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| UNM SOM EXPERIMENTAL BIOTECHNOLOGY LABORATORY: MICROARRAYS Standard Operating Procedure | | |
| Title: cDNA synthesis and aminoallyl labeling (Cy3&Cy5 Targets) | | |
| SOP#: M 5.1a | Revision level: 4 | Effective Date: 3/24/2004 |
| Author: V. Bain | Primary Reviewers: B. Griffith | |

1. PURPOSE

This protocol describes the reverse transcription of RNA using aminoallyl nucleotides to synthesize a cDNA which can be fluorescently labeled by coupling with Cyanine 3 or 5 (aka Cy3 or Cy5). The fluorescently labeled cDNAs are used as targets in microarray hybridizations. Before starting this procedure, the lab bench and the racks should all be cleaned and free from RNase. Be sure to use RNase free tubes, water, tips, etc. to assure maximal cDNA synthesis and fluorescent dye coupling.

2. SCOPE

This procedure is used by the Experimental Biotechnology Laboratory under the direction of Drs. Rick Lyons and Tom Williams. This protocol is adapted from prior protocols written by Chris Seidel, formerly of Operon, and from John Quackenbush of TIGR.

3. MATERIALS

- 3.1 Random Hexamer primers (Invitrogen, 3ug/ul stock, Cat# 48190-011)
Stored at -20 C, non-frost free freezer
- 3.2 NaOH (Fisher Cat# S318-500)
- 3.3 Tris (Sigma Cat# T8524, 99.9%, Trizma Base)
- 3.4 NaOAC (Sigma Cat# S-1304, trihydrate)
- 3.5 Na₂CO₃ (EM Science Cat# SX0400-1, anhydrous)
- 3.6 K₂HPO₄ (Sigma Cat# P-5504, dibasic, trihydrate)
- 3.7 KH₂PO₄ (Mallinkrodt Cat# 7100, monobasic)
- 3.8 Superscript II RT, 5X First Strand Buffer, 0.1 M DTT (Invitrogen kit, 200U/ul stock, Cat#18064-014).
Stored at -20 C, non-frost free freezer
- 3.9 dATP, dGTP, dCTP, & dTTP set [100mM] (Amersham Pharmacia, Cat# 27-2035-02)
Stored at -20 C, non-frost free freezer
- 3.10 aminoallyl dUTP (Sigma, cat# A0410).
Stored at -20 C, non-frost free freezer
- 3.11 0.5M EDTA and RNase free water (DEPC treated) (Ambion buffer set Cat# 9010)
- 3.12 Qiagen QiaQuick PCR Kit with PB, PE, and EB buffers (Cat# 28104)
- 3.13 Cy3 monoester dye (Amersham Pharmacia, cat# PA23001)
- 3.14 Cy5 monoester dye (Amersham Pharmacia cat# PA25001)
Prior to resuspension, store at 4C in refrigerator
- 3.15 100% DMSO (Sigma 50mls, cat# D8418)
Stored at room temperature.
- 3.16 Rnase Zap (Ambion Cat# 9780-9782)
Stored at room temperature

4. REAGENT PREPARATION

- 4.1 1 M NaOH
FW= 40gms/mole

Dissolve 4 gms in 85mls of autoclaved double deionized water (aka MilliQ water) and then bring up to final volume of 100mls for 1M final concentration.

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4.2 1M Tris (pH 7.5)

FW= 121.1 gms/mole

Dissolve 12.11 gms in approx 70 mls double deionized water and then pH to 7.5. Bring final volume up to 100mls. Autoclave.

4.3 1 M and 100mM NaOAc (pH 5.2)

NaAcetate trihydrate FW= 136.08 gms/mole

Dissolve 13.6 gms in minimum volume of autoclaved double deionized water and then use acetic acid to bring down to pH 5.2. Bring into final volume of 100 mls for 1 M NaOAc (pH 5.2) stock. Autoclave.

For a 0.1 M NaOAc (pH 5.2) solution, dilute the 1 M stock 1:10 with autoclaved double deionized water

4.4 aminoallyl dUTP 100mM stock

FW acid free= 523.2 gm/mole

Desire 100mM final concentration so resuspend 5mg in 96 ul of 0.1 M KPO₄. Gently vortex to mix and transfer to new sterile microfuge tube. Store at -20C

Check concentration by diluting an aliquot 1:5000 in 0.1M KPO₄ pH 7.5 and measuring the OD₂₈₉. The stock concentration in mM = OD₂₈₉ x 704. If your stock is below 100 mM, then adjust the volume added to the 50x aminoallyl-dNTP mix accordingly.

