

Agilent miRNA Microarray

1. Preparing the labeling reaction

Dephosphorylation

- Prepare 100ng total RNA in 2 ul volume for each sample and place on ice.
Use a vacuum concentrator to reduce sample volume if necessary.
- Prepare the CIP master mix on ice immediately prior to use:

Component	Volume / 1 reaction	Volume / 9 reactions
10x CIP buffer	0.4 ul	3.6 ul
Nuclease-free water	1.1 ul	9.9 ul
CI Phospatase	0.5 ul	4.5 ul

- Add 2 ul of the CIP master mix to each sample for a total volume of 4 ul, mix by pipetting.
- Incubate at 37°C in a heat block for 30 minutes
- Dephosphorylated samples can be stored at -80°C if required or else continue directly with the denaturation step.

Denaturation

- Add 2.8 ul of 100% DMSO to each sample.
- Incubate samples at 100°C in a heat block for 5 to 10 minutes.
- Immediately transfer samples to an ice-water bath.
- Continue with the ligation step immediately.

Ligation

- Warm the 10x T4 RNA Ligase buffer at 37°C for a few minutes and vortex to dissolve precipitate. Allow the buffer to cool to room temperature before proceeding.
- Prepare the Ligation master mix on ice immediately prior to use:

Component	Volume / 1 reaction	Volume / 9 reactions
10x T4 RL buffer	1.0 ul	9.0 ul
Cyanine3-pCp	3.0 ul	27.0 ul
T4 RNA Ligase	0.5 ul	4.5 ul

- Add 4.5 ul Ligation master mix to each sample and mix by pipetting and gently spin down.
- Incubate at 16°C in a cool block for 2 hours.
- Transfer samples to a vacuum concentrator pre-heated to 45-55°C and completely dry the samples. This step may take up to 3 hours, ensure samples are completely dry by flicking the side of the tube hard to make sure pellets do not move.

2. Prepare Hybridization Samples

- Pre-heat a heat block to 100°C
- Re-suspend each dried sample in 18 ul of nuclease-free water
- Add 4.5 ul of 10x GE Blocking Agent to each sample
- Add 22.5 ul of 2x Hi-RPM Hybridization buffer to each sample making a total volume of 45 ul.
- Vortex gently until samples are well mixed and incubate at 100°C for 5 minutes
- Immediately transfer to an ice waterbath for 5 minutes then centrifuge briefly to collect condensation. Keep samples on ice until ready to load.

3. Prepare Hybridization Assembly

- Load a clean gasket slide into the base of the hybridization chamber with the label facing up.
- Slowly dispense all of the sample onto the gasket well in a “drag and dispense” manner, avoid creating bubbles.
- Once all samples are loaded carefully place an array active side down (numeric barcode facing up) onto the gasket slide.
- Place the cover of the hybridization chamber on top of the sandwiched slides and seal the chamber with the clamp.
- Vertically rotate the assembled chamber to wet the gaskets and check that the air bubbles are moving freely. If necessary firmly tap the chamber on a hard surface to move any stationary bubbles.
- Place the slide chamber into the hybridization oven set at 55°C, set the rotator speed to 20 rpm and incubate for 20 hours.

4. Wash the microarray slide

- Pre-heat Wash Buffer 2 in a 37°C water bath overnight
- Half fill a slide-staining dish with Wash Buffer 1
- Place a slide-rack into a second slide-staining dish and $\frac{3}{4}$ fill with Wash Buffer 1
- Place a third slide-staining dish on a heating plate and $\frac{3}{4}$ fill with Wash Buffer 2 and maintain the temperature at 37°C.
- Remove the hybridization chamber from the oven, inspect the wells to check bubbles were rotating freely and there was no significant loss of volume. Unclamp the chamber and quickly submerge the sandwiched slides in Wash Buffer 1 (the first staining dish). Separate the slides by inserting plastic forceps between them at the barcode end.
- Place the microarray slide into the staining rack in Wash Buffer 1 (second staining dish), using a magnetic plate and stirrer set to speed 4, wash slide for 5 minutes.
- Transfer slide rack to the Wash Buffer 2 at 37°C (third staining dish), wash for 5 minutes agitating gently.
- Slowly remove the slide rack from Wash Buffer 2 to minimize liquid droplets on the slide. Touch the edge of the slide against some paper towel to remove excess liquid and scan as soon as possible.