UHN Microarray Centre

Aminoallyl (Indirect) Labelling Protocol from the University Health Network Microarray Centre (UHNMAC)

This is the two-step method to generate labelled cDNA from 10 µg total RNA. In the first step, aminoallyl-dUTP (AA-dUTP), an amine-modified nucleotide, is incorporated during reverse transcription. In the subsequent step, monofunctional forms of cyanine3 and cyanine5 (or equivalent) dyes react chemically with the amine-modified cDNA.

Protocol Timeline:
Reverse Transcription: 2.5 hr
Purification of aminoallyl-labelled cDNA: 0.4 hr
Dye conjugation: 1.3 hr
Purification of fluor-labelled cDNA: 0.4 hr
Hybridisation: 0.5 hr (set-up); overnight
Washing arrays: 1 hr

RNA Isolation
There are a variety of protocols available that will yield high quality RNA. Many microarray users rely on the RNeasy® RNA Isolation kit (Qiagen) for the isolation of total RNA from cultured cells and TRIZOL® Reagent (Invitrogen) for RNA isolation from tissue samples. Determine the final concentration of purified RNA by measuring A260 and A280. Prepare RNA to a final concentration of at least 1 µg/µL. Labelling 10 µg of total RNA is recommended, however, 5-10 µg of total RNA can be labelled.

Reverse Transcription
1. For 10 µg of total RNA (or 0.1-0.5 µg of mRNA), the reverse transcription reaction volume is 40 µl. Combine the following on ice:

    8.0 µL 5X First Strand buffer (SuperScript II, Invitrogen, catalogue # 18064-014)
    1.5 µL AncT primer (5'-T_{20}VN, 100 pmol/µL, custom order)
    3.0 µL dNTP-dTTP (6.67 mM each of dATP, dCTP, dGTP; GE Healthcare, # 27-2035-01)
    3.0 µL 2 mM dTTP (GE Healthcare, # 27-2035-01)
    3.0 µL 2 mM aminoallyl-dUTP (Enzo, catalogue # 42861)
    4.0 µL 0.1 M DTT (Invitrogen, catalogue # 18064-014)
    1.0 µL control RNA, artificial Arabidopsis transcripts (2-10 ng/µL; optional)
    10 µg total RNA
    to 40 µL nuclease-free water (Sigma, catalogue # W4502)

2. To denature the total RNA, incubate the labelling reaction at 65˚C for 5 minutes.

3. To cool the solution prior to adding the enzyme, incubate the labelling reaction at 42˚C for 5 minutes.

4. Add 2 µL reverse transcriptase (SuperScript II, Invitrogen catalogue # 18064-014) and incubate at 42˚C for 2 hours.

Stop Reaction
1. To stop the reaction and hydrolyse RNA, add 8 µL 1 M NaOH and heat to 65˚C for 15 minutes.

2. To neutralise the solution, add 8 µL 1 M HCl and 4 µL 1 M Tris-HCl, pH 7.5.

Purification of aminoallyl-labelled cDNA using CyScribe™ GFX™ Purification kit
(GE Healthcare, catalogue # 27-9606-02)

For this purification, each sample (labelling reaction) should be purified in one GFX column. Place the purification columns into the tubes provided, as described in the CyScribe™ GFX™ Purification kit manual. Please review the manual supplied by the manufacturer.

1. Add 500 µL of capture buffer to each column.

2. Transfer cDNA product (approx. 62 µL) to the column, pipette up and down several times to mix, spin at 13,800 x g for 30 seconds and discard flow-through.

3. Add 600 µL 80% ethanol and spin at 13,800 x g for 30 seconds and discard flow-through; repeat this step for a total of 3 washes.

4. Spin the column for an additional 30 seconds to ensure all ethanol is removed.

5. Transfer the GFX column to a fresh tube and add 60 µL 0.017M sodium bicarbonate, pH 9 (it is crucial that the buffer covers the membrane; 0.017M is 1 in 6 dilution of 0.1M sodium bicarbonate, so that when dried down in SpeedVac, resuspended with 7 µL water, and added to 3 µL dye/DMSO, the final sodium bicarbonate concentration is 0.1M in the 10 µL dye conjugation reaction).

