Signosis
BioSignal Capture

Cluster Real-Time PCR Assay Kit

(For Research Use Only)

Introduction

Based on its proprietary technology, Signosis has developed a highly sensitive and discriminative real-time PCR assay method for measuring multiple miRNA expression simultaneously. It implements oligo-ligation and SYBR green based real-time PCR. The assay can be used for quantitative analysis of miRNA expression of miR-126, miR-148a, miR-181b, miR-181d, miR-196a, miR-204, miR-23a, miR-95 and U6 in either total RNA or cell lysate without cDNA conversion.

Principle

In the assay, a target miRNA molecule is hybridized with two oligos to form a RNA/DNA duplex. When the sequences are perfectly matched, these two oligos are aligned with the miRNA target. The joint of the two oligos can be ligated with DNA ligase. A single nucleotide difference among miRNAs will block either the hybridization or the ligation. After the pair of oligos is ligated, the ligated molecules are subjected to real-time PCR analysis. In addition, a unique tag sequence is assigned to one specific miRNA. Different isoforms such as miR-19a and miR-19b can be differentiated during PCR when the tag sequence is used as one of amplification primers.

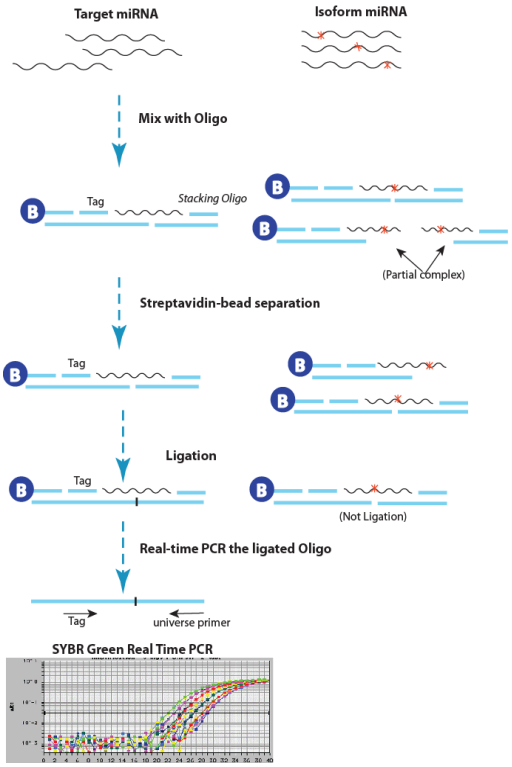

Diagram of miR-17-92 Real-time PCR Assay

Material required but not provided

Cell lysis buffer (CL-0001, Signosis)
Magnetic stand
RNase free water
Real time PCR machine
0.2ml PCR tube or plate

Materials provided

- Oligo Mix
- Annealing buffer (RT)
- Magnetic streptavidin beads (4 °C)
- Beads binding buffer (RT)
- Bead wash buffer (RT)
- T4 DNA Ligase (-20°C)
- Ligation buffer (-20°C)
- SYBR Green PCR Master Mix (-20°C)
- miRNA specific tag primers (-20°C)
- DNA polymerase (-20°C)

1. Sample preparation procedure

Either total RNA or cell lysate can be used for the assay.

(1) Total RNA preparation
We recommend using Trizol reagent or similar products to prepare total RNA. Small RNAs including miRNAs are co-precipitated with other sizes of RNA during isopropanol or ethanol of total RNA.

(2) Cell lysate
a. Estimate the number of cells. The number of cells should be between 10^4-10^5 cells. Wash the cells with 200ul ice cold 1XPBS and add 100ul ice-cold Cell lysis buffer and then subject to snap-frozen at -80°C. If the cell number is between 2000-10,000 cells, add 50ul Cell lysis buffer instead.
Notes: Keep the cells on ice during the procedure.

b. Incubate with cell lysis buffer on ice for 10 minutes, and centrifuge at 10,000g for 2 minutes. Transfer the supernatant to a fresh tube.
Optional: Add 0.25-1u DNAse I, and incubate at 37 °C for 10 minutes and inactivate at 75 °C for 10 minutes.

c. Heat the supernatant for 75 °C for 15 minutes, and put on ice. The cell lysate is ready for use or can be stored at -80°C for the future usage.

2. Annealing of oligos with miRNA

(1) Annealing reaction:

X µl 50ng-1ug total RNA or cell lysate
3 µl miRNA oligo mix
15 µl Annealing buffer
X µl ddH2O

30ul

(2) Incubate on a PCR machine at 72°C for 5 minutes and 53°C for 60 minutes.

(3) Beads washing
- Resuspend the beads by gently tapping the tube to obtain a homogeneous suspension.
- Transfer 4 µl beads to a 0.2ml PCR tube (the size of the tube that should fit into the magnetic stand.
- Add 50 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.

(4) Beads selection
- Add 30 µl of Bead binding buffer to 30 µl annealed miRNA/oligo hybrid from Step 2-(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 the buffer. The beads will remain on the side of tube.
- Remove the tube from the magnetic stand, add 100 µl of Bead wash buffer, pipette gently up and down to resuspend the beads, place the tube on the magnetic stand for 30 seconds and then aspirate the buffer. Repeat the washing step once.

3. Ligation of annealed oligos

(1) Equilibrate beads with 50 µl of Ligation buffer and 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 1µl ligase to the resuspended beads and incubate at 37° for 90 minutes.
(3) Add 100 µl Bead washing buffer directly to 20 µl ligation reaction mix from Step 3-(2), place the tube on the magnetic stand for 30 seconds, and aspirate the buffer.
(4) Remove the tube from the magnetic stand, add 20ul ddH_2O and resuspend the beads. Heat at 95°C for 3 minutes on a PCR machine with heated lid to release the ligated molecule from the beads.
(5) Place the reaction tube on the magnetic stand for 30 seconds. Immediately transfer the solution to a fresh tube. The ligation mixture is ready to use.

4. Real-time PCR

(1) Mix the following components for one reaction
20 µl SYBR Green PCR Master Mix
1 µl ligation mixture
0.2 µl DNA polymerase
1ul Specific miRNA primer
Note: The master mix can be made by multiplying the volume with reaction number, then add specific miRNA primer

(2) Proceed PCR cycles:
Heating the reaction at 82 °C for 60 seconds.
Proceed PCR 35 cycles as follows:
95 °C 40 seconds
55°C 50 seconds
72 °C 50 seconds