Kit Improvement, Revised Protocol

The *mir*Vana™ miRNA Probe Set has been improved; as a result, this Instruction Manual includes major protocol changes.

Ambion is committed to producing the highest quality reagents and kits for for analysis of miRNA. As part of that commitment, the *mir*Vana miRNA Probe Set has beeen significantly improved and updated. The highlights of these improvements are listed below:

- Improved probe design
- Includes probes for an exclusive set of new miRNAs
- Updated probe content to include recently discovered miRNAs reported in the literature
- Modified miRNA annotations

Ongoing experiments at Ambion have also resulted in significant protocol improvements that are detailed in this Instruction Manual. If you have previous versions of the *mir*Vana miRNA Probe Set instructions, discard them, and use these instructions instead.

Finally, we want to announce the new miRNA Array Resource on the web. Resource on the web at: www.ambion.com/miRNA/array
The miRNA Array Resource includes frequently updated miRNA annotation files, miRNA array tips, troubleshooting, and support for data analysis. Visit this resource frequently to ensure you have the most up-to-date miRNA, and miRNA analysis information.

*mir*Vana™ miRNA Probe Set (Cat #1564)

Instruction Manual

Manual Version 0508

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the *mir*Vana™ miRNA Probe Set.

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.

Warranty and Liability Ambion is committed to providing the highest quality reagents at competitive prices. Ambion warrants that the products meet or exceed the performance standards described in the product specification sheets. If you are not completely satisfied with any product, our policy is to replace the product or credit the full purchase price and delivery charge. No other warranties of any kind, expressed or implied, are provided by Ambion. Ambion's liability shall not exceed the purchase price of the product. Ambion shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products. This product is intended for research use only. This product is not intended for diagnostic or drug purposes.

Patents, Licensing, and Trademarks The *mir*Vana™ miRNA Probe Set is covered by US patents pending.

The purchase of Ambion products does not convey any license under any patent rights owned by Oxford Gene Technology IP Limited ("OGT") including any right to make or use oligonucleotide arrays under OGT's patents, i.e. US, European, Japanese patents and patent applications deriving from WO 89/10977, WO 90/03382 and WO 96/31622. Please contact OGT to enquire about a license under OGT's patents at licensing@ogt.co.uk. Further details can be found at www.ogt.co.uk.

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I. Introduction

A. Description of the *mir*Vana™ miRNA Probe Set

The *mir*Vana miRNA Probe Set (patent pending) is a collection of amine-modified DNA oligonucleotides representing a comprehensive panel of the human, mouse, and rat microRNAs (miRNAs) in the miRNA Registry:

microrna.sanger.ac.uk/sequences/index.shtml

In addition, the *mir*Vana miRNA Probe Set includes probes to detect an exclusive set of newly identified human miRNAs. Called Ambi-miRs, these novel miRNAs have been identified through a combination of bioinformatic prediction, cloning, and detection in human total RNA samples.

Developed in conjunction with the *mir*Vana miRNA Labeling Kit, the *mir*Vana miRNA Probe Set is designed for preparation of glass slide "miRNA arrays". Using the *mir*Vana Array System (see Figure 1 on page 2), you can analyze global miRNA expression profiles using commercially available microarray analysis instruments and software.

The probes in the *mir*Vana miRNA Probe Set are 42–46 nucleotides (nt) long, of which an 18–24 nt segment targets a specific known human, mouse, or rat miRNA, and the remaining sequence serves as spacer and attachment sequence for coupling to the surface of microarray slides. Probes are synthesized with an amine-modification for compatibility with both epoxy and aldehyde slide surface attachment chemistries. We recommend SCHOTT Nexterion® Slide E microarray slides because they are convenient and easy to use and they provide consistent results. The instructions in this protocol describe preparation of arrays using epoxy-coated slides only. In theory, however, the *mir*Vana miRNA Probe Set is compatible with all common array attachment chemistries except 3-D gel matrix slides (gel matrix slides are incompatible with the spacer and linker sequences on the miRNA Probe Set oligonucleotides).

