

cDNA Microarray Indirect Labeling

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

Reagents

Amino-allyl dUTP

Sigma, Cat. A0410

BSA

Sigma, Cat. A-9418

Cot I –DNA

Life Technologies, Cat. 25279-011

Deionized Formamide, 99%

Ambion, Cat. 9342

DEPC water

Research Genetics, Cat. 750024 and 750023

DMSO

dNTP Set

Amersham-Pharmacia, Cat. 27-2035-01

500mM EDTA

Research Genetics, Cat. 750009

Eppendorf tubes

1M HCl

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

Life Technologies, Cat. 48190-011

RNase Away

VWR, Cat.72830-022

10% SDS

Research Genetics, Cat. 750008

Sodium Carbonate (Na₂CO₃)

Sigma, Cat. S7795

SSII reverse transcriptase

Life Technologies, Cat. 18064-014

20x SSC

Research Genetics, Cat. 750020

Preparations

Prepare a 100mM aminoallyl dUTP by dissolving 1 mg in 19.1 μ l 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 = OD₂₈₉ / 0.04.

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

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

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 μ l DMSO. The dye esters can spontaneously hydrolyze, so this must either be used immediately or stored at -80°C as 4.5 μ l single-use aliquots, parafilm around each tube. Avoid moisture!

Prepare a 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

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

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.

Prehybridization

1. Prepare prehybridization buffer containing 5₂SSC, 0.1% SDS and 1% bovine serum albumin, BSA

10μl 10% BSA (or 1g BSA powder)

25ml 20xSSC

1ml 10% SDS

74ml DEPC water

100ml total

2. Prepare 1₂ hybridization buffer containing 50% formamide, 5₂SSC, and 0.1% SDS.

50μl deionized formamide

25μl 20x SSC

1μl 10% SDS

24μl DEPC water

100μl total

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 minutes or in -20oC freezer overnight.

- Centrifuge for 20-30 minutes 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. 1xSSC + 0.2% SDS

10ml	20xSSC
4ml	10% SDS
186ml	water

2. 0.1xSSC + 0.2% SDS

1ml	20xSSC
4ml	10% SDS
195ml	water

3. 0.1xSSC

1ml	20xSSC
199ml	water

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 and spin down and resuspend in water just before usage.

(1) RT Reaction:

After Trizol extraction and RNeasy purification, RNA is resuspended in H₂O. After taking samples out from -80C freezer, heat RNA samples to 50-65°C (water bath) for 10 min and cool to RT for 10min. before starting. (bring water bath down to 42 C).

Take 20µg total RNA and add DEPC H ₂ O 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
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Total volume	18.4µl

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

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

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

(2) Labeling Reaction:

1. to each labeling reaction add of 500mM EDTA (pH8.0) 5 μ l
2. vortex
3. add of 1M NaOH 10 μ l
4. vortex and spin briefly
5. incubate at 65°C (heat block) for 20 minutes to hydrolyze RNA, then cool to R/T
6. spin briefly
7. to neutralize, add of 1M HCL 10 μ l

vortex and spin briefly

8. perform probe purification with Qiagen PCR spin columns:
 - b. add 300 μ l PB buffer to reaction, mix with pipet, and transfer to QIAquick column
 - c. spin at maximum speed for 1 minute, discard flow through
 - d. wash with 740 μ l phosphate wash buffer (home-made buffer, NOT from kit)
 - e. spin at maximum speed for 1 minute
 - f. empty collection tube and repeat wash wash steps c and d
 - g. empty collection tube and spin at maximum speed for 1 minute
 - h. transfer column to a fresh tube (cut off caps), elute with 30 μ l elution buffer (home-made KPO₄, NOT from kit) with 1 minute incubation followed by spin at maximum speed for 1 minute
 - i. repeat elution step g, transfer to new tube
 - j. speedvac dry (~ 40 minutes) (heater on middle)
9. Cy dye coupling
 - a. resuspend the cDNA in 4.5 μ l 0.1M carbonate buffer, pH9.0 (< 1 month old)
 - b. add 4.5 μ l NHS-Cy dyes
 - c. incubate the reactions in dark for 1 hour in room temperature
 - make prehyb. buffer and warm to 42 C
 - heat block to 95 C

(3) Purification:

1. add 35 μ l 100mM NaOAc pH 5.2 to the reaction
2. add 250 μ l 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 30 μ l EB using 1 minute incubation time before centrifugation (eluted sample should look colored and filter should be white)
- g. take 1 μ l of sample for dye incorporation analysis
- h. speedvac dry sample (~ 40 minutes) (heater of middle)

(4) Dye Incorporation Analysis:

1. take 1 μ l of sample for dye incorporation analysis
2. measure absorbance at 260nm, 550nm (Cy3), and 650nm (Cy5)
- >> for Cy3 incorporation: nucleotide/dye = $17.1 * OD_{260} / OD_{550}$
- >> for Cy5 incorporation: nucleotide/dye = $28.5 * OD_{260} / OD_{650}$
- >>>> 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 42°C for 45 minutes
2. wash slides in room temperature DEPC-H₂O, shaker for 10min.
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 one hour
4. place slides in hybridization chamber adding 35 μ l of water to ends of the chamber

(6) Hybridization:

1. resuspend each labeled probe in 12 μ l of 1xHybridization buffer (home-made, make fresh)
2. combine 12 μ l of each of purified Cy3- and Cy5-labeled probes, mix well
3. add 2 μ l COT1-DNA (10 μ g/ μ l)
4. add 2 μ l Poly(A)-DNA (10 μ g/ μ l)
5. heat probe mixture at 95°C for 3 minutes to denature
6. centrifuge probe for 1 minute at maximum speed
7. apply labeled probe to prehybridized microarray slide and coverslip (22x50mm)
8. place sealed chamber in a 42°C water bath and incubate for 16-20 hours

(7) Washing:

1. remove array from chamber and place in staining dish containing low-stringency wash buffer (1xSSC + 0.2% SDS) at 42°C
2. gently remove coverslip while slide is in solution and agitate for 2x 10 minutes, changing solution in between.

3. wash slide at high-stringency (0.1xSSC + 0.2% SDS) in a Coplin jar agitating for 2x 10 minutes at room temperature, changing solution in between
4. wash slide in 0.1xSSC in Coplin jar agitating for 2x 10 minutes at room temperature, changing solution in between.
5. dry slides while centrifuging at 500rpm for 3 minutes
6. scan slide if possible the same day