

cDNA Labeling with Aminoallyl- dUTP (& generic buffers)

A. Probe labeling and purification

1. Remove all reagents required for each reaction from the freezer. Place the enzyme solutions on ice immediately. Thaw the other reagents at 37°C for ~ 5 minutes mix and store on ice until use (except the water, which should be stored at room temperature).
2. Combine the following to a final volume of 15.5 µl

Reagent	Volume(µl)
Primer P2	2.0 (2ug/ul)
Total RNA	(1-10 ug)
RNasin (inhib)	1.0
DEPC water	To 15.5 ul

Heat the mixture at 70°C for 10 min, then place on ice to chill for 10 minutes. Briefly centrifuge to collect all components to the bottom of the tube.

3. Add the following reagents in the order listed (if desired, a stock can be made up that will support several labeling reactions):

REAGENT	Volume(µl)
5X buffer	6.0
50X aa dUTP/dNTPs	0.6
DTT (0.1 M)	3.0
SuperScript RT	1.9
DEPC water	3.0
Total	14.5

50X aa dUTP/dNTPs: 10 ul each of dATP (100 mM), dGTP (100mM), and dCTP (100mM); 4 ul of aa dUTP (100 mM), 6 ul of dTTP (100mM).

4. Pipet up and down gently to thoroughly mix the reaction mixture and incubate at 42 °C for 2 hour
5. Briefly centrifuge the reaction and add 10µl of 0.5M EDTA to each reaction mixture to stop the reaction. (Note: you can interrupt the procedure at this point and keep the reaction mixture at 4°C overnight).
6. Add 10µl of 1M NaOH to each reaction mixture and mix.
7. Incubate at 65°C for 30 minutes.
8. Neutralize with 10µl of 1M HCl.

9. Concentrate and purify cDNA using Qiagen MinElute PCR Purification Kit:

- 9-1. Fill the microcentrifuge tube (1.5 ml) with 300 µl Buffer PB.
- 9-2. Add 60 µl of the neutralized reaction to Buffer PB.
- 9-3. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
- 9-4. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min.
For maximum recovery, transfer all traces of sample to the column.
- 9-5. Pour the flow-through back onto the top of the column and centrifuge again for 1 min.
- 9-6. Discard flow-through. Place the MinElute column back into the same tube.
- 9-7. To wash, add 750 µl Buffer PE to the MinElute column, incubate for 5 min at room temperature and centrifuge for 1 min.
- 9-8. Discard flow-through and place the MinElute column back in the same tube.
Centrifuge the column for an additional 1 min at maximum speed.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
- 9-9. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.
- 9-10. To elute DNA, add 10 µl of H₂O (pH between 7.0 and 8.5) to the center of the membrane, let the column stand for 1 min, and then centrifuge for 5 min.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.
The average eluate volume is 9 µl from 10 µl elution buffer volume.
- 9-11. Repeat step 9-10 two more times, collecting 27 µl of purified cDNA .
(Note: You can interrupt the procedure at this point and keep the reaction mixture at -20°C overnight).
- 9-12. Add 3 µl of 1M sodium bicarbonate, pH 9.3 to the cDNA solution.
Add cDNA solution to the dye, if dye has been aliquoted and dried in a SpeedVac; or add 1-2 µl of the dye directly to cDNA solution, if the dye was resuspended in 16 µl DMF or DMSO.
- 9-13. Mix the dye and cDNA; incubate at RT for 1-2 hours in a dark on the shaker (60 rpm)

10. Stop the labelling reaction by adding 4.5 µl of 4M hydroxylamine hydrochloride to the reaction mixture. Mix and briefly centrifuge.

Incubate at RT for 30 min in the dark.