Ambion's miRNA Array Web Resource

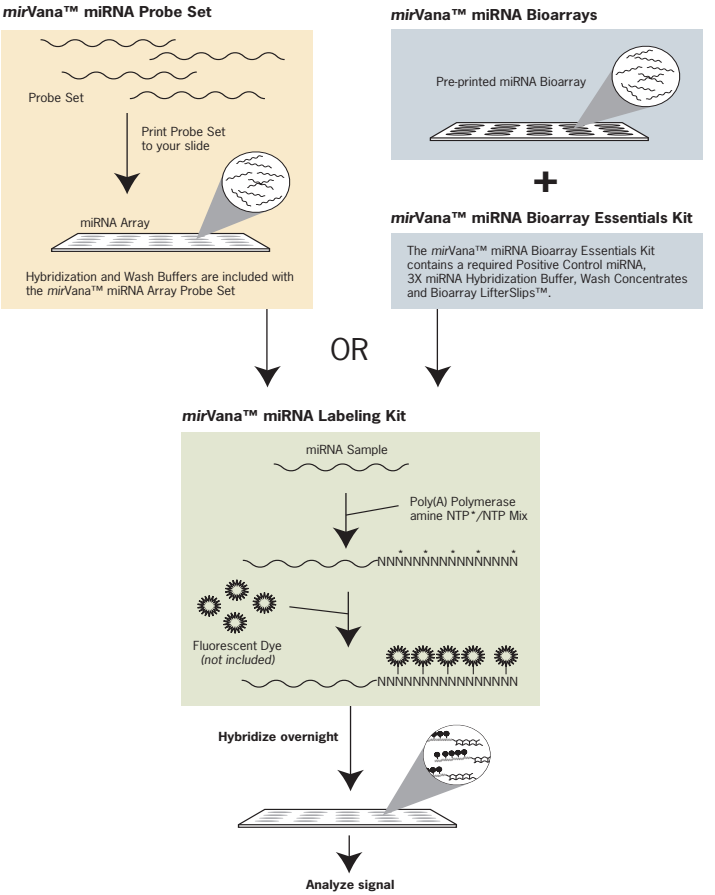
Both the miRNA and microarray fields are rapidly evolving. To provide you with the most up-to-date information on important developments in the miRNA field, and the latest recommendations for analyzing array data, Ambion provides a dynamic and comprehensive miRNA Array Resource on the web at: www.ambion.com/miRNA/array

The miRNA Array Resource includes frequently updated miRNA annotation files, miRNA array tips, troubleshooting, and support for data analysis. Visit this resource frequently to ensure you have the most up-to-date miRNA and miRNA analysis information.

B. Overview of the *mir*Vana Array System

Ambion's *mir*Vana™ Array System includes the *mir*Vana miRNA Labeling Kit, the *mir*Vana miRNA Probe Set, *mir*Vana miRNA Bioarrays, and the *mir*Vana miRNA Bioarray Essentials Kit. The system is designed to facilitate analysis of miRNA expression profiles in human, mouse, and rat RNA samples. Figure 1 shows how the system can be used to simplify sample labeling and purification, and miRNA array hybridization and washing, providing a robust, reproducible platform for miRNA expression profile analysis.

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



C. Reagents Provided with the Kit and Storage Conditions

Properly stored kits are guaranteed for 6 months from the date received. Note that the kit is shipped at room temperature, which will not affect its stability.

Amount	Component	Storage
1	miRNA Probe Set	–20°C
200 ml	Detergent Concentrate	room temp
1 L	Salt Concentrate	room temp
10 ml	3X miRNA Hybridization Buffer	4°C

Description of miRNA probes and controls

The *mir*Vana miRNA Probe Set is provided in 96 well plates; each well contains 500 pmol of dried amine-modified oligonucleotide. A link for the complete list of miRNA probes and controls included in the kit is posted at the following web address:

www.ambion.com/catalog/CatNum.php?1564v1

Ambion's miRNA Array Resource has information on the miRNAs targeted by each probe in the *mir*Vana miRNA Probe Set:

www.ambion.com/miRNA/array

D. Equipment and Reagents Not Provided with the Kit

Glass array slides and coverslips

Recommended slides: **SCHOTT Nexterion Slide E**

We recommend printing the *mir*Vana miRNA Probe Set onto SCHOTT Nexterion Slide E microarray slides, Cat #1066643. These epoxy-coated glass slides provide excellent signal-to-background and are easily processed after printing the *mir*Vana miRNA Probe Set. The epoxy surface coating permits efficient and very stable attachment of the amine-modified oligonucleotides through a covalent bond. Find more information on SCHOTT Nexterion Slide E microarray slides at their website:

www.us.schott.com/nexterion/english/products/coated_substrates/slide_e/index.html

Alternative slide coatings

The *mir*Vana miRNA Probe Set can alternatively be printed onto aldehyde-coated (SCHOTT Nexterion Slide AL) and amine-coated (SCHOTT Nexterion Slide A+) slides.

