

## Direct RNA Labeling for Microarray Profiling using the *LabelIT*<sup>®</sup> $\mu$ Array<sup>™</sup> Dual Labeling Kit

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### Introduction

Microarrays represent an established genomics technology that allows thousands of genes to be simultaneously hybridized and quantified in a single experiment. Gene expression profiling, a prominent microarray application, measures the presence and relative amount of specific RNA transcripts from the fluorescent signal generated by the hybridization of a complex labeled sample to a microarray. Traditional labeling methodologies employ enzymatic incorporation of modified nucleotides, which requires replication of the sample and has associated enzymatic replication and incorporation biases. Mirus Bio Corporation offers an alternative chemical labeling method with the *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit, optimized for direct labeling of any nucleic acid sample for microarray analysis.

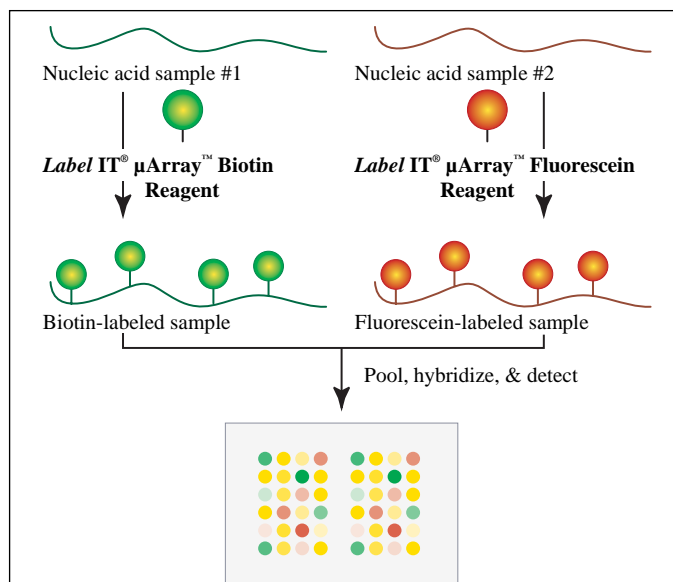
Mirus Bio's *LabelIT*<sup>®</sup> technology is designed to covalently attach marker molecules to nucleic acids in a simple one-step chemical reaction, allowing sensitive and precise detection of labeled samples. *LabelIT*<sup>®</sup> Reagents directly label RNA bases, and the labels do not impact hybridization performance. Since *LabelIT*<sup>®</sup> Reagents allow the

direct chemical labeling of nucleic acids, modified nucleotides and enzymatic incorporation biases are eliminated from the labeling process. The ability to simply, reproducibly, and uniformly label both RNA and DNA samples with a variety of detectable markers makes *LabelIT*<sup>®</sup> Reagents ideal for hybridization applications.

The *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit is designed for dual-channel microarray applications (Figure 1) and includes *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Reagents for labeling samples with either biotin or fluorescein. The use of biotin and fluorescein as marker molecules, combined with a post-hybridization detection step that introduces fluorophores (such as Cy<sup>™</sup>3 and Cy<sup>™</sup>5), provides a robust and flexible detection system. Here, we demonstrate the *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit in a variety of microarray applications.

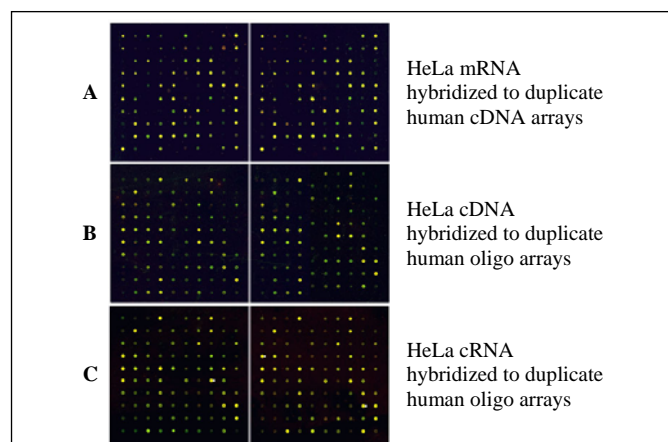
### Results and Discussion

The versatility of the *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit allows any nucleic acid sample to be labeled in a single step for microarray analysis. Traditional expression profiling applications require enzymatic replication of an RNA sample (to either cDNA or cRNA) in the presence of labeled nucleotides, followed by hybridization to a microarray. Since *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Reagents allow the direct chemical modification of nucleic acids, the original mRNA sample can be labeled and hybridized to a microarray (Figure 2A), as well as cDNA and cRNA derivatives (Figure 2B, C).



