Tsai Lab Microarray Protocols

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A. Total RNA Extraction

1. We recommend the CTAB protocol for total RNA extraction. This protocol works well in our hands for a wide range of tissues, including:

   Populus: apices, young and old leaves (normal and stressed), stems (primary and secondary), xylem, phloem, floral tissues (various stages, male and female), seedlings, etiolated seedlings, calli, cell suspensions (normal and elicited), and roots.

   Salix: leaves (normal and stressed)

   Pinus: xylem

   Eucalyptus: xylem

2. DNA contamination should be avoided. Check the RNA quality (and assess DNA contamination) by electrophoresis. If DNA contamination occurs, repeat the LiCl precipitation step. Alternatively, perform a DNase digestion to remove the contaminating DNA. We use the Turbo DNA-free kit from Ambion (Cat #1907R) for this purpose.

3. References:

   Chang S., Puryear J. and Cairney J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11: 113-116. **(original protocol)**

   Tsai C.-J., Cseke L.J. and Harding S.A. (2003) Isolation and purification of RNA. *In*: Cseke L.J., Kaufman P.B., Podila G.K., Tsai C.-J. (*eds*) Handbook of Molecular and Cellular Methods in Biology and Medicine. 2nd ed. CRC Press, Boca Raton, FL. pp. 25-44.

B. The aspen 7K array

1. **Array design:** The aspen 7K array contains replicate subarrays of 7,008 elements in a 4×4 grid with a spot diameter of ~135 µm and a spacing of 200 µm (Fig 1). These elements represent ~5,000 unique aspen sequences (Ranjan et al. 2004), and include various positive and negative controls (Lucidea Universal ScoreCard, Amersham) to monitor target labeling and hybridization efficiency, as well as many known function genes derived from our previous and ongoing research.

2. **Print area:** The replicate subarrays occupy an ~18×37 mm area **on the non-barcode side**. The green rectangular area on the image depicts our hybridization chamber gasket area (20×63.5 mm) using an automated hybridization station. For manual hybridization, a 22×50 mm cover slip (or parafilm piece) will safely cover the print area.

3. **Spot layout:** Each grid contains 12 spike controls on the top left corner (Fig 2). The first 5 are *calibration* controls (from high to low concentrations, green box), followed by 1 *negative* control (white box), 3 or 5 *ratio* controls (different ratio combinations between Cy3 and Cy5 targets, yellow box), and another *negative* control (bottom grid), or *negative-ratio-negative* controls (top grid). Each grid contains 21×21 spots, except for the last row which has only 16 elements for the top 8 grids, or 20 elements for the bottom 8 grids in each subarray (blue boxes depict empty spots).

4. **Spikes:** To take advantage of the spike controls, appropriate spike mRNA samples need to be included during target labeling (see Section C below and Amersham manual). However, the use of spike mRNA in hybridization is optional, and will not affect the array analysis (e.g., using global per chip normalization etc).

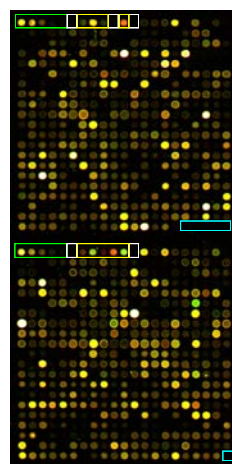

Fig 2. Layout of two representative grids.

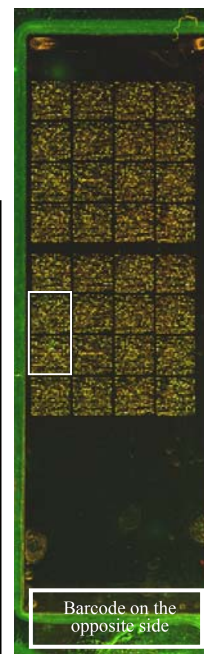

Fig 1. A full slide view of the array.

5. **Clone information:** A gene list can be downloaded from our website (www.aspenDB.mtu.edu). Sequence information can be obtained by searching the GenBank or our ESTDB with the MTU clone IDs. As is the case for other large-scale projects, there are likely errors in our array system. Due to funding limit, we have only re-sequenced a number of randomly selected array-ESTs. Other potential errors include mis-annotation, or mis-classification with regard to the functional groups. Therefore, additional confirmation for array-selected candidate genes will be necessary.

References: Ranjan P, Kao Y-Y, Jiang H, Joshi CP, Harding SA, Tsai C-J (2004) Suppression subtractive hybridization-mediated transcriptome analysis from multiple tissues of aspen (*Populus tremuloides*) trees altered in phenylpropanoid metabolism. Planta 219: 694-704.