IMPORTANT

The mirVana miRNA Probe Set is ***not*** *compatible with 3-D gel matrix slides.*

Recommended coverslips: **Erie Scientific LifterSlips™**
We strongly recommend using raised-edge coverslips such as LifterSlips™ available from Erie Scientific (Cat #22x22I-2-4788). They provide more robust and reproducible results than flat coverslips.

Reagents

Spotting solution
We routinely spot the *mir*Vana miRNA Probe Set in 3X SSC or 3X SSC, 0.01% SDS onto epoxy-coated glass slides such as SCHOTT Nexterion Slide E slides. Other spotting solutions such as betaine- and DMSO-based solutions are also compatible with the miRNA Probe Set.

POPO™-3 iodide dimeric cyanine nucleic acid stain
POPO-3 (Molecular Probes Invitrogen Detection Technologies) provides a simple and direct method for analyzing the quality of miRNA arrays made with the miRNA Probe Set.

Reagents for preparation of hydration and blocking buffers

- Nuclease-free water; high quality water that has not been DEPC-treated, e.g., Ambion Cat #9930
- 0.5X SSC; most researchers prepare a 20X SSC solution, and dilute to 0.5X SSC as needed

Table 1. 20X SSC

Component	Concentration
NaCl	3 M
sodium citrate, pH 7	0.3 M

- 1 M Tris, pH 9, molecular biology grade
- Ethanolamine, molecular biology grade. Ethanolamine is extremely hygroscopic; in this procedure, use only ethanolamine that has been handled to avoid introducing water.
- 20% sodium dodecyl sulfate (SDS), for blocking buffer (20% SDS is available from Ambion, Cat #9820). Prepare 20% SDS in nuclease-free water.

CAUTION
To avoid inhaling SDS, weigh it in a fume hood or while wearing a mask.

Equipment and supplies

Microarrayer

Any microarrayer capable of printing to glass slides

Incubators

- Heat block capable of 130°C
- 42°C waterbath incubator
- 50° C incubator, to preheat the 1 M Tris
- 50° C incubator (hybridization oven recommended), to block the slides

General laboratory equipment and supplies

- Microcentrifuge for microcentrifuge tubes
- Nuclease-free microcentrifuge tubes
- Adjustable pipettors, and nuclease-free tips

Array handling and scanning equipment

- Containers for array post-processing; we use sterile, disposable, Pap-Fix Plus™ Pap Jars with screw-cap lids, Evergreen Scientific #240-5400
- Lint-free or low-lint laboratory wipes, to clean and dry coverslips for hybridization of miRNA arrays.
  We recommend using lint-free wipes, such as those used in clean-rooms, because it considerably speeds up the process of removing dust particles from coverslips. However, ordinary low-lint laboratory wipes are adequate.
- Humidity chamber for rehydrating slides
- Array hybridization chamber; Corning® Hybridization Chamber #2551 recommended
- Slide racks and wash dishes
- Centrifuge or microcentrifuge with a slide carrier capable of drying slides
- Microarray scanner and image processing software

E. Related Products Available from Ambion

mirVana™ miRNA Labeling Kit Cat #1562	The *mir*Vana miRNA Labeling Kit employs a simple and highly efficient labeling strategy for universal fluorescence labeling of microRNA (miRNA) samples in preparation for microarray analysis. The kit was developed using CyDye™ fluorescent dyes (GE Healthcare), and is fully compatible with other amine reactive dyes. Reagents for purification of labeled miRNA from unincorporated nucleotides and other labeling reaction components are included, resulting in low background signal on miRNA arrays.
flashPAGE™ Fractionator Cat #13100	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.
mirVana™ miRNA Isolation Kit Cat #1560	The *mirVana* 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.
mirVana™ PARIS™ Kit Cat #1556	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 Cat #1975	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.

II. *mir*Vana™ miRNA Probe Set Protocol

A. Printing the *mir*Vana miRNA Probe Set

1. Resuspend each probe in 10 µl printing buffer

Resuspend the contents of each well of the *mir*Vana miRNA Probe Set in 10 µl of 3X SSC or other printing buffer appropriate for the glass substrate chemistry or array platform requirements. The final concentration of each probe will be 50 µM with a resuspension volume of 10 µl.

2. Print the miRNA array(s)

Configure the microarrayer according to the manufacturer's recommendations and print the desired number of arrays. Printed arrays can be stored in a dessicator until labeled miRNA samples are ready for hybridization.

