*mir*Vana™ miRNA Labeling Kit

(Part Number AM1562)

Instruction Manual

Manual 1562M Revision C **Revision Date: February 5, 2008**

For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *mir*Vana™ miRNA Labeling Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this instruction manual, along with a copy of the paper.

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I. Introduction

A. Description of the Ambion® *mir*Vana™ miRNA Labeling Kit

The *mir*Vana™ miRNA Labeling Kit (patent pending) is designed to prepare microRNA (miRNA) samples for microarray analysis using a simple and effective end-labeling strategy (see Figure 1 on page 3). Start with total RNA that contains the miRNA fraction. Ambion offers "miRNA certified" FirstChoice® prepared Total RNA and kits for isolating RNA that includes small RNAs: the *mir*Vana miRNA Isolation Kit (P/N AM1560) and the *mir*Vana PARIS™ Kit (P/N AM1556). If the experimental design requires small RNA enrichment, obtain the miRNA fraction using the Ambion flashPAGE™ Fractionator and flashPAGE Reaction Clean-Up Kit (P/N AM13100, AM12200). Alternatively, miRNA can be obtained using traditional PAGE.

NOTE

Plant miRNAs cannot be labeled with this kit due to endogenous 3' methylation. For more information, see Yu B, et al. (2005) Methylation as a crucial step in plant microRNA biogenesis. Science. 307:932–5.

In the first step of the *mir*Vana miRNA Labeling Kit procedure, *E. coli* Poly(A) Polymerase and a mixture of unmodified and amine-modified nucleotides are used to add a 20–50 nucleotide tail to the 3' end of each miRNA (and other RNA) in the sample. The amine-modified miRNAs are then cleaned up and coupled to amine-reactive labeled moieties such as NHS-ester CyDye™ fluors (Amersham Biosciences) to produce a fluorescently-labeled sample for microarray analysis. An important benefit of the tailing strategy is that each miRNA obtains multiple fluorescently-labeled molecules. Since most miRNAs comprise less than 10^{-6} of a total RNA sample, this high degree of labeling is very important for obtaining sensitive and accurate miRNA microarray data. miRNA samples labeled using the *mir*Vana miRNA Labeling Kit can be hybridized to a *mir*Vana miRNA Bioarray or to miRNA arrays prepared using the *mir*Vana miRNA Probe Set.

Ambion miRNA Array Web Resource

Both the miRNA and microarray fields are rapidly evolving. To provide you with the most up-to-date information on important developments in the miRNA field, and the latest recommendations for analyzing array data, see the dynamic and comprehensive Ambion miRNA Array Resource on the web at: www.ambion.com/miRNA/array

The miRNA Array Resource includes frequently updated miRNA annotation files, miRNA array tips, troubleshooting, support for data analysis, and current versions of all the data files that are included with Ambion *mir*Vana miRNA Bioarrays and *mir*Vana miRNA Probe Set. Visit this resource frequently to ensure you have the most up-to-date miRNA and miRNA analysis information.

B. Overview of the Ambion *mir*Vana Array System

The Ambion *mir*Vana Array System includes: the *mir*Vana miRNA Labeling Kit, *mir*Vana miRNA Bioarrays, the *mir*Vana miRNA Bioarray Essentials Kit, the *mir*Vana miRNA Bioarray Spike-In Controls, the *mir*Vana miRNA Reference Panel v9.1, and the *mir*Vana miRNA Probe Set. The system is designed to facilitate analysis of miRNA expression profiles in human, mouse, and rat RNA samples. Figure 1 on page 3 shows how the *mir*Vana miRNA Bioarrays procedure simplifies sample labeling and purification, and miRNA array hybridization and washing, providing a robust, reproducible platform for miRNA expression profile analysis. Bioarray results for individual targets can be validated using real-time RT-PCR with TaqMan® MicroRNA Assays.

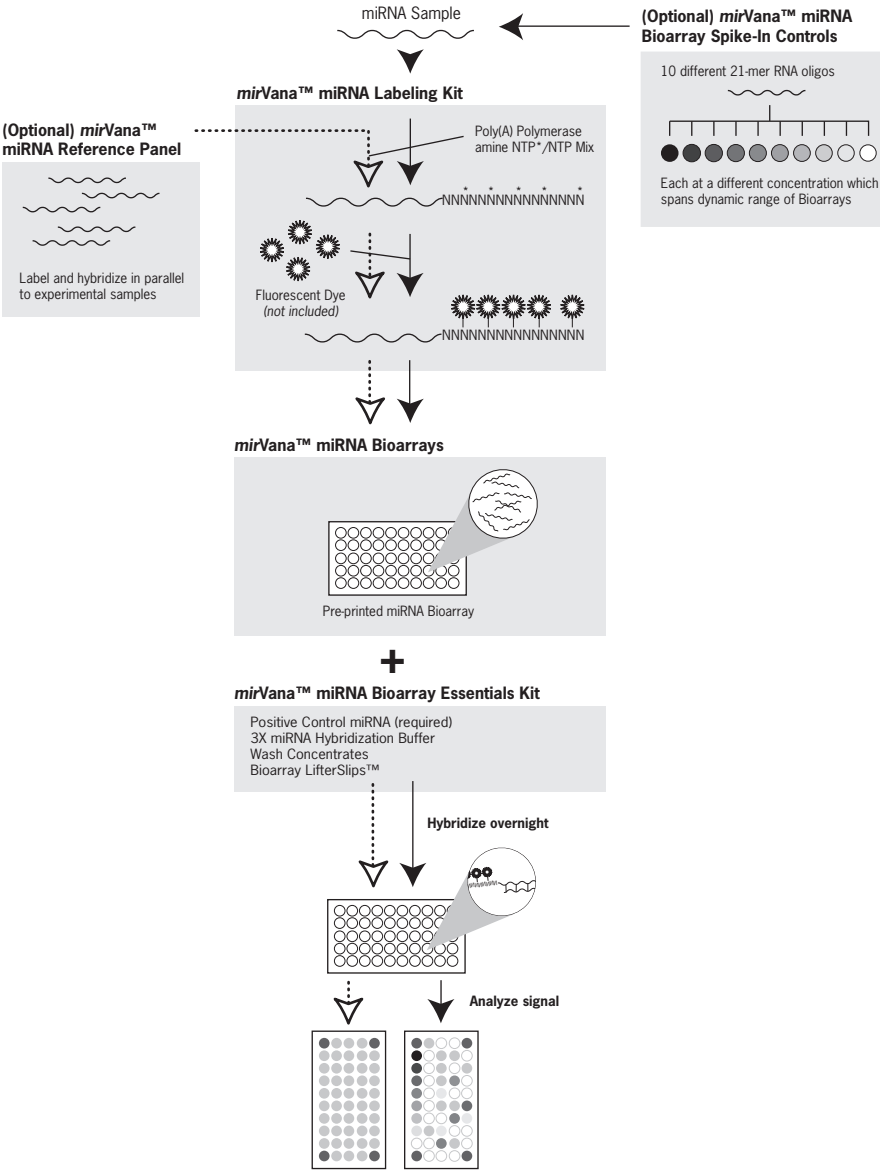
C. Reagents Provided with the Kit and Storage Conditions

