StonyBrook Arrays Protocol
Title: Aminoallyl Labeling of Genomic DNA
Version 1.0
Last updated 1/9/04 by Haiying Chen / Anna Oliva

1. Purpose

To convert glass bead sheared *S. pombe* Genomic DNA into labeled DNA for array hybridization. This protocol incorporates aminoallyl dUTP (aadUTP) into random hexamer primed DNA followed by coupling of the aminoallyl groups to either Cyanine 3 or 5 (Cy3/Cy5) fluorescent molecules. The final product is fluorescently labeled DNA, purified and lyophilized, that is ready for re-suspension and array hybridization.

2. Reagent Preparation

2.1. Labeling Mix(dNTPs, 3mM)

2.1.1. Mix the following reagents:

		Final Concentration
dATP (100 mM)	2.5 µl	3mM
dCTP (100 mM)	2.5 µl	3mM
dGTP (100 mM)	2.5 µl	3mM
dH_2O	76 µl	
Total:	**83.5 µl**	

2.1.2. Store unused solution at -20 °C.

2.2 aa-dUTP, 15mM

Dilute 50mM Ambion aa-dUTP to 15mM with 10mM Tris(pH 7.5)

2.3 Phosphate Buffers

2.3.1 Prepare 2 solutions: 1M K_2HPO_4 and 1M KH_2PO_4

2.3.2 Phosphate buffer, 1 M (KPO_4, pH 8.5-8.7), combine:

1 M K_2HPO_4 9.5 mL
1 M KH_2PO_4 0.5 mL
filter sterilize

2.3.3 Phosphate wash buffer (5mM KPO_4, pH 8.0, 80% EtOH), mix:

1 M KPO_4 pH 8.5 0.5 mL
dH_2O 15.25 mL
95% EtOH 84.25 mL

2.3.4 Phosphate Elution Buffer,
Made by diluting 1 M KPO_4, pH 8.5 to 4 mM with dH_2O.

2.4 Sodium Carbonate Buffer (Na_2CO_3): 0.1 M, pH 9.0:

2.4.1 Dissolve 2.12 g Na_2CO_3 in 15 mL of dH_2O

2.4.2 Need ~ 2 mL conc. HCl to bring pH to 9, bring total volume to 20ml with dH_2O, filter sterilize.

2.4.3 Dilute 1:10 with distilled water

Note: Na_2CO_3 buffer changes composition over time so make it fresh every two weeks.

2.5 NHS-ester Cy dye

2.5.1 Resuspend a tube of dye(from Amersham) in 50 µl of anhydrous DMSO

2.5.2 Aliquote 4.5µl fractions into screw-cap tubes.

2.5.3 Wrap all reaction tubes in foil in order to prevent photo-bleaching of the Cy dyes.

2.5.4 Store in desiccant at -20°C.

Note: Any water introduced to the dye esters will result in a lower coupling efficiency due to the hydrolysis of the dye esters. Since anhydrous DMSO is hygroscopic (absorbs water from the atmosphere) store DMSO well sealed at -80°C in desiccant unless a dry atmosphere can be maintained in the stock bottle.

3. Protocol

3.1 Aminoallyl Labeling

3.1.1. Make Genomic DNA / primer mix:

- Purified genomic DNA 4 μg
- Random hexamer (1μg/μl) 10 μl
- Filtered sterilized H_2O bring to 36.5 μl

3.1.2. Mix well and incubate at 100°C for 5 min.

3.1.3. Snap cool on ice/water for 5 min, spin briefly at >10,000 rpm.

3.1.4. Add the following:

	Volume	**Final Concentration**
Genomic DNA / primer	36.5 μl	
Klenow buffer (10x)	5 μl	
Labeling mix (dNTPs, 3mM)	6 μl	360μM
dTTP (5 mM)	1.2 μl	120μM
aa-dUTP (15mM)	0.8 μl	240μM
Klenow fragment(exo)	0.5μl	
Total volume:	**50 μl**	

3.1.5. Mix and incubate at 37°C overnight (or 4-5 hrs).

3.1.6. Stop DNA synthesis by adding 5μl 0.5 M EDTA (pH 8.0)

3.2 Reaction Purification I: Removal of unincorporated aa-dUTP and free amines. (Qiagen PCR purification kit)

Note: This purification protocol is modified from the Qiagen QIAquick PCR purification kit protocol (07/2002, pg 18). The phosphate wash and elution buffers (prepared in 2.3.3 and 2.3.4) are substituted for the Qiagen supplied buffers because the Qiagen buffers contain free amines which compete with the Cy dye coupling reaction. Other modifications from the Qiagen protocol are underlined.