Visual examination of array quality as the array is spotted

Experienced microarrayer operators can often evaluate array print runs as the spots are deposited on the slide. It is a good idea to evaluate arrays for the following quality criteria:

- Appropriate spot size (not too large or too small)
- Uniform spot size
- Presence of a spot at every intended location
- Absence of extra spots or other arrayer errors

Alternative printing buffer to create slightly larger spots

If you suspended the *mir*Vana miRNA Probe Set in 3X SSC, and the spot size is smaller than optimal, you can add SDS to the Probe Set for a final concentration of 0.01% SDS. This will result in slightly larger spots on Schott Nexterion Slide E slides than 3X SSC alone.

IMPORTANT

Once SDS is added to the miRNA Probe Set, it cannot be removed.

B. Storage and Re-Use of the *mir*Vana miRNA Probe Set

Storage of the *mir*Vana miRNA Probe Set after resuspension

Resuspended in printing buffer, the *mir*Vana miRNA Probe Set can be stored at –20°C overnight.

To store the *mir*Vana miRNA Probe Set longer than overnight, dry it to completion in a vacuum concentrator and seal the plate. Store the dried Probe Set at –80°C.

Re-use of the *mir*Vana miRNA Probe Set

To rehydrate the *mir*Vana miRNA Probe Set a second time, add nuclease-free water to bring each probe to its final volume before vacuum concentration. For example, if the Probe Set was initially dissolved in 10 µl, and 3 µl were used to print the first set of arrays, then it should now be dissolved in 7 µl of nuclease-free water.

If you freeze the *mir*Vana miRNA Probe Set in 3X SSC, incubate the plate at room temperature for 2 hr after thawing before use.

C. Array Post-Processing for Epoxy-Coated Slides

IMPORTANT

This post-processing protocol is suitable ONLY for arrays on epoxy-coated glass slides (such as SCHOTT Nexterion Slide E) printed with the miRNA Probe Set suspended in 3X SSC or in 3X SSC, 0.01% SDS. For other slide types and other spotting buffers, use associated post-processing procedures.

For best results, post-process arrays just before they will be hybridized for analysis; after hydration and blocking, arrays should not be stored for more than 2 days.

Array post-processing consists of two steps: rehydration and blocking.

Preheat solutions and incubators

- Preheat 0.5X SSC to 42°C, to hydrate the arrays in step 1
- Preheat a heat block to 130°C, to dry the arrays in step 2
- Preheat 1M Tris, pH 9 to 50°C, to prepare the blocking buffer in step 3
- Preheat an incubator, such as a hybridization oven, to 50°C, to block the slides in step 4

1. Rehydrate arrays in a humidity chamber with 0.5X SSC at 42°C

a. Place arrays in a humidity chamber containing 0.5X SSC that has been warmed to 42°C. Position the slides so that the spotted side is facing the SSC.

b. Incubate the arrays in the chamber until the spots glisten. This typically takes 30 sec–5 min.

2. Immediately dry arrays for 3 sec on a 130°C heat block

Immediately snap-dry the arrays by carefully, but quickly, placing the slides, array-side up, onto a heating block set at 130°C. Dry the slides on the heating block for ~3 sec, or just until all visible condensation disappears. It is important to avoid overdrying the arrays. Then, place the arrays in a slide rack for further processing.

3. Prepare blocking buffer

NOTE

We recommend using the blocking buffer defined in Table 2 because we find that it provides the cleanest, most robust data from miRNA arrays. For Nexterion Slide E slides, SCHOTT has developed and optimized Block E blocking buffer, which also produces adequate blocking.

Table 2. Blocking Buffer

Concentration	Component
1 M	Tris, pH 9, preheated to 50°C
100 mM	Ethanolamine
0.1 %	SDS

- Prepare the blocking buffer immediately before using it.
- We typically use sterile, single-use Pap Jars with screw cap lids for array post-processing. These jars hold 4–5 slides, and have a capacity of ~30–40 ml. Determine the volume of blocking buffer needed to fill your jars so that the slides will be completely immersed in solution.
- Blocking buffer cannot be re-used, so prepare enough blocking buffer for all of the arrays you intend to hybridize that day.

4. Submerge arrays in blocking buffer, then incubate 20 min at 50°C with frequent mixing

a. Fill a Pap Jar with freshly prepared 50°C blocking buffer.

b. Block the arrays by quickly submerging the slides in the blocking buffer. It is important to submerge the slides in the blocking buffer quickly for uniform blocking.

c. Incubate the arrays for 20 min at 50°C in blocking buffer, with constant agitation if possible. We typically clamp the Pap Jars into the roller bottle holders of a hybridization oven for this incubation.

If your incubator does not support constant agitation, agitate or invert the container at least every 5 min to mix well.

