Signosis
BioSignal Capture

Human MicroRNA Array IV

Catalog Number AP-0009 **(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. Systematic profiling of miRNA expression displays unique signatures in a number of cancers.

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 miRNA array IV that targets 100 miRNAs to expand our miRNA array product line.

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.

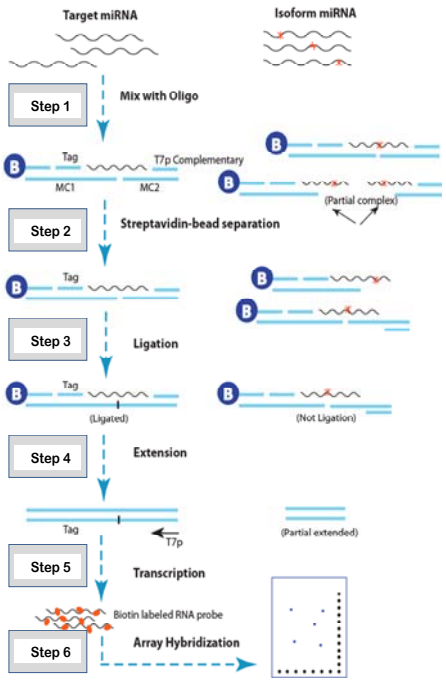


Figure 1: Diagram of miRNA array assay

Materials provided with the kit

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

Assay Procedure

1. Annealing of miRNA with Oligo mix

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

40ul

(2) Incubate on a PCR machine at 72°C for 5 minutes and 53°C 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 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, place onto the magnetic stand for 30 seconds.
- Aspirate out the liquid.
- Remove the tube from magnetic stand.

(2) Beads selection
- Add 40 µl of Bead binding buffer to 40 µl annealed miRNA/oligo hybrid mix from Step 1, transfer to the washed beads and resuspend the beads in the solution.
- Incubate at 37°C 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 tube.
- Remove the tube from the magnetic stand and add 100 µl of 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 Ligation buffer to resuspend the beads, pipette gently up and down, 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 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 ligation reaction mix from Step 3, place the tube on the magnetic stand for 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 94°C for 2 minutes, 54°C for I minute, 72°C for 1.5 minute, and 94°C 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 37°C for 1 hour.
(7) The transcribed RNA is ready for hybridization.

5. Pre-hybridization and hybridization

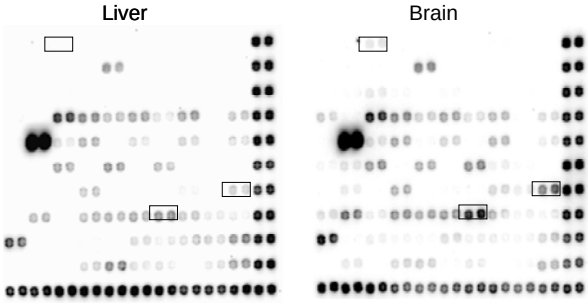
(1) Place each array membrane into a 50 ml tube. Wet the membrane by filling the tube with dH_2O, then carefully decant the water. The side of the membrane with the spotted oligos should face into the middle of the tube.
(2) Add 4 ml of prewarmed 1x Hybridization buffer to each tube. Incubate the tubes in a hybridization oven at 42°C 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 42°C.
(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 42°C 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



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

Schematic diagram of human miRNA array IV

miR-138	miR-139	miR-105	miR-130a	miR-135a	miR-147	miR-152	miR-154#	miR-184	miR-187	
miR-189	miR-211	miR-212	miR-217	miR-22	miR-220	miR-299-3p	miR-299-5	miR-301	miR-302a	
miR-302a#	miR-302b	miR-302b#	miR-302c	miR-302c#	miR-302d	miR-30d	miR-30e-3	miR-30e-5	miR-32	
miR-320	miR-323	miR-324-3p	miR-324-5p	miR-325	miR-326	miR-328	miR-329	miR-33	miR-330	
miR-331	miR-335	miR-337	miR-338	miR-339	miR-33b	miR-340	miR-345	miR-346	miR-34b	
miR-34c	miR-361	miR-362	miR-363	miR-363#	miR-365	miR-367	miR-369-3	miR-369-5	miR-370	
miR-371	miR-373#	miR-374	miR-376a	miR-376a#	miR-376b	miR-377	miR-378	miR-379	miR-380-3p	
miR-380-5p	miR-381	miR-382	miR-383	miR-384	miR-409-5p	miR-410	miR-411	miR-412	miR-422a	
miR-422b	miR-423	miR-424	miR-425	miR-425-5p	miR-429	miR-432	miR-432#	miR-433	miR-448	
miR-449	miR-449b	miR-450	miR-451	miR-452	miR-452#	miR-453	miR-455	miR-483	U6	

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