4.5 50X aminoallyl-dNTP mix :

contains final concentrations thus: 25mM each of dATP, dCTP & dGTP, 15mM dTTP and 10mM aminoallyl dUTP

Combine

10ul of 100mM dATP

10ul of 100mM dCTP

10ul of 100mM dGTP

6ul of 100mM dTTP

4ul of 100mM aminoallyl dUTP (or 5.3 ul of 76mM aadUTP)

Aliquot into 4 aliquots at 10 ul/sterile tube. Store at -20C.

Will use 0.6ul/cDNA synthesis

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4.6 1M Na₂CO₃ (pH 9.0) and 0.1M Na₂CO₃ (pH 9.0)
anhydrous Na₂CO₃ FW = 105.99 gms/mole

The 1M Na₂CO₃ should be made fresh monthly

For the 1 M stock, dissolve 10.6 gms into approx 50-60 mls and pH to 9.0 with 12N HCl. Bring final volume into 100ml for a 1 M stock

The 0.1 M Na₂CO₃ can be made from the 1 M and heated to 60 C for 15 min to inactivate Dnase

4.7 Dye monoester dissolved in 100% DMSO
dissolve 1 tube of dye into 72 ul of 100% DMSO

Aliquot into 16 sterile tubes @ 4.5 ul/tube

Store tubes wrapped in foil at -70C, inside a container with dessicant and with the container wrapped also in foil

Keep dye tubes wrapped in foil to prevent photobleaching

4.8 Phosphate buffers: 1M K₂HPO₄ and 1M KH₂PO₄

4.8.1 1M KH₂PO₄

FW of KH₂PO₄ = 136.09 gms/mole (Mallinkrodt AR)

Dissolve 13.6 gms into final volume of 100 ml of sterile double distilled water for 1 M stock

4.8.2 1M K₂HPO₄

FW of K₂HPO₄ trihydrate = 228.2 gms/mole (Sigma P5504, 99%+, dibasic)

Dissolve 22.8gms into final volume of 100ml sterile double distilled water for 1 M stock

4.8.3 To make 1M Potassium Phosphate buffer (KPO₄ pH 8.5 to 8.7) combine
9.5mL 1M K₂HPO₄
0.5ml 1M KH₂PO₄

4.8.4 To make 100 ml of 5mM KPO₄ Phosphate wash buffer with 80% ethanol, combine

0.5ml of 1 M KPO₄ pH 8.5 to 8.7

15.25 ml of autoclaved double deionized water

84.25 ml of 95% ethanol

This solution will be slightly cloudy

4.8.5 To make KPO₄ Phosphate Elution buffer, combine

4 ul of 1 M KPO₄ buffer pH 8.5-8.7

996ul of sterile double deionized water

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5 PROCEDURE

5.1 First Strand cDNA synthesis

- 5.1.1 Make a Primer/ RNA mixture:
 10 ug of total RNA (2 ug of mRNA)
 2 ul of Random Hexamer primers (Invitrogen, 3ug/ul stock, Cat# 48190-011)
 Bring volume up to 18.5 ul with RNase free water (DEPC treated)
- 5.1.2 Pre-annealing of primer to the RNA:
 Vortex briefly and gently
 Incubate at 70 C for 10 min
 Place on ice for 30 sec to 1 min
 Centrifuge briefly
- 5.1.3 Adding additional reverse transcription reaction components:
 6ul 5X First Strand Buffer supplied with Superscriptase II
 3ul 0.1 M DTT supplied with Superscriptase II
 0.6ul 50X aminoallyl-dNTP mix (stored at -20C)
 2ul Superscript II RT
 (Can make a mastermix of these components)
- 5.1.4 cDNA synthesis reaction incubation:
 Vortex briefly and gently
 Incubate at 42 C for 2 to 2 ½ hours
 Centrifuge briefly

5.2 RNA hydrolysis post cDNA synthesis

- 5.2.1 To hydrolyze the RNA, add:
 10 ul 0.5M EDTA (always add the EDTA first and then add the NaOH)
 10ul 1 M NaOH
 (Do not make a mastermix of EDTA/NaOH; add them separately and sequentially)
 Vortex
 Incubate at 65 C for 15 min
 Neutralize by adding 25 ul of 1M Tris (pH 7.5) or 10ul of 1M HCl. (Use Tris if you select Microcon filters for next step and use HCl if you select Qiagen columns for next step.)