The *mir*Vana miRNA Labeling Kit contains reagents to label 20 miRNA-containing RNA samples. See section *IV.A. Safety Information* starting on page 20 for chemical safety guidelines.

Amount	Component	Storage
1.75 mL	Nuclease-free Water	any temp*
2 x 1 mL	Elution Solution	any temp*
40	miRNA Collection Tubes	room temp
40	miRNA Labeling Cartridges + Tubes	room temp
200 µL	DMSO†	room temp
400 µL	miRNA Carrier	–20°C
40 µL	Poly(A) Polymerase	–20°C
200 µL	2X Poly(A) Polymerase Reaction Buffer	–20°C
40 µL	25 mM $MnCl_2$‡	–20°C
40 µL	10X Amine-NTP Mix	–20°C
400 µL	Coupling Buffer	–20°C
180 µL	4 M Hydroxylamine	–20°C
70 mL	miRNA Binding/Wash Buffer Add 38 mL 100% ethanol before use	–20°C
10 µL	Positive Control miRNA	–20°C

* Store at –20°C, 4°C, or room temp.
† Store the DMSO in a desiccator at room temp.
‡ Keep 25 mM $MnCl_2$ in the dark.

Figure 1. Overview of the *mir*Vana Array System



D. Materials Not Provided with the Kit

Reagents

- miRNA-containing RNA sample: Detailed information for obtaining miRNA is provided in section II.A starting on page 7. Briefly, we recommend the following:
  - Start with total RNA that contains the miRNA fraction; we recommend isolating RNA with either of the Ambion *mir*Vana RNA Isolation Kits (P/N AM1560, AM1556). Alternatively, you can purchase Ambion FirstChoice Total RNAs from normal human, mouse, and rat tissue that is "miRNA Certified". A list is available at: www.ambion.com/prod/fcrna.

    NOTE

    Note that plant RNA cannot be labeled using this procedure due to endogenous 3' methylation that interferes with the tailing reaction.

  - If the experimental design requires small RNA enrichment, for example, samples devoid of precursor miRNAs, isolate the miRNA fraction using the innovative Ambion flashPAGE rapid electrophoresis system (P/N AM13100, AM9015, AM10010, AM12200). Alternatively miRNA can be obtained using traditional PAGE; a protocol, *Gel Purification of miRNA from Total RNA* is available from the *mir*Vana miRNA Labeling Kit web catalog page:
    www.ambion.com/catalog/CatNum.php?1562
- 100% ethanol, ACS grade or higher quality
- NHS esters of fluorescent dyes
  This kit was developed using CyDye™ fluorescent dyes from Amersham Biosciences (GE Healthcare), but mono-reactive NHS esters of any label moiety should be capable of coupling to the amine-modified miRNA generated with this kit. Compatible dyes are listed below.
  - CyDye Post-Labelling Reactive Dye Pack, GE Healthcare Product Codes: RPN 5661, 25-8010-80, 25-8010-79
  - Molecular Probes' AlexaFluor® Reactive Dye Decapacks for Microarray Applications
    AlexaFluor 555, Cat #A32756
    AlexaFluor 594, Cat #A32751
    AlexaFluor 647, Cat #A32757
- (Optional) Ambion *mir*Vana miRNA Bioarray Spike-In Controls (P/N 4382205), for positive controls that span the signal dynamic range of the *mir*Vana miRNA Bioarray

- (Optional) *mir*Vana miRNA Reference Panel (P/N 4388891), for processing and quality control of the *mir*Vana miRNA Bioarray procedure (requires a labeling reaction and *mir*Vana miRNA Bioarray in addition to that used for experimental samples)

Equipment and supplies

- Heat block set to 95–100°C
- Incubators set to 37°C and 65°C
- Microcentrifuge capable of at least 10,000 x g
- (Optional) Vacuum manifold: to pull solutions through the Filter Cartridges
- RNase-free 1.5 mL or 0.5 mL polypropylene microcentrifuge tubes, adjustable pipettors, and RNase-free tips

