



Human Apoptosis-Associated miRNA Array

Catalog Number AP-0004

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Introduction

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death, including changes to the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. It plays important roles in removing unwanted cells during development and homeostasis. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. MicroRNAs are newly discovered small and non-coding RNA molecules that regulate up to 30% of mammalian gene expression [1]. Numerous miRNAs have been revealed to influence on the regulation of apoptotic cell signaling such as overexpression of miR-145, miR-216, miR-182, and miR-96 miRNA to decrease caspase-3 activation [2]. Signosis developed a miRNA array for targeting 52 miRNAs that have been shown in literature to involve in apoptosis [2-5]. Systematic profiling of the expression of these miRNAs will facilitate in uncovering the regulation of miRNA in apoptosis.

Based on its proprietary technology, Signosis has developed a highly sensitive and discriminative array technology for monitoring miRNA. By combining oligo-ligation assay-based detection and T7 transcription-based linear amplification, we are 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 the array monitored. In assigning unique tag sequences to individual isoforms, they can be easily differentiated. The whole procedure is simple and straightforward.

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 microarray 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, one miRNA molecule is targeted by two oligos, each of which hybridizes with the target miRNA to form an 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 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 human apoptosis miRNA array kit to profile the expression of 52 apoptosis-associated miRNAs and isoforms. The procedure is simple and straightforward, including three steps: (1) mix the total RNA with the provided oligos to form miRNA/oligo hybrids; (2) select the hybrids and remove the free oligos, and ligate the miRNA-directed pairing of oligos to become a single DNA; and (3) Amplify the ligated DNA with T7 transcription.

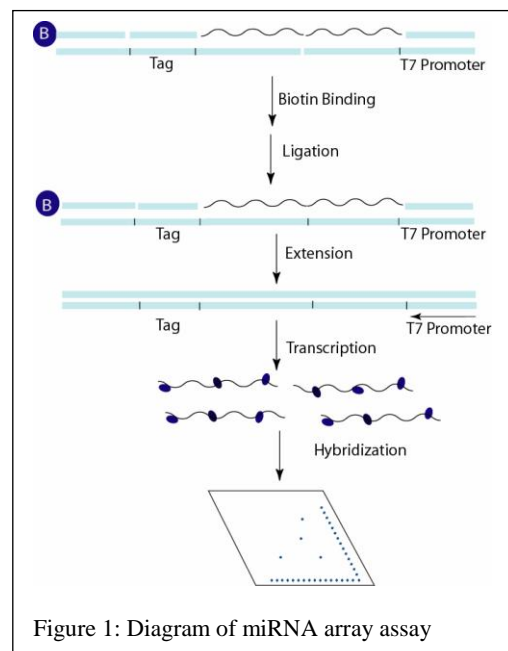


Figure 1: Diagram of miRNA array assay

Materials provided with the kit

15 µl Apoptosis Oligo Mix (-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 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)
1.8 ml Substrate A (4 °C)
1.8 ml Substrate B (4 °C)
3 Apoptosis 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 42°C 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 Apoptosis Oligo Mix
1 µl Array Detection Oligo
20 µl Annealing buffer
X µl ddH₂O

40ul

(2) Incubate on 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 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 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 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 37° for 90 minutes.

4. T7 RNA transcription of ligated molecule

- (1) Add 100 μ l Bead washing buffer directly to 20 μ l 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 μ l of Extension mix to resuspend the beads.
- (3) Incubate the mixture on PCR machine at 94°C for 2 minutes, 54°C for 1 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 μ l of the extension mix to a fresh tube (keep the solution and toss the beads).
- (5) Add 20 μ l of Labeling mix and 1 μ l 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 dH₂O, 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 μ l 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 μ l 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 μ l 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

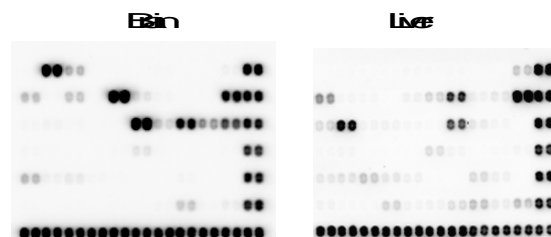
Trouble Shooting

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



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

References

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5. Wang, Y., et. al. (2005) Profiling MicroRNA Expression in Hepatocellular Carcinoma Reveals MicroRNA-224 Up-regulation and Apoptosis Inhibitor-5 as a MicroRNA-224-specific Target. *JBC.* 283:13205-13215.

Schematic Diagram of Human Apoptosis-Associated miRNA Array

Let-7a	Let-7b	Let-7c	Let-7d	Let-7e	Let-7f	Let-7g	Let-7i	miR-1	miR-7
miR-10a	miR-15a	miR-15b	miR-16	miR-21	miR-25	miR-28	miR-133b	miR-145	miR-148
miR-155	miR-182	miR-204	miR-216	miR-224	miR-368	miR-24	miR-101	miR-142	miR-150
miR-151	miR-153	miR-188	miR-196a	miR-197	miR-202	miR-210	miR-214	miR-218	miR-296
miR-144	miR-152	miR-184	miR-337	miR-338	miR-345	miR-371	miR-96	miR-369-5p	miR-369-3p
miR-193a	miR-193b	RUN48							