Signosis
Innovative Plate Assay Solutions

Human MicroRNA Array II

Catalog Number AP-0002 **(For Research Use Only)**

Introduction

Newly discovered microRNAs (miRNAs) are important to the regulation of gene expression (1). Up to 30% of mammalian genes might be regulated by miRNAs. So far, more than 400 miRNAs have been identified in the human genome and many of them are different only in one or a few nucleotides. Expression of mature miRNAs is tissue-specific (2) and the abundance of miRNAs varies in several orders of magnitude (3). More importantly, mis-regulation of miRNA expression might contribute to human cancers (4). Systematic profiling of miRNA expression displays unique signatures in a number of cancers (5).

Based on its proprietary technology, Signosis has developed a highly sensitive and discriminative array technology for monitoring miRNA. It combines oligo-ligation assay-based detection and T7 transcription-based linear amplification, providing a highly sensitive and specific array assay. Via ligation, selection, and transcription of a pair of oligos that are hybridized to each specific miRNA, the targets are amplified and array monitored. By assigning unique tag sequences to individual isoforms, they can be easily differentiated. The whole procedure is simple and straightforward. We are currently offering an array that targets the 60 most well studied miRNAs, each with cited literature, which is able to facilitate the comparison and discovery of differentiated miRNA expression in different samples.

Principles of the technology

miRNAs are different from large messenger RNAs in three aspects; (1) miRNAs are small size molecules with quite a big difference in abundance, (2) mature miRNAs co-exist with their precursor pre-miRNA and pri-miRNA, differing only in length, and (3) many miRNAs are very closely related in sequences, such as isoforms, differing by only one or a few nucleotides. Therefore, the conventional mircoarray technologies cannot directly be applied to analyzing these molecules. A number of miRNA microarray products are commercially available, but they are either tedious in requiring pre-isolation of microRNA, lack discriminative power to differentiate isoforms, or are not sensitive enough to monitor low abundant miRNAs.

In our array assay, each miRNA molecule is targeted by two oligos, each that hybridizes a half molecule of the target miRNA to form a RNA/DNA duplex. When the sequences are perfectly matched, they are aligned with the miRNA and the joint can be ligated by DNA ligase (figure 1). A single nucleotide difference among miRNAs will block either the hybridization or the ligation, so that miRNA isoforms can be differentiated. Due to the small size of miRNA, the hybrid might not be stable; therefore we introduce the stacking sequences. By extending these two oligos along with their complementary oligos the stability is increased. Once the pair of oligos is ligated, the ligated molecules are subjected to linear amplification via T7 transcription into RNA in the presence of biotin-UTP, which are used as probes for array hybridization. To differentiate each isoform, we assigned unique tag sequences to the ligation oligos, so that single nucleotide differences are converted into unique tag sequences. Therefore, each isoform can be easily distinguished by array hybridization.

We offer the miRNA profiling assay kit to profile the expression of the 60 most popular miRNAs and their isoforms. The procedure is simple and straight forward, including three steps: (1) mix the total RNA with provided oligos to form miRNA/oligo hybrids; (2) select the hybrids and remove free oligos, and ligate miRNA-directed pairing of oligos to become a single DNA; and (3) amplify the ligated DNA with T7 transcription.

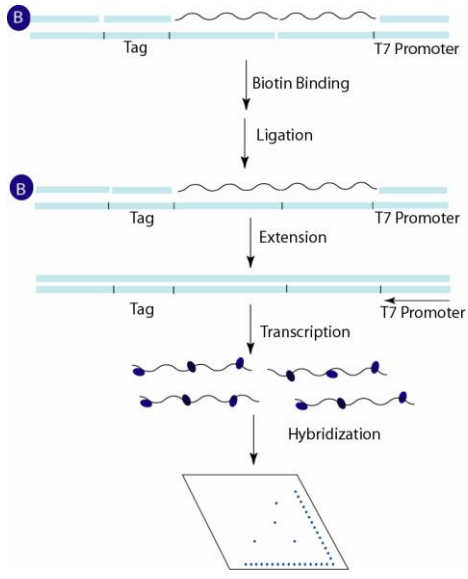


Figure 1: Diagram of miRNA array assay

Materials provided with the kit

15 µl Oligo Mix II (-20ºC)
5 µl Array Detection Oligo (-20ºC)
400 µl Annealing buffer (RT)
15 µl Magnetic streptavidin beads (4 ºC)
120 µl Beads binding buffer (RT)
1.0 ml Bead wash buffer (RT)
6 µl Ligase (-20ºC)
250 µl Ligation buffer (-20ºC)
60 µl Extension mix (-20ºC)
60 µl Labeling mix (-20ºC)
6 µl T7 RNA polymerase (-20ºC)
30ml 1x Hybridization buffer (RT)
30ml 5x Hybridization wash buffer (RT)
60ml Blocking buffer (4 ºC)
50 µl Streptavidin-HRP conjugate (4 ºC)
40ml 5x Detection wash buffer (RT)
1.8ml Substrate A (4 ºC)
1.8ml Substrate B (4 ºC)
3 Array membranes (RT)
3 Detection sheets (RT)