E. Related Products Available from Ambion

FirstChoice® Total RNA See web or print catalog for P/Ns	Ambion FirstChoice Total RNA is available from many different human, mouse, and rat tissues, and from human cell lines. These high quality RNAs are shown to be intact and free of DNA by stringent quality control standards. FirstChoice Total RNA is certified to contain small RNAs (miRNA, siRNA, and snRNA).
flashPAGE™ Fractionator P/N AM13100	The flashPAGE Fractionator is a specialized electrophoresis instrument for rapid PAGE-purification of small nucleic acids. Designed for use with flashPAGE Pre-Cast Gels and the optimized running buffers supplied in the flashPAGE Buffer Kit, the flashPAGE Fractionator purifies small nucleic acid molecules more quickly, easily, and efficiently than traditional PAGE purification.
flashPAGE™ Reaction Clean-Up Kit P/N AM12200	The flashPAGE Reaction Clean-Up Kit is a fast and convenient filter-based purification/concentration system for small nucleic acids obtained using the flashPAGE Fractionator. It is a rapid and simple alternative to overnight precipitation.
*mir*Vana™ miRNA Isolation Kit P/N AM1560	The *mir*Vana miRNA Isolation Kit (patent pending) is designed especially for the isolation of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and small nuclear RNA (snRNA), from tissues and cells. The kit uses a fast and efficient glass-fiber filter (GFF) based procedure to isolate total RNA ranging in size from kilobases down to 10-mers. It also includes a procedure to enrich the population of RNAs that are 200 bases and smaller, which enhances the sensitivity of small RNA detection by solution hybridization and Northern blot analysis.
*mir*Vana™ PARIS™ Kit P/N AM1556	The *mir*Vana PARIS Kit employs a unique and versatile procedure for quantitative recovery of native protein and all RNA species, including small RNAs such as microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), from the same sample. The kit also includes a procedure to enrich the population of RNAs <200 nt, which can dramatically enhance sensitivity in downstream applications.

RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE P/N AM1975	The RecoverAll Total Nucleic Acid Isolation Kit for FFPE is designed to extract total nucleic acids (RNA, miRNA, and DNA) from formaldehyde- or paraformaldehyde-fixed, paraffin-embedded (FFPE) tissues, using a straightforward procedure that requires little hands-on time. By facilitating isolation of nucleic acid from archived tissue samples in a form that is suitable for downstream applications such as microarray analyses, qRT-PCR, and mutation screening, the RecoverAll Kit enables retrospective studies of diseased tissue at both the genomic and gene expression level.
*mir*Vana™ miRNA Bioarrays See web or print catalog for P/Ns	*mir*Vana miRNA Bioarrays are high-quality microarrays preprinted with probes targeting an extensive collection of currently known human, mouse, and rat microRNAs (miRNAs) using Applied Microarrays' CodeLink 3-D Gel Matrix slide surface for maximum interaction between probe and target. *mir*Vana miRNA Bioarrays are designed for analysis of miRNA samples labeled with the *mir*Vana miRNA Labeling Kit, using the positive control, hybridization, and wash components supplied with the *mir*Vana miRNA Bioarray Essentials Kit (P/N AM1567).
*mir*Vana™ miRNA Bioarray Spike-In Controls P/N 4382205	The *mir*Vana™ miRNA Bioarray Spike-In Controls are a set of 10 synthetic 21-mer RNA oligonucleotides designed for use as positive controls with *mir*Vana miRNA Bioarrays. Add *mir*Vana miRNA Bioarray Spike-In Controls, which are premixed at concentrations that span the signal dynamic range of the *mir*Vana miRNA Bioarrays, to miRNA-containing RNA samples before labeling and hybridization to bioarrays. Use *mir*Vana miRNA Bioarray Spike-In Controls to monitor sample labeling and bioarray processing and to indicate the high and low signal limits on each bioarray, thus providing an indicator of confidence in *mir*Vana miRNA Bioarray data.
*mir*Vana™ miRNA Reference Panel v9.1 P/N 4388891	The *mir*Vana™ miRNA Reference Panel v9.1 is a mixture of synthetic RNA oligonucleotides representing an extensive collection of known human, mouse, and rat microRNAs (miRNAs), as annotated in miRBase v9.1 (microrna.sanger.ac.uk/sequences/). The *mir*Vana miRNA Reference Panel is designed for use as a positive control and a quality control sample, validated extensively for microarray analysis and real-time PCR using *mir*Vana miRNA Bioarrays (P/N 4392878) and available TaqMan® MicroRNA Assays, respectively.
*mir*Vana™ miRNA Probe Set P/N AM1564V2	The *mir*Vana miRNA Probe Set V2 comprises an extensive collection of highly specific probes targeting human, mouse, and rat microRNAs (miRNAs) annotated in miRBase v8.0. Designed for accurate microarray profiling, the *mir*Vana miRNA Probe Set is a collection of amine-modified oligonucleotides suited for spotting onto epoxy and aldehyde slide surface chemistries such as SCHOTT Nexterion™ Slide E and Slide AL (except slides with 3-D gel matrices). The *mir*Vana miRNA Probe Set produces accurate and highly reproducible data, and it is provided in a flexible, cost-effective format that includes controls, to accommodate various experimental designs.

II. miRNA Labeling and Cleanup

IMPORTANT

Plan to prepare labeled miRNA on the day that you will hybridize the array. Note, however, that there is one potential stopping point in this procedure (after step C.6 on page 11).

A. Preparation of miRNA-containing Samples

Input RNA requirements

The first step in miRNA analysis using the *mir*Vana Array System is to obtain miRNA-containing RNA. The two principal isolation strategies are to prepare total RNA containing the small RNA fraction (e.g., using the Ambion *mir*Vana miRNA Isolation Kits), or to physically separate the miRNA fraction from total RNA using gel electrophoresis (e.g., with the Ambion flashPAGE Fractionator).

