Application Forum

MicroRNA Labeling Methods Directly Influence the Accuracy of Expression Profiling Detection

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MicroRNA (miRNA) expression profiling is a powerful and commonly used methodology for assessing the relative levels of individual miRNAs in specific tissues. To achieve accurate results using miRNA microarray detection strategies, it is important that all sample miRNA species are representatively labeled prior to the hybridization step. In this study, we compared the expression profiles of miRNAs labeled using enzymatic methods and a direct chemical labeling method. Using miRNA samples isolated from mouse brain and heart in a comparative microarray analysis, we show that some specific miRNAs are not detected when enzymatic tailing methods are used; however they are detected with the Label IT® *chemical labeling method. The presence of the discrepant miRNAs in the model mouse tissues was corroborated by northern blot analysis and confirmed by qRT-PCR. Thus, miRNA expression profiles generated by enzymatic labeling methods (poly(A) polymerase tailing) may not be representative for all miRNAs that are present in a given sample. The* Label IT *technology may offer a more sensitive and universal miRNA labeling method.*

INTRODUCTION

MicroRNAs (miRNAs) are phylogenetically conserved, small non-coding RNAs (~22 nucleotides) characterized by distinct expression and biogenesis criteria [1]. MicroRNAs modulate the expression of genes through post-transcriptional effects on target mRNA stability and translational efficiency in both plants and animals. Microarray expression profiling can be used to determine if specific miRNAs are present in different cell types, stages of development, and disease states [2]. To generate accurate and reproducible expression profiles using microarrays, it is critical that the labeling method effectively label all miRNA species in the sample regardless of specific sequence or structure.

Due to the small size of miRNAs and the lack of a common sequence tag (e.g. a poly(A) tail), specialized labeling methods are required to achieve consistent and representative labeling. To date, the prominent commercially available miRNA labeling methods are enzyme-based and involve the addition of nucleotides to the 3′ end of purified miRNAs. *E. coli* poly(A) polymerase I is used to catalyze the addition of approximately 20 – 50 nucleotides to the miRNA resulting in a total length between 40 and 70 nucleotides. Next, the extended miRNA is purified and fluorescent labels are attached through a direct chemical linkage to enzyme incorporated modified nucleotides or, is hybridized to a tag sequence that is linked to fluorophores. In contrast, the *Label* IT chemical labeling method facilitates the covalent attachment of fluorescent labels directly onto nucleic acids without an enzyme dependent step.

The goal of this study was to determine if miRNA expression profiles (using microarrays) are affected by the type of miRNA labeling method used. Three different miRNA labeling methods were directly compared for their ability to detect differentially expressed miRNAs from two different tissue sources (mouse brain and mouse heart). To validate the miRNA expression in these tissues, quantitative RT-PCR and published northern blot data were used to confirm and corroborate the miRNA profiles.

MATERIALS AND METHODS

miRNA-enriched RNA isolation

MicroRNA-enriched samples were prepared from heart and brain tissue from adult ICR mice (Harlan Laboratories, Indianapolis, IN, USA) using the *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX, USA).

miRNA labeling and hybridization

MicroRNA-enriched samples were chemically labeled via alkylation (*Label* IT miRNA Labeling Kit, Mirus Bio Corporation, Madison, WI, USA) or enzymatically labeled with the *mir*Vana miRNA Labeling Kit (Ambion) or the NCode miRNA Labeling System (Invitrogen, Carlsbad, CA, USA). Labeled miRNAs were then hybridized to NCode™ Multi-Species miRNA microarray slides (Invitrogen). Cy™3 and Cy™5 fluorophores were detected when the *Label* IT chemical or *mir*Vana enzymatic method was used. Alexa Fluor® 3 and Alexa Fluor® 5 were detected when the NCode enzymatic labeling system was used. To increase the stringency of our data analysis, a "dye swap" setup was used for each hybridization experiment performed. Each brain and heart miRNA-enriched sample was labeled for detection with

each of the provided fluorophores before determining relative expression (e.g. heart with Cy5, brain with Cy3, and vice versa).