3.2.1 To the 50 μl of above reaction add 3.5 μl 3M NaOAc pH 5.2 and 275 μl of PB buffer, mix and transfer to Qiaquick column.

3.2.2 Place the column in a 2 ml collection tube and spin @ > 13,000 rpm (~13,000 x g) for 1 min. Empty collection tube.

3.2.3 To wash, add 750 μl phosphate buffer to the column and spin at ~ 13,000 rpm (~13,000 x g) for 1 min. Incubate 1 min after adding buffer at room temperature.

3.2.4 Empty the collection tube and repeat the wash and centrifugation step 2 more times.

3.2.5 Empty the collection tube and spin column 1 min at max speed.

3.2.6 Transfer column to a new 1.5 ml tube and carefully add 35 μl phosphate elution buffer (preheated at 37°C) to the center of column membrane.

3.2.7 Incubate 5 min at room temperature.

3.2.8 Elute by centrifugation @ ~ 13,000 rpm (~13,000 x g), 1 min.

3.2.9 Elute a second time in the same way into the same tube, the final volume should be ~ 65 ul.

3.2.10 Dry the eluted sample (aminoallyl DNA, or aa-DNA) in a speed vac. This is a stopping point.

3.3. Coupling aa-DNA to Cy Dye Ester

3.3.1. Most important step! Resuspend the aa-labeled DNA in 4.5 µl 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0 -look at eppendorf and wash side with the tiny drop of buffer until see no pellet on wall -- up and down at least 10 times.

3.3.2. Turn off the lights (and hope your labmates don't complain)... keep the Cy dyes in the dark! Add 4.5 µl -NHS-ester Cy dye to your resuspended aa-DNA

3.3.3. Incubate the rx for 1 hr in the dark at room temperature.

3.4. Reaction Purification II: removal of uncoupled dye (Qiagen PCR purification kit)

3.4.1. As for the previous purification (3.2) bring the volume to 30 µl with dH_2O

3.4.2. Add 2 µl 3M NaOAC pH 5.2 and 150 µl of PB buffer (supplied in the kit).

3.4.3. Follow the same steps as before except using the Qiagen buffers (note that differences from Qiagen protocol are underlined in protocol above). Elute twice with 40 µl of Qiagen elution buffer, the final elution volume should be ~ 75 µl.

3.5. Analysis of Labeling Reaction

If you have a fluorimeter, use it. Otherwise, use a 50 µl quartz cuvette to analyze the entire undiluted sample in a spectrophotometer:

3.5.1 Soaked the cuvette for 1 hr in 1:1 methanol:conc. HCl, then wash with distilled water (lots and lots) and blow dry with compressed air duster.

3.5.2 Pipette the whole sample into the cuvette, for each sample, measure abs @ 260 nm for DNA, 550 nm for Cy3 and 650 nm for Cy5.

3.5.3 Pipette sample from the cuvette back to the original sample tube.

3.5.4 For each sample calculate the total picomoles of DNA synthesized using:

pmol nucleotides: $\frac{[OD260*volume(\mu l)*37\ ng/\mu l*1000\ pg/ng]}{324.5\ pg/pmol}$

Note: 1 OD_{260} = 37 ng/µL for DNA, 324.5 pg/pmol is average molecular weight of a dNTP)

3.5.5 For each sample calculate the total picomoles of dye incorporation (Cy3 or Cy5 accordingly) using:

pmol Cy3 = OD_{550}*volume(µl) / 0.15

pmol Cy5 = OD_{650}*volume(µl) / 0.25

nucleotides/dye ratio: = pmol DNA / pmol Cy dye

For an average reaction get approx 230-350 pmol dye incorporated.

For a good reaction get 1 dye for 20-45 nucleotides you should certainly have not less than 1 dye/60 nt.

4. Materials, suppliers, and ordering information

4.1	dNTP set (100 mM)	Invitrogen,	10297-018
4.2	Klenow Fragment (DNA poly. I)	New Eng. Biolabs	M0212M
4.3	Random Hexamer	MWG	
4.4	Cy5 monoreactive dye pack	Amersham	PA25001
4.5	Cy3 monoreactive dye pack	Amersham	PA23001
4.6	Aminoallyl deoxyuridine triphosphate	Ambion	8439

5. Detailed Protocol notes/discussion

5.1. Unless otherwise specified, all centrifugations are done in a bench top micro centrifuge at room temperature. For this centrifuge 13,000 rpm is roughly 13,000 x g.

6. Protocol adapted from sources

6.1 The Institute for Genomic Research, Standard Operating Procedure (SOP # M004)

7. Related web-links and reference