5. Rinse arrays 2 x 1 min in nuclease-free water

a. Pour off the blocking buffer, and fill the jar with room temperature nuclease-free water.

b. Wash thoroughly by inverting or gently shaking the container constantly for 1 min.

c. Decant the water and repeat this rinsing step with fresh nuclease-free water.

6. Centrifuge the arrays to dry them

a. Transfer the miRNA array slides to a centrifuge equipped with a slide holder. Alternatively, the entire slide rack can be centrifuged in a microtiter plate rotor adapter.

b. Start the centrifugation immediately after transferring the slides to the centrifuge.

- In a picofuge centrifuge for 1–2 min (speed is not adjustable).
- In a tabletop centrifuge, spin at 600 x g for 3 min.

D. (Optional) Print Validation

Instructions for dying the array to check quality

After hydration and blocking, the dimeric cyanine dye POPO-3 (Molecular Probes Invitrogen Detection Technologies) can be used to evaluate the quality of arrays before using them for hybridization. POPO-3 does not impact sample hybridization and thus, after removing it, the arrays can be used for hybridization. Also, POPO-3 has an absorption maximum of 532 nm and emission maximum at 570 nm, making it well suited for most microarray scanner platforms.

Stain the array following the "Staining DNA Microarrays for Quality Control" protocol suggested by the dye manufacturer:

www.probes.com/media/pis/mp03600.pdf

Scan the array using the settings for Cy3. POPO-3 should stain each spot on the array, making it easy to verify that all the spots are present, and that they are of uniform size and fluorescence intensity. Remove the POPO-3 dye following the manufacturer's instructions before using the array for miRNA analysis.

III. miRNA Array Hybridization and Washing

A. Preparation of Labeled Sample

Obtaining miRNA

Detailed information on obtaining miRNA samples for analysis on *mir*Vana miRNA arrays is provided in the Instruction Manual sent with the *mir*Vana miRNA Labeling Kit and is available for download from our website: www.ambion.com/catalog/CatNum.php?1562

In brief, our recommendations are the following:

1. 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 (Cat #1560) and the *mir*Vana PARIS™ Kit (Cat #1556).
2. Isolate the miRNA fraction using Ambion's innovative flashPAGE rapid electrophoresis system (Cat #13100, 9015, 10010, 12200). Alternatively miRNA can be obtained using traditional PAGE electrophoresis; we provide a protocol link on our website: www.ambion.com/catalog/CatNum.php?1562

Labeling miRNA with fluorescent dye(s)

We strongly recommend using the *mir*Vana miRNA Labeling Kit to prepare labeled miRNA samples for analysis on miRNA arrays spotted with the *mir*Vana miRNA Probe Set.

The *mir*Vana miRNA Probe Set was developed and extensively tested with CyDye™-labeled samples, but samples labeled with any of the following fluorescent dyes are compatible with analysis using miRNA arrays spotted with the *mir*Vana miRNA Probe Set:

- CyDye Post-Labelling Reactive Dye Pack, GE Healthcare, Product Codes: RPN 5661, 25-8010-80, 25-8010-79
- Molecular Probes Alexa Fluor® Reactive Dye Decapacks for Microarray Applications
  – Alexa Fluor 555, Cat #A32756
  – Alexa Fluor 594, Cat #A32751
  – Alexa Fluor 647, Cat #A32757

B. miRNA Array Hybridization

Incubators needed:

65°C incubator (for microcentrifuge tubes)

95°C incubator (for microcentrifuge tubes)

42°C waterbath incubator for array hybridization

1. Preheat 3X miRNA Hybridization Buffer to 65°C for >5 min

Heat the 3X miRNA Hybridization Buffer to 65°C for at least 5 min immediately before use. Vortex the tube a few times at maximum speed for ~15 sec during this preheating incubation. Even after heating and vortexing, you may see precipitate in the bottom of the tube; it will not affect hybridization.

Keep the solution at 65°C until use.

2. Clean a coverslip and place it over the printed miRNA array

We strongly recommend using raised-edge coverslips such as LifterSlips™ from Erie Scientific (Cat #22x22I-2-4788) for hybridization. They provide more robust and reproducible results than flat coverslips.

a. Clean a coverslip with 70% ethanol, and wipe it dry with a lint-free laboratory wipe. (Ordinary low-lint laboratory wipes can be used, but they will deposit some dust particles that must be removed in the next step.)

b. Using the same wipe, remove all dust particles from the coverslip.

