



Cancer MicroRNA Array

Catalog Number AP-0003

(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 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, 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.

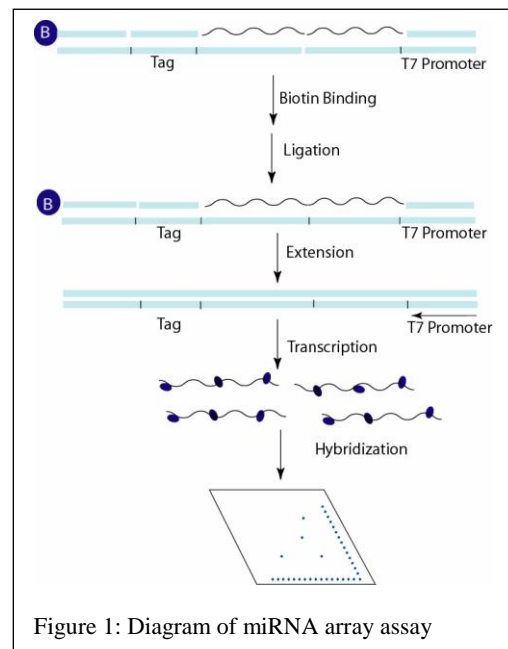


Figure 1: Diagram of miRNA array assay

Materials provided with the kit

15 µl Cancer 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)
1.0 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 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.

11. Remove the sealing film from the plate.

1. Annealing of miRNA with Oligo mix

(1) Sample preparation
X µl 5ug total RNA or 10ng isolated miRNA
5 µl Cancer 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

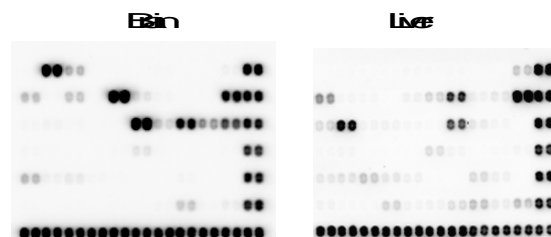
Trouble Shooting

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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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 cancer miRNA array