NOTE

Plant miRNAs cannot be labeled with this kit due to endogenous 3' methylation. For more information, see Yu B, et al. (2005) Methylation as a crucial step in plant microRNA biogenesis. Science. 307:932–5.

We have found that expression profiles on *mir*Vana miRNA Bioarrays using Ambion FirstChoice Total RNA (certified to contain miRNA) are similar to those obtained with RNA enriched for miRNA using the Ambion flashPAGE Fractionator. Both sample preparation methods provided high sensitivity, accuracy and reproducibility on *mir*Vana miRNA Bioarrays.

User-printed arrays spotted with the *mir*Vana miRNA Probe Set may show higher background signal with miRNA-containing total RNA than with flashPAGE Fractionator-purified miRNA.

We recommend preparing flashPAGE Fractionator-purified miRNA samples if your array platform or experimental design requires highly purified RNA samples or samples devoid of precursor miRNA.

Refer to the web catalogue page for the *mir*Vana miRNA Labeling Kit for the most up-to-date information about miRNA isolation methods for producing input RNA suitable for this labeling method:
www.ambion.com/catalog/CatNum.php?1562

Options for obtaining miRNA samples

See *Suggested starting total RNA mass* starting on page 8 for more detailed information on the recommended mass amount of RNA.

miRNA-containing total RNA

Isolate or purchase total RNA that includes the full complement of miRNAs.


NOTE

~0.01% of total RNA is miRNA. flashPAGE purification achieves ~10,000 fold enrichment for miRNA, whereas enrichment using the mirVana RNA isolation kits results in ~10 fold enrichment for miRNA and other small RNAs.

Typically, RNA isolation methods that use RNA-binding glass-fiber filters do not quantitatively recover RNA species <200 nt. We recommend using the Ambion *mir*Vana miRNA Isolation Kit or *mir*Vana PARIS™ Kit to purify total RNA or to isolate RNA fractions enriched for small RNA species. Total RNA from other commercial suppliers may or may not contain miRNAs. All of Ambion FirstChoice Total RNAs from normal human, mouse, and rat tissue are "miRNA certified". A list is available at:

www.ambion.com/prod/fcrna

miRNA-enriched fraction of total RNA

The small RNA enrichment protocol in the *mir*Vana miRNA Isolation Kit or *mir*Vana PARIS Kit, provides ~10-fold enrichment for miRNA and other small RNA. If satisfactory results are not obtained using miRNA-containing total RNA, labeling RNA enriched for the small RNA fraction may be sufficient.

flashPAGE Fractionator-purified miRNA

If highly purified RNA samples, or samples devoid of precursor miRNAs, are desired, purify the miRNA fraction using the Ambion flashPAGE Fractionator and flashPAGE Reaction Clean-Up Kit. Alternatively miRNA can be isolated using traditional gel electrophoresis; a detailed protocol is available on at the following address:

www.ambion.com/catalog/CatNum.php?1562

Suggested starting total RNA mass

We recommend labeling 3–10 µg total RNA, or the miRNA fraction obtained from 5–20 µg of total RNA, for analysis with *mir*Vana miRNA Bioarrays. These ranges are also useful starting points for user-printed arrays spotted with the *mir*Vana miRNA Probe Set, but the optimal starting mass of RNA and need for miRNA fractionation will depend upon the quality of the array, the array surface chemistry, and the array hybridization and processing protocol.

We recommend a broad range of input miRNA because, with some targets, saturation of signal may be seen with only 3 µg of labeled total RNA, whereas other targets may be difficult to detect with 10 µg of labeled total RNA. In addition, the relative mass of miRNA varies considerably among different sample types. For example, 10 µg of total RNA from most tissues contains approximately 1 ng of miRNA, whereas 10 µg of total RNA from cultured cells contains only ~250 pg of miRNA.

For total RNA samples that contain relatively little miRNA, label up to 20 µg of total RNA for array hybridization. Conversely, for samples that contain a relatively high miRNA content, as little as 1–2 µg of labeled total RNA may be sufficient.

B. Appending 3' Amine-Modified Tails to miRNA

Before you start

- Warm non-enzyme reaction components to room temp.

Keep $MnCl_2$ in the dark!

Remove the non-enzyme reagents that will be used in the Poly(A) Polymerase reaction (step 4 below) from the freezer, and allow them to equilibrate to room temp for 2 hours. [Leave the Poly(A) Polymerase at –20°C.]

- Preheat a 37°C incubator.

1. Dry miRNA-containing RNA samples

Dry miRNA-containing RNA samples to completion by vacuum centrifugation.

(Optional) If you are using *mir*Vana miRNA Bioarray Spike-In Controls, add them to your miRNA-containing RNA samples ***before*** drying them down, following the instructions supplied with the controls.

*(*Optional) If you are using the *mir*Vana miRNA Reference Panel, dry an aliquot of the *mir*Vana miRNA Reference Panel ***in parallel*** with your experimental samples, following the instructions supplied with the reference panel.

2. Dilute the Positive Control miRNA in Nuclease-free Water

The Positive Control miRNA supplied with the *mir*Vana miRNA Labeling Kit and the *mir*Vana miRNA Bioarray Essentials Kit (P/N AM1566) are identical. The control is designed to hybridize with the *mir*Vana miRNA Bioarrays Control_1 probe (BA10001) and the *mir*Vana miRNA Probe Set Control_1 probe (PS20001). (Use the Positive Control miRNA even if you are using *mir*Vana miRNA Bioarray Spike-In Controls.)