C. Target preparation

C.1. Random hexamer-primed cDNA synthesis and labeling

This protocol is adapted from TIGR using an indirect, aminoallyl-labeling protocol (Hughes et al. 2001), coupled with amine-modified random hexamer-primed cDNA synthesis (Xiang et al. 2002). Ambion offers an Amino Allyl cDNA labeling Kit (#1705) for similar purpose.

Chemicals and Reagents

Amine-modified random primer: 2 μg/μL in ddH_2O
5'-amino C6dT modified hexamers: 5'-[AC6T]NNNNNN (Sigma-Genosys)

Cy-3 and Cy-5 NHS ester (Amersham RPN 5661)

mRNA spikes: Lucidea Universal ScoreCard (Amersham RPK3161 or RPK3164)

RNase inhibitor: 40 U/μL (Ambion 2682)

SuperScript II Reverse Transcriptase (Invitrogen 18064-022)

QIAquick PCR Purification Kit (Quiagen 28106)

Aminoallyl-dUTP (5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate; Sigma A0410, 1 mg)

Stock preparation: Add 19.1 μL of 0.1 M KPO_4 buffer (pH 7.5) to the aa-dUTP vial (for a *theoretical* final concentration of 100 mM). Gently vortex to mix. Transfer the solution to a new microfuge tube and store at -20°C. Check the aa-dUTP concentration by diluting 1:5000 in 0.1 M KPO_4 (pH 7.5), and measuring A_{289}. Stock concentration in mM = $A_{289} \times 704$.

50X nucleotide mix (aa-dUTP: dTTP = 2:3).

Mix 5 μL each of dATP, dCTP and dGTP (100 mM), 3 μL of dTTP (100 mM) and 2 μL of aa-dUTP (100 mM). Total = 20 μL. Aliquot and store at –20°C.

1 M Phosphate buffer, pH 8.5

Prepare 1 M K_2HPO_4 and 1 M KH_2PO_4.To make a 1 M Phosphate buffer (KPO_4, pH 8.5-8.7), combine 9.5 mL of 1 M K_2HPO_4 and 0.5 mL of 1 M KH_2PO_4.

5 mM phosphate wash buffer (5 mM KPO_4, pH 8.0, 80% EtOH)

Mix 0.5 mL of 1 M KPO_4 (pH 8.5), 15.25 mL of sterile ddH_2O and 84.25 mL of 95% EtOH.
The wash buffer may be slightly cloudy.

4 mM phosphate elution buffer: dilution of 1 M KPO_4 (pH 8.5) to 4 mM with ddH_2O

0.1 M Sodium Carbonate Buffer (Na_2CO_3), pH 9.0

Dissolve 0.265 g Na_2CO_3 in 25 mL ddH_2O, adjust pH to 9.0 with 12 N HCl (~200 μL).
Make fresh every 1-2 weeks (check to be sure pH does not go above 9.1).

cDNA synthesis and aminoallyl labeling:

<u>RT reaction</u>

C.1.1. Add the following:

Total RNA	5-10 µg
RNase inhibitor (40 U/µL)	0.5 µL
5X First strand buffer	0.5 µL
0.1 M DTT	0.5 µL
AC6T-N6 primer (2 µg/µL)	1 µL
Amersham mRNA spike mix*	3 µL
RNase-free water	to 19 µL

* For each pair of Cy3-Cy5 targets, one should receive the *<u>Reference</u>* mix and the other the *<u>Test</u>* mix.

C.1.2. Mix and incubate at 70°C for 5 min. Quick chill on ice and centrifuge briefly at >10,000 rpm.

C.1.3. Add the following:

5X First strand buffer	5.5 µL
0.1 M DTT	2.5 µL
RNase inhibitor (40 U/µL)	0.5 µL
50X nucleotide mix	0.6 µL
SuperScript II RT (200 U/µL)	2 µL

C.1.4. Mix and incubate at room temperature for 10 min and then 42°C for 3 hr, followed by 46°C for 20 min and 42°C overnight.

C.1.5. Hydrolyze the RNA by adding 10 µL of 1 M NaOH and 10 µL of 0.5 M EDTA. Mix and incubate at 65°C for 15 min.

C.1.6. Neutralize the solution to pH 5.6-6.2 with 12-15 µL of 1 M HCl and 20 µL of 100 mM NaOAc (pH 5.2). Check the pH with pH paper. The total volume is ~85 µL.