**Figure 1. Overview of the *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit**

The *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit offers a simple, versatile chemical labeling procedure for microarray applications. Any nucleic acid sample (RNA or DNA) can be directly labeled in one hour with the *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Biotin or Fluorescein Reagents, resulting in permanent and uniform labeling of the original samples. The resulting labeled samples are then pooled, purified, and hybridized to a microarray. A post-hybridization detection step using antibody- or streptavidin-conjugated Cy<sup>™</sup>3 and Cy<sup>™</sup>5 introduces fluorophores for signal generation.



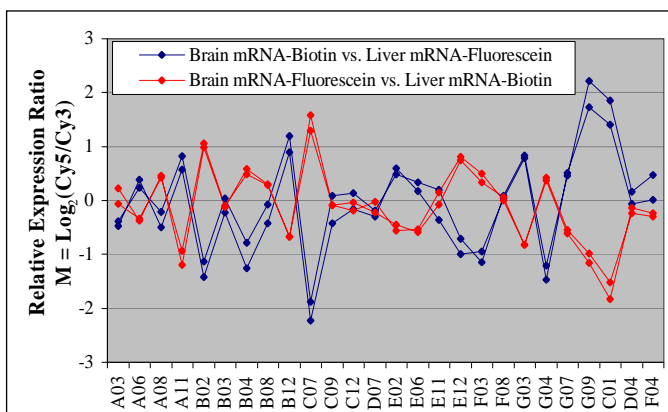
**Figure 2. Direct Labeling of a Variety of Nucleic Acid Samples**

The *LabelIT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit was used to label mRNA directly for hybridization to double-stranded cDNA arrays (A), or cDNA and cRNA samples for hybridization to sense-strand oligo arrays (B, C). For each microarray hybridization, a single sample was divided, labeled independently with biotin and fluorescein, and then pooled; yellow represents the overlap of the green (Cy3) and red (Cy5) channels. Labeled samples were detected with a streptavidin-Cy<sup>™</sup>3 conjugate and an anti-fluorescein antibody-Cy<sup>™</sup>5 conjugate on each duplicate array.

The primary advantage of the *Label IT*<sup>®</sup> direct labeling technology is the ability to covalently attach labels to mRNA, eliminating the requirement for a reverse transcription reaction to make cDNA. Traditional enzymatic labeling methods have inherent biases introduced by the properties of replication and incorporation of modified nucleotides.<sup>1</sup> Direct labeling of biological samples reduces handling steps and allows the original material to be used directly in microarray hybridization and analysis. Enzymatic biases such as the 3' bias associated with oligo(dT) priming, sequence-specific differences in replication, and differential dye incorporation are all eliminated with direct mRNA labeling.

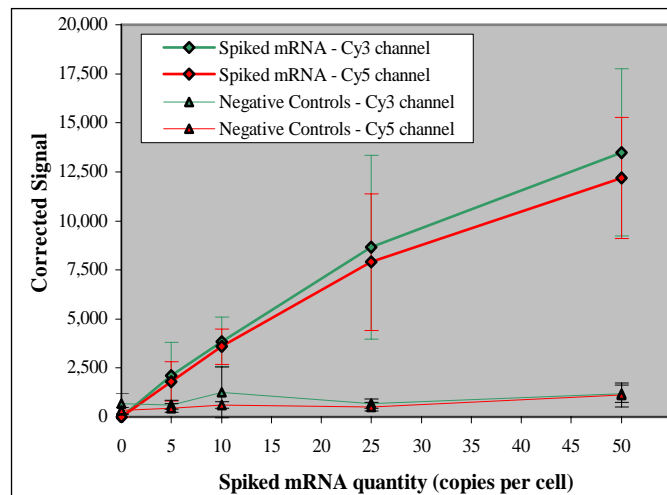
The sensitivity of direct labeling with the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit to detect small changes in gene expression levels is demonstrated by a control experiment in which two mRNA samples are each labeled for analysis in both channels, then pooled appropriately for a "label-swap" experiment (Figure 3). In this experiment, the expectation is a sign change (e.g. from negative to positive, or vice versa) in the average  $\log_2(\text{Cy5}/\text{Cy3})$  ratio determined for every gene represented on the microarray, representing the "swap" between channels for the labeled samples. The *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> technology also allows sensitive detection of low copy number transcripts, lower than 10 copies per cell (Figure 4). Overall, the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit generates low background and high signal-to-noise ratios in microarray hybridizations with the added benefit of direct mRNA labeling and hybridization.