6. Incubate the GFX column at room temperature for 5 minutes.

7. To elute aminoallyl-labelled cDNA, spin at 13,800 x g for 1 minute.

8. Use Speed Vac (heat setting on high) to completely dry sample; be careful not to over-dry. Resuspend in 7 µL nuclease-free water.

Preparing Monofunctional Reactive Cyanine Dye
Cyanine5 and cyanine3 monofunctional reactive dyes can be purchased from GE Healthcare (catalogue # PA 23001 and PA 25001). Add 45 µL DMSO to the vial of dye. This vial (dye/DMSO) can be stored at -20°C for up to three months. For each labelling reaction, add 3 µL dye/DMSO.

Alternatively, Enzo cyanine dyes (ESBE, catalogue # 42541/42542) can be used. These fluors are single use. Thus, add 3 µL of DMSO per tube to resuspend the dye. See ESBE catalogue for details.

Labelling Reaction with Monofunctional Reactive Cyanine Dyes

1. Add 3 µL dye/DMSO to 7 µL aminoallyl-labelled cDNA, mix by pipetting up and down, and incubate in the dark at room temperature for 1 hour.

2. To quench non-conjugated dye, add 4.5 µL 4M hydroxylamine. Incubate in the dark at room temperature for 15 minutes.

Purification of fluorescent-labelled cDNA Using CyScribe™ GFX™ purification columns

1. To bring the volume of each labelling reaction to 50 µL, add 35 µL of water to each reaction.

2. If you are not measuring dye incorporation, combine the cyanine5- and cyanine3-labelled samples that will be co-hybridised. (If you are measuring the dye incorporation of each labelling reaction, the fluor-labelled samples should be purified separately).

3. Add 500 µL of capture buffer to each column.

4. Transfer labelled-cDNA product (approx. 100 µL) to the column, pipette up and down several times to mix, spin at 13,800 x g for 30 seconds and discard flow-through.

5. Add 600 µL 80% ethanol and spin at 13,800 x g for 30 seconds and discard flow-through; repeat this step for a total of 3 washes.

6. Spin the column for an additional 30 seconds to ensure all ethanol is removed.

7. Transfer the GFX column to a fresh tube and add 60 µL elution buffer (provided with the kit).

8. Incubate the GFX column at room temperature for 5 minutes.

9. Spin at 13,800 x g for 1 minute to elute purified fluor-labelled cDNA.

10. Use Speed Vac (heat setting on high) to completely dry sample; be careful not to over-dry. Resuspend in 5 µL nuclease-free water.

Note: If you wish to stop the protocol at any point and resume the next day, do so after either of the purification stages. Simply freeze the aminoallyl-labelled or fluor-labelled cDNA at –20°C for several days.

Hybridisation
A prehybridisation step is not required.

1. Prepare hybridisation solution:
   To each 100 µL of DIG Easy Hyb solution (Roche, catalogue # 1603558), add 5 µL of yeast tRNA (10 mg/ml; Invitrogen, catalogue # 15401-011) and 5 µL of calf thymus DNA (10 mg/ml; Sigma, catalogue # D8661). Incubate the mixture at 65°C for 2 minutes and cool to room temperature. Make enough solution for all hybridisations (estimate about 100 µL per slide).

2. Add the appropriate volume of the prepared hybridisation solution to each pooled pair of fluor-labelled cDNA (about 5 µL). The appropriate volumes of prepared DIG Easy Hyb solution (with yeast tRNA and calf thymus DNA added) are based on the size of the coverslip you are using.
   24x 60mm coverslip: 85 µL
   2 slides hybridised face-to-face (no coverslip): 85 µL

3. Mix the hybridisation solution with the fluor-labelled cDNA, incubate at 65°C for 2 minutes, and cool to room temperature.