<u>cDNA purification</u>

C.1.7. Add 425 µL (5X volume) of Qiagen buffer PB to the cDNA.

C.1.8. Transfer the solution to a QIAquick column placed in a 2 mL collection tube. Centrifuge at 13,000 rpm for 1 min. Reapply the flow-through to the same column and spin again. Repeat once more. Discard the flow through.

C.1.9. Wash the column with 600 µL of 5 mM phosphate wash buffer, spin at 13,000 rpm for 1 min. *Do not use the wash and elution solutions provided by the Qiagen PCR purification kit for cDNA purification. They contain free amines that compete with the Cy dye coupling reaction.*

C.1.10. Discard the flow through and repeat the wash one more time. Empty the collection tube and centrifuge the column for another minute at 13,000 rpm.

C.1.11. Transfer the column to a new microcentrifuge tube, and carefully add 30 µL of 4 mM phosphate elution buffer to the center of the column.

C.1.12. Incubate at room temp for 2 min. Elute the target by centrifugation at 13,000 rpm for 1 min. Repeat the elution using the same collection tube. The final elution volume should be ~ 60 µL.

C.1.13. Measure A_{260} and calculate the cDNA concentration ($A_{260}/\mu L \times 37$ = cDNA ng/µL). Make 2 µg cDNA aliquots. Dry in a Speed Vac and store the aliquots in **amber** microfuge tubes at -20°C.

Cy Dye coupling

C.1.14. Resuspend 1 Cy dye vial in 10 µL of 0.1 M sodium carbonate buffer (pH 9.0), and mix it with 2 µg of dried, aa-cDNA in the amber microfuge tube.

C.1.15. Incubate the Cy3 tube for 1 hr and the Cy5 one for 1.5 hr in the dark at room temp on a shaker.

Note: To prevent photobleaching of the Cy dyes, use amber tubes (or wrap all reaction tubes with foil) and perform all steps in a dark room with dim light.

Dye-labeled target purification

C.1.16. To the cDNA-Cy dye coupling mix, add 35 µL of 100 mM NaOAc (pH 5.2) and 250 µL of the Qiagen PB buffer.

C.1.17. Transfer the solution to a QIAquick column placed in a 2 mL collection tube. Centrifuge at 13,000 rpm for 1 min. Reapply the flow-through to the same column and spin again. Repeat once more. Discard the flow through.

C.1.18. Wash the column with 600 µL of the Qiagen PE buffer, centrifuge at 13,000 rpm for 1 min.

C.1.19. Discard the flow through and repeat the wash one more time. Empty the collection tube and centrifuge the column for another minute at 13,000 rpm to dry.

C.1.20. Transfer the column to a new microcentrifuge tube, and carefully add 30 µL of Qiagen EB elution buffer to the center of the column.

C.1.21. Incubate at room temp for 2 min. Elute the labeled target by centrifugation at 13,000 rpm for 1 min. Repeat the elution using the same collection tube. The final elution is ~ 60 µL.

Target quantification using the Nanodrop spectrophotometer

C.1.22. Measure A_{260} and calculate the cDNA concentration: $A_{260}/\mu L \times 37$ = cDNA ng/µL

C.1.23. Measure A_{550} for Cy3 and A_{650} for Cy5 to calculate pmol of incorporated dye per µL (the values are calculated by the Nanodrop program).

C.1.24. Calculate the frequency of incorporation (FOI) for each Cy dye-labeled target.

FOI = pmol of dye incorporated × 324.5 ÷ ng of cDNA

Best results are obtained with targets having an FOI of 20-30 (20-30 labeled nt per 1000 nt)

C.1.25. Divide the target into 30-50 pmol aliquots, dry in a Speed Vac and store at –20°C until use.

References:

Hughes, Mao et al. (2001) Nature Biotechnol 19: 342-347
Xiang, Kozhich et al. (2002) Nature Biotechnol 20: 738-742

C.2. Oligo dT-primed cDNA synthesis and labeling

This protocol is modified from the Invitrogen's SuperScript Indirect cDNA Labeling Kit (L1014-01/-02).