The *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit includes two different *Label IT*<sup>®</sup> reagents for dual channel microarray experiments. The use of biotin and fluorescein as labeling markers, followed by a post-hybridization detection step that introduces the fluorophores for scanner detection, creates a

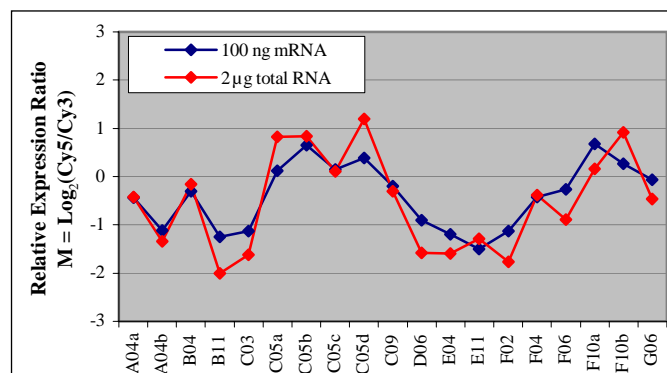


**Figure 3. Direct mRNA Labeling for Gene Expression Analysis**  
Brain and liver mRNA samples were directly labeled for analysis in each channel and pooled appropriately for a "label-swap" experiment. Equivalent gene expression ratios were determined for every gene from both analyses indicating the reproducibility of gene expression results and the absence of label incorporation bias. In this experiment, the average  $\log_2(\text{Cy5}/\text{Cy3})$  signal ratio for each gene is expected to change sign (e.g. from +1 to -1) when samples are labeled for detection in the opposite channel. Each microarray hybridization was conducted in duplicate; a streptavidin-Cy<sup>™</sup>3 conjugate and an anti-fluorescein antibody-Cy<sup>™</sup>5 conjugate were used for detection.

robust system that is less sensitive to variations in labeling density and is inherently less prone to photobleaching and quenching. A variety of commercially available detection reagents, including Cy<sup>™</sup> dye-conjugated antibodies and strept-avidin as well as signal amplification methods such as the TSA system (Perkin Elmer), are compatible with the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit. The option to amplify the hybridization signal allows small amounts of labeled mRNA, much less than 1  $\mu\text{g}$  per channel, to be analyzed in a microarray hybridization. With the TSA detection system, a total RNA sample with equivalent copy number of mRNA sequences can also be labeled and hybridized directly, allowing the mRNA isolation step to be eliminated (Figure 5).

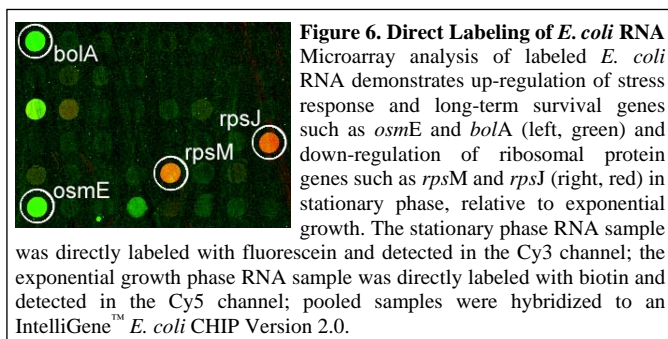


**Figure 4. Sensitivity of mRNA Detection**  
Sensitivity of detection with the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit was determined from "spike-in" experiments in which a specific quantity of an *Arabidopsis* mRNA sample was added to HeLa mRNA before labeling. An estimated mRNA copy number of ~10 copies per cell<sup>2</sup> (30 pg of the spiked mRNA in 1  $\mu\text{g}$  HeLa mRNA) can be detected in each channel above non-specific hybridization signal (negative controls). Fluorescent signal detected for the spiked mRNA and negative controls is corrected by subtracting the median local background value; error bars represent one standard deviation.



**Figure 5. Detection of Labeled RNA Samples with Signal Amplification**  
Equivalent copy number samples of mRNA (100 ng per channel) and total RNA (2  $\mu\text{g}$  per channel) derived from the same original source of A549 total RNA were directly labeled, hybridized, and detected with the TSA amplification system from Perkin Elmer. The average  $\log_2(\text{Cy5}/\text{Cy3})$  signal ratio is indicated for genes with detectable signal (at least 3 of 6 replicate features detected in each channel) on each array.

