UHN Microarray Centre

MEEBO Array Labelling and Hybridisation Protocols

This document provides two protocols for RNA labelling followed by hybridisation to the Mouse Exonic Evidence Based Oligonucleotide (MEEBO) array. There are two different protocols; the first involves labelling with the Genisphere 3DNA Array 900™ kit and the second protocol is an aminoallyl indirect labelling method that is based on the UCSF Indirect Labelling of Total RNA for Oligonucleotide arrays. The Genisphere protocol is quicker, yet more expensive, than the Indirect Labelling protocol.

Protocol Timeline:

Genisphere Protocol for MEEBO Arrays:

cDNA synthesis:	1.5 hr (set-up), 2 hr incubation
cDNA hybridisation and wash:	0.5 hr (set-up), overnight incubation
3DNA hybridisation and wash:	1.5 hr (set-up), 4 hr incubation

Indirect Labelling Protocol for MEEBO Arrays:

cDNA synthesis:	1 hr (set-up), 2 hr incubation
Purification of aminoallyl-labelled cDNA:	0.75 hr
Labelling with monofunctional dyes:	2 hrs
Pre-hybridisation with BSA:	1 hr incubation
Hybridisation:	0.25 hr set up; overnight incubation
Wash slides	0.4 hr

Genisphere Protocol for MEEBO Arrays

1. cDNA Synthesis from total RNA :

a. Preparation of RNA-RT primer mix
   Take about 10 µg “sample” and “control” into 2 separate new tubes
   Add 1 µL RT primer (Vial 11, 5 pmole/µL) (Cy5 for sample, Cy3 for control)
   Add Nuclease Free Water (Vial 10) to a final volume of 11 µL
b. Mix the RNA-RT primer with vortex and spin to collect to the bottom of the tube
c. Heat to 80^{o}C degree for 5 minutes and immediately transfer to ice for 2-3 minutes, quick spin, collect, keep it in ice
d. Prepare the cDNA synthesis master mix, as below:

Reaction Components	Volume (µL)
5X SuperScript II 1st Strand Buffer	4 µL
0.1M DTT (supplied with enzyme)	2 µL
Superase-in RNase inhibitor (vial 4)	1 µL
dNTP mix (vial 3)	1 µL
SuperScript II enzyme, 200 U/ µL	1 µL
Total volume	9 µL

Gently mix, quick spin, keep it in ice until ready to use

e. Add 9 µL reaction Master Mix into the "sample" (20 µL final volume), add 9 µL reaction Master Mix into the "control" (20 µL final volume)
f. Gently mix, quick spin and incubate at 47°C for 2 hours
g. Quick spin, add 2 µL of 1.0 M NaOH /100mM EDTA, for stopping the reaction
h. Incubate at 65°C for 10 minutes to denature the DNA/RNA and degrade the RNA
i. Add 2.4 µL of 2M Tris-HCl, pH 7.5, and spin at maximum speed for 10 seconds

2. cDNA Hybridisation and Wash (use with the 25x60 mm Lifter slip):

a. Combine "sample" with "control" together
b. Add 44.4 µL 2X Hybridisation buffer (Vial 7) (pre-heat for 2-3 minutes, 50°C)
c. Add 1 µL Cot-1 DNA
d. Gently mix it, incubate for 10 minutes at 80°C
e. In the meantime, assemble the hybridisation chamber (UHNMAC slide box set-up; pre-warm the chamber to the hybridisation temperature, 45°C)
f. Cool hybridisation buffer/Cot-1 DNA/cDNA at room temperature (about 2-3 minutes)
g. Gently vortex, spin down
h. Load the cDNA mix on the microarray
i. Apply slight pressure on the Lifter slip if the hybridisation mix does not cover the whole array.
j. Hybridise the array in 45°C degree in hybridisation oven overnight in dark.
k. Next day, pre-warm the 2X SSC/0.2% SDS wash buffer to 40°C
l. Slide off the Lifter slip in pre-warmed 2X SSC/0.2% SDS by gently jiggling the array under the wash buffer
m. Wash for 10 minutes in the pre-warmed 2X SSC/0.2% SDS, 40°C
n. Wash for 10 minutes in the 2X SSC at room temperature
o. Wash for 10 minutes in the 0.2X SSC at room temperature
p. Spin array at 600 rpm for 10 minutes to dry

3. 3DNA Hybridisation and Wash:

a. Prepare Reagent:
3DNA array 900 Capture Reagent (Vial 1)
2X Hybridisation Buffer (Vial 7, pre-warmed at 50°C for 2- 3 minutes)
b. Prepare 3DNA Hybridization Mix:
Take 2.5 µL Cy3/Alexa Fluor 546 3DNA Array 900 Capture Reagent (Vial 1)

Take 2.5 µL Cy5/Alexa Fluor 647 3DNA Array 900 Capture Reagent (Vial 1)
Add 35 µL 2X Hybridization Buffer (pre-warmed)
Add 40 µL Nuclease Free water (Vial 10)
Add 1 µL Cot-1 DNA

c. Gently vortex the 3DNA Hybridization Mix, incubate at 80°C for 10 minutes
d. Cool down the 3DNA Hybridization Mix for 2-3 min.
e. Load the 3DNA Hybridization Mix on the array
f. Hybridise the array in the dark at 45°C for 4 hours
g. Pre-warm wash solution (2X SSC/0.2% SDS) at 40°C
h. Slide off the Lifter slip in pre-warmed 2X SSC/0.2% SDS by gently jiggling the array under the wash buffer
i. Wash for 10 minutes in pre-warmed 2X SSC/0.2% SDS, 40°C
j. Wash for 10 minutes in 2X SSC at room temperature
k. Wash for 10 minutes in 0.2X SSC at room temperature
l. Spin the array at 80 x g for 10 minutes to dry