Chemicals and Reagents

Anchored oligo dT_{20} primer: 2.5 µg/µL in ddH_2O

5'-TTTTTTTTTTTTTTTTTTTTVN (can be custom synthesized)

Cy-3 and Cy-5 NHS ester (Amersham RPN 5661)

mRNA spikes: Lucidea Universal ScoreCard (Amersham RPK3161 or RPK3164)

SuperScript III Reverse Transcriptase (Invitrogen 18080-094)

RNase inhibitor: 40 U/µL (Ambion 2682)

QIAquick PCR Purification Kit (Quiagen 28106)

Aminoallyl-dUTP (5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate; Sigma A0410, 1 mg)

Stock preparation: Add 19.1 µL of 0.1 M KPO_4 buffer (pH 7.5) to the aa-dUTP vial (for a *theoretical* final concentration of 100 mM). Gently vortex to mix. Transfer the solution to a new microfuge tube and store at -20°C. Check the aa-dUTP concentration by diluting 1:5000 in 0.1 M KPO_4 (pH 7.5), and measuring A_{289}. Stock concentration in mM = $A_{289} \times 704$.

50X nucleotide mix (aa-dUTP: dTTP = 2:3).

Mix 5 µL each of dATP, dCTP and dGTP (100 mM), 3 µL of dTTP (100 mM) and 2 µL of aa-dUTP (100 mM). Total = 20 µL. Aliquot and store at –20°C.

1 M Phosphate buffer, pH 8.5

Prepare 1 M K_2HPO_4 and 1 M KH_2PO_4.To make a 1 M Phosphate buffer (KPO_4, pH 8.5-8.7), combine 9.5 mL of 1 M K_2HPO_4 and 0.5 mL of 1 M KH_2PO_4.

5 mM phosphate wash buffer (5 mM KPO_4, pH 8.0, 80% EtOH)

Mix 0.5 mL of 1 M KPO_4 (pH 8.5), 15.25 mL of sterile ddH_2O and 84.25 mL of 95% EtOH.
The wash buffer may be slightly cloudy.

0.1 M Sodium Carbonate Buffer (Na_2CO_3), pH 9.0

Dissolve 0.265 g Na_2CO_3 in 25 mL ddH_2O, adjust pH to 9.0 with 12 N HCl (~200 µL).
Make fresh every 1-2 weeks (check to be sure that pH does not go above 9.1).

cDNA synthesis and aminoallyl labeling:

RT reaction

C.2.1. Add the following:

Total RNA	20 µg
RNase inhibitor (40 U/µL)	0.5 µL
5X First strand buffer	0.5 µL
0.1 M DTT	0.5 µL
Anchored oligo dT_{20} primer (2.5 µg/µL)	2 µL
Amersham mRNA spike mix*	2 µL
RNase-free water	to 19 µL

* For each pair of Cy3-Cy5 targets, one should receive the ***Reference*** mix and the other the ***Test*** mix.

C.2.2. Mix and incubate at 70°C for 5 min. Chill on ice and centrifuge briefly at >10,000 rpm.

C.2.3. Add the following:

5X First Strand buffer	5.5 µL
0.1 M DTT	2.5 µL
RNase inhibitor (40 U/µL)	0.5 µL
50X nucleotide mix	0.6 µL
SuperScript II RT (200 U/µL)	2 µL

C.2.4. Mix and incubate at room temperature for 10 min, 46°C for 3 hr and then 50°C for 3 hr.

C.2.5. Hydrolyze the RNA by adding 10 µL of 1 M NaOH and 10 µL of 0.5 M EDTA. Mix and incubate at 65°C for 15 min.

C.2.6. Neutralize the solution to pH 5.6-6.2 with 12-15 µL of 1 M HCl and 20 µL of 100 mM NaOAc (pH 5.2). Check the pH with pH paper. The total volume is ~85 µL.

cDNA purification

C.2.7. Add 425 µL (5X volume) of Qiagen buffer PB to the cDNA.

C.2.8. Transfer the solution to a QIAquick column placed in a 2 mL collection tube. Centrifuge at 13,000 rpm for 1 min. Reapply the flow-through to the same column and spin again. Repeat once more. Discard the flow through.

C.2.9. Wash the column with 600 µL of 5 mM phosphate wash buffer, spin at 13,000 rpm for 1 min. *Do not use the wash and elution solutions provided by the Qiagen PCR purification kit for cDNA purification. They contain free amines that compete with the Cy dye coupling reaction.*

C.2.10. Discard the flow through and repeat the wash one more time. Empty the collection tube and centrifuge the column for another minute at 13,000 rpm.

C.2.11. Transfer the column to a new microcentrifuge tube, and carefully add 30 µL of Qiagen EB elution buffer to the center of the column.

C.2.12. Incubate at room temp for 2 min. Elute the target by centrifugation at 13,000 rpm for 1 min. Repeat the elution using the same collection tube. The final elution volume should be ~ 60 µL.

