Direct labeling of mouse genomic DNA for microarray hybridization using the Klenow fragment of E. coli DNA polymerase I and Cy3 dCTP.

This is a detailed version of the protocol published in "Genomic DNA as a cohybridization standard for mammalian microarray measurements", Brian A. Williams, Richele M. Gwirtz and Barbara J. Wold. Nucleic Acids Research, 2004, Vol. 32, No. 10, e81.

This protocol is designed to produce a cohybridization standard for use on microarrays that represent genomes with relatively low gene density (such as the mouse genome). To effectively label a single array with this standard, 3 aliquots (2 µgs each) of sonicated mouse genomic DNA (see our associated protocol for preparing mouse genomic DNA) should be labeled as described below. These can then be combined for cohybridization with a single, labeled cDNA sample.

(This protocol is an adaptation of the protocol found on the Brown Lab website http://cmgm.stanford.edu/pbrown/protocols/4_genomic.html).

Equipment and reagents

Denaturation and labeling

A) Thermal cycler
B) Incubation oven capable of holding steady 37°C
C) Invitrogen BioPrime DNA labeling system (catalog # 18094-011).
D) Amersham Pharmacia Cy3 dCTP (catalog # PA53021).
E) *Roche Applied Science dNTP set (catalog # 1 277 049).
*Note: these dNTPs work well, but be certain to call Roche and ask for the most recent lot with the furthest expiration date. Performance declines as time passes.

F) 10X dNTP solution for labeling
(final concentrations in the reaction are 200 µM dATP, dGTP, and dTTP, 100 µM unlabeled dCTP and 60 µM Cy3 dCTP. Ratio of (unlabeled dCTP : labeled dCTP) is (5 : 3).

50 µl of 100 mM dATP
25 µl of 100 mM dCTP
50 µl of 100 mM dGTP
50 µl of 100 mM dTTP
2325 µl of ddH_2O

Final volume = 2500 µls
Store in single use aliquots at -80°C.

Prior to labeling reactions, be sure that the following reagents are also stored in single use aliquots at minus 80°C:

G) 20 µL aliquots of 2.5X random primer solution from BioPrime DNA labeling system.
H) 3 µL aliquots of 1mM Cy3 dCTP

I) 2 µgs sonicated genomic DNA (see our protocol for mouse genomic DNA prep.).

Cleanup and probe preparation

J) Eppendorf 5417C microcentrifuge with variable RPM/RCF adjustment at room temperature (any good variable speed lab microcentrifuge should work, make sure that it is capable of speeds greater than 10,000 g).
K) Qiagen Qiaquick PCR cleanup columns (catalog # 28104)
L) Speed Vac
M) Heating block at 85°C.
N) Hybridization solution (50% formamide, 5X SSC, 0.1% SDS, 1 µg/µL yeast tRNA).

Miscellaneous

O) Thin-walled PCR tubes, standard microcentrifuge tubes, wet ice.

Yield and incorporation estimation

P) Molecular Probes PicoGreen DNA quantitation kit (catalog # P-7589).
Q) BioRad Cuvettes (catalog # 170-2415)
R) BioRad VersaFluor Fluorometer (catalog # 170-2402)
S) BioRad excitation filter for PicoGreen (catalog # 170-2427)
T) BioRad emission filter for PicoGreen (catalog # 170-2424)
U) Chroma (www.chroma.com) excitation filter for Cy3 (535 nm)
V) Chroma (www.chroma.com) emission filter for Cy3 (565 nm)

Labeling

1) Combine in a thin-walled PCR tube:
2 µg aliquot of genomic DNA (reduce the volume to 10 µL or less in a Speed Vac)
20 µL of random octamers in buffer (BioPrime kit – Invitrogen)

2) Heat to 97°C for 3 minutes and 50 seconds in a ThermoCycler.

3) Plunge into ice water bath (adding salt to the ice brings the temperature to -5°C) for 3 minutes.

4) Spin down in centrifuge, place on wet ice.

5) Add on ice:
3 µL of Cy3 dCTP 1 mM. (Amersham)
5 µL of 10X dNTPs (10X concentration is 2mM for dATP, dGTP and dTTP; 1mM dCTP).
X µL of chilled ddH_2O to bring the reaction to a volume of 49 µL.

6) Mix the reagents and move to the benchtop for 2 minutes at room temperature.

7) Add 1 µL Klenow enzyme (BioPrime kit, high activity concentration). Mix gently with pipette.

8) 5 minutes at room temperature.

