Indirect labeling of mouse genomic DNA using Klenow and aminoallyl-dUTP


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.

Equipment and reagents

BioRad VersaFluor Fluorometer (catalog # 170-2402)
BioRad excitation filter for PicoGreen (catalog # 170-2427)
BioRad emission filter for PicoGreen (catalog # 170-2424)
Chroma (www.chroma.com) excitation filter for Cy3 (535 nm)
Chroma (www.chroma.com) emission filter for Cy3 (565 nm)
Incubation oven capable of holding steady 37°C
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).
Speed Vac
Heating block at 70°C and 85°C.

Zymo DNA Clean and Concentrator columns (catalog #s D4003 or D4005)
Invitrogen BioPrime DNA labeling system (catalog # 18094-011).
Sigma aminoallyl dUTP (catalog # A-0410).
*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.
Roche Applied Science pyrophosphatase (catalog # 108 987)
100 mM sodium acetate, pH 5.2.
100 mM Na₂CO₃ pH 9.0
4M hydroxylamine solution.
Molecular Probes PicoGreen DNA quantitation kit (catalog # P-7589).
USA Scientific G-50 Sephadex cleanup columns (catalog # 1415-1602)
Qiagen Qiaquick PCR cleanup columns (catalog # 28104)
BioRad P30 BioGel cleanup columns (catalog # 732-6223)
BioRad Cuvettes (catalog # 170-2415)
Thin-walled PCR tubes, standard microcentrifuge tubes, wet ice.
Hybridization solution (50% formamide, 5X SSC, 0.1% SDS, 1 μg / μl yeast tRNA).

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Phosphate elution buffer

Phosphate elution buffer is made by diluting 1M KPO$_4$ solution to 4 mM with double distilled water.

To make 1 M KPO$_4$ solution pH 8.5-8.7, combine:

- 9.5 mL 1M K$_2$HPO$_4$
- 0.5 mL 1M KH$_2$PO$_4$

Before beginning labeling protocol be sure that the following reagents are available as single use aliquots stored at -80°C:

- 2.5 μg aliquots of sonicated mouse genomic DNA
- 20 μL aliquots of 2.5X random primer solution from BioPrime DNA labeling system.
- 10X AAdUTP/dNTPS labeling mix
- 4.5 μL aliquots of monofunctional Cy Dye ester in DMSO.

Protocol for resuspension of dry CyDye in DMSO

Add 73 μL DMSO (DMSO is hygroscopic, keep in dessicatator) to 1 tube (1mg) monofunctional reactive dye.
Mix thoroughly, spin down.
Aliquot at 4.5 μL per tube.
Store in dessicator at -80°C.

10X dNTP solution for labeling

Optimization experiments for yield and incorporation indicated that Klenow enzyme works best at a 900 μM concentration and with a (2:3) ratio of labeled to unlabeled precursor.

Resuspend 1 mg of AAdUTP as a 20 mM stock solution:

Combine:
- 85 μL of ddH$_2$O
- 0.8 μL of 0.1 N NaOH

Add this to 1 mg of AAdUTP
Draw up 5 μL to check pH on a pH strip. pH should be approximately 7.0

Prepare a 9 mM 10X stock solution of dNTPs for labeling.

Final concentrations in the reaction are 900 μM dATP, dCTP, and dGTP, 360 μM AAdUTP and 540 μM unlabeled dTTP. Ratio of (labeled AAdUTP : unlabeled dTTP) is (2:3).

- 40 μL of 100 mM dATP
- 40 μL of 100 mM dCTP
- 40 μL of 100 mM dGTP
80 μL of 20 mM AAaUTP
24 μL of 100 mM dTTP
220 μL of ddH₂O

Final volume = 444 μL
Store in single use aliquots at -80°C.

**Denature template sonicated genomic DNA with NaOH**

X μL (2.5 μg aliquots) sonicated genomic DNA (*try to bring the volume to 10 μL).
30 μL ddH₂O
10 μL 1N NaOH

Incubate at room temperature for 20 minutes.
Wet ice/NaCl bath for 3 minutes.
Spin down.
Wet ice.
Neutralize with 10 μl 1N HCl *important step*.

**Cleanup over Zymo columns (either 5 μg or 25 μg capacity)**

Add a volume of Zymo DNA binding buffer that is equivalent to twice the sample volume, to the sample. Mix lightly and spin down.

The entire volume of the sample is passed over the column twice, in order to bind as much DNA as possible to the column. If your sample volume is greater than 400 μL, you can load the column serially.

Set the centrifuge speed to maximum, and watch the RCF counter. When it hits 10,000g, begin timing for 10 seconds. Then stop the centrifuge.

Repeat with same volume, then discard and bind the second volume with 2 spins.

Wash with 200 μL of wash buffer. Time the spin as above.

Repeat the wash, but this time spin for 30 seconds after 10,000g is reached in order to dry the column, then stop the centrifuge.

Elute the DNA in the appropriate volume of ddH₂O that has been adjusted to pH 8.0 (*important step*).
Incubate for 5 minutes at room temperature.

Spin for 30 seconds total elapsed time with the centrifuge set at maximal speed. Do not wait until the centrifuge hits maximal speed and then start timing.

Repeat elution as above.

Place on wet ice
Recount on UV spec. Remember that OD of 1 for single-stranded DNA is 40 μg/ml.

Re-aliquot approximately 2 μg per reaction.

**Annealing to random primers.**

Combine:
2 μg denatured sonicated mouse genomic DNA
20 μL of 2.5X random primers

Heat to 70°C for 10 minutes
Plunge into wet ice/NaCl bath for 3 minutes.
Spin down and place on wet ice.

