SCHNABLE LAB
Plant Genomics

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INDIRECT LABELING OF AMPLIFIED RNA FOR cDNA MICROARRAY EXPERIMENTS

(Last Revised: Jan 2016)

This protocol was optimized by Dr. David Skibbe (edited by Dr. Kazuhiro Ohtsu) and is used by the Schnable Laboratory (Iowa State University) for cDNA microarray experiments. Please contact Dr. Patrick S. Schnable (schanble@iastate.edu) regarding questions or corrections.

NOTE: Before performing a microarray experiment, the quality of the starting material should be assessed on a 1.2% denaturing RNA gel.

Procedure:

Mix high quality amplified RNA (aRNA) and reverse transcription primer.

~2 μg aRNA	x	μl
3 μg random hexamer (Roche Cat#1034731)	y	μl
dH2O (nuclease-free)	18.4-x-y	μl
	18.4	μl

1. Denature the mixture at 70°C for 10 minutes. Cool on ice for 2 minutes.

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2. Add the following reverse transcription mix to each sample:

5X 1st Strand Buffer	6.0 µl
0.1 M DTT	3.0 µl
50X aa-dNTP mix*	0.6 µl
	9.6 µl

* recipe for making 50X aa-dNTP

	Vol.	Final concentration
dATP (100mM)	5ul	(25mM)
dCTP (100mM)	5ul	(25mM)
dGTP (100mM)	5ul	(25mM)
dTTP (100mM)	3ul	(15mM)
aa-dUTP (100mM)	2ul	(10mM)
Total	20ul	

Store the mix solution at -20 freezer until used.
Aminoallyl-dUTP(aa-dUTP) 1mg (Sigma, Cat# A0410), adding 17ul DEPC-treated water to dissolve it which makes 100mM stock aa-dUTP.

3. Add 2 µl of Superscript II (200 U/µl), mix gently, and incubate the samples at 42°C for 3 hours.
4. Add 1.5 µl of 20 mM EDTA pH 8.0 and mix.
5. Add 15 µl of 0.1N NaOH and incubate at 65°C for 20 minutes
6. Add 15 µl of 0.1N HCl to neutralize
7. Purify the cDNA using a Qiagen QiaQuick PCR Purification Kit (it is recommended to use a kit that is less than a year old)
    a. Add 35 µl of 100 mM Sodium Acetate pH 5.2 to each tube.
    b. Add 500 µl of Buffer PB to each tube.
    c. Proceed as per manufacturer's instructions until elution
    d. Elute the cDNA from the column by adding 15µl of water, incubating at room temperature for 1 minute, and spinning at maximum speed for 1 minute.
    e. Repeat step 8d once (30 µl total eluent).
8. Dry the samples to completion in a concentrator/evaporator under vacuum at 50°C.
9. Resuspend the lyophilized cDNA in 10.5 µl of 0.1 M sodium bicarbonate pH 9.0.
10. Add 6.0 µl of Cy dye* to each tube, mix, and incubate at room temperature in the dark for 1 hour.

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11. * Resuspend a stock Cy dye tube (Amersham, cat. numbers; Cy3: PA23001, Cy5: PA25001) in 72 µl anhydrous DMSO, aliquot into 12 tubes and store at -80°C until used.
12. Purify the labeled cDNA using the Qiagen QiaQuick PCR Purification Kit (it is recommended to use a kit that is less than a year old)
    a. Add 35 µl of 100 mM Sodium Acetate pH 5.2 to each tube.
    b. Add 250 µl of Buffer PB to each tube.
    c. Proceed as per manufacturer's instructions until elution
    d. Elute the fluorescently labeled target from the column by adding 20 µl of EB (10 mM Tris pH 8.5), incubating at room temperature for 1 minute, spinning at maximum speed for 1 minute.
    e. Repeat step 12d once (40 µl total eluent).

NOTE:

A. The column bed should be visibly pink or blue if incorporation is successful.
B. The labeled cDNA is sense strand.

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Quantify the cDNA (A260), Cy3 (A550), and Cy5 (A650) incorporation using a spectrophotometer (Nanodrop may also be used).

