

STANDARD OPERATING PROCEDURE		
J. David Gladstone Institutes Genomic Core Laboratory		
Title: Probe Synthesis and Fluorescent Labeling		Page Number: 1
SOP #P004	Version: #2	Date: May 2, 2002
Author: Yanxia Hao		Reviewer: Chris Barker

MATERIALS

1. 20 µg total RNA
2. RNasin (40U/µl) (Promega, Catalog # N211A)
3. Superscript II RT (200 units/µl, Invitrogen, Catalog# Y02226, the kit includes both 5X First Strand cDNA Synthesis Buffer and 0.1M DTT)
4. Aminoallyl-dUTP (Sigma, Cat# 0410)
5. Anchored oligo-dT [(dT)₂₄-V], (Operon, HPLC purified) or Oligo(dT)₁₂₋₁₈ (Invitrogen, # N420-01)
6. 1N NaOH (Sigma, #S-0899)
7. 1 M HEPES pH 7.55 (Invitrogen, Catalog# 15630106)
8. 3M NaOAC pH 5.5 (Sigma, #S-7899)
9. EtOH (Rossville Goldshield, 200 Proof)
10. 70% EtOH
11. NaHCO₃ (Sigma, #S-6297)
12. Monofunctional NHS-ester Cy3 (Amersham Pharmacia, # PA23001)
13. Monofunctional NHS-ester Cy5 (Amersham Pharmacia, # PA25001)
14. Cy5Dye Post-labelling Reactive Dye Pack (Amersham Pharmacia, #RPN5661)
15. DMSO (Clontech, #7898-1)
16. Hydroxylamine (Sigma, #H-9876)
17. QIA-Quick PCR purification kit (QIAGEN, # 28106)
18. 100mM dATP, dGTP, dCTP, dTTP (Amersham Pharmacia, 27-2035-02)
19. RNase free water (Ambion #9920)

NOTE: Use items 12 and 13 OR item 14 (contains single use packets of Cy3 and Cy5 dyes).

EQUIPMENT

1. Waterbath (42°C)
2. Eppendorf microfuge, Model 5417C
3. ThermoSavant SpeedVac system
4. Heating Block (70°C)
5. -20°C freezer
6. Vortex genie
7. Pipetman adjustable micropipettors (various)

REAGENT PREPARATION

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1. 50X aa-dUTP/dNTP (7:3) Mix
 - a. Dissolve 1 mg aminoallyl-dUTP in 17µl RNase free water to make a 100 mM solution.
 - b. Prepare a mix of the following nucleotides in the indicated proportions
 - i. 10µl each 100mM dATP, dGTP, dCTP
 - ii. 7µl 100 mM aminoallyl-dUTP
 - iii. 3µl 100 mM dTTP
 - c. Store -20°C
 - d. Altering the ratio of aminoallyl-dUTP to dTTP will affect the incorporation of Cy dye in different system.

2. 0.1 M Sodium Bicarbonate Buffer (NaHCO₃)
 - a. Dissolve 0.84 g NaHCO₃ in 100 mL RNase free water.
 - b. To make a 0.05M solution for dye coupling reaction, dilute 1:2 with RNase free water.
 - c. Confirm pH is approximately 9 with pH paper (range 1-12),

3. Cy-dye esters (Amersham Pharmacia, # PA23001, # PA25001)
 - a. Each pouch contains one tube of dye residue sufficient for 6-8 labeling reactions and should be used immediately or aliquoted and stored. If you will be preparing one or two sets of probes at a time, consider using the single-use packs instead (below).
 - b. Resuspend monofunctional NHS-ester Cy3 or Cy5 dye. Open one pouch of dye. Add 12 or 16 µl DMSO directly to the dye vial. Vortex and briefly spin down.
 - c. Aliquot 2µl of the rest of the dye into single use tubes, then dry immediately in SpeedVac and store desiccated at 4°C.
 - d. If you have already made aliquots of dye, simply transfer your cDNA in NaHCO₃ to the aliquot of the dye.
 - e. NOTE: Dye esters must either be used immediately or aliquoted and stored at 4°C. Any introduced water to the dye esters will result in a lower coupling efficiency due to the hydrolysis of the dye esters. Since DMSO is hygroscopic (absorbs water from the atmosphere), store it well sealed or in single use aliquots.