5.3 Purification post cDNA first strand synthesis to removed unincorporated dNTPs and aadUTP: use substituted Potassium Phosphate buffers with Qiagen QiaQuick columns

- 5.3.1 **Purify with Qiaquick PCR columns**, Use with Potassium Phosphate buffers substituted for the wash and elution buffers as listed below: (The Potassium Phosphate buffers are substituted because the kit supplied Qiagen buffers contain free amines which would compete with the Cy dye coupling reaction steps). For 10 rxns, the column purification takes approx 1 hr and the lyophilization takes approx 1 hr.
- Neutralize the RNA with 10ul 1M HCl
 Mix cDNA rxn with 300ul buffer PB (supplied with Qiagen kit)
 Add to QIAquick column
 Place column in collection tube.

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Spin at 13,000 rpm for 1 min in Eppendorf centrifuge
 Empty collection tube
 Add 750ul Phosphate wash buffer
 Spin at 13,000rpm for 1 min in Eppendorf centrifuge.
 Empty collection tube
 Repeat wash and spin
 Spin column again for an additional min at max speed to dry column
 Place column in new 1.5ml tube
 Add 30ul phosphate elution buffer to center of membrane
 Incubate for 1 min at room temperature
 Spin for 1 min at 11,000 rpm (13,000x G) in Picofuge
 Elute into same tube with another 30ul (perform 1 min incubation and 1 min spin as with the first elution)
 The final volume should be 60ul
 Dry down in speed vac on low.

5.4 Coupling Cy Dye Ester to aminoallyl labeled cDNA

- 5.4.1 Resuspend aa-cDNA in 4.5 ul of 0.1M Na₂CO₃ (pH 9.0)
The 1M Na₂CO₃ should be made fresh monthly
 A 0.1 M Na₂CO₃ can be made from that and heated to 60 C for 15 min to inactivate DNase
- 5.4.2 Add 4.5 ul of Dye Ester (prepared in 100%DMSO)
Keep rxns in foil to prevent photobleaching
Note: Axon scanner defaults to Cy3 as the control or reference and Cy5 as the experimental. It is easiest if you follow this order for your cDNA labelings
- 5.4.3 Incubate at Room Temperature for one hour (in the dark)

5.5 Purify away unincorporated Cy dye with Qiagen QiaQuick PCR Kit (use buffers supplied with the Qiagen kit)

Add 35 ul of 100mM NaOAc (pH 5.2) to the dye labeled cDNA
 Add 500ul Buffer PB (supplied with kit)
 Place spin column in collection tube
 Apply sample to column
 Centrifuge at 13,000 g for 1 min in Eppendorf centrifuge (13000rpm)
 Empty collection tube
 Wash with 750ul of Buffer PE (supplied with kit)
 Centrifuge at 13,000 g for 1 min in Eppendorf centrifuge (13,000rpm)
 Empty collection tube
 Centrifuge at 13,000 g for 1min to dry membrane in Eppendorf centrifuge(13,000 rpm)
 Place column in clean tube
 Add 30ul of Buffer EB to center of column (EB supplied with kit)
 Incubate at Room Temperature for 1 min
 Centrifuge 13,000g (11,000 rpms in picofuge) for 1 min to elute
 Elute again using 30ul of EB, incubate, and spin. Final vol. should be 60ul.

5.6 Analyze with Spectrophotometer

Read the wavelengths from 200nm to 800nm, using clean microcuvette
 Measure the 260, 280, and 550 (Cy3) or 650 (Cy5) of the full 60ul of rxn
 Do not throw sample away!! Place it back into tube!
 Determine incorporation by calculations available at this website:

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http://www.pangloss.com/seidel/Protocols/percent_inc.html

5.7 Yield

5.7.1 In general, 10ug's of input total RNA incorporates approx 300-500 pmoles of Cy3 dye or 300 to 500 pmoles of Cy5 dye. The desired ratio of pmoles cDNA /pmoles of dye is 50 or below. (10/2003 review of incorporation noted an average of 406pm of Cy5 and 366pm of Cy3 per 10ug input total RNA)

5.8 Storage conditions:

5.8.1 Dry down labeled cDNAs in Speed Vac (cover speed vac to keep light out) and store at -20 C or use for hybridizations. Keep tubes wrapped in foil and in a covered, opaque box.