IMPORTANT

Follow these instructions only if you are using a raised-edge coverslip, such as LifterSlips. If you are using a non-raised coverslip (not recommended), do not place the coverslip until after you pipet the miRNA hybridization mixture onto the array (see step 5.b on page 13).

c. Examine the coverslip to determine on which side the raised edges are located. We do this by carefully examining the reflection of overhead lights on the glass; the white strips that "lift" the coverslip away from the slide appear dull because they do not reflect the light.

d. Gently place the coverslip (raised edges down) over the miRNA array on the slide so that the raised edges align with the long edges of the slide.

3. Assemble the miRNA hybridization mixture with labeled miRNA and 1X final concentration miRNA Hybridization Buffer to cover the array

a. Using sample prepared with the *mir*Vana miRNA Labeling Kit, bring the volume of the labeled sample to 20 µl with nuclease-free water.

b. Add 10 µl of preheated 3X miRNA Hybridization Buffer.

When using LifterSlips from Eric Scientific as we suggest, ~30 µl of miRNA hybridization mixture will completely fill the space under the coverslip.

IMPORTANT

It is important to prepare enough miRNA hybridization mixture to completely fill the area under the coverslip. Also, the final concentration of miRNA Hybridization Buffer in the mixture must be 1X for optimal hybridization.

NOTE

If you are using another type of coverslip and are unsure of the volume required, use an extra cover slip and a microarray slide identical to the ones used for spotting your miRNA Probe Set to determine the volume of miRNA hybridization mixture needed to completely cover the array.

4. Heat to 95°C for 3 min, then let the mixture cool to room temp

a. Heat the miRNA hybridization mixture to 95°C for 3 min.

b. Briefly centrifuge to bring the sample to the bottom of the tube, and leave the mixture in the dark for ~1 min to allow it to cool to room temperature.

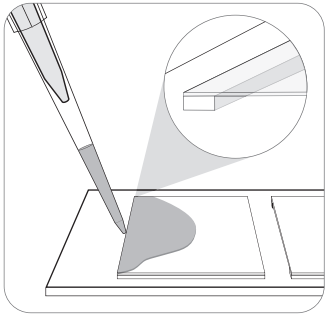
5. Hybridize for 12–16 hr at 42°C

a. Place the array slide(s) into a hybridization chamber (e.g., Corning® Product #2551).

b. Position the pipette tip at an open side of the coverslip and slowly pipette the entire miRNA hybridization mixture under the LifterSlip onto the array (see Figure 2). Do not reposition the pipette tip while you are dispensing the miRNA hybridization mixture as this may result in a trapped bubble.

If you are using a non-raised coverslip (not recommended), pipette the miRNA hybridization mixture onto the array first and then gently place a coverslip on top of the mixture.

Figure 2. Pipetting miRNA Hybridization Mixture Under the Coverslip



c. Add 1X Hybridization Buffer (dilute the 3X Hybridization Buffer to a 1X concentration with nuclease-free water) to the designated places in the chamber to maintain humidity during hybridization. This is critical for avoiding problems caused by drying out of the miRNA hybridization mixture.

d. Check the position of the coverslips, and gently push them back into position if they have drifted. Then seal the hybridization chamber completely.

e. Place the sealed hybridization chamber in a 42°C waterbath (resting on the floor of the waterbath) and incubate for 12–16 hr.

If the waterbath has a clear cover, or a hole for a thermometer, cover the lid to prevent light from entering the waterbath during hybridization.

C. Washing miRNA Arrays

IMPORTANT

During the wash procedure, carefully avoid exposing the slides to air for more than 1–2 sec. Longer exposures typically result in drying, which causes the formation of precipitates on the array. It is also very important to complete the wash procedure quickly (i.e., in 15 min or less), and in as little light as possible (to avoid photobleaching of the labeled sample).

1. Prepare array wash solutions

- Assemble 2 slide racks and 3 slide wash containers, and determine the volume of wash solution needed to fill the containers so that the slides in a slide rack will be completely immersed in solution.
- You will need 1 container of Low Stringency Wash, and two containers of High Stringency Wash.
- Starting with the nuclease-free water, assemble the wash solutions and mix thoroughly.

Table 3. Wash Solution Recipes for 400 ml Wash Containers

Low Stringency Wash	High Stringency Wash	
376 ml	796 ml	**Nuclease-free Water**
4 ml	– –	**Detergent Concentrate**
20 ml	4 ml	**Salt Concentrate**

2. Submerge miRNA array in Low Stringency Wash and wash for ~30 sec

a. Remove the hybridized miRNA array slide from the hybridization chamber and submerge it in Low Stringency Wash at room temperature. The cover slip will disengage from the slide and fall to the bottom of container. Quickly place the slide in a slide rack in the wash container.

b. Leave the slide in the Low Stringency Wash, and wash at room temperature for ~30 sec by dipping the slide rack up and down in the container of wash solution or with gentle stirring (150–200 rpm).