4. CyDye Post-Labeling Reactive Dye Pack
 - a. Each pack contains enough Cy3 or Cy5 dye for one reaction. If you will be preparing more than one or two sets of probes at a time, consider using the Cy-dye ester pouches (above).
 - b. Add 9 µl cDNA in NaHCO₃ directly to each Cy dye tube as indicated below (see section D, Procedures).

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5. 4M Hydroxylamine (NH₂OH.HCL, FW=69.49)
 - a. Resuspend 13.90g Hydroxylamine in a final volume of 50 ml RNase free water.
 - a. Filter solution through 0.22-micron filter. Store room temperature.

PROCEDURE

A. Reverse Transcription Reaction

1. To 20 µg of total RNA (for each Cy3 and Cy5 reaction) add 2 µg anchored oligo-dT [(dT)24-V] (preferred), or 2 µg of oligo(dT)12-18 and then adjust the volume to 15 µl with RNase free water.
2. Incubate for 10 minutes at 70°C. Hold on ice for 5 minutes.
3. Add:
 - 6 µl 5X First Strand cDNA Synthesis Buffer
 - 3 µl 0.1mM DTT
 - 1 µl 50X aa-dUTP/dNTP (7:3) Mix
 - 0.5 µl RNasin (40U/µl)
 - 1.5 µl Superscript II RT (200 units/µl)
 Add RNase free water to reaction to a final volume of 30 µl.
4. Incubate at 42°C for 1 hour.
5. Add an additional 1 µl Superscript II RT and continue incubation for an additional 1 hour (2 hours total reaction time at least).

B. Hydrolysis

1. Add 10 µl of 1N NaOH, incubate at 70°C for 10 min.
2. Add 25 µl of 1 M HEPES pH 7.5 to neutralize.
3. Sample may be stored at 4°C overnight at this point.

C. Cleanup

1. Add 1/10 volume of 3M NaOAc, 2.5 volume of EtOH to precipitate cDNA.
2. Keep in -20 °C for 20 min or overnight.
3. Spin at maximum speed for 30 min at 4 °C, wash with 1ml of 70% EtOH twice, sample can be stored in 70 % EtOH at -20°C or air dry pellet, continue coupling reaction.

D. Fluorescent Dye Coupling

1. Resuspend cDNA pellet in 9µl of 0.05M NaHCO₃ (pH8.5-9.0), incubate 15 min at RT.

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2. Add 2µl of dye (Cy3 or Cy5) to each reaction.
3. Incubate in dark at RT for 1 hour. Mix gently every 15-minute.
Or if you have dye aliquot, you can simply add your 9 ul of cDNA to it.

E. Quenching and Cleanup

1. Add 4.5µl of 4M Hydroxylamine to each reaction. Incubate for 15 min in dark at RT.
2. Add 35 µl of 100mM NaOAC, pH 5.2.
3. To remove unincorporated/quenched Cy dyes, process with QIA-Quick PCR purification kit:
 - a. Combine Cy3 and Cy5 reactions and add 500 µl Buffer PB.
 - b. Load the sample into the column. Spin at maximum speed for 1 min at room temperature. Discard the flow-through.
 - c. Add 750µl of Buffer PE to the column. Centrifuge at maximum speed for 1 min.
 - d. Discard the flow-through, repeat once.
 - e. Spin at maximum speed for 1 min to dry the column.
 - f. Place the column in a clean 1.5-ml microcentrifuge tube. Add 30µl of Buffer EB, and let stand 1 min. Spin at maximum speed for 1 min.
 - g. To ensure complete elution of cDNA, add a second 30µl of Buffer EB to the column. Let stand 1 min, and centrifuge at maximum speed for 1 min. Elute to the same tube.

D. Analysis of Probe Quality

1. Measure A₂₆₀ and A₅₅₀ for Cy3 probe, A₆₄₉ for Cy5 probe.
2. Refer to <http://www.pangloss.com/seidel/Protocols/probecheck.html> to check your probe incorporation efficiency.
 - a. An acceptable probe will incorporate a minimum of 50 pM of Cy dye.