Sequences of RNA oligonucleotides used for spike-in and labeling experiments were determined using miRBase Sequence Database (http://microrna.sanger.ac.uk/sequences) [3-5].

The labeling density (pmol fluorophore per µg RNA) generated by the *Label* IT labeling method was estimated using spectrophotometric (Beckman DU 530, Beckman Coulter, Fullerton, CA, USA) measurements and the molar extinction coefficient (ε), 150,000 M^{-1} cm^{-1}, for the Cy3 fluorophore. The λ_{max} for Cy3 was 550 nm. RNA molar concentration was determined using absorbance at 260 nm and the calculated molecular weight and extinction coefficient for each RNA oligonucleotide.

Image analysis and data processing

Microarray data was obtained using the Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) for image acquisition and associated GenePix Pro 5.0 software for signal measurement. Photomultiplier tube measurements (PMTs), or laser intensities, for each hybridization slide were adjusted so that the highest expressing miRNAs (brightest features) exhibited measurements at the top of the signal linear range. Each of the data sets used was filtered using signal-to-noise criteria and the demonstration of differential expression for the given miRNA based on $\log_2$ calculations of heart:brain signal ratios. Quality data was defined to have signal greater than the average signal of the array negative controls in the relevant channel plus 1.5 standard deviations of the negative control signal. Measurements below this signal cutoff were recorded as "Not Detected". Signal measurements from either channel for brain or heart labeled samples were averaged before calculating heart/brain ratios and $\log_2$ transformation for each miRNA. Differentially expressed miRNAs were required to have a calculated $\log_2$(heart signal/brain signal) greater than 1 or less than –1 to represent greater than two fold differential expression between the two tissues. To fulfill the "dye swap" requirement, log transformed expression ratios were required to switch sign (e.g. from 3 to –3) when samples were detected using the opposite pairing, ensuring consistent differential expression regardless of which dye or method was used to label the samples.

RESULTS AND DISCUSSION

MicroRNA-enriched brain and heart samples were labeled by chemical or enzymatic methods. The *Label* IT direct alkylation chemistry is based on an aromatic nitrogen mustard reactive group. This chemistry facilitates the direct covalent attachment of Cy3 or Cy5 labels onto miRNAs [6].

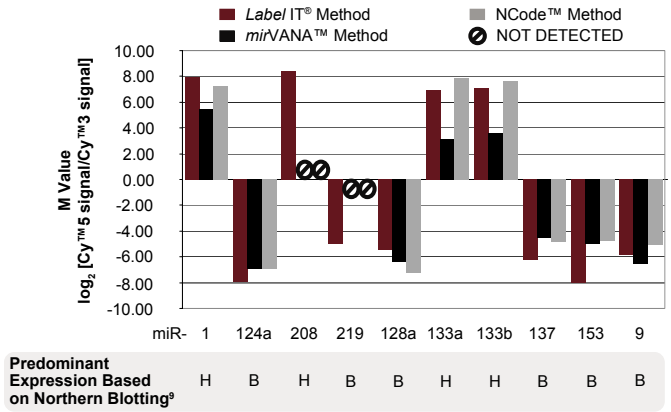
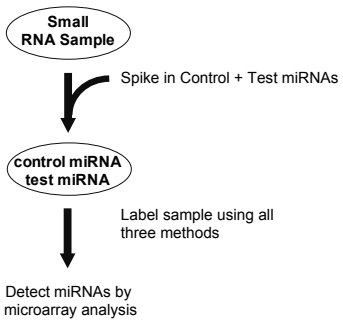


Figure 1. Discrepant microarray miRNA profiles obtained from chemical and enzymatic labeling methods. miRNA-enriched mouse heart and brain samples were hybridized to miRNA microarrays after labeling via *Label* IT alkylation (n=14), mirVana (n=3) or NCode (n=2) enzymatic methods. Positive relative expression values expressed as the $\log_2$ transformed ratio of heart/brain signal represent mouse miRNAs differentially expressed in heart tissue, while negative values correspond to miRNAs differentially expressed in brain. The tissue specificity of each miRNA, as determined by northern blot analysis [9], is also presented.