3. Change slide rack and wash in High Stringency Wash for 30 sec with gentle stirring

a. Transfer the miRNA array to a clean slide rack that is already submerged in the High Stringency Wash, being careful not to expose the array to the air for more than 1–2 seconds.

b. Wash in High Stringency Wash at room temperature for 30 sec by dipping the slide rack up and down in the container of wash solution or with gentle stirring (150–200 rpm).

4. Wash in fresh High Stringency Wash for 30 sec

Transfer the slide rack with the miRNA array to a clean container of fresh High Stringency Wash, and wash for 30 sec by dipping the slide holder up and down in the container of wash solution or with gentle stirring (150–200 rpm).

5. Centrifuge the slide to dry it

a. Rapidly transfer the miRNA array slide to a centrifuge equipped with a slide holder. Alternatively, the entire slide rack can be centrifuged in a microtiter plate rotor adapter.

b. Start the centrifugation immediately after transferring the slides to the centrifuge. At Ambion, we typically cover the top of the centrifuge to keep out the light.

- In a picofuge, centrifuge for 1–2 min (speed is not adjustable).
- In a tabletop centrifuge, spin at 600 x g for 3 min.

D. Image Acquisition

Digital images are made up of many small square pixels, each with its own intensity value. The resolution of an image is the total number of pixels and is expressed as the number of rows and columns of pixels in the image (e.g., 1600 x 1200). The number of possible values for an individual pixel is determined by the color depth; 16 bits/pixel is typical for array analysis systems. This means that individual pixels can have intensity values ranging from 0 to 65,535. Pixels exhibiting the maximum intensity value or higher are "saturated" and are assigned the intensity value 65,535. In other words, all signals at the maximum intensity value and higher are assigned the same value.

Scanning is the process of illuminating a microarray with laser light and collecting the light emitted by bound fluorescent dye. The laser is set to emit the wavelength that excites the fluorescent dye used to label the sample, and the resulting fluorescence is then measured. Any of a num-

ber of commercially available scanners can be used to collect and analyze microarray data. Ambion uses the Axon® GenePix 4000B scanner and associated GenePix software.

Scan Settings

Resolution setting
Scan arrays at 5–10 µm resolution.

Power Setting
Scan at 100% power.

PMT settings
For hybridization experiments using two different fluorescent dyes on one array, it is important to adjust the laser settings to maximize the dynamic range of the data. To do this, adjust the photomultiplier tube (PMT) settings of your scanner in each channel to achieve the following results.

- The highest intensity spots are just below saturation, or contain only a few saturated pixels.
- The overall signals from the two samples are approximately equal. For two color analysis with Cy3 and Cy5, the Cy3 (green) channel is usually scanned at a slightly lower PMT setting than the Cy5 (red) channel. Do not scan the two channels at PMT settings that differ by more than 20% or the resulting data may be skewed. If the PMT settings for the two channels need to be set more than 20% apart to yield approximately equal signal from the two samples, it indicates that there may be a fundamental problem with the hybridization experiment. See section IV.A on page 17 for troubleshooting suggestions.

NOTE
If you are using GenePix analysis software, you can use the "Histogram" tab to show the overall signal intensities from each channel.

E. Evaluating *mir*Vana miRNA Array Data

Currently accepted best practice for evaluating array data is constantly being refined. To provide the most timely information, we provide suggestions and guidelines for data evaluation on our miRNA Array Web Resource at: www.ambion.com/miRNA/array

IV. Troubleshooting *mir*Vana miRNA Microarray Analysis

A. Low Overall Fluorescence Signal Intensity

Consider these suggestions if the average signal intensity from both your sample RNA and the Positive Control miRNA are very low (<500 RFU).

1. Troubleshoot miRNA sample labeling

The most common cause of low overall fluorescence signal is poor labeling of the miRNA sample. Troubleshooting suggestions for the *mir*Vana miRNA Labeling Kit are provided in the Instruction Manual supplied with the product, and available on our website at

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

2. Excessive exposure of the labeled sample to light

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 clean-up, array hybridization, and array washing in the dark when possible.

3. 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. Mark your slides as you spot them so that orientation can be easily determined later.