C.2.13. Measure A_{260} and calculate the cDNA concentration ($A_{260}/\mu L \times 37$ = cDNA ng/µL). Make 200 ng cDNA aliquots. Dry in a Speed Vac and store the aliquots in **amber** microfuge tubes at -20°C.

Cy Dye coupling

C.2.14. Resuspend 1 Cy dye vial in 10 µL of 0.1 M sodium carbonate buffer (pH 9.0), and mix it with 200 ng of dried, labeled aa-cDNA in the amber microfuge tube.

C.2.15. Incubate the reaction for 1-1.5 hr in the dark at room temp on a shaker.

Note: *To prevent photobleaching of the Cy dyes, use amber tubes (or wrap all reaction tubes with foil) and perform all steps in a dark room with dim light.*

Dye-labeled target purification

C.2.16. To the cDNA-Cy dye coupling mix, add 35 µL of 100 mM NaOAc (pH 5.2) and 250 µL of the Qiagen PB buffer.

C.2.17. Transfer the solution to a QIAquick column placed in a 2 mL collection tube. Centrifuge at 13,000 rpm for 1 min. Reapply the flow-through to the same column and spin again. Repeat one more time. Discard the flow through.

C.2.18. Wash the column with 600 µL of the Qiagen PE buffer, centrifuge at 13,000 rpm for 1 min.

C.2.19. Discard the flow through and repeat the wash one more time. Empty the collection tube and centrifuge the column for another minute at 13,000 rpm to dry.

C.2.20. Transfer the column to a new amber microcentrifuge tube, and carefully add 23 µL of Qiagen EB elution buffer to the center of the column.

C.2.21. Incubate at room temp for 2 min. Elute the labeled target by centrifugation at 13,000 rpm for 1 min. Reapply the flow through to the column and repeat the incubation and centrifugation steps. The final elution volume is ~ 20-21 µL.

Target quantification using the Nanodrop spectrophotometer

C.2.22. Measure A_{260} and calculate the cDNA concentration: $A_{260}/\mu L \times 37$ = cDNA ng/µL

C.2.23. Measure A_{550} for Cy3 and A_{650} for Cy5 to calculate pmol of incorporated dye per µL (the values are calculated by the Nanodrop program).

C.2.24. Calculate the frequency of incorporation (FOI) for each Cy dye-labeled target.

FOI = pmol of dye incorporated × 324.5 ÷ ng of cDNA

Best results are obtained with targets having an FOI of 20-30 (20-30 labeled nt per 1000 nt)

C.2.25. Divide the target into 10 pmol aliquots, dry in a Speed Vac and store at -20°C until use.

D. Array hybridization

D.1. Manual hybridization (no mixing)

Reagents and Supplies

Prehybridization Solution (filter sterilized)
5X SSC
0.1% SDS
1% BSA

Hybridization solution (filter sterilized)
50% formamide
5X SSC
0.1% SDS
0.1% BSA

Wash solution I
1X SSC
0.2% SDS

Wash solution II
0.1X SSC
0.2% SDS

Wash solution III
0.1X SSC

Microarray Air Jet
(TeleChem International Cat # MAJ)

Slide Processing

D.1.1. Immobilize the spotted cDNA by UV crosslinking at 300 mJ. DNA side (non-barcode side) up.

Prehybridization

D.1.2. Prehybridize the slide in a new 50-mL Falcon tube at room temp on an orbital shaker for 15 min.

D.1.3. Rinse the slide with autoclaved ddH$_2$O 20 times.

D.1.4. Rinse the slide with ethanol for a few seconds. Dry slides by spinning (in Falcon tubes) at 2,500 rpm for 2 min at room temperature, or under heavy stream of filtered air using the Microarray Air Jet. Dry immediately. The slides are ready for hybridization. Do not leave them exposed for more than 1 hr

Hybridization

D.1.5. Resuspend the labeled cDNAs in an appropriate volume (e.g., 25 µL each) of hybridization buffer and combine.

Ideally, the Cy3 and Cy5 targets should have similar FOI and cDNA concentration after labeling. Mix equal amount of cDNA targets (each with approximately 30 to 50 pmol of incorporated dye when using the random hexamer labeling, or 10 pmol when using the oligo dT labeling).

D.1.6. Denature the targets at 42°C for 5 minutes. Spin briefly to collect the content.

D.1.7. Apply hybridization mixture to the slide (non-barcode side), and cover with a clean glass cover slip or a piece of parafilm.