Table 1. Absorbance readings for samples (according to the TIGR protocol)

Dye	**Cy3**	**Cy5**
Sample		
A_{260}		
A_{550}		
A_{650}		

Table 2. pmoles quantifications for each sample

Sample		
cDNA		
Cy3		
Cy5		

pmoles cDNA= (A_{260} x 40 µl x 37 ng/µl for1 OD unit x 1000 pg/ng)/324.5 pg/pmole dNTP
pmoles cDNA=(A_{260} x 4561)

pmoles Cy3= (A_{550} x 40 µl)/0.15 Extinction coefficient for Cy3 is 150,000 $M^{-1}cm^{-1}$
pmoles Cy3= A_{550} x 267

pmoles Cy5= (A_{650} x 40 µl)/0.25 Extinction coefficient for Cy5 is 250,000 $M^{-1}cm^{-1}$
pmoles Cy5= A_{650} x 160

* Greater than 50 pmoles Cy dye, 3000 pmoles cDNA and one dye molecule per 60 bases are ideal for hybridization.

13. Dry the samples to completion in a concentrator/evaporator under vacuum at 50°C (requires ~ 1 minute/1.5 µl)
14. Prehybridize the slide in 50 ml solution containing 5X SSC, 0.1% SDS, and 0.01% BSA for 45 minutes at 42°C
    - For 50 ml of prehybridization solution, mix 36.75 ml autoclaved Millipore dH_2O, 12.5 ml of 20X SSC, 0.25 ml of 20% SDS, and 0.5 ml of 10 mg/ml BSA (New England BioLabs, Purified BSA 100X; 10 mg/ml)

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15. Wash the slide by dipping five times in room temperature Millipore dH2O.
16. Dip the slide five times in room temperature isopropanol.
17. Dry the slide by centrifuging at 500 rpm* for 5 minutes at room temperature.
18. (Centrifuge the slide immediately after dipping in isopropanol. Even a few minutes of delay can result in higher background.)
19. * A small bench top centrifuge may also be used. 30 sec is sufficient to dry slides with a bench top centrifuge.
20. Prepare 1X hybridization buffer containing 5X SSC, 0.1% SDS, 0.2 mg/ml of yeast tRNA (Invitrogen, Cat# 15401-029), 0.2 mg/ml of polyadenylic acid (Sigma, Cat# P9403), and 25% super-pure formamide (Fisher, Cat# BP228-100).
    - For 100 µl of hybrididation buffer, mix 42 µl of autoclaved Millipore dH_2O, 25 µl of 20X SSC, 5 µl of 2% SDS, 1 µl of 20 µg/µl yeast tRNA (Invitrogen, Cat# 15401-029), 2 µl of 10 µg/µl polyadenylic acid (Sigma, Cat# P9403), and 25 µl of super-pure formamide (Fisher, Cat# BP228-100).
21. Resuspend each probe in 30 µl of hybridization buffer and combine the two probes

    Note: Choose the correctly sized hybrislip according to the size of the spotted area. 60 µl hybridization buffer is sufficient for a 25 mm x 60 mm hybrislip. Experiments using smaller/larger hybrislips will require less and more volume, respectively. It is best to test for the best volume on a plain glass slide prior to hybridizing.

22. Heat the samples at 95°C for 3 minutes
23. Spin the samples at maximum speed in a microcentrifuge for 1 minute.
24. Apply the probe to the slide and cover with a 25 mm x 60 mm hybrislip (LifterSlip, Fisher, Cat# 22035809).
25. Fill each slot with 10 µl of 3X SSC.
26. Seal the chamber and incubate (immersed) at 42°C for 12-18 hours.
27. Wash the slide in 200 ml of 1X SSC and 0.2% of SDS for 2 minutes.
28. Wash I=188 ml of Millipore dH2O, 10 ml of 20X SSC, and 2 ml of 20% SDS
29. Wash the slide in 200 ml 0.1X SSC and 0.2% of SDS for 2 minutes.
30. Wash II=197 ml of Millipore dH2O, 1 ml of 20X SSC, and 2 ml of 20% SDS
31. Wash the slide in 200 ml of 0.1X SSC for 2 minutes.
32. Wash III=199 ml of Millipore dH2O and 1 ml of 20X SSC
33. Dip the slide in 50 ml of autoclaved Millipore dH2O five times.
34. Dry the slide by spinning at 500 rpm* for 5 minutes at room temperature.
35. (Centrifuge the slide immediately after dipping in isopropanol. Even a few minutes of delay can result in higher background.)

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36. * A small bench top centrifuge may also be used. 30 sec is sufficient to dry slides with a bench top centrifuge.
37. Scan the slide.