Klenow Label Genomic DNA Cy3 &/or Cy5

NOTES:

For each reaction label 1ug fragmented genomic DNA (fragment by sonication or other method to average size of 500-1000bp. This will improve labeling efficiency).

We use high concentration Klenow (50units/ul) from New England Biolabs. This provides better incorporation of labeled dNTP (10X Eco/Pol labeling buffer should be supplied with enzyme).

Use a mixture of dATP, dCTP, dGTP each at 5mM as a 10X stock (500uM final), and 1mM Cy3-dUTP or Cy5-dUTP when setting up the reaction.

PROTOCOL

1. Mix DNA and random hexamer.

   X ul frag genomic DNA (~1ug)
   2 ul random $Pd(N)_6$ hexamer @ 5mg/ml (Pharmacia)
   Z ul MQH_2O
   20 ul total volume

2. Heat sample to 95°C for 2 min. and transfer to ice water for 5 min.
3. Set up Labeling Mix

1X	3X
5 ul 10X klenow buffer (Eco/Pol, NEB)	15 ul 10X klenow buffer (Eco/Pol, NEB)
5 ul 10X dNTP (no dUTP)	15 ul 10X dNTP (no dUTP)
17 ul MQ-H_2O	51 ul H_2O

4. To DNA/hexamer on ice add 27ul of labeling mix
5. Add 1.5 to 2 ul Cy dye labeled dUTP + 1ul Klenow DNA polymerase (NEB 50U/ul)
6. Incubate 2-6 hours at 37°C in dark
7. Add 2.5 ul 0.5M EDTA pH 8.0 to stop the reaction.

PURIFY PROBES PRIOR TO HYBRIDIZATION!

You must remove unincorporated dNTPs prior to hybridization to minimize background fluorescence.

- Add 470ul MQ H_2O to each labeling reaction.
- transfer this to Microcon 30 (Amicon) filtration unit in a collection tube.
- Spin at 12,000 RPM 8-10 minutes (trying to achieve a volume of 20-30ul)
- Add additional 450ul MQ H2O and repeat spin
- Add one more volume (450ul) MQ H2O and concentrate probe to <10ul

Note- if you are hybridizing Cy3 AND Cy5 labeled probes to the same array you should spin one probe into a clean tube after the first wash. Do this by inverting the filtration unit into a clean tube and spinning at max speed for 1 min. Mix this probe with the second probe and proceed with the final washes. You need to do this so that your final volume of the mixed probes is <10ul.

Proceed with hybridization as you would for a cDNA probe (see below) or store at 4°C in dark until ready to use. We have stored our labeled target up to one week with no obvious loss in signal strength.

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SETUP HYBRIDIZATION

We have tested 2 buffers for hybridization. One involves an SDS/SSC hybridization solution (that which is typically reported in the literature) while the second uses a commercial hybridization buffer from Sigma. Both methods are described below. We find that the most common problem encountered during hybridization is drying of probe to the array leading to very high background which is typically more pronounced at the edges of the cover slip (this is usually more of a problem for RNA/cDNA hybridization's than for genomic hybridization's). We believe that the commercial buffer mitigates this somewhat due to its viscous nature.

Sigma PerfectHyb method:
Mix in 0.5ml microfuge tube

x ul labeled probe
y ul milliQ H_2O
1 ul 10mg/ml salmon sperm DNA (BRL)
1 ul 4mg/ml yeast tRNA
10 ul Sigma Perfect Hyb™ Plus Hybridization Buffer
Total probe volume = **20 *ul***

SSC/SDS method:
Mix in 0.5ml microfuge tube

x ul labeled probe
y ul milliQ H_2O
1 ul 10mg/ml salmon sperm DNA
1 ul 4mg/ml yeast tRNA
3.5ul 20X SSC (3.5X final conc.)
1.2ul 5% SDS (0.3% final conc.)
Total probe volume = **20 *ul***

2- Heat this mixture to 100°C 5 minutes
3- Spin for 5 minutes at 14,000 RPM to pellet any particulate material
4- Place the Microarray(s) in a sealed box to avoid exposure to dust in the air.
5- TeleChem or GeneMachines
To prevent arrays from drying, pipette 20-25 ul of H_2O into the wells located on either end of the hybridization chamber. Also add a thin strip of whatman paper, 2.5 inches long, saturated with milliQ H_2O (60-70ul) to the chamber alongside where the microarray will go.
6- After the probe is done spinning, carefully transfer the probe/hybridization solution to a new tube avoiding any pelleted material.
7- Carefully pipette the probe/hybridization mix onto a plastic cover slip (Sigma) and immediately over lay with the microarray *with array side down* (ensure you are between both etched marks!) Avoid bubbles!!!
8- Gently turn the microarray ride side up and place in the Hybridization chamber. Seal chamber and place in 60°C H_2O bath. (Do Not place directly on bottom of water bath. Cushion bottom with a pipette box or some other material.) Incubate 8-16 hours.

Reagents and Suppliers

Cy3-dUTP:	1 mM	Amersham	Cat # PA53022
Cy5-dUTP:	1 mM	Amersham	Cat # PA55022
Klenow	50U/ul	NEB	Cat # M0210M
$Pd(N)_6$ (Hexamer)	5mg/ml*	Amersham/Pharmacia	Cat# 27-2166-01
100 mM dNTP set	10X**	Pharmacia	Cat # 27-2035-01
Yeast total tRNA	4 µg/µl*	Sigma	Cat # R8759
Microcon YM-30 columns		Amicon	Cat # 42410
Hybridization Chambers		Telechem	Cat # AHC-1
Perfecthyb Plus buffer		Sigma	Cat # H7033
Hybri-Slip™ (22x22mm)		Sigma(Grace Bio Labs)	Cat# Z36,590-4

Other reagents: 20X SSC, TE pH7.4, 10% SDS, 500 mM EDTA, sterile dH_2O

* comes lyophilized, must be resuspended at specified concentration
**for 10X stock: 5 mM each of dA, dG, dC in MQ H_2O

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