D.1.8. Incubate in a humid hybridization chamber (with hybridization buffer-soaked filter papers) at 42°C for 20-36 hours.

Note: *Longer hybridization times can significantly improve the hybridization results as reported by Sartor et al. (2004) and confirmed in our hands with the Tecan automated hybridization station. We used to perform hybridization overnight (~16 hr), but are now doing this step for 36 hr with mixing.*

Reference: *Sartor M, Schwanekamp J, Halbleib D, Mohamed I, Karyala S, Medvedovic M, and Tomlinson CR (2004) Microarray results improve significantly as hybridization approaches equilibrium. BioTechniques 36:790-796.*

Washing

D.1.9. Immerse the slide in Wash solution I prewarmed to 42°C using a large petri dish. Shake the slide gently to loose the cover slip (approximately 4 min).

Note: *Do not allow the arrays to dry out between washes, as this will result in high background. Protect the slides from light at all times.*

D.1.10. Wash the slide in Wash solution II on an orbital shaker for 4 minutes at room temp.

D.1.11. Rinse twice in Wash solution III for 4 minutes at room temp on an orbital shaker.

D.1.12. Rinse with sterile ddH_2O water for 5 seconds.

D.1.13. Dry the slide by spinning at 2,500 rpm at room temperature for 2 min, or under heavy stream of filtered air.

D.1.14 Scan immediately.

We have also used the Corning Pronto! Hybridization Kit, following the manufacturer's protocols, with success.

D.2. Hybridization with the Tecan hybridization station

Reagents and Supplies

Blocking Solution (filter sterilized)
- 5X SSC
- 0.2% SDS
- 1% BSA

Hybridization solution (filter sterilized)
- 50% formamide
- 5X SSC
- 0.4% SDS
- 0.1% BSA

Wash solution I
- 0.1X SSC
- 0.2% SDS

Wash solution II
- 0.1X SSC

Microarray Air Jet (TeleChem International Cat # MAJ)

Slide Processing

D.2.1. Immobilize the spotted cDNA by UV crosslinking at 300 mJ. DNA side (non-barcode side) up.

D.2.2. Snap bake the slide on a hot plate for 1 min at 120°C (DNA side up).

Prehybridization

D.2.3. Prehybridize the slide in a large petri dish for 10 min at room temp with gentle shaking.

D.2.4. Rinse the slide with autoclaved ddH_2O 20 times.

D.2.5. Rinse the slide with 100% ethanol for a few seconds. Dry slides by spinning (in Falcon tubes) at 2,500 rpm for 2 min at room temperature, or under heavy stream of filtered air using the Microarray Air Jet. Dry immediately. The slides are ready for hybridization. Do not leave them exposed for more than 1 hr

Hybridization and Washing

D.2.6. Place the slide on the Tecan hybridization station according to manufacturer's instruction. Non-barcode side up.

D.2.7. Resuspend each of the labeled cDNAs in 50 µL of hybridization buffer at room temp for 10 min. Spin down briefly and combine into one tube (final volume is ~ 100 µL).

Ideally, the Cy3 and Cy5 targets should have similar FOI and cDNA concentration after labeling. Mix equal amount of cDNA targets (each with approximately 30 to 50 pmol of incorporated dye when using the random hexamer labeling, or 10 pmol when using the oligo dT labeling).

D.2.8. Begin hybridization with the following program:

[Wash] with sterile ddH_2O at 42^oC for 5 cycles of 30-sec wash and 5-sec soak

[Probe injection] at 55 ^oC

[Denaturation] at 65^oC for 2 min

[Hybridization] at 39-42^oC for 36 hr with medium agitation

[Wash] with solution I at 40^oC for 4 cycles of 1-min wash and 30-sec soak

[Wash] with solution II at 23 ^oC for 2 cycles of 1-min wash and 30-sec soak

[Drying] at 30^oC for 2 min with filtered nitrogen gas

Note: *Longer hybridization times can significantly improve the hybridization results as reported by Sartor et al. (2004) and confirmed in our hands with the Tecan automated hybridization station. We used to perform hybridization overnight (~16 hr), but are now doing this step for 36 hr with mixing.*

Reference: *Sartor M, Schwanekamp J, Halbleib D, Mohamed I, Karyala S, Medvedovic M, and Tomlinson CR (2004) Microarray results improve significantly as hybridization approaches equilibrium. BioTechniques 36:790-796.*

D.2.9. Slides are ready for scanning

We have also used the Corning Pronto! Hybridization Kit reagents in the above program with success.