Material required but not provided

Magnetic stand (96 well plate)
RNase free water
PCR machine
Hybridization oven
Washing tray
50ml Centrifuge tubes (Corning tubes are recommended, cat#430290) or hybridization bottles
0.2ml PCR tube
Alpha Innotech image or equivalent image system or X-ray film

Reagent preparation before starting experiment

- Dilute the 5x Hybridization washing buffer and 5x Detection washing buffer to 1x buffer
  1x Hybridization washing buffer:
  30ml 5x Hybridization washing buffer
  120ml ddH2O
  1x Detection washing buffer:
  40ml 5x Detection washing buffer
  160 ml ddH2O
- Prewarm 1x Hybridization buffer, Blocking Buffer, 1x Hybridization wash buffer at 42ºC for 1 hour or until the buffers are clear without visible precipitation before using.
- Pre-hybridization can be done during T7 RNA transcription at the Step 4.

1. Annealing of miRNA with Oligo mix

(1) Sample preparation
X µl 5ug total RNA or 10ng isolated miRNA
5 µl Oligo mix II
1 µl Array Detection Oligo
20 µl Annealing buffer
X µl ddH2O

40ul

(2) Incubate on PCR machine at 72ºC for 5 minutes and 53ºC for 90 minutes.

2. Selection of miRNA/oligo hybrids

(1) Beads washing

- Resuspend the beads by gently tapping the tube to obtain a homogeneous suspension.
- Transfer 5 µl of the beads to a 0.2ml PCR tube (the size of the tube that should fit into the magnetic stand.
- Add 100 µl of annealing buffer to the tube and then place onto the magnetic stand for 30 seconds.
- Aspirate out the liquid.
- Remove the tube from the magnetic stand.

(2) Beads selection

- Add 40 µl of the Bead binding buffer to 40 µl annealed miRNA/oligo hybrid mix from Step 1, transfer to the tube containing the washed beads from Step 2(1) and resuspend the beads in the solution.
- Incubate at 37ºC for 30 minutes.
- Place the bead mixture on the magnetic stand for 30 seconds, and aspirate out the buffer. The beads will remain on the side of the tube.
- Remove the tube from the magnetic stand and add 100 µl of the Bead wash buffer to resuspend the beads, pipette gently up and down, and place the tube on the magnetic stand for 30 seconds, aspirate the buffer.
- Repeat the washing step once.

3. Ligation of miRNA-directed oligos to form a single molecule

(1) Add 50 µl of the Ligation buffer to resuspend the beads, pipette gently up and down, then place the tube on the magnetic stand for 30 seconds and aspirate the buffer.
(2) Remove the tube from the magnetic stand. Add 20 µl of ligation buffer to resuspend the beads, then add 2µl of Ligase to the resuspended beads and incubate at 37º for 90 minutes.

4. T7 RNA transcription of ligated molecule

(1) Add 100 µl Bead washing buffer directly to 20 µl ligat reaction mix from Step 3, place the tube on the magnetic stand 30 seconds, and aspirate the buffer.
(2) Remove the tube from the magnetic stand and add 20 µl of Extension mix to resuspend the beads.
(3) Incubate the mixture on PCR machine at 94ºC fr 2 minutes, 54ºC for I minute, 72ºC for 1.5 minute, and 94ºC for 30 seconds.
(4) Place the reaction tube on the magnetic stand for 30 seconds. Immediately transfer the 20 µl of the extension mix to a fresh tube (keep the solution and toss the beads).
(5) Add 20µl of Labeling mix and 1 µl of T7 RNA polymerase to the tube.
(6) Incubate the mixture at 37ºC for 1 hour.
(7) The transcribed RNA is ready for hybridization.

5. Pre-hybridization and hybridization

(1) Place each array membrane into a 50 ml tube. Wet the membrane by filling the tube with dH_2O, then carefully decant the water. The side of the membrane with the spotted oligos should face into the middle of the tube.
(2) Add 4 ml of prewarmed 1x Hybridization buffer to each tube. Incubate the tubes in a hybridization oven at 42ºC for at least 30-60 minutes.
(3) Decant the hybridization buffer and replace with 4ml of prewarmed 1x Hybridization buffer. Add 40 µl of transcribed RNA to prehybridized membrane and incubate overnight in a hybridization oven at 42ºC.
(4) Decant the hybridization mixture from each tube and wash each membrane as follows:

- Rinse the membrane with 20 ml Hybridization washing buffer, and decant liquid.
- Incubate the membrane with 20 ml Hybridization wash buffer at 42ºC for 20 minutes. Decant liquid.