For *mir*Vana miRNA Bioarrays, dilute 1 µL of Positive Control miRNA with 99 µL Nuclease-free Water.

To prepare samples for analysis on *mir*Vana miRNA Bioarrays, dilute 1 µL of the Positive Control miRNA into 99 µL Nuclease-free Water.

The Positive Control miRNA ***must*** be added to each miRNA sample that will be hybridized with *mir*Vana miRNA Bioarrays; it serves as a positive control for sample labeling and hybridization, and also produces fiducial spots that are recognized by array scanning equipment and are necessary for array analysis.

For *mir*Vana miRNA Probe Set arrays, dilute 1 µL of Positive Control miRNA with 499 µL Nuclease-free Water.
(Optional) For *mir*Vana miRNA Probe Set arrays, dilute 1 µL of the Positive Control miRNA with 499 µL Nuclease-free Water.

The Positive Control miRNA can be added to each sample miRNA as a spike control for samples that will be hybridized with miRNA arrays made using the *mir*Vana miRNA Probe Set.

Store the diluted Positive Control miRNA in small aliquots at –70°C between experiments.

3. Resuspend miRNA samples in 3 µL Nuclease-free Water

Add 3 µL Nuclease-free Water to each dried miRNA sample prepared in step 1, and resuspend the RNA by vortexing briefly and/or by pipetting up and down a few times.

4. Add Poly(A) Polymerase reaction mix to each miRNA sample

At room temp, add the tailing reaction reagents to each miRNA sample in the order shown below and mix well by gently flicking the tube a few times. For experiments that include >2 samples, it is a good idea to prepare a master mix.

Amount	Component
1 µL	diluted Positive Control miRNA*
10 µL	2X Poly(A) Polymerase Reaction Buffer
2 µL	25 mM $MnCl_2$
2 µL	10X Amine-NTP Mix
2 µL	Poly(A) Polymerase

* If you are preparing sample for analysis on an miRNA array prepared using the *mir*Vana miRNA Probe Set, and you choose not to include the Positive Control miRNA in your reaction, then replace it with Nuclease-free Water.

5. Incubate at 37°C for 2 hr

Incubate the tailing reaction at 37°C for 2 hr.


NOTE

Begin heating the Column Elution Buffer to 95°C towards the end of this incubation. It will be used in step C.5 below.

C. Post-Tailing Sample Cleanup

This cleanup procedure removes unincorporated nucleotides from the tailed miRNA by filter purification with the miRNA Labeling Columns.

Before you start

- Add 38 mL ACS-grade or higher quality 100% ethanol to the bottle of miRNA Binding/Wash Buffer, mix well, and mark the label to indicate that the ethanol was added.
- Preheat the Elution Solution to 95°C
- Preheat an incubator to 65°C

1. Add miRNA Carrier and miRNA Binding/Wash Buffer and incubate 5 min at room temp

a. Add miRNA Carrier and miRNA Binding/Wash Buffer to each sample according to the table below.
Make sure that the ethanol has been added to the miRNA Binding/Wash Buffer before use.

Amount	Component
10 µL	miRNA Carrier
350 µL	miRNA Binding/Wash Buffer

b. Mix by vortexing briefly, then incubate at room temp for 5 min.

2. Pass mixture through an miRNA Labeling Cartridge

a. Pipet the mixture from the previous step into an miRNA Labeling Cartridge (in an miRNA Collection Tube) and centrifuge at ~10,000 x g for 15 sec, or until the mixture is through the filter.

b. Discard the flow-through and place the miRNA Labeling Cartridge back in the Collection Tube.

3. Wash with 2 x 300 µL miRNA Binding/Wash Buffer

a. Pipet 300 µL miRNA Binding/Wash Buffer into the miRNA Labeling Cartridge and centrifuge at ~10,000 x g for 15 sec, or until the buffer is through the filter.

b. Discard the flow-through and place the miRNA Labeling Cartridge back in the Collection Tube.

c. Repeat steps a–b to wash with a second 300 µL of miRNA Binding/Wash Buffer.

4. Dry the filter

Dry the filter by centrifuging at ~10,000 x g for 1 min.

5. Elute tailed miRNA with 30 µL hot Elution Solution

a. Transfer the cartridge to a fresh miRNA Collection Tube.

b. Add 15 µL of 95°C Elution Solution to the miRNA Labeling Cartridge and incubate at 65°C for 5–10 min.

c. Centrifuge at ~10,000 x g briefly (just until the centrifuge comes up to speed) to collect the tailed miRNA in the tube.

d. With the miRNA Labeling Cartridge still in the tube, add a second 15 µL of 95°C Elution Solution, and incubate at 65°C for 5–10 min.

e. Centrifuge at ~10,000 x g for 1 min to collect all of the tailed miRNA in a single tube.

6. Dry samples in a vacuum concentrator

Dry samples to completion in a vacuum concentrator.

STOPPING POINT

If desired, the dried sample can be stored at –20°C for 1–2 days.

D. Labeling the Tailed miRNA with Amine-Reactive CyDye

Amine-modified miRNA-containing samples prepared with this kit can be labeled with any of a number of detectable moieties. We have used Cy5 and Cy3 extensively, and we recommend them for array experiments. We have also labeled with Alexa dyes and found them to work well. Similar fluorescent dyes and detectable moieties such as digoxigenin and biotin are also expected to be compatible with this procedure. Most amine-reactive dyes are provided in convenient sizes to facilitate nucleic acid labeling. Here we provide instructions for using Cy3, Cy5, and Alexa Fluor dyes. For other labeling moieties, follow the manufacturer's recommendation to resuspend and aliquot the dye prior to use.