Indirect Labelling and Hybridisation Protocol for MEEBO Arrays:

(modification of UCSF Indirect Labelling (and Hybridisation) of Total RNA for Oligo Arrays Protocol)

1. cDNA synthesis from total RNA (aminoallyl-labelled):

a. Label 10 µg total RNA
b. Set up Master Mix as follows:

Reaction Components	Volume (µL)
5X 1st strand reaction buffer	8
AncT mRNA primer (5'-T20VN, 100pmol/ µL)	1.5
Random Primer (Hexamer 3 µg/ µL)	1.5
20 mM dNTP (minus dTTP) (6.67 mM each of dATP, dGTP, dCTP)	3
2 mM dTTP	3
2 mM aminoallyl-dUTP	3
0.1 M DTT	4
Spot Report Alien 1 (1 ng/ µL)	1
Spot Report Alien 2 (1 ng/ µL)	1
Universal total RNA (2 µg/ µL)	5
Sigma H_2O	9
Total Volume	40

c. Quick spin
d. Incubate at 65°C for 5 minutes
e. Incubate at 42°C for 5 minutes
f. Add 2 µL SuperScript per reaction; incubate at 42°C for 2 hours
g. Inactivate the enzyme, hydrolyse the RNA, and neutralise the reaction by adding:
8 µL 1M NaOH, 65°C for 15 minutes
8 µL 1M HCl and 4 µL 1M Tris-Cl, pH7.5

2. Purification of aminoallyl-labelled cDNA with CyScribe™ GFX™ Purification Columns

The CyScribe™ GFX™ column (GE Healthcare, cat # 27-9606-02) uses a glass fiber matrix packed into a spin column format for highly efficient purification of labelled cDNA. Aminoallyl-labelled and fluorescent-labelled cDNA probes are captured by the matrix while unincorporated Cy dye and primers are removed by washing. Bound probes are eluted with a quick spin in elution buffer.

a. Place one GFX column into a clean collection tube and add 500 µL of capture buffer to each column. (One column can be used for 2 reactions)
b. Transfer labelled-cDNA products (volume ranging from 20-100 µL) into each GFX column and mix gently by pipetting up and down 5 times
c. Centrifuge each column in a microcentrifuge at 13,800 X g for 30 seconds
d. Remove GFX column and discard the liquid at the bottom of each collection tube; re-use the collection tube
e. Add 600 µL 80% ethanol to each column and centrifuge at 13,800 X g for 30 seconds
f. Discard collected liquid and repeat step e twice more (for a total of 3 washes).
g. Centrifuge each column at 13,800 X g for an additional 10-30 seconds to remove any traces of ethanol in the tip of the column; discard collection tube
h. Transfer each column to a fresh 1.5 mL tube and add 60 µL of 0.017M $NaHCO_3$ (pH 9.0) buffer f or cold cDNA (OR 60 µL elution buffer for fluorescently labelled-cDNA) on the top of the glass fiber matrix
i. Incubate column at room temperature for 2 minutes. Centrifuge at 13,800 X g for 1 minute to elute purified labelled-cDNA
j. Concentrate the purified labelled-cDNA using speed vac (high heat for 20-30 minutes until it is dry); reconstitute in Sigma water (7 µL water for aminoallyl-labelled cDNA and 5 µL for fluorescently-labelled cDNA)

3. Label with monofunctional Cy dyes and Purify with CyScribe™ GFX™ columns

a. To 7 µL of aminoallyl-labelled cDNA (in 0.1M Sodium bicarbonate, pH 9.0), add 3 µL dye to the reaction (each tube of lyophilised dye initially dissolved in 45 µL DMSO)
b. Incubate at room temperature for 1 hour (keep in the dark)
c. Quench with 4.5 µL 4M hydroxylamine
d. Incubate at room temperature to 15 minutes
e. Purify Cy-labelled cDNA using CyScribe™ GFX™ purification columns (as described above)

4. Pre-hybridisation with BSA

a. Prepare pre-hybridisation buffer as follows:

Buffer Component (stocks)	Buffer - final concentrations	For 50 mL
BSA	1%	0.5 g
20X SSC	5X SSC	12.5 mL
10% SDS	0.1% SDS	500 µL

b. Pre-warm buffer to 42°C then incubate the slides for at least 45 minutes at 42°C
c. Rinse once in milli-Q water (invert 5 times)
d. Rinse once in isopropanol (invert 5 times)
e. Spin dry at 60 x g for 2 minutes

5. Hybridisation

a. Prepare hybridisation buffer as follows:

Hybridisation Buffer Components	Buffer - final concentrations	Volume (µL)
20X SSC	3X SSC	12.75
Calf thymus DNA (10 mg/mL)	0.75 mg/mL	6.38
0.5M HEPES, pH 7	25 mM	4.25
10% SDS	0.225%	1.91
water to 80 µL		54.74

b. Add 80 µL of hybridisation buffer to the labelled-cDNA (5 µL) and denature for 2 minutes at 100°C, allow it to cool to room temperature.
c. Incubate slides in 63°C water bath overnight, if using hybridisation box, or use SlideBooster (Advalytix) Hybridisation Station (set for overnight hybridisation at 63°C)

6. Wash Slides

a. Remove coverslip by dipping the slide in 2X SSC/0.03% SDS, 55°C
b. Wash slide in 2X SSC/0.03%SDS, 55°C for 2 minutes
c. Rinse slide in 1X SSC, plunge slide up and down several times (1 minute)
d. Spin dry at 60 x g for 2 minutes