let7a	let7b	let7c	let7d	let7e	let7f	let7g	let7i	miR1	miR7
miR9	miR9a	miR9a	miR9b	miR9c	miR9d	miR9e	miR9f	miR9g	miR9h
miR9a	miR9b	miR9c	miR9d	miR9e	miR9f	miR9g	miR9h	miR9i	miR9j
miR9k	miR9l	miR9m	miR9n	miR9o	miR9p	miR9q	miR9r	miR9s	miR9t
miR9u	miR9v	miR9w	miR9x	miR9y	miR9z	miR9aa	miR9ab	miR9ac	miR9ad
miR9ae	miR9af	miR9ag	miR9ah	miR9ai	miR9aj	miR9ak	miR9al	miR9am	miR9an
miR9ao	miR9ap	miR9aq	miR9ar	miR9as	miR9at	miR9au	miR9av	miR9aw	miR9ax
miR9ay	miR9az	miR9ba	miR9bb	miR9bc	miR9bd	miR9be	miR9bf	miR9bg	miR9bh
miR9bi	miR9bj	miR9bk	miR9bl	miR9bm	miR9bn	miR9bo	miR9bp	miR9bq	miR9br
miR9bs	miR9bt	miR9bu	miR9bv	miR9bw	miR9bx	miR9by	miR9bz	miR9ca	miR9cb
miR9cc	miR9cd	miR9ce	miR9cf	miR9cg	miR9ch	miR9ci	miR9cj	miR9ck	miR9cl
miR9cm	miR9cn	miR9co	miR9cp	miR9cq	miR9cr	miR9cs	miR9ct	miR9cu	miR9cv
miR9cw	miR9cx	miR9cy	miR9cz	miR9da	miR9db	miR9dc	miR9dd	miR9de	miR9df
miR9dg	miR9dh	miR9di	miR9dj	miR9dk	miR9dl	miR9dm	miR9dn	miR9do	miR9dp
miR9dq	miR9dr	miR9ds	miR9dt	miR9du	miR9dv	miR9dw	miR9dx	miR9dy	miR9dz
miR9ea	miR9eb	miR9ec	miR9ed	miR9ee	miR9ef	miR9eg	miR9eh	miR9ei	miR9ej
miR9ek	miR9el	miR9em	miR9en	miR9eo	miR9ep	miR9eq	miR9er	miR9es	miR9et
miR9eu	miR9ev	miR9ew	miR9ex	miR9ey	miR9ez	miR9fa	miR9fb	miR9fc	miR9fd
miR9fe	miR9ff	miR9fg	miR9fh	miR9fi	miR9fj	miR9fk	miR9fl	miR9fm	miR9fn
miR9fo	miR9fp	miR9fq	miR9fr	miR9fs	miR9ft	miR9fu	miR9fv	miR9fw	miR9fx
miR9fy	miR9fz	miR9ga	miR9gb	miR9gc	miR9gd	miR9ge	miR9gf	miR9gg	miR9gh
miR9gi	miR9gj	miR9gk	miR9gl	miR9gm	miR9gn	miR9go	miR9gp	miR9gq	miR9gr
miR9gs	miR9gt	miR9gu	miR9gv	miR9gw	miR9gx	miR9gy	miR9gz	miR9ha	miR9hb
miR9hc	miR9hd	miR9he	miR9hf	miR9hg	miR9hh	miR9hi	miR9hj	miR9hk	miR9hl
miR9hm	miR9hn	miR9ho	miR9hp	miR9hq	miR9hr	miR9hs	miR9ht	miR9hu	miR9hv
miR9hw	miR9hx	miR9hy	miR9hz	miR9ia	miR9ib	miR9ic	miR9id	miR9ie	miR9if
miR9ig	miR9ih	miR9ii	miR9ij	miR9ik	miR9il	miR9im	miR9in	miR9io	miR9ip
miR9iq	miR9ir	miR9is	miR9it	miR9iu	miR9iv	miR9iw	miR9ix	miR9iy	miR9iz
miR9ja	miR9jb	miR9jc	miR9jd	miR9je	miR9jf	miR9jg	miR9jh	miR9ji	miR9jj
miR9jk	miR9jl	miR9jm	miR9jn	miR9jo	miR9jp	miR9jq	miR9jr	miR9js	miR9jt
miR9ju	miR9jv	miR9jw	miR9jx	miR9jy	miR9jz	miR9ka	miR9kb	miR9kc	miR9kd
miR9ke	miR9kf	miR9kg	miR9kh	miR9ki	miR9kj	miR9kk	miR9kl	miR9km	miR9kn
miR9ko	miR9kp	miR9kq	miR9kr	miR9ks	miR9kt	miR9ku	miR9kv	miR9kw	miR9kx
miR9ky	miR9kz	miR9la	miR9lb	miR9lc	miR9ld	miR9le	miR9lf	miR9lg	miR9lh
miR9li	miR9lj	miR9lk	miR9ll	miR9lm	miR9ln	miR9lo	miR9lp	miR9lq	miR9lr
miR9ls	miR9lt	miR9lu	miR9lv	miR9lw	miR9lx	miR9ly	miR9lz	miR9ma	miR9mb
miR9mc	miR9md	miR9me	miR9mf	miR9mg	miR9mh	miR9mi	miR9mj	miR9mk	miR9ml
miR9mn	miR9mo	miR9mp	miR9mq	miR9mr	miR9ms	miR9mt	miR9mu	miR9mv	miR9mw
miR9mx	miR9my	miR9mz	miR9na	miR9nb	miR9nc	miR9nd	miR9ne	miR9nf	miR9ng
miR9ni	miR9nj	miR9nk	miR9nl	miR9nm	miR9no	miR9np	miR9nq	miR9nr	miR9ns
miR9nt	miR9nu	miR9nv	miR9nw	miR9nx	miR9ny	miR9nz	miR9oa	miR9ob	miR9oc
miR9od	miR9oe	miR9of	miR9og	miR9oh	miR9oi	miR9oj	miR9ok	miR9ol	miR9om
miR9on	miR9oo	miR9op	miR9oq	miR9or	miR9os	miR9ot	miR9ou	miR9ov	miR9ow
miR9ox	miR9oy	miR9oz	miR9pa	miR9pb	miR9pc	miR9pd	miR9pe	miR9pf	miR9pg
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