Fluorescein Labeling Reaction	
Purified cRNA 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> ® µArray™ Fluorescein 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 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 cRNA to be labeled. If the cRNA 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 A is 1X.

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

NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of each reaction.
4. Stop the labeling reactions with the addition of 0.1 volume of the 10X STOP Reagent (10 µl to a 100 µl labeling reaction). Place on ice or at -80°C until ready to proceed with purification.

5. **Recommended:** Pool the *Label IT*[®] μ Array[™] Biotin and *Label IT*[®] μ Array[™] Fluorescein cRNA labeling reactions.
6. **Optional:** The Biotin- and Fluorescein-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 volume of 5X Fragmentation Buffer (55 μ l to a 220 μ l pooled sample) and incubate at 94°C for 15 minutes. Place immediately on ice.

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

5.5 Purification of Biotin- and Fluorescein-labeled cRNA

1. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] μ Array[™] Dual Labeling Kit. Post-labeling purification is highly recommended for improved signal-to-noise ratios and lower background levels following hybridization.
 - a. Ethanol precipitation
 - i. **Recommended:** Add blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon DNA) required for the hybridization. This will increase the recovery of the labeled cRNA.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2.5 volumes of ice-cold 100% ethanol. Mix and store at –20°C (or colder) for at least 30 minutes. Recommended: store precipitated sample at –20°C (or colder) until immediately before hybridization to a microarray.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C (plus blockers) for at least 30 minutes to pellet the labeled cRNA. Aspirate the ethanol, being careful not to disturb the pellet.

NOTE: Orient the tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small RNA quantities can be invisible to the naked eye.
 - iv. Gently wash the pellet once with 500 μ l MB-grade 70% ethanol. Centrifuge at full speed for at least 15 minutes at 4°C and remove 70% ethanol wash. Pulse-spin and 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.
 - v. Resuspend the labeled cRNA pellet in hybridization buffer, or buffer of choice (see Section 6.0 for recommendations).
 - b. Microcon Centrifugal Filter Unit (Millipore Corp.) purification, using size YM-30 (size YM-10 if the cRNA is fragmented), according to the manufacturer's directions for desalting samples
 - b. 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 protocol. See Section 6.0 for recommendations.

6.0 HYBRIDIZATION PROCEDURE

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

1. Before hybridization, concentrate, dry down, or precipitate the pooled biotin- and fluorescein-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.
2. Dilute or resuspend the pooled biotin- and fluorescein-labeled samples in the desired volume of hybridization buffer (see below).
3. When using formamide based hybridization buffers, denature labeled sample at 65°C for 1-3 minutes. Spin the sample at maximum speed for 1 minute to pellet any particulates.
4. 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 scientists, using arrays fabricated in-house on a variety of slide substrates. Other conditions are also compatible with samples labeled with the *Label IT*[®] μ Array[™] Dual Labeling Kit, and should be optimized for the type of sample, microarray, and surface in the particular application. Please see APPENDIX Section 10.1 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 (1 µg from each labeled sample)	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. 4. Proceed immediately with detection
cDNA	≥ 2 µg (1 µg from each labeled sample)	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. 4. Proceed immediately with detection
cRNA (unfragmented)	≥ 2 µg (1 µg from each labeled sample)	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. 4. Proceed immediately with detection

^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 Microarrays:

Labeled Sample	Recommended Mass per Array ^a	Hybridization Buffer	Hybridization Temp./Duration	Post-Hybridization Washes ^b
mRNA ^c				
cDNA	≥ 2 µg (1 µg from each labeled sample)	100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween-20	45°C (50mer oligos) ~16 hours (overnight)	1. 6X SSPE, 0.01% Tween 20, 2 x 5 min. at RT 2. 100 mM MES, 0.1 M [Na+], 0.01% Tween 20, 1 x 5 min. at 50°C 3. Proceed immediately with detection
cRNA (fragmented)	≥ 4 µg ^d (2 µg from each labeled sample)	100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween-20	45°C (50mer oligos) ~16 hours (overnight)	1. 6X SSPE, 0.01% Tween 20, 2 x 5 min. at RT 2. 100 mM MES, 0.1 M [Na+], 0.01% Tween 20, 1 x 5 min. at 50°C 3. Proceed immediately with detection

^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 DETECTION PROCEDURE

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. There are also a variety of anti-fluorescein antibody fluorescent conjugates that can be used to detect hybridized fluorescein-labeled samples on glass slides. A general detection protocol using CyTM5-conjugated anti-biotin and CyTM3-conjugated anti-fluorescein monoclonal antibody is provided that is compatible with a majority of microarray scanners. Optimization of detection parameters may be required. Please see Appendix Section 10.1 for the preparation of the recommended buffers.