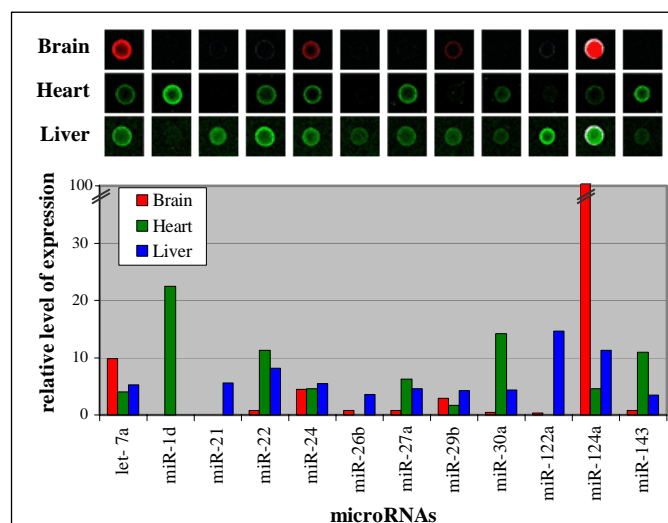
In addition to standard eukaryotic samples labeled for analysis of gene expression, the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit will efficiently label alternative samples for microarray analysis. For example, bacterial RNA samples can be directly labeled for hybridization, allowing analysis of gene expression or microbial detection/identification and diagnostics. Hybridization of directly labeled stationary phase and exponential growth phase *E. coli* RNA samples generates comparable results to published reports using standard enzymatic labeling methods.<sup>2,3</sup> Genes upregulated in stationary phase included stress response and long-term survival genes; down-regulated genes included ribosomal protein genes and other genes associated with cell growth, such as cell membrane synthesis genes (Figure 6 and data not shown).



Small RNAs represent another alternative sample type that, because of their short length, are difficult to label by enzymatic incorporation of modified nucleotides but easy to directly label with *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> reagents. A novel class of small RNAs implicated in RNA interference phenomena, namely microRNAs and related siRNAs (~22 nucleotides), can be labeled for microarray analysis with the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit (Figure 7). The quantification of microRNA expression in several mouse tissues matches literature reports of the prevalence of different microRNA sequences in each tissue (including high expression of miR-124 in brain, miR-1d in heart, and miR-122a in liver).<sup>4</sup> The *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit, and its ability to directly label small RNA samples, is the ideal alternative to cumbersome enzymatic labeling protocols.

## Summary

Ideal requirements for labeled microarray samples include minimal introduction of bias, sensitivity to small changes in gene expression levels, and detection of low copy number transcripts. In addition to all of these benefits, the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit provides a one-step direct labeling technology that is simple to use. For example, *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Reagents can be used to label mRNA directly for gene expression analysis, eliminating enzymatic steps, biases, and costs from the labeling process. Alternatively, these reagents can directly label cDNA and cRNA for standard oligo microarrays as well as alternate sample types for specialized applications (bacterial RNA, microRNA,



**Figure 7. Direct Labeling of microRNA: Tissue Specific Expression**

Expression profiles of microRNAs were determined from microarray hybridizations using mouse RNA samples enriched for small RNAs that were labeled with the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit. Top: 250 ng (heart) or 1  $\mu$ g (brain, liver) “small RNA” samples spiked with 250 pg control oligo were directly labeled with biotin (heart, liver) or fluorescein (brain), hybridized to an oligo microarray containing microRNA capture sequences, and detected with a Streptavidin-Cy<sup>™</sup>3 conjugate (heart, liver) or an anti-fluorescein antibody-Cy<sup>™</sup>5 conjugate (brain). Bottom: To quantify microRNA expression levels, average corrected signals for each microRNA were scaled to the control oligo for each array and the relative level of expression of miR-124a in brain was defined as 100.

ribosomal RNA, and genomic DNA). The labeling and detection scheme, using biotin and fluorescein as labeling markers and a post-hybridization detection step to introduce fluorophores such as Cy<sup>™</sup>3 and Cy<sup>™</sup>5, is robust and provides an option for signal amplification as needed.

In summary, features and benefits of the *Label IT*<sup>®</sup>  $\mu$ Array<sup>™</sup> Dual Labeling Kit include:

- One-step covalent labeling chemistry
- Direct labeling of any nucleic acid, including mRNA, for hybridization
- Elimination of enzymatic replication and incorporation biases
- Sensitive detection of low copy number transcripts and small changes in gene expression
- Easy integration into existing microarray platforms
- Optional post-hybridization signal amplification