1. Resuspend in 7 µL Nuclease-free Water

Add 7 µL Nuclease-free Water to each dried sample and vortex briefly to resuspend the tailed miRNA.

2. Resuspend Cy3- or Cy5-NHS ester with 16 µL of DMSO

CyDyes: Resuspend one vial of Cy3 or Cy5 Post Labelling Reactive Dye (Amersham Biosciences) with 16 µL of DMSO.

Alexa Fluor dyes: Resuspend Alexa Fluor dyes in 4 µL DMSO.

- Prepare dye immediately before starting the dye coupling procedure.
- It is very important that dye compounds remain dry both before and after dissolving in DMSO, because any introduced water will cause hydrolysis of the NHS esters, lowering the efficiency of coupling.
- Store any unused solubilized dye in the dark at –80°C. Note that Amersham Biosciences reports that solubilized dye has a very limited shelf life.

3. Add 9 µL Coupling Buffer and 4 µL prepared CyDye to each sample

Set up each labeling reaction as follows:

Amount	Component
9 µL	Coupling Buffer
7 µL	amine-modified miRNA
4 µL	prepared Cy3 or Cy5 in DMSO

Vortex briefly to mix, and shield from light as much as possible.

4. Incubate 1 hr at room temp in the dark

This 1 hr incubation at room temp allows the dye coupling reaction to occur. To keep the samples in the dark, simply put the tubes in a closed drawer.

5. Add 4.5 µL 4M Hydroxylamine and mix

To quench the reaction, add 4.5 µL 4M Hydroxylamine and mix well by vortexing gently.

6. Incubate 15 min at room temp in the dark

Incubate the reaction in the dark at room temp for 15 min.

During this incubation, the large molar excess of hydroxylamine will quench the amine-reactive groups on the unreacted dye molecules.

E. Post-Labeling Cleanup

IMPORTANT

Keep your sample in the dark as much as possible to avoid photobleaching.

Before you start

- Preheat the Nuclease-free Water to 95°C
- 65°C incubator needed

1. Add 350 µL miRNA Binding/Wash Buffer and incubate 5 min at room temp in the dark

a. Add 350 µL miRNA Binding/Wash Buffer to each labeled sample and vortex briefly to mix thoroughly.

b. Incubate at room temp in the dark for 5 min.

2. Pass mixture through an miRNA Labeling Cartridge

a. Pipet the mixture from the previous step into the miRNA Labeling Cartridge (in an miRNA Collection Tube) and centrifuge at ~10,000 x g for 15 sec, or until the mixture is through the filter.

b. If you plan to cohybridize two samples labeled with different dyes to a single array: add the second sample to the same miRNA Labeling Cartridge as the first sample, and centrifuge as in the previous step.

3. Wash with 2 x 300 µL miRNA Binding/Wash Buffer

a. Pipet 300 µL miRNA Binding/Wash Buffer into the miRNA Labeling Cartridge and centrifuge at ~10,000 x g for 15 sec, or until the buffer is through the filter.

b. Discard the flow-through and place the miRNA Labeling Cartridge back in the Collection Tube.

c. Repeat steps a–b to wash with a second 300 µL miRNA Binding/Wash Buffer.

4. Dry the filter

Dry the filter by centrifuging at ~10,000 x g for 1 min.

5. Elute tailed miRNA with 26 µL 95°C Nuclease-free Water and 10 min incubation at 65°C

a. Transfer the column to a fresh miRNA Collection Tube

b. Add 26 µL of 95°C Nuclease-free Water to the miRNA Labeling Cartridge and incubate at 65°C for 10 min.

c. Centrifuge at ~10,000 x g for 1 min to collect the labeled miRNA in the tube.

6. Immediately proceed to array hybridization

It is important to use the labeled sample for miRNA array hybridization within 30 min after elution from the cartridge.

III. Troubleshooting miRNA Labeling

Check the Ambion miRNA Array Web Resource

As new information on troubleshooting the *mir*Vana miRNA Array System becomes available, we will post it on the miRNA Array Resource on the web at: www.ambion.com/miRNA/array

A. Low Signal from Samples, Adequate Signal from Positive Control miRNA

1. miRNA was lost during purification

Many total RNA isolation systems do not recover the entire miRNA population. Use miRNA-certified Ambion FirstChoice Total RNA, or use the Ambion *mir*Vana miRNA Isolation Kit or *mir*Vana PARIS Kit to isolate miRNA-containing total RNA.

If your experimental design requires highly purified RNA samples, or miRNA devoid of precursor miRNA, isolate the miRNA fraction using the flashPAGE Fractionator. Alternatively you can purify miRNA from total RNA using traditional gel electrophoresis; see the protocol on our website at: www.ambion.com/catalog/CatNum.php?1562.

If you are using miRNA samples that have been fractionated from larger RNA, the control experiment described in section *III.E. Positive Control miRNA* on page 18 can help differentiate between problems with the miRNA isolation and labeling processes.

2. Poor quality RNA samples

Poor quality total RNA may contain impurities that can inhibit the poly(A) tailing reaction. Since the Positive Control miRNA is in great excess over the sample miRNA, moderate inhibition of the labeling reaction might go undetected on the bioarray, even in the control experiment described in section *III.E. Positive Control miRNA* on page 18. Because the *mir*Vana miRNA Bioarray Spike-In Controls controls are used at concentrations that span the signal dynamic range of the bioarrays, moderate inhibition of the labeling reaction should be evident by lower overall signal from each control.

Some inhibitors associated with the sample RNA, such as high carbohydrate, can result in poor labeling of the sample compared to the Positive Control miRNA.