1. Immediately after the post-hybridization washes, incubate the slide(s) in 6X SSPE-T for 5 minutes at RT.
2. Prepare sufficient Detection Solution (200 μ l per slide):
 - 0.5 μ g/ml CyTM5-conjugated anti-biotin monoclonal antibody (Jackson ImmunoResearch Labs Inc., www.jacksonimmuno.com, Cat# 200-172-096)
 - 0.5 μ g/ml CyTM3-conjugated anti-fluorescein monoclonal antibody (Jackson ImmunoResearch Labs Inc., Cat# 200-162-037)
 - 2 mg/ml BSA (Sigma-Aldrich, www.sigmaaldrich.com; Cat # A9418)
 - 10% mouse serum (Sigma cat# M5905)
 - 6X SSPE-T

NOTE: The antibody concentrations provided are recommended starting conditions. Titration of the antibody may be necessary for optimal detection. Reconstitute and store detection reagents according to manufacturer's instructions.

3. One at a time, remove a slide from the 6X SSPE-T wash and briefly blot edge of slide to wick off excess buffer (do not allow the slide to dry). Immediately overlay ~200 μ l Detection Solution on the array and gently cover with a large coverslip to evenly distribute the Detection Solution. Incubate under humidified conditions at 37°C for 20 minutes, protected from light.
4. Remove Detection Solution/coverslips in 6X SSPE-T and wash three times for 5 minutes in 6X SSPE-T at RT with gentle shaking.
5. Dip the slide in water very briefly, spin or blow (compressed air) dry, and scan using appropriate CyTM3 and CyTM5 filters.
6. Store hybridized and detected slides at RT, protected from light.

8.0 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*[®] μ Array[™] Dual Labeling Kit. In some applications, the hybridization of purified labeled total RNA may provide satisfactory hybridization performance. However, Mirus recommends that the sample be enriched for labeled mRNA via polyA⁺ purification, for better hybridization sensitivity. In Section 3.0, Mirus recommends the isolation of polyA⁺ RNA prior to labeling with the *Label IT*[®] μ Array[™] Dual 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, 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).

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 materials required for the microarray hybridization and then labeling as described in the cDNA labeling protocol (Section 4.0).

E. Adjusting the Density of Biotin and Fluorescein Labels

The labeling protocols in Sections 3.0, 4.0 and 5.0 have been optimized for microarray hybridization and detection 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.

F. Use of Reagent D1 and Buffer N1

To ensure that there is no cross-labeling of samples, use the described procedures to terminate the labeling reactions prior to pooling and purifying samples. 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).

G. Label/Fluor Exchange Experiments

To verify expression profiling results, it may be advantageous to perform additional hybridizations with the same nucleic acid samples labeled with the opposite reporter molecule – that is, perform a ‘label swap’ experiment. Although we have found very consistent hybridization results using Cy[™]5-conjugated anti-biotin and a Cy[™]3-conjugated anti-fluorescein monoclonal antibody compared to Cy[™]3-conjugated anti-biotin and a Cy[™]5-conjugated anti-fluorescein monoclonal antibody (all reagents are available from Jackson ImmunoResearch Labs Inc.), a detection-swap experiment can also be performed.

9.0 TROUBLESHOOTING GUIDE
Poor Hybridization Signal

Problem	Solution
Suboptimal amount of sample applied to microarray	<ul style="list-style-type: none"> - label and hybridize more sample to microarray - label and pool equal amounts of the two nucleic acid samples - add blockers before purifying sample by precipitation to increase recovery
Poor quality RNA samples	<ul style="list-style-type: none"> - 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
Improper detection strategy	<ul style="list-style-type: none"> - optimize detection procedure with biotin- and fluorescein-labeled DNA spotted on glass - verify that detection instrumentation is compatible with detection reagents
Signal lost by exposure to light, environmental conditions	<ul style="list-style-type: none"> - minimize exposure to light
Poor quality microarray	<ul style="list-style-type: none"> - use verified capture sequences - use verified strand sequence - optimize microarray production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted arrays
Poor biotin or fluorescein detection	<ul style="list-style-type: none"> - 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 in labeling reactions - increase the duration of labeling reactions - ensure that the detection reagents have been stored properly - ensure that the detection solution was prepared properly, immediately before use
Improper treatment of biotin- or fluorescein-labeled cDNA	<ul style="list-style-type: none"> - ensure that the D1/N1 steps are performed as described
Hybridization signal 'stripped' from microarray	<ul style="list-style-type: none"> - decrease stringency of hybridization incubation or post-hybridization washes by increasing salt concentration and/or decreasing temperature
Suboptimal hybridization time	<ul style="list-style-type: none"> - extend hybridization time

