

Labeling Protocol

A. Synthesis of cyanine-labeled cDNA

1. Program a thermocycler as follows:

Program CMT

Temperature	Time	Reaction
70°C	10 min	Denaturation of template RNA
4°C	5 min	Can pause thermocycler at this point
23°C	10 min	Annealing of primer to RNA
42°C	2 h	Extension of cDNA chain
4°C	hold	

2. Depending on the RNA template, prepare the appropriate RNA/primer annealing mix on ice:

For Total RNA Templates

Total RNA	20 µg
Oligo(dT) primer	2 µl (stock 1µ/µl)
<i>A. thaliana</i> spikes	1µl
Nuclease-free water to:	23 µl (total vol.)

For mRNA Templates

mRNA	1.5 µg
Random primers (9-mers)	3.3 µg
<i>A. thaliana</i> spikes	1µl
Nuclease-free water to:	23 µl

3. Place tubes in the thermocycler and initiate the cycling program. **The *A. thaliana* spikes should be at a final concentration of 0.25ng/µl per reaction for each spike.**
4. Perform this step and the next during incubation of the RNA/primer mix. Prepare working stock of dNTPs having the following concentrations: 0.5 mM dATP, dGTP, dTTP, and 0.25 mM dCTP in DEPC-H₂O.
5. Keeping all reagents on ice, add in the following order (if performing multiple cDNA synthesis reactions, a single labeling mix for each dye can be prepared by scaling up the volumes proportionally):

Labeling Master Mix:

5 X first-strand buffer
0.1 M DTT
dNTPs (from working stock prepared in step A4)

Cy3™ labeling

8 µl
4 µl
<u>2 µl</u>
14µl

1 mM FluoroLink™ Cy3™-dCTP or Cy5™-dCTP	1 µl
SuperScript™ II Reverse Transcriptase (200 U/µl)	2 µl

6. Initiate the cDNA synthesis reaction by adding in the following order: the labeling mix, dye, and SSII RT from step A5 to the RNA/primer mix of step A3, during the initial 5 minute hold at 4°C. The final reaction volume of the reaction is **40 µl**. Continue the thermocycler program (10 min at 23°C followed by 2 h at 42°C).
7. To the labeling reaction from step A6 add 1 µl of RNase H (2 U/µl) and 0.5 µl of RNase A (3.2 U/µl). Incubate at 37°C for 15 min.

B. Probe Purification

1. Transfer labeling reactions to 1.5ml E-tubes to perform the precipitation and cleanup.
2. To precipitate the labeled cDNA, add 4 µl of 3 M sodium acetate, pH 5.2, to the 41.5-µl cDNA-synthesis reaction from step B2, mix briefly, then add 100 µl of 100% ethanol. Briefly mix the contents of the tube and incubate at -20°C for at least 30 min. The cDNA may be stored at -20°C for

up to one week. This is a good stopping point if the purification procedure cannot be completed within the day. Following incubation at -20°C , centrifuge at $12,000 \times g$ for at least 30 min to pellet the cDNA.

3. Carefully remove the supernatant and dissolve the pellet in $40 \mu\text{l}$ of DEPC treated water. Add $4 \mu\text{l}$ of 3 M sodium acetate, pH 5.2, and $200 \mu\text{l}$ of QIAquick™ Buffer PB. Incubate at 37°C for 15 min (incubation at 95°C for 30 sec is sometimes necessary to fully dissolve the pelleted cDNA).
4. Place a QIAquick™ column in a 2-ml collection tube. If combining several labeling reactions, keep in mind that these columns have a binding capacity of approximately $10 \mu\text{g}$. For the purpose of purification, assume a yield of $1 \mu\text{g}$ of cDNA per reaction. Apply the cDNA/Buffer PB mixture to the center of the column.
5. Centrifuge column at 14,000 rpm for 60 sec in a microcentrifuge.
6. Discard flow-through and place column back in the collection tube.
7. Add $600 \mu\text{l}$ of QIAquick™ Buffer PE to the column. Centrifuge at 14,000 rpm for 1 min or until buffer has passed through column.
8. Discard flow-through and place column back in the collection tube.
9. Repeat steps C6 and C7 once more for a total of two $600\text{-}\mu\text{l}$ washes with Buffer PE.
10. Centrifuge the column for 2 min at 14,000 rpm to dry off the ethanol.
11. Place the column in a clean 1.5-ml microfuge tube. Apply $35 \mu\text{l}$ of elution buffer (we recommend the use of distilled water with pH adjusted to 8.0 by addition of a small volume of NaOH) preheated to 37°C to the center of the resin bed and let stand for 1 min.
12. Centrifuge column at 14,000 rpm for 1 min.
13. Repeat the elution step with a second $35 \mu\text{l}$ of elution buffer (see C10). Optionally, DTT can be added to the eluate at a final concentration of 1 mM, to stabilize the cDNA.

Note: The QIAquick™ PCR columns eliminate single-stranded DNA molecule less than 200 nucleotides long (100 bp for double-stranded DNA). Failure to remove these small fragments from the probe may result in higher backgrounds.

C. Determination of the Frequency of Incorporation of Fluorescent Nucleotide into the Probe

The frequency of incorporation (FOI) is defined as the number of labeled nucleotides incorporated per 1,000 nucleotides of cDNA. Use the following formulae to calculate the FOI:

Amount of cDNA (ng) = $A_{260} \times 37 \times \text{total volume of probe } (\mu\text{l})$ (Usually 70 μl)

Total pmol of dye incorporated =

for Cy3™-cDNA: $A_{550} \times \text{total volume of probe} / 0.15$

for Cy5™-cDNA: $A_{650} \times \text{total volume of probe} / 0.25$

FOI = pmol of dye incorporated $\times 324.5$ / ng of cDNA

Note: Measurements should be done using undiluted probe directly in an ultra microcuvette. The probe should not be diluted for spectrophotometry because of its low initial concentration. Diluting the probe prior to assay may give inaccurate readings because of the low absorbance. The probe used for spectrophotometry should be recovered from the cuvette and used in the hybridization reaction. Clean the cuvette thoroughly between samples with 0.1 N HCl and nuclease-free water to prevent cross contamination.