Use the Ambion *mir*Vana miRNA Isolation Kit to isolate total RNA. To further purify the miRNA fraction, use the flashPAGE Fractionator. Alternatively you can purify miRNA from total RNA using traditional gel electrophoresis; see the protocol on our website at: www.ambion.com/catalog/CatNum.php?1562.

B. Low Overall Signal Intensity

1. Poor quality RNA samples

Inefficient labeling of sample miRNA can result from low quality or poorly purified total RNA. Low quality total RNA may contain impurities that can inhibit the poly(A) tailing reaction.

Use the Ambion *mir*Vana miRNA Isolation Kit to isolate total RNA. To further purify the miRNA fraction, use the flashPAGE Fractionator. Alternatively you can purify miRNA from total RNA using traditional gel electrophoresis; see the protocol on our website at: www.ambion.com/catalog/CatNum.php?1562.

2. Problems with recovery of samples from filter cleanup steps

Make sure ethanol was added to the miRNA Binding/Wash Buffer

There are two filter purification steps in the miRNA labeling process, and both use miRNA Binding/Wash Buffer. The ethanol concentration of this buffer is critical for efficient recovery of miRNA. Confirm that 38 mL of 100% ethanol was added to the bottle supplied before using the Binding/Wash Buffer and be sure to keep the bottle tightly capped between uses to prevent the ethanol from evaporating.

Check that the filters in the Filter Cartridges are seated properly

It may also be a good idea to check that the filters in the Filter Cartridges are seated firmly at the bottom of the column. Occasionally these filters can be dislodged during shipping.

Measure the Nuclease-free Water used for sample elution carefully

In step II.E.5 on page 13, the labeled miRNA sample is eluted with Nuclease-free Water that is preheated to 95°C. At that temperature, it may be difficult to measure the volume accurately.

Visually estimate the volume of the eluate collected in the tube after centrifugation, and if it appears that there is less than 26 µL, measure it with a pipettor. If necessary, bring the final volume to 26 µL by adding Nuclease-free Water.

3. Problems with the fluorescent dye ester

The fluorescent dye is "bad"

We have observed variability between different lots of NHS-ester fluorescent dyes. Consider trying a new lot of dye.

Photobleaching of the fluorescent dye

Fluorescent dyes are subject to photobleaching. Exposing them to light, either before or after coupling, will reduce signal intensity. Limit the exposure of fluorescent dyes to light by conducting coupling reactions, labeled miRNA cleanup, and array hybridization in the dark when possible.

4. Incubation temperatures for miRNA labeling were incorrect

The incubation temperatures are critical for effective labeling and hybridization of your miRNA sample.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.
- If a thermal cycler is used for incubation, check the accuracy of the adjustable temperature lid. If the lid temperature cannot be adjusted to match the reaction temperature, use the lid with the heat turned off, or do not use it to cover the reaction vessel(s).

5. Problems with the miRNA purification process

If you are using miRNA samples that have been size-separated from larger RNA:

- Check the efficiency of miRNA purification by running the experiment described in section III.E on page 18.
- When using gel purification to purify miRNA, you can confirm that the miRNA fraction is migrating as expected by 5' end radiolabeling the Positive Control miRNA, and running it on a gel in a lane adjacent to your sample RNA. You can then identify the position of miRNAs on the gel by autoradiography.

C. Low Signal Intensity from the Positive Control miRNA

1. Troubleshoot preparation of the miRNA array

Inefficient coupling of probes to the microarray support, and problems with array post-processing can easily result in low overall signals from arrays. Users of the *mir*Vana miRNA Probe Set can find troubleshooting suggestions in the Instruction Manual supplied with the product and available on Ambion's miRNA Array Resource:
www.ambion.com/miRNA/array

2. The array slide was placed in the scanner incorrectly

Improper placement of slides in the scanner, for example, upside down or backwards, can result in no signal from the scanned region. *mir*Vana miRNA Bioarrays have writing on them that is legible when they are array-side-up. If you are using miRNA arrays made using the *mir*Vana miRNA Probe Set, be sure to mark the slides for orientation.

3. Hybridization problems

a. Suboptimal hybridization stringency

The formamide concentration of the hybridization mixture typically is the most important component in achieving the proper hybridization stringency. The *mir*Vana miRNA Probe Set and the *mir*Vana miRNA Bioarray Essentials Kit include a 3X miRNA Hybridization Buffer that must be used at precisely 1X to achieve optimal stringency for miRNA array hybridization.

b. Hybridization and/or wash temperatures were higher than recommended

With the *mir*Vana miRNA array system, the recommended hybridization temperature is 42°C. If in doubt, verify the incubator temperature with a calibrated thermometer.

Conduct post-hybridization washes at room temperature. If the ambient room temperature in your lab is over 25°C, washing may be too stringent and could result in loss of legitimate signal.

4. Nuclease-contaminated tubes, tips, or equipment

Using pipette tips, tubes, or other plasticware that is contaminated with nucleases during purification and/or handling of miRNA samples can degrade miRNA, reducing yield and the size of miRNA. Both RNases and DNases can be removed from surfaces using Ambion RNase*Zap* (P/N AM9780).

D. High Signal from Most Probes

1. Incomplete reactive dye quenching and removal

If the labeling moiety is not removed by column purification, it can interact nonspecifically with elements on the array. Check the color of the solution of the labeled, purified sample; it should be clear or lightly tinted. If the solution is more than just lightly tinted, it is an indication that uncoupled dye is present, and it cannot be used for miRNA array hybridization. This could occur if too much fluorescent dye is used in the labeling reaction, or if the glass-fiber filter inside the miRNA Labeling Column is not properly positioned and sample bypasses the filter during the purification.