High Background

Problem	Solution
Excess detection reagents remaining on microarray	<ul style="list-style-type: none"> - centrifuge detection reagents before use to remove particulates - increase number and duration of post-detection washes - decrease the concentration of the fluor-conjugate in detection solution - increase the concentration of BSA in detection solution - ensure that antibody host-specific serum is used in detection solution - do not allow slides to dry out during detection protocol - do not touch microarray directly or forcibly remove coverslip at any time
Excess sample applied to microarray	<ul style="list-style-type: none"> - quantify the amount of labeled sample and use less in hybridization
Insufficient blocking of the microarray	<ul style="list-style-type: none"> - perform a pre-hybridization blocking step
Labeled samples not efficiently purified	<ul style="list-style-type: none"> - repeat purification - use alternate purification strategy
Suboptimal blocker/suppressor DNA used in the hybridization	<ul style="list-style-type: none"> - add more or alternate blocker and/or suppressor DNA to hybridization buffer
Ink or marker used to identify microarray	<ul style="list-style-type: none"> - avoid using markers or stickers to identify slide; use a diamond scribe pen
Low stringency hybridization or wash conditions	<ul style="list-style-type: none"> - increase hybridization temperature - increase stringency of post-hybridization washes by decreasing salt concentration and/or increasing temperature
Salt from wash buffer remaining on microarray	<ul style="list-style-type: none"> - dip rapidly in water before drying slide
Poor quality microarray	<ul style="list-style-type: none"> - optimize microarray production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted microarrays
Punctate background	<ul style="list-style-type: none"> - assure the labeled sample has been denatured and spun as indicated in Section 6.3

10.0 APPENDIX
Stock Solutions
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	

20X SSPE

3 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.4	
NaCl	175.3 g
NaH ₂ PO ₄ ·H ₂ O	27.6 g
EDTA	7.4 g
Water	800 ml
Mix well and adjust pH to 7.4 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	

12X MES Stock

1.22 M MES, 0.89 M [Na+]	
MES-free acid monohydrate	70.4 g
MES sodium salt	193.3 g
Water	800 ml
Mix well and adjust volume to 1000 ml. Ensure pH is between 6.5 and 6.7. Filter through a 0.2 µm filter.	
Total Volume	1000 ml
Store at 4°C; protect from light	

Hybridization Buffer (cDNA Microarrays)

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 -20°C	

Post-Hybridization Buffers (cDNA Microarrays)

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, 0.1% SDS	
20X SSC	5 ml
10% SDS	10 ml
Water	985 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	

Hybridization Buffer (Oligo Microarrays)

100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20	
12X MES Stock	83 µl
5 M NaCl	177 µl
0.5 M EDTA	40 µl
10% Tween 20	1 µl
Water	699 µl
Total Volume	1000 µl
Store at 4°C; protect from light	

Post-Hybridization Buffers (Oligo Microarrays)

6X SSPE, 0.01% Tween 20	
20X SSPE	300 ml
10% Tween 20	1 ml
Water	699 ml
Total Volume	1000 ml
Store at RT	

100 mM MES, 0.1 M [Na+], 0.05% Tween 20	
12X MES Stock	83.3 ml
5M NaCl	5.2 ml
10% Tween 20	1 ml
Water	910.5 ml
Total Volume	1000 ml
Store at 4°C; protect from light	

Wash and Detection Solutions

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

Detection Solution	
6X SSPE-T	170.7 µl
BSA (50 µg/µl)	8 µl
mouse serum	20 µl
Cy ⁵ -conjugated anti-biotin* (0.7 µg/µl)	1.4 µl (spin before use)
Cy ³ -conjugated anti-fluorescein mAb* (0.6 µg/µl)	1.7 µl (spin before use)
Total Volume	200 µl
Use 200 µl per slide; make fresh immediately before use.	

***Dilute antibodies 10 fold in 6xSSPE-T before preparing Detection Solution.**

10.2 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>

10.3 Related Products

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

Label IT® μ Array™ Cy³/Cy⁵ Labeling Kit (MIR, MIR 8205, MIR 8225)

This product is sold to the Buyer with a limited license to use this product for research only. This product, or parts from this product, may not be re-packaged or re-sold without written permission from Mirus Bio Corporation.

Cy3 and Cy5 are trademarks of GE Corp.

μ Array is a trademark of Mirus Bio Corporation. *Label IT* is a registered trademark of Mirus Bio Corporation.

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

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