UHN Microarray Centre

Aminoallyl (Indirect) Genomic DNA Labelling Protocol for UHN CpG Arrays

This protocol can be used with UHNMAC CpG-island (CGI) arrays.

The Human CGI 8.1K array contains 8,109 CpG-island clones, consolidated from the UHN's HCGI12K set and an independent 8.5K set obtained from Tim Huang at the Ohio State University (Columbus, OH). Redundant clones, clones with internal repeat sequences and clones mapping to chromosome M (Mitochondria) have been excluded. Compared to the HCGI12K array, the HCGI8.1K array has consistently lower signal intensities.

The Mouse CGI 4.6K array contains 4,642 CpG-island clones obtained from the Sanger Institute. This array set replaces the Mouse CGI 7.3K set.

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

Synthesis of aminoallyl-labelled DNA
1. Combine the following reagents on ice:

x μL digested Genomic DNA (5 μg)
20 μL 2.5X Random Primer Mix (see solution recipe below)
0.5 μL Spot Report Alien 1 (1 ng/ μL) (optional; Stratagene, cat # 1-252551)
0.5 μL Spot Report Alien 2 (1 ng/ μL) (optional; Stratagene, cat # 2-252552)
1 μL Arabidopsis DNA (10 ng/ μL) (optional; plasmid from UHNMAC)
nuclease-free water to a final volume of 40 μL

2. Incubate at 99°C for 5 minutes, followed immediately by incubation on ice for 10 minutes.

3. On ice, add the following reagents to each reaction:

    5 µL NEB buffer 2
    5 µL Aminoallyl-dUTP-dNTP mix (see recipe solution below)
    1.5 µL Klenow (50 U/µL; NEB, catalogue # M0212M)
    (final volume approximately 50 µL)

4. Incubate at 37°C overnight (16-18 hours).

Purification of Aminoallyl-dUTP-labelled DNA using CyScribe™ GFX™ Purification kit
(GE Healthcare, catalogue # 27-9606-02)
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. Add 50 µL nuclease-free water to sample.

3. Transfer aminoallyl-labelled DNA to the column, pipette up and down several times to mix.
4. Centrifuge at 13,800 x g for 30 seconds and discard flow-through.

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

6. Centrifuge 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 nuclease-free water (pre-warmed at 65°C) to the top of the glass fiber matrix.

8. Incubate the GFX column at room temperature for 10 minutes.

9. To elute purified labelled-DNA, centrifuge at 13,800 x g for 1 minute.

10. Do a second elution by adding 60 µL 0.1M sodium bicarbonate (pH 9) to the column; incubate the column at room temperature for 5 minutes; centrifuge at 13,800 x g for 1 minute.

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

Dye Conjugation
In this step, monofunctional forms of Alexa Fluor 555 and Alexa Fluor 647 dyes react chemically with the amine-modified DNA. Prepare dyes by resuspending fluors (Invitrogen, catalogue # A-20006, A-20009) in 200 µL of methanol; prepare aliquots of 5 µL each; dry the dye in the SpeedVac and store aliquots at -20°C. Resuspend each aliquot with 2 µL DMSO prior to use.

Add 2 µL of fluor/ DMSO to sample and incubate in the dark at room temperature for 2 hours.

Purification of fluor-labelled DNA using CyScribe™ GFX™ Purification kit

1. Add 45 µL of nuclease-free water to each sample (samples that will be co-hybridised can be pooled at this point).

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

3. Transfer fluor-labelled genomic DNA product (50 µL - 100 µL) to the column, pipette up and down several times to mix.

4. Centrifuge 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 of elution buffer (pre-warmed at 65°C) to the top of the glass fiber matrix.

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

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

10. Add 5 µL of species-specific CotI DNA [1 µg/ µL; Invitrogen catalogue # 15279-011 (human), 18440-016 (mouse)]

11. Use Speed Vac (heat setting on medium) to completely dry sample; be careful not to over-dry. Resuspend in 2.5 µL nuclease-free water.

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). 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) 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 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. To remove all trace SDS after the washes, 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 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).

Solutions and Reagents

2.5X Random Primer Mix:

125 mM Tris pH 6.8
12.5 mM MgCl2
1250 µg/mL Random Primers (Invitrogen, catalogue # 48190-011)
25 mM 2-mercaptoethanol

aa-dUTP-dNTP mix:

5 mM dATP (dNTPs from GE Healthcare, catalogue # 27-2035-02)
5 mM dCTP
5 mM dGTP
1 mM dTTP
4 mM aminoallyl-dUTP (Enzo, catalogue # 42861)

Reagent	Supplier	Product Code
Random Primer	Invitrogen	48190-011
Spot Report Alien™ 1 and 2	Stratagene	#1-252551 #2-252552
Arabidopsis DNA	Plasmid available from UHNMAC	
Klenow Fragment (3'→5' exo)	New England Biolabs	M0212M
Alexa Fluor 647 and Alexa Fluor 555	Invitrogen	A20006 and A20009
CyScribe™ GFX™ purification columns	GE Healthcare	27-9606-011
Aminoallyl-dUTP	Enzo	42861
dNTP nucleotides	GE Healthcare	27-2035-02
Human Cot1 DNA	Invitrogen	15279-011
Mouse Cot1 DNA	Invitrogen	18440-016
Yeast tRNA	Invitrogen	15401-011
DIG Easy Hyb solution	Roche	1 603 558
Staining Dishes	Diamed	E/S 258-4100-000
Slide Boxes	VWR	48444-004
Sigma Water	Sigma	W 4502