BRI MicroArray Laboratory Direct- cDNA Labeling Synthesis Protocol

Required Reagents

5X First Strand Reaction Buffer (SuperScript II Invitrogen Cat no.180604-014)
0.1 M DTT
SuperScript II (200 U/µl) (SuperScript II, Invitrogen Cat no. 18064-014)
20 mM dNTP mix (6.67 mM each dATP, dGTP, dTTP) (Invitrogen, Cat. No. 10297-018)
2 mM dCTP (Invitrogen, Cat. No. 10297-018)
1.5 µl AncT mRNA primer (p54, p55 and p56 (1:1:1) 100 pmoll
4-8 ng Control RNA (we are an artificial *Arabidopsis* transcripts)
RNase A (0.05 mg/ml) (RNase A; USB, Cat. No. 70194Y)
RNase H (0.05 units/µl) (RNase H; Life Technologies, Cat. No. 18021-014)
Dig Ease Hybridization buffer (Roche)
Cyanine 3-dCTP (1.0 mM) (Perkin-Elmer/NEN, cat. no. NEL 576)
Cyanine 5-dCTP (1.0 mM) (Perkin-Elmer/NEN, cat. no. NEL 577)
DNase/RNase-free distilled water pH 7-8.5 (e.g., Sigma, Cat. No. W-4502)
100% isopropanol (Fisher Scientific Cat. No. A451-1)
NaOAc pH 5.2 3M
QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
Cover slips, No. 1½ x 24 x 60mm (Fisher Scientific, Cat. No. 12-548-5P, Grace Biolabs, Cat. No. HS6024)
10% SDS
20X SSC
1% BSA
Bakers tRNA (10mg/ml)
Salmon Sperm DNA (10mg/mL)
Hybridization Chamber (Corning Cat. No. 2551)
50ml Coplin jar (e.g., Sigma, Cat. No. S5516)

** Note*

We are using home-made AncT primers for our labeling rxns. Random or oligo(dT) primers can also be used in the labeling reaction with mRNA. Random primers are recommended if some degradation of the RNA sample is suspected.

If total RNA is used as a template for cDNA synthesis, oligo(dT) primers must be used to avoid labeling the rRNA and the tRNA present in the total RNA preparation.

The yield of cDNA using mRNA and random primers is generally greater than with total RNA with oligo(dT) primers.

Possible sources:
Random primers (9-mers; Statagene, Cat. No. 300309) ***for mRNA use 2.5µg***
Oligo(dT) primer (Oligo(dT)12-18 Primer, Life Technologies, Cat. No. 18418-012) ***for total RNA use 2µg***

Corning Hyb chambers are usable in both the humidity chamber and water bath. Be sure to follow manufacturers recommendations for use. There are many other commercially available chamber, home made versions are also acceptable.

Required Equipment

Micropipettors to pipette a range of 1 µl to 1 ml volumes
Sterile, nuclease-free 1.5 ml microcentrifuge tubes
Sterile, nuclease-free aerosol barrier pipet tips
Heating block or waterbath temperature set to 70°C and 95 °C
Microcentrifuge
Circulating waterbath, temperature set to 42°C or Humid chamber at 42°C
Vortex mixer
SpeedVac rotory dessicator
UV spectrophotometer and 0.1 ml volume quartz cuvettes (1 cm path length) *optional*

Critical Guidelines for Synthesis of Fluorescent-Labeled cDNA Probes

- The critical step in making fluorescent-labeled cDNA is the quality of the starting material. The RNA must be clean. If phenol is used in the RNA isolation, column purification must follow. All traces of phenol must be removed from the RNA prior to probe synthesis. Please refer to our protocol on Cell Preparation for details.
- We recommend a final column purification and DNase treatment of RNA. For total RNA use Qiagen RNeasy (Cat no. 74104). or Stratagene Absolutely RNA RT-PCR Miniprep kit (Cat no. 400800) . For mRNA we use Oligotex mRNA Mini Kit from Qiagen (Cat. no. 70022), or Micro-FastTrack mRNA isolation kit by Invitrogen (Cat no. 45-0036).
- The RNA concentration and purity should be determined by the absorbance at A_{260} and A_{280}. Analyze 5-10 µl RNA (in 1 ml TE-SDS 0.1%) on spectrophotometer. 1 OD260nm = 40 µg RNA. Calculate OD 260/280 (should be 1.8 or higher).
- The purity and integrity of the RNA should be confirmed by gel electrophoresis. For total RNA, specific bands of highly abundant ribosomal RNA should be visible at approximately 1.9 and 5 kb.. The bands should be sharp and clear; smearing of the rRNA bands on the gel indicates that the RNA has degraded. In addition, high molecular weight bands (>9000 kb) indicate DNA contamination of the RNA sample. Please refer to our protocol on Cell Preparation for details.
- For Poly A+mRNA, a faint smear in the range of 0.5 to 2 kb should be detectable.
- mRNA concentration should be deterimined using the RiboGreen method, outlined in the protocol for cell preparation.
- Quantitative and qualititative analysis of both mRNA and total RNA can also be determined using the Agilent 2100 Bioanalyzer. We are currently preparing protocols for the use of this instrument.
- If the buffer used to bind the DNA to the Qiagen PCR Purification column is not slightly acidic (less than pH 7) the cDNA will bind poorly, resulting in low yields.
- Exposure to light will cause photobleaching of the cyanine dyes. Cy 3 is particularly sensitive therefore, wherever possible, protect the cyanine-labeled nucleotides, labeled probe, and hybridization probe from light. Once the cyanine-labeled cDNA is synthesized it is stable for several weeks at 4°C if protected from light.
- Labeling Poly A+ mRNA requires **2-4 µg**, total RNA requires **20 µg**
- The RNA should be stored at –80° C until used.