Both of the enzymatic methods used in this investigation are based on 3′ tailing of small RNAs using *E.coli* poly(A) polymerase I. The *mir*Vana enzymatic method uses the polymerase and a mixture of unmodified and amine-modified nucleotides to append a 20-50 polyuracil tail to the 3′ end of the miRNAs [7]. The 3′ extended miRNAs are then purified and chemically coupled with NHS ester-activated Cy3 or Cy5 labels. After purification, the labeled sample is ready to be hybridized to the microarray. The NCode enzymatic method uses poly(A) polymerase and adenosine triphosphate (ATP) to add a similar length of adenines to the 3′ end of small RNA samples [8]. The polyadenylated miRNAs are then sequence tagged by ligation to two prehybridized oligonucleotides – a capture sequence tag and the corresponding bridging oligonucleotide. After ligation, the sequence-tagged sample is purified and hybridized to the miRNA-specific microarray. The hybridized miRNAs are then detected by a second hybridization step where the array is incubated with Alexa Fluor labeled dendrimers containing the capture sequence specific for the immobilized sequence-tagged miRNAs.

To compare the effects of different labeling methods on miRNA expression profiling signatures, miRNA-enriched brain and heart samples were labeled by chemical or enzymatic methods and then hybridized to miRNA microarrays (NCode arrays). Significant differences in miRNA expression profiles were observed using the different miRNA labeling methods to label the same miRNA-enriched samples. Ten miRNAs previously shown by northern analyses to be differentially expressed in these tissues were identified [9] and their relative abundance was determined in microarray profiling experiments (Figure 1). Eight of the ten miRNAs exhibited the expected tissue specific expression pattern [9]. However, two of the ten miRNAs (miR-208 and miR-219) were only detected with the *Label* IT labeling

A.



B.

	Brain Small RNA Sample		Heart Small RNA Sample	
	Control miRNA miR-1	Test miRNA miR-208	Control miRNA miR-124a	Test miRNA miR-219
Label IT® Method	Detected	Detected	Detected	Detected
*mir*Vana™ Method	Detected	NOT DETECTED	Detected	NOT DETECTED
NCode™ Method	Detected	NOT DETECTED	Detected	NOT DETECTED

Figure 2. Specific miRNAs are not detected by enzymatic labeling methods. Synthetic RNA oligonucleotides representing control and test miRNAs were spiked into miRNA-enriched samples before chemical or enzymatic labeling and hybridization (Panel A). Control spike-in sequences represented miRNAs that were consistently detected on microarrays by all three labeling methods used. Test spike-in sequences represented the miRNAs that exhibited different profiles depending on the labeling method used. In each case, 10 fmol of each RNA oligonucleotide was spiked into the sample that did not endogenously contain the specific miRNA. Accordingly, miR-219 (test) and miR-124a (control) were each spiked into heart RNA, and miR-208 (test) and miR-1 (control) were each spiked into brain RNA. Hybridization results are presented in Table B.

method. A hybridization signal was not detected for either miR-208 or miR-219 in any of the replicates in either of the enzymatic labeling methods tested.