2. Hybridization stringency was too low

The recommended hybridization temperature is 42°C. Lower temperatures can lead to nonspecific interactions between miRNAs in the sample and probes on the array. If in doubt, verify the incubator temperature with a calibrated thermometer.

3. Nonspecific binding of the labeled RNA

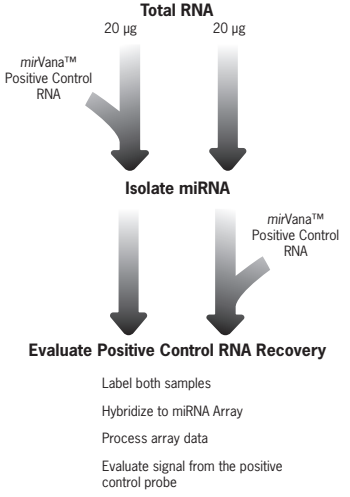
We have found that expression profiles on *mir*Vana miRNA Bioarrays obtained with Ambion FirstChoice Total RNA (which is certified to contain miRNA) are similar to those obtained with RNA enriched for miRNA using the Ambion flashPAGE Fractionator. In some cases, however, labeling total RNA may lead to nonspecific binding to the relatively short probes used for miRNAs.

To further purify your miRNA samples, and to size-separate your miRNA from larger RNAs, we recommend using the flashPAGE Fractionator before labeling. Alternatively, miRNA can be isolated using traditional gel electrophoresis; a detailed protocol is available at the following address:

www.ambion.com/catalog/CatNum.php?1562

E. Positive Control miRNA

Using the Positive Control miRNA to troubleshoot miRNA purification



If you are using miRNA samples that have been purified from larger RNAs, the following experiment can help differentiate between problems with the miRNA isolation and labeling processes. In this simple experiment, the Positive Control miRNA is added to your experimental RNA either before or after miRNA isolation. The two samples are then labeled and hybridized to microarrays, and the hybridization signal of the Positive Control Probe between the two samples provides a measure of the efficiency of miRNA isolation.

1. Start with 40 µg of total RNA that contains miRNAs and split it into two 20 µg samples.
2. Add 1 µL of diluted Positive Control miRNA to one of the two samples (dilution instructions are in step II.B.2 on page 9).
3. Isolate miRNA from the two samples using the flashPAGE Fractionator or by traditional gel electrophoresis. (The protocol for isolating miRNA from total RNA is available on our web site at: www.ambion.com/catalog/CatNum.php?1562
4. Add 1 µL of the diluted Positive Control miRNA to the purified, second miRNA sample (which did not receive the control before isolating miRNA).
5. Label one miRNA sample with one fluorescent dye (e.g., Cy3), and label the other sample with a different fluorescent dye (e.g., Cy5) and hybridize to a single miRNA array spotted with the *mir*Vana miRNA Probe Set.
6. Measure the signal from the positive control element for the two samples.

Expected results and interpretation

The desired result is that both samples should have ~100-fold signal above background for the positive control probe spots; this indicates that miRNA isolation worked well.

Low fluorescence from both samples

If neither sample produces good fluorescence from the positive control target spot, it suggests that there was a problem with labeling or hybridization. Troubleshooting suggestions are in sections III.A through III.C. Additional troubleshooting suggestions are provided in the Instruction Manuals for the *mir*Vana miRNA Probe Set and *mir*Vana miRNA Bioarrays.

Lower fluorescence from sample spiked with the Positive Control miRNA before miRNA isolation

If the sample that was spiked with the Positive Control miRNA before miRNA isolation elicits at least 3-fold lower fluorescence from the positive control probe spot, some of the Positive Control miRNA was lost during miRNA isolation. If this is the case, troubleshoot the miRNA purification process or consider using an alternative procedure for obtaining miRNA.

F. Optional Controls

*mir*Vana™ miRNA Bioarray Spike-In Controls

The high and low detection limits of each bioarray can be measured by mixing the *mir*Vana miRNA Bioarray Spike-In Controls (P/N 4382205) with your miRNA-containing samples prior to labeling (see *II.B. Appending 3' Amine-Modified Tails to miRNA* on page 9). The *mir*Vana miRNA Bioarray Spike-In Controls hybridize to individual probes on the *mir*Vana miRNA Bioarrays and are premixed at concentrations that span the signal dynamic range of the bioarrays. Along with the Positive Control miRNA, they also enable monitoring of sample labeling and bioarray processing (see section III.A starting on page 14). However, the *mir*Vana miRNA Bioarray Spike-In Controls should not be used for signal intensity normalization across multiple bioarrays.

*mir*Vana miRNA Reference Panel V9.1

*mir*Vana miRNA Reference Panel V9.1 provides a positive control and quality control sample for *mir*Vana miRNA Bioarrays. The reference panel is a mixure of synthetic RNA oligonucleotides representing an extensive collection of the known mature miRNAs in human, mouse, and rat, as annotated in miRBase v9.1. Label and hybridize an aliquot of the miRNA Reference Panel in parallel with your experimental samples. Each miRNA in the miRNA Reference Panel v9.1 will show a characteristic fluorescence signal on *mir*Vana miRNA Bioarrays. Typically, a 2–3 log range of fluorescence signal intensity is measured. Follow the instructions provided with the reference panel, available at:

www.ambion.com/techlib/spec/sp_4388891.pdf

IV. Appendix

A. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address:
  www.ambion.com/techlib/msds
- Alternatively, e-mail your request to MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

B. Quality Control

Functional testing

miRNA is isolated from a reference tissue source and labeled according to the instructions in section II starting on page 7. The labeled miRNA is hybridized to a *mir*Vana miRNA Bioarray and signal intensities are correlated with expected results.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 40 ng labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.