A. Synthesis of Cyanine 3- and Cyanine 5-labeled cDNA

1. Combine on ice in 1.5 ml eppendorf tubes:

   2-4 µg mRNA or 20 µg total RNA
   2-8 ng Control RNA (we are using artificial *Arabidopsis* transcripts)
   1.5 µl AncT mRNA primer (p55, p56 and p57 (1:1:1) 100 pmol
   DEPC dH_20 to final volume of 18.5 µl (Cy3) or 19.5 µl (Cy5)

   Incubate at 70 °C for 10 min

2. Add on ice:
   8 µl 5 X first strand buffer
   4 µl 0.1 M DTT
   3 µl 20 mM dNTP-dCTP (6.67 mM each dATP, dGTP, dTTP)
   1 µl 2 mM dCTP
   2 µl 1mM Cy3 or 1µl 1mM Cy5 + 1 µl DEPC H_20
   2 µl fresh SuperScript II reverse transcriptase

Important!

For efficiency and to minimize pipetting errors when setting up the cDNA synthesis reactions, prepare one master mix of the components above for all reactions. Keep the master mix on ice until ready to aliquot into each reaction tube.

Both total RNA and mRNA can be used as a template for cDNA probe synthesis. Total RNA is easier and less expensive to prepare than mRNA.

Label the two different samples to be analysed (i.e. control and experimental) on an array with different fluors (i.e. Cyanine 3 or Cyanine 5). It is also suggested that you perform reciprocal labelling reaction. One labeling reaction generates enough probe for one yeast slide.

3. Mix gently, give a quick spin and incubate in a prewarmed H_20 bath at 42°C
4. After 2 hours give the reaction a quick spin to collect evaporated solution and add 2 µl of SuperScript II reverse transcriptase. Incubate for an additional hour.
5. Give solutions a quick spin and add 1 µl Rnase A (0.05 mg/ml) and 1 ul Rnase H (0.05 units/µl) Incubate at 37° C for 15-30 min.
7. Purify the probes using Qiagen columns:

   Top up the reaction to 50µl with DEPC H_20

   Add 2.7 µl of 3M NaOAc pH 5.2

   Add 200 µl PB buffer

   Apply solutions to columns

   Centrifuge for 15 sec at 13000 g

   Wash 4x with 600 µl of PE buffer (centrifuge for 15 sec each time)

   Centrifuge tubes for 1 min to eliminate traces of buffer

   Transfer the column to a new microcentrifuge tube

   To elute the sample add 30 µl of H_20 (pH 7.0 – 8.5) to the center of the column, leave at RT for 1 min or longer, and then centrifuge for 1 min at 13000g

   Repeat with an additional 30 µl and elute in the same tube second elution the same way

   Final eluted volume should be should be approximately 60 µl
8. At this point you may want to determine the efficiency of incorporatation of the Cy dyes into the probe, in order to determine whether you wish to proceed with the experiment. You may choose to skip this option and proceed with the concentration and hybridization of the probe. Eluted probes are usually slightly blue (Cy 5) or pink (Cy 3) if the labeling was successful, for a more accurate determination, the following steps should be performed.

Important!

Elution efficiency is dependant on pH. The maximum elution efficiency is achieved between 7.0 and 8.5. Make sure that the pH value of the H_20 is within this range.

B. Determination of the Effeciency of Incorporation of the Probe

1. The optical density of the purified probes should be measured on a spectrophotometer at 260nm, 550nm (for Cy3) and 650nm (for Cy5). 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 amount of probe required for spectrophotometry will depend on the cuvette used. The probe used for spectrophotometry should be recovered from the cuvette and used in the hybridization reaction. Clean the cuvette thoroughly between samples with 0.1N HCl to prevent cross contamination.

2. Calculate the amount of cDNA produced, pmole of dye incorporated, and the efficience of incorporation. If measuring a probe that was previously stored (i.e., contains 1mM DTT), the blank used for spectrophotometry should also contain 1mM DTT. Frequency of incorporation in this application is defined as the number of labeled nucleotides incorporated per 1000 nucleotides.