6. Detection

(1) Using forceps, carefully transfer the membrane from the hybridization tube to a container (an empty 200 µl pipette tips box). Each box could have two membranes, one at each side of the box.
(2) Rinse the membrane with 10 ml of 1X Detection wash buffer.
(3) Block the membrane with 15 ml of Blocking buffer for 30 minutes at room temperature with moderate shaking.
(4) Dilute 15 µl of Streptavidin-HRP conjugate with 1 ml of the 1X Blocking buffer and transfer to the container. **Do not** add HRP diluted solution directly onto the membrane.
(5) Continue shaking the membrane for 45 min at room temperature.
(6) Decant the Blocking buffer and wash three times at room temperature with 15 ml of 1x Detection washing buffer, 10 minutes each wash.
(7) Mix equal amounts of Substrate A and B. Place the membrane on the bottom side of detection sheet on a flat surface and overlay the membrane with 1 ml of substrate solution. To ensure that the solution remains evenly distributed over the membrane when enveloped by the detection sheet: gently lower the top side of the detection sheet halfway over the membrane then pull back up slightly to allow the solution to flow back over the membrane. Then slowly lay the top sheet down completely without trapping air bubbles. Incubate at room temperature for 5 minutes.
(8) Remove excess substrate by gently applying pressure over the top sheet using a paper towel. Expose the membranes using either Hyperfilm ECL (2-10 min) or a chemiluminescence imaging system (i.e., FluorChem imager from Alpha Innotech). With either method, experiment with different exposure times.
(9) Use the schematic diagram of human miRNA array I to identify the spots on the array.

Example of miRNA array analysis

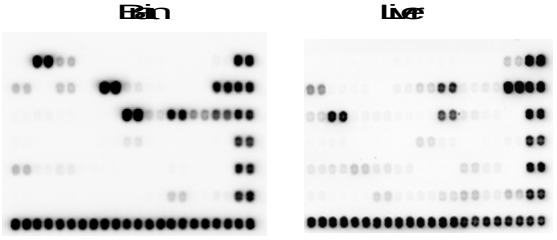
Trouble Shooting

Signals are too weak

- Total RNA may not contain small RNA
- RNA may be degraded
- If signals of the alignment spots are weak as well, the incubation of the membrane with Streptavidin HRP conjugate may be too short or the exposure time may be too short.

Uneven background

- Substrate was not evenly overlaid on the membrane



5µg total RNA was used for miRNA array assay and hybridization was detected with a chemiluminescence imaging system.

References

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2. Poy, et. al. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature.* 432:226–230.
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4. Calin, et. al. (2002) Frequent deletions and down-regulation of micro-RNA genes *miR15* and *miR16* at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. USA.* 99**:**15524–15529.
5. Calin, et. al. (2004) MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc. Natl Acad. Sci. USA.* 101**:**11755–11760.

Schematic diagram of human miRNA array II

miR-9-1-5p	**miR-10b-5p**	**miR-17-3p**	**miR-22-3p**	**miR-23a-3p**	**miR-24-3p**	**miR-26a-5p**	**miR-26b-5p**	**miR-27a-3p**	**miR-27b-3p**
miR-29a-3p	**miR-29b-3p**	**miR-29c-3p**	**miR-30a-3p**	**miR-30a-5p**	**miR-30b-5p**	**miR-30c-5p**	**miR-92-3p**	**miR-92b-3p**	**miR-92-3p**
miR-95-3p	**miR-101-1-3p**	**miR-103-3p**	**miR-106a-5p**	**miR-106b-5p**	**miR-107**	**miR-128a-3p**	**miR-128b-3p**	**miR-132-3p**	**miR-134-5p**
miR-135b-5p	**miR-136-5p**	**miR-137**	**miR-140-5p**	**miR-141-3p**	**miR-142-3p**	**miR-149-5p**	**miR-150-5p**	**miR-151-3p**	**miR-153-3p**
miR-154-5p	**miR-181d-5p**	**miR-183-5p**	**miR-185-5p**	**miR-186-5p**	**miR-188-5p**	**miR-190-5p**	**miR-191-5p**	**miR-196a-5p**	**miR-196b-5p**
miR-197-3p	**miR-198**	**miR-200b-3p**	**miR-202-5p**	**miR-203a**	**miR-205-5p**	**miR-210-3p**	**miR-214-3p**	**miR-215-5p**	**miR-218-5p**
miR-219	**miR-221-3p**	**miR-222-3p**	**miR-296-5p**	**miR-372-3p**	**miR-373-5p**	**miR-488-5p**	**miR-100-5p**	**miR-127**	**miR-142-5p**
miR-31-5p	**miR-181a-3p**	**U6**							