To further investigate whether the sequences of miR-208 and miR-219 could be detected following enzymatic labeling, we implemented a spike-in experiment using synthetic RNA oligonucleotides (Figure 2A). Prior to labeling, RNA oligonucleotides representing miRNAs that were known not to be present in that specific tissue were added into the miRNA-enriched samples. Two different spike-in miRNA sequences were used – control sequences represented miRNAs that were consistently detected by all three labeling methods, while test sequences represented the miRNAs that exhibited different profiles depending on the labeling method used. Heart specific miR-208 (test) and miR-1 (control) were each spiked into brain miRNA-enriched small RNA, and brain specific miR-219 (test) and miR-124a (control) were each added to the heart sample, each at a 10 fmol level. This spike-in amount is readily detectable by microarray analysis using each of the labeling methods (data not shown). Each spiked sample was labeled using the three different methods. Consistent with the original profile spiked miR-208 and miR-219 were not detectable using either of the poly(A) polymerase-based enzymatic labeling methods (Figure 2B). The enzymatic methods did detect the control spike-in miRNA sequences (miR-1 and miR-124a) in all samples, indicating the inability to detect miR-208 and miR-219 with enzymatic methods was not due to overall poor labeling of the sample. As previously observed, the *Label* IT chemical labeling method detected the miR-208 and miR-219 test spike-in sequences. Also consistent with these findings, a recent study [7, supplemental data], using microarrays and the *mir*Vana labeling method to survey miRNA expression patterns across 26 different human tissues, miR-208 and miR-219 were not identified as heart and brain miRNAs, respectively. Furthermore, to independently validate the tissue specific expression profiles of the different miRNAs, qRT- PCR analysis was performed using the same miRNA enriched brain and heart samples. The qRT-PCR results (data not shown) corroborate the published northern data, which indicate that miR-1 and miR-208 are expressed in heart tissue, and miR-124a and miR-219 are expressed in brain tissue [9].

The *Label* IT reagent (Figure 3) contains an alkylating reactive group coupled with strong nucleic acid binding capability facilitated via electrostatic interaction. With this reagent, direct covalent modification of the RNA takes place, during a one hour incubation at 37°C, on any reactive heteroatom in the polynucleotide. Preferred heteroatoms are predicted to be N^7 of guanine, N^3 of adenine and N^3 of cytosine. To assess the efficiency and specificity of labeling, the *Label* IT labeling method was tested using synthetic RNA oligonucleotides representing four mammalian miRNA sequences each lacking a specific nucleotide. The labeling density for each oligonucleotide was estimated by measuring the spectrophotometric absorbances of the nucleic acid and fluorophore at the appropriate wavelengths. Consistent labeling was observed with each of the four miRNA sequences indicating that the alkylation reaction was neither dependent on, nor preferential for a specific nucleotide (Figure 4). Furthermore, to assess the universality of the *Label* IT technology, a wide panel of synthetic RNA oligonucleotides representing different miRNA sequences have all been comparably labeled by the *Label* IT reagents, and appropriately detected after microarray hybridization experiments (data not shown).

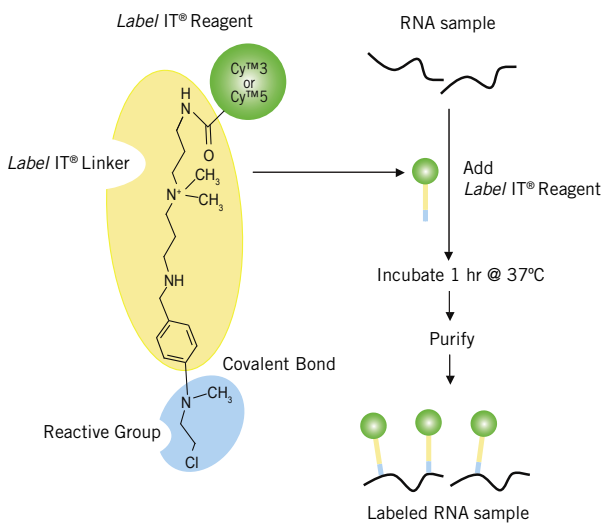


Figure 3. Chemical structure of the chemical labeling reagent. The *Label* IT labeling reagent consists of 3 parts: the label or fluorophore (for example, Cy3 or Cy5), the positively charged linker, and the reactive (alkylating) moiety.

