cDNA Microarray Indirect Labeling

Section of Cancer Genomics, Genetics Branch, NCI
National Institutes of Health

Reagents

Amino-allyl dUTP
Sigma, Cat. A0410
Bovine Serum Albumin (BSA)
Sigma, Cat. A-9418
Cot I –DNA
Invitrogen Corp., Cat. 25279-011
Deionized Formamide, 99%
Ambion, Cat. 9342
DEPC water
Research Genetics, Cat. 750024 and 750023
Dimethyl-sulfoxide (DMSO)
dNTP Set
Amersham-Pharmacia, Cat. 27-2035-01
500mM EDTA
Research Genetics, Cat. 750009
Eppendorf tubes
HCl, 1N and 12 N
3M NaOAc pH 5.2
Quality Biological, Cat. 351-035-060
1M NaOH
Sigma, Cat. 930-65
NHS-Cy dyes
Amersham-Pharmacia, Cat. PA23001 and PA25001
Plugged pipet tips
Poly dA (40-60)
Amersham-Pharmacia, Cat. 27-7988-01
Potassium Phosphate, dibasic (K2HPO4)
Sigma, Cat. P8584
Potassium Phosphate, monobasic (KH2PO4)
Sigma, Cat. P8709
QIAquick PCR purification kit
Qiagen, Cat. 28106
Random hexamer
Invitrogen Corp., Cat. 48190-011
RNase Away
VWR, Cat.72830-022
10% SDS
Research Genetics, Cat. 750008

Sodium Carbonate (Na2CO3)
Sigma, Cat. S7795
SSII reverse transcriptase
Invitrogen Corp., Cat. 18064-014
20X SSC
Research Genetics, Cat. 750020

Preparations

Prepare a 100mM aminoallyl dUTP by dissolving 1 mg in 19.1 µl of 0.1 M KPO4 buffer, pH 7.5. The concentration of this stock solution can be measured by diluting an aliquot 1:5000 in the same buffer and measuring OD289. Stock concentration in mM = OD289x704.

Prepare 50X aa-dNTP labeling mix containing a 2:3 ratio of aminoallyl-dUTP to unlabeled dTTP containing a final concentration of 25 mM dATP, 25 mM dCTP, 25 mM dGTP, 15 mM dTTP ,10 mM aa-dUTP:

		final conc.
dATP (100mM)	5µl	25mM
dCTP (100mM)	5µl	25mM
dGTP (100mM)	5µl	25mM
dTTP (100mM)	3µl	15mM
aa-dUTP (100mM)	2µl	10mM
Total:	20µl	

Unused solution can be stored at -20°C.

Prepare the Cy-dye esters for labeling. The Cy3-ester and Cy5-ester are each provided by AmershamPharmacia as dried samples in 5 tubes. Resuspend the dye ester in 73 µl DMSO. The dye esters can spontaneously hydrolyze, so this must either be used immediately or stored at -80°C as 4.5 µl single-use aliquots, parafilm around each tube. Avoid moisture!

Prepare a 0.1 M Na2CO3, pH 9.0 carbonate buffer for the coupling reaction. Dissolve 10.8 g Na2CO3 in 80 ml water and bring the pH to 9.0 with 12 N HCl; bring the final volume to 100 ml with water. Dilute 1:10 with water to make the 0.1M solution used in the coupling reaction. Carbonate buffer changes composition over time so make sure you make it fresh every couple of weeks to a month.

Preparation of Phosphate buffers

1. Prepare 2 solutions: 1 M KH2PO4 and 1M K2HPO4.

2. Combine

1M K2HPO4	9.5 ml
1M KH2PO4	0.5 ml
Total	10 ml

to make 1 M KPO4 (Phosphate buffer). The pH of this solution should be 8.5-8.7.

3. For 100 ml Phosphate wash buffer, mix:

1M KPO4 pH 8.5	0.5 ml
MilliQ water	15.25 ml
95% ethanol	84.25 ml
Total	100 ml

Note: this solution will be slightly cloudy.