4. For the hybridisations involving the use of coverslips, the hybridisation mixture should be pipetted directly onto the coverslip. Place coverslip onto a reliable surface (the corner of a tip box works well) and add the hybridisation mixture. Lay the slide “array-side” down on top of the coverslip.

5. If you want to use two slides in a “face-to-face” hybridisation, coverslips are not required. The two slides which make up the pair should be faced together slightly offset to create a lip along one edge. The barcodes on the arrays will create a small space between the slides. The hybridisation solution should be slowly and carefully applied along the lip. The hybridisation solution should evenly occupy the space between the slides and yield no bubbles. 85 µL is sufficient to cover the arrays.

6. Carefully place the slide(s) into hybridisation chamber(s) (VWR, catalogue # 48444-004). The hybridisation chambers that we use are plastic microscope slide boxes containing a small amount of DIG Easy Hyb solution in the bottom to keep a humid environment. Clean plain microscope slides are placed at every second or third slide position in the slide box to create rails or a platform onto which the hybridisation arrays can be placed. Each hybridisation chamber can hold two arrays. Carefully place the lid on the box and wrap with plastic wrap.

7. Incubate on a level surface in a 37 °C incubator overnight (about 16-18 hours).

Washing arrays
Use MilliQ water to prepare wash and rinse solutions.

1. Prepare wash solutions. Four staining dishes (Diamed, catalogue # E/S 258-4100-000) containing room temperature 1X SSC; three staining dishes containing 1X SSC/0.1% SDS pre-warmed to 50°C; one staining dish containing room temperature 0.1X SSC. Each staining dish holds about 250 mL.

2. Hold the array at the bar-code end with forceps and remove the coverslip by quickly but gently dipping the array in 1X SSC. The coverslip will slide off gently. For arrays used in a "face-to-face" hybridisation, carefully work the pair of arrays free of one another by immersing in 1X SSC and sliding the arrays past one another. It is critical that powder-free gloves be worn during this procedure.

3. Place the array into a staining rack and place into a staining dish with fresh 1X SSC.

4. When all of the arrays have been removed from the hybridisation chambers, wash for 3 sets of 15 minutes each at 50°C in staining dishes containing pre-warmed (50°C) 1X SSC/0.1% SDS. Gently agitate every 5 minutes.

5. After the washes are complete, rinse the arrays twice in room temperature 1X SSC (plunging 4-6 times) and then in 0.1X SSC.

6. Place the arrays in a slide box lined with blotting paper and spin slides dry at 80 x g for 5 minutes. Alternatively, slides can be placed in a clean and dry 50 mL Falcon tube and spun at 80 x g for 5 minutes.

7. Store arrays in the dark until scanned. It is recommended that arrays be scanned as soon as possible after they are washed (at least within two days).

Reagent list and suggested suppliers

Reagent	Supplier	Product Code
Superscript II, 5X First Strand Buffer, 0.1M DTT	Invitrogen	18064-014
dNTP nucleotides	GE Healthcare	27-2035-01
Aminoallyl-dUTP	Enzo	42861
AncT primer ($5'$-T_{20} VN)	Custom	5'-TTT TTT TTT TTT TTT TTT TTV N -3' (V=A,C,G N=A,C,G,T)
DIG Easy Hyb solution	Roche	1 603 558
Yeast tRNA	Invitrogen	15401-011
Calf Thymus DNA	Sigma	D 8661
Monofunctional dyes	GE Healthcare	PA 23001/PA 25001
Monofunctional dyes	ESBE	42541/42542
CyScribe™ GFX™ Purification kit	GE Healthcare	27-9606-02
Staining Dishes	Diamed	E/S 258-4100-000
Slide Boxes	VWR	48444-004
Sigma Water	Sigma	W 4502
Artificial Arabidopsis transcript	Plasmid from UHNMAC (transcript must be generated by user)	Arabidopsis plasmid