Since both enzymatic methods involve the step of nucleotide addition using *E.coli* poly(A) polymerase I, it is possible that this step is the cause of the lack of detection of certain miRNA species. While the exact cause of the enzymatic detection discrepancy is not known, selective bias of incorporation by enzymes is not unprecedented and it has previously been shown that fluorophore-conjugated nucleotides are unevenly incorporated during labeling [10]. The structure of the miRNA species, particularly under the *in vitro* conditions of the tailing reaction, may affect poly(A) polymerase binding affinity; it has been reported that RNA molecules terminating with a stem-loop structure are poor substrates [11]. As another example of the importance of miRNA structure, it has recently been reported [12] that the *mir*Vana kit cannot be used to label plant miRNA for expression profiling applications since endogenous plant miRNAs are methylated at the 3′ end [13] (Figure 5). With *in vitro* assays, poly(A) polymerase has been demonstrated to require a short single-stranded overhang of at least 2 nucleotides [11]. Because miRNAs may have such stem-loop structures and may not have this overhang, miRNAs may not be optimum substrates for poly(A) polymerase-based labeling systems. Conversely, the bulk of the appended tails (Figure 5) may impact the hybridization performance of particular miRNAs. Further investigation is required to discern whether the enzymatic labeling, hybridization or detection steps are compromised with specific miRNA species.

Expression profiling is a powerful technique that allows a researcher to obtain a global snapshot of the expression patterns of all known miRNAs in a given tissue at a particular point in time. MicroRNA expression profiling may play an important role in plant, animal and human disease diagnostics and therapeutics in the future. To be of value however, it is imperative that consistent and representative detection of all miRNAs is achieved. The consistent inability to detect certain miRNA candidates is a serious impediment for the use of enzymatic labeling methods, especially if those miRNAs play important roles in plant or animal biology. miR-219, for instance, has been associated with ovarian and breast cancer [14]. We demonstrate that the method of labeling miRNAs for microarray analysis can have a significant effect on the expression profile outcome. Therefore, care must be taken in choosing an appropriate miRNA labeling system.

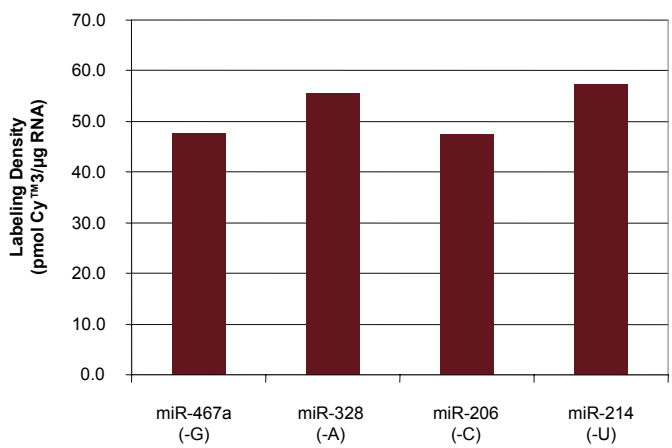


Figure 4. Chemical labeling of selected miRNA sequences. Synthetic RNA oligonucleotides representing miRNAs with no Gs (miR-467a), no As (miR-328), no Us (miR-214), and no Cs (miR-206) were chemically labeled in triplicate with Cy3, purified and spectophotometrically measured to estimate labeling density (pmol Cy3 / µg RNA). Average labeling densities are plotted. Similar results were observed with Cy5 labeling reactions (data not shown).

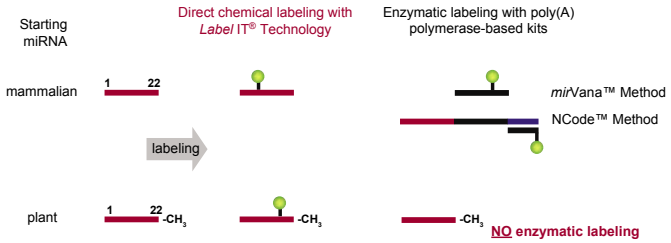


Figure 5. Internal chemical labeling of any miRNA species. Mammalian miRNAs can be labeled using direct chemical labeling or enzymatic methods. The poly(A) polymerase-based methods generate long 3′ tails which dramatically extend the length of the miRNA species. The *Label* IT reagents covalently modify plant and animal miRNAs at internal sites. Conversely, plant miRNAs, due to their endogenous 3′ methylation, cannot be efficiently labeled using poly(A) polymerase-based methods.

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