9) Place in a 37°C oven for 2.5 hours. (Note – this is an improvement over the thermocycler).

10) Respike with 1 µL Klenow after 2.5 hours. Mix gently with pipette.

11) Place in a 37°C oven for 2.5 hours.

12) Stop the reaction with 5 µL stop solution (BioPrime kit).

Cleanup

Cleanup according to the protocol in the Qiagen QiaQuick PCR cleanup kit.

13) Pass the labeled sample over the column twice to bind as much labeled DNA as possible before washing. Spin at 10,000g for 1 minute each time.

14) Wash the column at least twice with 750 µL of PE buffer. Spin at 10,000g for 1 minute each.

15) Be sure to dry the column thoroughly by spinning at top speed for 2 minutes after the last wash.

16) Elute with 50 µL of elution buffer, incubating for 5 minutes on the benchtop. Spin at top speed for 1 minute.

17) Repeat with another 50 µL of elution buffer.

18) Reserve 5 µL of labeled sample for counting using PicoGreen. Can be stored at -80°C.

Quantifying yield and incorporation efficiency

Cy3 measurements

19) Prepare two sets of fluorometric standards in the BioRad cuvettes using Cy3 dUTP and the reagents in the PicoGreen kit:

Cy3 Standards (2ml final volume each)

A) 0 ng of Cy3 dUTP, 400ng of lambda DNA in 2mL TE.
B) 50 ng of Cy3 dUTP, 400ng of lambda DNA in 2mL TE.
C) 5ng of Cy3 dUTP, 400ng of lambda DNA, in 2mL TE.
D) 500 pg of Cy3 dUTP, 400ng of lambda DNA, in 2mL TE.
E) 50 pg of Cy3 dUTP, 400ng of lambda DNA, in 2mL TE.

20) Add the 5 µL sample of labeled genomic DNA (the unknown) to 2 mL of TE in a BioRad cuvette. Mix with pipette.

21) Count the Cy3 standards and unknowns according to the directions for the BioRad VersaFluor fluorometer.

22) Be sure to insert the appropriate Cy3 excitation and emission filters.

23) Use the LOW Gain setting.

24) Zero the instrument with Cy3 standard A, and set the range of 19,999 RFU using Cy3 standard B.

25) Continue reading the rest of the standards (which should decrease linearly from 19,999) and then read the (unknown) samples.

(Note: Standard E (50 pg Cy3) is close to the limits of the machine at low gain and may be erratic. Also, the Cy3 dye will bleach if read multiple times or left in the machine for too long).

DNA yield measurements

Note: Do not add the PicoGreen fluorescent DNA binding reagent until after the Cy3 measurements have been taken. We have found some spectral overlap between the two fluors.

DNA Standards (2mL final volume each)

A) 0 ng lambda DNA, 1mL TE, 1mL 1:200 PicoGreen Reagent in TE.
B) 400 ng lambda DNA in 1mL TE, 1mL 1:200 PicoGreen Reagent in TE
C) 40 ng lambda DNA in 1 mL TE, 1mL 1:200 PicoGreen Reagent in TE
D) 4 ng lambda DNA in 1 mL TE, 1mL 1:200 PicoGreen Reagent in TE
E) 400 pg lambda DNA in 1 mL TE, 1mL 1:200 PicoGreen Reagent in TE

Note: Add PicoGreen to standards and unknowns simultaneously.

26) Add the 1 mL diluted Picogreen Reagent in TE to each of the standards and mix well.

27) Add 5 µL PicoGreen dsDNA Quantitation Reagent (undiluted) to each of the unknowns and mix well.

28) Incubate 10 minutes in the dark at room temperature.

29) Count the standards and unknowns as before using PicoGreen excitation and emission filters, the LOW gain setting, DNA standard A to zero and DNA standard B to set the range to 19,999.

Good incorporation will have RFU for Cy3 between 3000 and 5000, and a good DNA yield will have RFU for PicoGreen between 8000 and 10,000.

Prepare the probe for the array

30) Dry the probe down in a speed vac until just dry. Make an effort to avoid overdrying.

31) Resuspend the probe in an appropriate volume of hybridization solution. We use 30 μL. Use the hybridization solution to wash down the sides of the tube to bring all of the dried product down into solution. Mix thoroughly with the pipette to disrupt all crystalline pieces.

32) Heat in the dark to 85°C for two minutes.

33) Immediately spin down for 1 minute in the dark to cool the probe.

34) Place the probe at hybridization temperature until ready to apply to your array.

35) We spin down again at top speed for 30 seconds immediately prior to loading the probe onto the array.