   Amount of cDNA probe:

   OD260 x 37 x total volume of probe (µl) = ng of probe

   pmol of dye incorporated:

   Cy3™: OD550nm x (total volume of probe) / 0.15 = pmol of Cy3™ dye incorporated

   Cy5™: OD650nm x (total volume of probe) / 0.25 = pmol of Cy5™ dye incorporated

   Efficiency of incorporation: (# labeled nucleotides per 1000 nucleotides)

   f.o.i = pmol of dye incorporated x 324.5 / amount of cDNA probe (ng)

Important!

Do not use the probe if there is less than 15 pmol of dye incorporated or if the efficiency of incorporation of the probe is less than 10. Optimal amounts are 20 pmol of dye incorporated with a efficiency of incorporation of between 20-50 labeled nucleotides per 1000 nucleotides.

If the measured concentration of probe is unusually high, there may still be RNA present in the probe. If this is a chronic problem increase the length of RNase digestion in step 5. Probes can also be checked by gel electrophoresis for quality and size.

C. Concentration of the Probe

1. Following purification with the QIAquick columns, the purified, labeled cDNA samples may be concentrated as follows:
2. Dry the solutions under vacuum in rotary dessicator until dry (approximately 60 minutes).
3. Do not use heat during drying to prevent degradation of the cyanine dyes, do not overdry the probes.
4. Either proceed directly to hybridization or freeze on dry ice and store at -80 °C.

D. Pre-Hybridization

1. Prepare a pre-hybridization buffer and sterilize using a syringe filter (0.22 µm). Preheat buffer to 42 °C before use.

   Pre-hybridization buffer

   5X SSC
   0.1% SDS
   1% BSA

2. Pipet 15 µl aliquots of the pre-hyb solution onto 3 separate areas of the printed slide surface (left center and right side) try to cover as much of the printed array area which will be used for the hybridization experiment.
3. Hold a 22mm x 60mm coverslip over the array, gradually lower one end of the coverslip and allow the solution to wick across the entire length of the slide. After the pre-hyb buffer has covered the entire array, adjust the coverslips position and the edge of the slide accordingly. Try to remove the air bubbles which may be trapped under the coverslip by gently tapping onto the coverslip, smaller bubbles will be removed during the pre-hyb process.
1. Place the array in 42 °C pre warmed humid chamber or water bath for at least 1 hour

E. Hybridization

1. Prepare a Hybridization buffer, filter using a syringe filter (0.22 µm).

2. Hybridization buffer:

   400 µl Dig Ease Buffer
   20 µl Bakers tRNA (10mg/ml)
   20 µl Sonicated Salmon Sperm DNA (10mg/ml)

3. Combine the appropriate Cy3-cDNA and Cy5-cDNA targets intended for hybridization to a single array in a 1.5 mL microcentrifuge tube

4. Add 30-60 µl of hybridization buffer, mix well and incubate at 95 for 5 minutes, then on ice for 1 min.

5. Do a quick spin to recover solution., incubate at 42 °C until ready to use.

6. Remove the coverlslip from the prehybridization by dipping the array in filtered H_20. Shake the slide gently to loosen the coverslip. With time the coverslip will slide free of the slide surface. Wash twice with H_20. Spin dry the slides in a centrifuge at 1000g for 5 min, or dry under a clean air stream.

7. Add the probe onto the coverslip in 3 aliquots onto separate areas of the printed slide surface (left center and right side) try to cover as much of the printed array area which will be used for the hybridization experiment.

8. Hold a 22mm x 60mm coverslip** over the array, gradually lower one end of the coverslip and allow the solution to wick across the entire length of the slide. After the hyb buffer has covered the entire array, adjust the coverslips position and the edge of the slide accordingly. Try to remove the air bubbles which may be trapped under the coverslip by gently tapping onto the coverslip, smaller bubbles will be removed during the hyb process.

9. For Candida arrays that are printed on 2 separate slides (Part A and B) use one of the slides as a coverslip, creating a "sandwich of the 2 slides"face to face on top of one another.

10. Incubate immediately at 42 °C overnight, or from 12-16 hours.

F. Washing

1. To remove coverslip submerge slide in a Coplin jar, or slide holder containing 1X SSC, 0.2% SDS wash buffer preheated to 42 °C. Shake the slide gently to loosen the coverslip. With time the coverslip will slide free of the slide surface.

2. After the coverslip is removed place slide in Coplin jar, or slide holder containing 1X SSC, 0.2% SDS preheated to 42 °C and agitate for 10 minutes.

3. Wash the slide once in a Coplin jar or slide holder with 0.1X SSC, 0.2% SDS at RT and agitate for 10 minutes.

4. Wash the slide twice in 0.1X SSC, agitating for 5 minutes at room temperature.

5. Finally dip the slide in a Coplin jar filled with water several times. Spin dry the slides in a centrifuge at 1000g for 5 min, or dry under a clean air stream.

6. Scan arrays as soon as possible. Stores slides in the dark, they may be stable for 2 or more weeks.

7. Use a laser power that will give a signal between 1000 and 60000 (approximately 1% white spots)

Important!

Once the slide has been hybridized it should be exposed to light as little as possible. Therefore, all staining dishes should be covered with foil to make them light tight

Tracey Rigby
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