UHN Microarray Centre

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

The following protocol is used at the UHNMAC for the direct labelling of total RNA samples. To compare two different samples (i.e. control and experimental), label each sample with a different fluor (for example, cyanine3 and cyanine5) and co-hybridise on an array.

Protocol Timeline
Reverse transcription/fluor incorporation: 2.5 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.

Reverse Transcription
It is important to keep the reaction tubes in the dark throughout this protocol as the fluors are light sensitive.

1. For 10-20 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 reaction buffer (Superscript II, Invitrogen, catalogue # 18064-014)
    1.5 µL AncT primer (5'-T20VN, 100 pmol/µL)
    3.0 µL dNTP-dCTP (6.67 mM each of dATP, dGTP, dTTP, GE Healthcare, cat # 27-2035-01)
    1.0 µL 2 mM dCTP (GE Healthcare, catalogue # 27-2035-01)
    1.0 µL 1 mM Cyanine 3-dCTP or Cyanine 5-dCTP (NEN, NEL 576, NEL 577)
    4.0 µL 0.1 M DTT (Invitrogen, catalogue # 18064-014)
    0.1 – 20 µg RNA (0.1-0.5 µg mRNA or 10-20 µg total RNA)
    1.0 – 5.0 ng Control RNA (artificial Arabidopsis transcripts)
    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 in the dark (fluors are light sensitive).

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

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

Note: This is a convenient place to stop if necessary. Labelling reactions can be frozen at –20°C overnight or until such time that you are ready to continue processing.

5. Briefly centrifuge the reaction and place on ice.

6. To stop the reaction and hydrolyse RNA, add 4 µL 50mM EDTA (pH 8.0) and 2 µL 10 N NaOH; incubate at 65˚C for 20 minutes.

7. To neutralise the solution, add 4 µL 5M acetic acid.

At this point, the two reactions that will be hybridised to the same slide (i.e. control and experimental) can be combined together, unless you are measuring dye incorporation following purification.

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

For this purification, each column can purify two labelling reactions (the labelled samples that will be co-hybridised). Place the purification columns into the tubes provided, as described in the CyScribe™ GFX™ Purification kit manual. The buffers and solutions referred to below are included in the kit. Please review the manual supplied by the manufacturer.

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

2. Transfer up to 100 µL cDNA products to column (samples that will be co-hybridised), pipette up and down several times to mix.

3. Spin at 13,800 x g for 30 seconds and discard flow-through.

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

5. Spin the column for an additional 30 seconds at 13,800 x g to ensure all wash buffer is removed.

6. Transfer the GFX column to a fresh tube and add 60 µL elution buffer (it is crucial that the elution buffer covers the membrane).

7. Incubate the GFX column at room temperature for 1 minute.

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

9. 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: The best results are often obtained if labelled-cDNA is hybridised immediately after labelling, however, it can be stored in the dark 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 your 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: 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 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 Whatman paper and spin slides dry at 80 x g for 5 minutes. Alternatively, slides can 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
AncT primer (5'-T20VN)	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
Cyanine dyes	NEN	NEL 576, NEL 577
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