**Labeling**

Add to primed genomic DNA on ice:

2 μL of 25 mM MgCl$_2$.
5 μL of 10X AAdUTP/dNTPs labeling mixture.
1 μL of pyrophosphatase diluted 1:10 in ddH$_2$O.
X μL of chilled ddH$_2$O to bring the reaction to a volume of 49 μL.

Mix the reagents and move to the benchtop for 2 minutes at room temperature
Add 1 μL Klenow enzyme (BioPrime kit, high activity concentration). Mix gently with pipette.
5 minutes at room temperature.

Place in a 37°C oven for 2.5 hours. (Note – this is an improvement over the thermocycler).
Respike with 1 μL Klenow and 1 μL of freshly diluted pyrophosphatase after 2.5 hours. Mix gently with pipette.
Place in a 37°C oven for 2.5 hours.
Stop the reaction with 5 μL stop solution (BioPrime kit).

**Cleanup**

Gel Filtration Chromatography column to remove excess unincorporated nucleotides from AA dUTP labeling reaction.
We are working with a high concentration of free nucleotides. This step is necessary to prevent column failure in the cleanup steps on the Qiagen columns.

Reduce the volume of the labeled sample to 25 μL in Speed Vac. The G-50 cleanup columns are more efficient at removing unincorporated nucleotides when the sample is in a smaller volume.
While waiting, bring Sephadex G-50 columns to room temperature.
Repack the Sephadex by spinning the column at 3500 RPM (1000g) for 3 minutes. Discard the buffer. Add 500 μL of phosphate elution buffer \(^{(2)}\). Spin again for 3 minutes. Discard the buffer. Repeat the addition of 500 μL of phosphate elution buffer, spin for 3 minutes. Discard the buffer.

Check the volume of the labeled probe to confirm that it is 25 μL. Add the 25 μL of labeled probe to the repacked column.

Spin for 3 minutes at 3500 RPM (1000g).
Dry down the sample until just dry in a speed vac.
Wrap the top of the tube in parafilm and store at -80°C overnight.

**Coupling to monofunctional reactive dye \(^{(2)}\).**

Sample is resuspended in 4.5 μL of 0.1M Na₂CO₃ pH 9.0. (*High pH is important for coupling*).
Sides of tube are washed with this volume to resuspend all dried material.
Incubate at 37°C for 20 minutes to resuspend dried DNA. Spin down.
4.5 μL of Cy Dye ester resuspended in DMSO is added to the resuspended sample and mixed thoroughly. Use the entire volume to wash the sides of the tube to resuspend all crystallized sample on the walls of the tube.

Incubate for 1.5 hours at room temperature in the dark.

Quench the reaction with 4.5 μL of 4M hydroxylamine.

Incubate 15 minutes at room temperature in the dark.

Add 45 μL of 100 mM NaOAc.

**Qiagen affinity column to remove enzyme and uncoupled dye from sample.**

Add 5X volume (300 μL) PB to the filtered sample
Vortex and spin down
Bind to the Qiagen column by passing the sample volume over the column twice.
Wash with PE wash buffer (750 μL)
Spin 10,000 g for 1 min
Repeat washes an additional 3 times.
After fourth wash, discard the wash buffer and respin at top speed for 2 min to dry the column.
Elute with 50 μL of elution buffer 5 minutes at room temperature
Spin 1 minute top speed
Elute again with 50 μL of elution buffer 5 minutes at room temperature
Spin 1 minute top speed.

**Final cleanup over P30 Biogel chromatography column.**
Reduce volume to 25 μL in Speed Vac. The gel filtration columns are more efficient at retaining small molecules when the sample volume is reduced.

While waiting, bring BioRad P30 columns to room temperature.

Do not use BioRad collection tubes; replace with 2 ml microfuge tubes.

Spin at 3400 RPM to repack column; discard buffer.

Repack columns by adding 500 μL EB buffer (Qiagen elution buffer) to matrix. Spin 3400 RPM, 2 minutes.

Discard buffer and repack again by adding another 500 μL EB buffer and spinning 2 minutes.

Add 25 μL sample to repacked P30 column.

Spin 3400 RPM 4 minutes.

**Prepare the probe for the array**

Combine the sample with alternately labeled cDNA or cRNA sample for cohybridization. Note: for mouse microarrays, we combine 3 labeled genomic DNA samples with a single alternately labeled experimental sample in a single hybridization experiment.

Dry the combined samples down in a speed vac until just dry. Make an effort to avoid overdrying.

Resuspend the sample in an appropriate volume of hybridization buffer. We use 30 μL. Use the buffer 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.

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

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

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

**Quantifying yield and incorporation efficiency**

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

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

Count the Cy3 standards and unknowns according to the directions for the BioRad VersaFluor fluorometer. Be sure to insert the appropriate Cy3 excitation and emission filters. Use the LOW Gain setting, zero the instrument with Cy3 standard A, and set the range of 19,999 RFU using Cy3 standard B. 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).

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

Note: Add PicoGreen to standards and unknowns simultaneously.

Add the 1 mL diluted Picogreen Reagen in TE to each of the standards and mix well. Add 5 μL PicoGreen dsDNA Quantitation Reagent (undiluted) to each of the unknowns and mix well.

Incubate 10 minutes in the dark at room temperature.

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.

A good yield will have RFU for Cy3 between 4000 and 5000, and RFU for PicoGreen between 9000 and 10,000.

References:

2) TIGR website. http://pga.tigr.org/sop/M004_1a.pdf