4. Phosphate elution buffer is made by dilution of the 1 M KPO4 pH 8.5 to 4 mM.

1 M KPO4 pH 8.5	0.4 ml
MilliQ water	99.6 ml
Total	100 ml

Prehybridization

1. Prepare prehybridization buffer containing 5X SSC, 0.1% SDS and 1% bovine serum albumin (BSA).

		final conc.
BSA powder	1 g	1%
20X SSC	25 ml	5X
10% SDS	1 ml	0.1%
DEPC H_2O	74 ml	
Total	100 ml	

2. Prepare 1X hybridization buffer containing 50% formamide, 5X SSC, and 0.1% SDS.

		final conc.
Deionized formamide	50 μl	50 %
20X SSC	25 μl	5X SSC
10% SDS	1 μl	0.1% SDS
DEPC H_2O	24 μl	
Total	100 μl	

Hybridization

Prepare Poly(A)-DNA by dissolving stock Poly(A)-DNA in a neutral buffer (i.e. 10 mM Tris, pH 7) to a final concentration of 10 μg/μL.

Prepare COT1-DNA (stock conc.1μg/μL) by ethanol precipitation:
- Add 2 to 3 volumes of ethanol and 0.1 volumes of 3 M Sodium Acetate (NaOAc) to the stock tube.

- Mix well and place on dry ice for 20-30 min or in –20°C freezer overnight.
- Centrifuge for 20-30 min in a cold room microfuge at maximum angular velocity.
- Remove supernatant and allow excess ethanol to dry off.
- Dissolve precipitated COT1 in a neutral buffer (i.e. 10 mM Tris, pH 7) to the final concentration of 10μg/μL.

Washing (for 200ml)

1. 1X SSC + 0.2% SDS at 42°C

10 ml	20X SSC
4 ml	10% SDS
186 ml	water
Total 200 ml	

2. 0.1X SSC + 0.2% SDS at room temperature

1 ml	20X SSC
4 ml	10% SDS
195 ml	water
Total 200 ml	

3. 0.1X SSC at room temperature

1 ml	20X SSC
199 ml	water
Total 200 ml	

Procedure: Indirect (amino-allyl) labeling of cDNA Arrays

High - concentrated RNA appears to be "gel like" most of the time and it does not interfere with it's quality. However, RNA is not very stable if you keep it concentrated in DEPC-water (which is slightly acidic). It degrades with time.
Our suggestion would be to keep your RNA under 95% EtOH in -80°C and spin down and resuspend in water just before usage.

(1) <u>RT Reaction:</u>

After Trizol extraction and RNeasy purification, RNA is resuspended in H_2O. After taking samples out from –80°C freezer, heat RNA samples to 50-65ºC (water bath) for 10 min and cool to RT for 10 min before starting. Bring water bath down to 42°C.

Take 20µg total RNA and add DEPC H_2O to get final amount of (One can try to use also as less as 10µg total RNA.)	16.4µl
Add Random hexamer (3µg/µl)	2µl

Total volume	18.4µl

Incubate at 70°C (heat block) for 10 min and cool to 42°C (by placing on ice for 5 min)

Add:	
5X First Strand Buffer	6µl
0.1M DTT	3µl
50X aa-dNTP mix	0.6µl
SSII Reverse Transcriptase	2µl
Total volume	30µl

Incubate at 42°C (water bath) for 3 hr. (Can be longer, e.g. over night)

(2) Labeling Reaction:

1. to each labeling reaction add of 500mM EDTA (pH8.0) 5µl
2. vortex
3. add of 1M NaOH 10µl
4. vortex and spin briefly
5. incubate at 65°C (heat block) for 20 min to hydrolyze RNA, then cool to R/T
6. spin briefly
7. to neutralize, add 1M HCL 10µl
   vortex and spin briefly
8. perform probe purification with Qiagen PCR spin columns:
   a. add 300µl PB buffer to reaction, mix with pipet, and transfer to QIAquick column
   b. spin at maximum speed for 1 min, discard flow through
   c. wash with 740µl phosphate wash buffer (home-made buffer, NOT from kit)
   d. spin at maximum speed for 1 min
   e. empty collection tube and repeat wash wash steps c and d
   f. empty collection tube and spin at maximum speed for 1 min
   g. transfer column to a fresh tube (cut off caps), elute with 30µl elution buffer (home-made KPO4, NOT from kit) with 1 min incubation followed by spin at maximum speed for 1 min
   h. repeat elution step g, transfer to new tube
   i. speedvac dry (~ 40 minutes) (heater on middle)

9. Cy dye coupling
   a. resuspend the cDNA in 4.5µl 0.1M carbonate buffer, pH9.0 (< 1 month old)
   b. add 4.5µl NHS-Cy dyes
   c. incubate the reactions in dark for 1 hour in room temperature
      - make prehyb. buffer and warm to 42°C
      - set heat block to 95°C

(3) Purification:

1. add 35µl 100mM NaOAc pH 5.2 to the reaction
2. add 250µl PB buffer to reaction and follow QIAquick PCR purification protocol:
    a. apply reaction volume to QIAquick spin column and centrifuge for 1 minute
    b. discard flow-through, should be colored
    c. add 0.74ml buffer PE (as provided with kit) to column and centrifuge for 1 minute
    d. discard flow-through and centrifuge for an additional 1 minute at maximum speed
    e. place QIAquick column in a clean 1.5ml microcentrifuge tube
    f. elute twice with 30µl EB using 1 minute incubation time before centrifugation (eluted sample should look colored and filter should be white)
    g. take 1µl of sample for dye incorporation analysis
    h. speedvac dry sample (~ 40 minutes) (heat setting to middle)

(4) Dye Incorporation Analysis:

1. take 1µl of sample for dye incorporation analysis
2. measure absorbance at 260nm, 550nm (Cy3), and 650nm (Cy5)

>> for Cy3 incorporation: nucleotide/dye = 17.1*OD260/OD550
>> for Cy5 incorporation: nucleotide/dye = 28.5*OD260/OD650
>>>> 150pmole/slide is optimal for hybridization, nucleotide/dye should be < 50

(5) Prehybridization:

1. place slides to be analyzed into a Coplin jar with pre-warmed prehybridization buffer and incubate at 42ºC for 45 min
2. wash slides in room temperature DEPC-H_2O, shaker for 10 min
3. dip slides in room temperature isopropanol and spin dry in centrifuge for 5 min
    >> slides should be used immediately and should not dry for more than 1 hr
4. place slides in hybridization chamber adding 35µl of water to ends of the chamber

(6) Hybridization:

1. resuspend each labeled probe in 12µl of 1xHybridization buffer (home-made, make fresh)
2. combine 12µl of each of purified Cy3- and Cy5-labeled probes, mix well
3. add 2µl COT1-DNA (10µg/µl)
4. add 2µl Poly(A)-DNA (10µg/µl)
5. heat probe mixture at 95ºC for 3 min to denature
6. centrifuge probe for 1 min at maximum speed
7. apply labeled probe to prehybridized microarray slide and coverslip (22x50 mm)
8. place sealed chamber in a 42ºC water bath and incubate for 16-20 hr

(7) <u>Washing:</u>

1. remove array from chamber and place in staining dish containing pre-warmed low stringency wash
   buffer (1X SSC + 0.2% SDS) at 42ºC
2. gently remove coverslip while slide is in solution and agitate for 2x 10 min, changing solution in between.
3. wash slide at high-stringency (0.1X SSC + 0.2% SDS) in a Coplin jar agitating for 2x 10 min at room temperature, changing solution in between
4. wash slide in 0.1X SSC in Coplin jar agitating for 2x 10 min at room temperature, changing solution in between.
5. dry slides while centrifuging at 500 rpm for 3 min
6. scan slide if possible the same day