4. Hybridization problems

a. Incorrect ratio of miRNA Hybridization Buffer to miRNA sample

The dilution of 3X miRNA Hybridization Buffer with labeled miRNA sample must be precise so that the final concentration is 1X miRNA Hybridization Buffer. This ensures the proper formamide concentration for optimal hybridization stringency.

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

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.

5. Nuclease-contaminated tubes, tips, or equipment

Using pipettte 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's RNase*Zap* (Cat #9780).

B. Array Signal Not Associated with Probe

If your array image shows blotchy spots, signal that is not associated with probe spots, and/or high background, consider the following troubleshooting suggestions.

1. Partial or complete drying of the hybridization reaction on the array slide

- Exposure to air during washing is a common problem encountered by people attempting array analysis for the first time. When miRNA arrays are exposed to air for more than 1–2 sec, it can allow partial drying of the array and problems with blotches, streaks, and dots of signal that are not associated with probe.
- Make sure that the repositories in the hybridization chamber are filled with 1X miRNA Hybridization Buffer to maintain humidity.
- Make sure that the area under the coverslip is completely filled with miRNA hybridization mixture before starting the hybridization.
- Be sure to check the position of the coverslip over each miRNA array just before sealing the hybridization chamber. If a coverslip gets too close to the edge of the slide, the miRNA hybridization mixture can wick under the slide, leaving the miRNA array with insufficient hybridization mixture.

2. Incomplete washing

If there was a problem with washing the array, then repeating the entire wash procedure may help remove the excess signal from the array. Following are some precautions to observe in subsequent experiments to avoid having to re-wash your array:

- Follow the washing procedure exactly, and be sure to completely submerge the miRNA array in wash buffer at each step.
- Be sure to put the array slides into a clean slide rack when switching them from the Low Stringency Wash to the High Stringency Wash. Otherwise detergent may be carried over, causing detergent residue on the slides.

C. High Signal from More Probes Than Expected

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 and purified miRNA. The solution should be clear or lightly tinted. More intense color likely means that uncoupled dye is contaminating the labeled miRNA sample, 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. Low stringency hybridization conditions

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. Total RNA was used to prepare labeled miRNA sample

Labeling total RNA can lead to nonspecific binding to the relatively short probes used for miRNAs. We recommend purifying miRNAs using the flashPAGE Fractionator before labeling to eliminate non-miRNAs from the sample before analysis with the *mir*Vana miRNA Array System. Alternatively miRNA can be isolated using traditional gel electrophoresis; a detailed protocol is available on our website at the following address:

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

V. Appendix

A. *mir*Vana miRNA Probe Set Specifications

Kit contents and storage conditions

Properly stored kits are guaranteed for 6 months from the date received. Note that the kit is shipped at room temperature, which will not affect its stability.

Amount	Component	Storage
1	miRNA Probe Set (more information below)	–20°C
200 ml	Detergent Concentrate	room temp
2 x 500 ml	Salt Concentrate	room temp
5 x 2 ml	3X miRNA Hybridization Buffer	4°C

The *mir*Vana miRNA Probe Set is provided in 96 well plates; each well contains 500 pmol of dried amine-modified oligonucleotide. A link for the complete list of miRNA probes and controls included in the kit is posted at the following web address:

www.ambion.com/catalog/CatNum.php?1564v1

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from our website by going to the following address and clicking on the link for the *mir*Vana miRNA Probe Set:
  www.ambion.com/techlib/msds
- Alternatively, e-mail us at MSDS@ambion.com to request MSDSs by e-mail, fax, or ground mail. Specify the Ambion catalog number of the kit(s) for which you want MSDSs and whether you want to receive the information by e-mail, fax, or ground mail. Be sure to include your fax number or mailing address as appropriate. If the mode of receipt is not specified, we will e-mail the MSDSs.
- Customers without internet access can contact our technical service department by telephone, fax, or mail to request MSDSs (contact information on the back of this booklet).

B. Quality Control

Functional testing

Each miRNA probe and control is evaluated by MALDI-TOF or ESI-analysis. The quantity of each miRNA probe and control is assessed before filling the *mir*Vana miRNA Probe Set.

Nuclease testing

Each component supplied as a solution is tested in Ambion's rigorous nuclease assays:

RNase activity

None detected after incubation with ^{32}P-labeled RNA; analyzed by PAGE.

Non-specific endonuclease/nickase activity

None detected after incubation with supercoiled plasmid DNA; analyzed on a 1% agarose gel.

Exonuclease activity

None detected after incubation with ^{32}P-labeled *Sau*3A fragments of pUC19; analyzed by PAGE.