11. To remove unincorporated/quenched cye-dyes proceed with QIAGEN Qia-quick PCR Purification Kit (11) or QIAGEN MinElute Purification Kit (12):

Qia-quick PCR purification kit:

- 11-1. Combine Cy3 and Cy5 reactions.
- 11-2. Add 30 µl of water to the mixture.
- 11-3. Add 500 µl of Buffer PB to the mixture of labelled cDNAs.
- 11-4. Place a QIAquick spin column in a provided 2 ml collection tube.
- 11-5. Apply the sample to the QIAquick column and centrifuge at 13,000 rpm for 30-60 sec

(continued on next page)

- 11-6. Discard flow-through. Place the QIAquick column back into the same tube.
- 11-7. To wash, add 750µl Buffer PE to the QIAquick column and centrifuge for 30-60 sec.
- 11-8. Discard flow-through and place the QIAquick column back into the same tube.
- 11-9. Repeat step 11-7.
- 11-10. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1min at maximum speed.
- 11-11. Place QIAquick column in a clean 1.5 ml microfuge tube
- 11-12. To elute Cy-labelled cDNA, add 30µl of EB buffer or ultra pure water to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge at 13,000 rpm for 1 min.
- 11-13. Repeat elution step one more time.

MinElute Purification Kit:

- 12-1. Combine Cy3 and Cy5 reactions.
- 12-2. Add 30µl of water to the mixture.
- 12-3. Add 500 µl of Buffer PB to the mixture of labelled cDNAs.
- 12-4. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
- 12-5. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min. *For maximum recovery, transfer all traces of sample to the column.*
- 12-6. Discard flow-through. Place the MinElute column back into the same tube.
- 12-7. To wash, add 750 µl Buffer PE to the MinElute column, incubate for 5 min at room temperature and centrifuge for 1 min.
- 12-8. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1min at maximum speed.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
- 12-9. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.
- 12-10. To elute DNA, add 10 µl of Buffer EB to the center of the membrane, let the column stand for 1 min, and then centrifuge for 5 min.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.
The average eluate volume is 9 µl from 10 µl elution buffer volume.

B. Hybridization

(steps 1 and 2 are for Qia-quick PCR purification kit users)

1. Dry the QIAquick eluate samples in a SpeedVac protecting them from the light
 2. Bring the volume up to 23 µl with ultra pure water.
 3. Add 4.5 µl of 20X SSC.
 4. Add 2 µl of polyA(10mg/ml)
 5. Add 0.6 µl of 10% SDS.
 6. Incubate the reaction mixture at 100° C for 2 min.
 7. Centrifuge the reaction mixture at 13,000-14,000 rpm for 5 min.
 8. Apply 12 µl to prepared microarray. Add 2x12ul of 3xSSC to the chamber.
 9. Incubate at conditions specified by array manufacturer.
- (Wash procedure on next page)*

C. Wash off unbound fluorescent cDNA

1. Remove the hybridization chamber from the water bath (or incubator), cool and carefully dry off (if needed). Unseal the chamber and remove the slide.

As there may be negative pressure in the chamber after cooling, it is necessary to remove water from around the seals so that it is not pulled into the chamber and onto the slide when the seals are loosened.

2. Place the slide, with the coverslip still affixed, into a Coplin jar filled with 0.5X SSC/0.01% SDS wash buffer. Allow the coverslip to fall from the slide and then remove the coverslip from the jar with a forceps. Allow the slide to wash for 2-5 minutes.
3. Transfer the slide to a fresh Coplin jar filled with fresh 0.5X SSC/0.01% SDS wash buffer. Allow the slide to wash for 10 minutes on the shaker (rpm 60).
4. Transfer the slide to a fresh Coplin jar filled with 0.06X SSC. Allow the slide to wash for 10 minutes on the shaker (rpm 60).
5. Transfer the slide to a slide rack and centrifuge at low rpm (700-1000) for 5 minutes in a clinical centrifuge equipped with a horizontal rotor for microtiter plates.

If the slide is simply air dried, it frequently acquires a fluorescent haze. Centrifuging off the liquids results in a lower fluorescent background. As the rate of drying can be quite rapid, it is suggested that the slide be placed in the centrifuge immediately upon removal from the Coplin jar.