## Methods

### Microarrays

All microarrays were printed at Mirus Bio Corporation, unless indicated otherwise, using the QArray<sup>lite</sup> robotic arrayer (Genetix Ltd., www.genetix.co.uk) and standard protocols; each array contained six replicate features per gene. Human cDNA microarrays consisting of a panel of human housekeeping gene cDNA I.M.A.G.E. clones (Research Genetics/Invitrogen Life Technologies, www.invitrogen.com) were printed on Takara Slide Glass (Takara Mirus Bio Inc.,

www.takaramirusbio.com). Human sense-strand oligo microarrays (oligo test sets, MWG Biotech, www.mwgbio.com) were printed on Takara Hubble Glass slides (Takara Mirus Bio). The bacterial mRNA expression profiling analysis used an IntelliGene™ *E. coli* CHIP Version 2.0 (Takara Mirus Bio), arrayed with DNA fragments covering 94% of total ORFs. The microRNA microarray oligos were designed in-house.

#### Sample Preparation

Total RNA was extracted from near-confluent tissue culture cells using TriReagent (Molecular Research Center Inc., www.mrcgene.com), followed by RNeasy column purification (Qiagen Inc., www.qiagen.com). For mRNA labeling, poly(A) RNA (mRNA) was extracted from total RNA using the Poly(A)Purist™ mRNA Purification Kit (Ambion Inc., www.ambion.com). For cDNA labeling, total RNA was used to synthesize first-strand cDNA using an oligo dT primer and SuperScript™ II or III RNaseH<sup>-</sup> reverse transcriptase (Invitrogen). The RNA template was hydrolyzed and the cDNA purified prior to biotin or fluorescein labeling (as directed in the Label IT® μArray™ Dual Labeling Kit, protocol #ML032). Similarly, cRNA was prepared and labeled as per the kit's instructions. In the 'dye swap' experiments, FirstChoice™ human brain and liver poly(A) RNA samples (Ambion) were directly labeled with biotin or fluorescein and then pooled appropriately.

For the sensitivity of detection experiments, specific amounts of an exogenous *Arabidopsis thaliana* mRNA sequence from the SpotReport™ Array Validation System (Stratagene, www.stratagene.com) were spiked into HeLa mRNA samples before labeling. The spike-in amounts were selected using a transcript copy number per cell calculation.<sup>5</sup>

For bacterial mRNA profiling, total RNA was isolated from *E. coli* DH10b cultures at both exponential growth and stationary phases using the RNAprotect™ Reagent and the RNeasy Kit (Qiagen). *E. coli* mRNA was isolated using the MICROExpress™ Kit (Ambion) and labeled according to the mRNA labeling section of the protocol.

For microRNA expression analysis, "small RNA" samples (enriched for microRNAs) were prepared from mouse organs using the MirVana™ miRNA Isolation Kit (Ambion) and labeled according to the mRNA labeling section of the protocol.

#### Hybridization and Dual Detection

Microarray hybridizations were performed as recommended in the kit protocol for the specific array type used (cDNA or oligo), except that post-hybridization washes for the microRNA microarrays were performed at 40°C instead of 50°C. Biotin/fluorescein detection steps were performed as recommended in the kit protocol; specifically, both channels were detected simultaneously when using standard biotin and fluorescein detection reagents such as Cy<sup>TM</sup>3 or Cy<sup>TM</sup>5 conjugated to streptavidin or to anti-biotin or anti-fluorescein

antibodies (Jackson ImmunoResearch Laboratories Inc., www.jacksonimmuno.com). TSA detection of RNA samples labeled with the Label IT® μArray™ Dual Labeling Kit used the detection reagents and protocol from the TSA system (Perkin Elmer, www.perkinelmer.com). All scans were acquired using the GenePix® 4000B microarray scanner (Axon Instruments Inc., www.axon.com). Data analysis was performed using GenePix® Pro Microarray Image Analysis (Axon) and Microsoft Excel software.

#### References

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Mirus Bio's Label IT® Technologies are covered by U.S. Patent Numbers 6,262,252 and 6,593,465.

#### Ordering Information:

Label IT® μArray™ Dual Labeling Kit  
with Biotin and Fluorescein labeling reagents

Product #	Quantity
MIR 8105	2 x 5 reactions*
MIR 8125	2 x 25 reactions*

Detection reagents are not included.

#### Related Products:

Label IT® μArray™ Biotin Labeling Kit

Product #	Quantity
MIR 8010	10 reactions*
MIR 8050	50 reactions*

Detection reagents are not included.

Label IT® μArray™ Cy<sup>TM</sup>3/Cy<sup>TM</sup>5 Labeling Kit

Product #	Quantity
MIR 8205	2 x 5 reactions*
MIR 8225	2 x 25 reactions*

\*Each reaction labels 1 μg of nucleic acid.

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