INTRODUCTION

Note: Following was adapted from the Invitrogen SuperScript™ Plus Indirect cDNA Labeling System instruction manual.

The SuperScript™ Plus Indirect cDNA Labeling System is a highly efficient system for generating fluorescently labeled cDNA for use on microarrays in gene expression studies. It uses an aminoallyl-modified nucleotide together with other dNTPs in a cDNA synthesis reaction with SuperScript™ III Reverse Transcriptase. After a purification step to remove unincorporated nucleotides, the amino-fluorescent modified cDNA is coupled with a monoreactive, N-hydroxysuccinimide (NHS)-ester fluorescent dye. A final purification step removes any unreacted dye, and the fluorescently labeled cDNA is ready for hybridization to microarrays.

PRECAUTIONS


Prevent particulate and fluorescent contamination. When working with microarrays it is essential to avoid fluorescent and particulate contamination. Please follow the these precautions. Filter solutions through 0.2 um filters to remove particles. Only use powder-free nitrile gloves (latex gloves can cause fluorescent background).

Prevent RNase contamination. When working with RNA it is essential to avoid contamination with RNase. Please follow these precautions: Use RNase-free virgin plastic ware. Use RNase-free solutions (Note: all traces of DEPC must be removed to avoid degradation of Cy dyes). Do not handle tubes or reagents with ungloved hands. Clean the inside and the outside of the barrel of micropipettes and/or use barrier tips. It is recommended to aliquot your solutions when receiving them to reduce multiple user contamination.
Reduce the degradation of dNTPs. Repeated freeze thaw cycles result in progressive breakage of phosphate bonds and consequently lower labeling efficiency. Store dNTP solutions at -20°C in small aliquots, and Cy-dNTP solutions in single use aliquots at -20°C protected from light.

Reduce the degradation of fluorescent dyes. Alexa Fluor® dyes are susceptible to photobleaching and oxidation. Protect dyes from light -- store in the dark, work in low ambient light, and wrap tubes in foil. If using DEPC-treated solutions make sure all traces of DEPC are removed by autoclaving. If using MilliQ water in post hybridization washes make sure the MilliQ system is well maintained - it has been reported that water from poorly maintained systems can increase background intensity readings when scanning your microarray.

Prevent physical damage of microarrays. The DNA spots are not visible on the surface of the slide and are very easily scratched. On DGRC microarrays the DNA is printed on the same face of the slide as the barcode label. Never touch surface of the microarray - handle slides by grasping the barcode end of the slide with clean forceps.

MATERIALS
This protocol makes use of the following Invitrogen packages: SuperScript™ Plus Indirect cDNA Labeling System (L1014-06), which includes the Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Deca Packs and the Low Elution Purification Module.

The following materials are supplied by the user:
- Daphnia cDNA microarrays.
- Daphnia total RNA - 20µg per reaction.
- 1 N NaOH (1M) - 15µl per reaction
- 1 N HCl (1M) - 15µl per reaction
- 100% ethanol
- 100% isopropanol
- I-Block (Applied Biosystems, Cat. # A1300)
- 20X SSC (Invitrogen, Cat. #15557-028); DGRC uses homemade 20X SSPE.
- UltraPure 10% SDS (Invitrogen cat. no. 16663-027)
- Water, DNase RNase free (Sigma, Cat. #W4502)
- 1.5µl microcentrifuge tubes
- 200µl Ultra Tubes or PCR tubes
- 50mL centrifuge tubes (Corning, Cat.# 430290)
- Bucket of ice
The following instruments are used:
- Water, Millipore treated ddH₂O
- Thermocycler (or heating block) set at 70°C and 95°C.
- Water bath set at 42°C - 50°C.
- Microcentrifuge
- Slide Pro Hybridization System
- Centrifuge with rotor for either microplates or 50 ml tubes.

The following materials are supplied with the Core Module (Catalog Number 46-6340)
- Anchored Oligo(dT)₂₀ primer - 2µl per reaction
- dNTP mix, including amino-modified nucleotides – 1.5µl per reaction
- 5X First-Strand buffer – 6µl per reaction
- RNaseOUT™ - 1µl per reaction
- SuperScript™ III Reverse transcriptase (RT) - 2µl per reaction
  - M DTT – 1.5µl per reaction
- DEPC treated H₂O
- 2X Coupling Buffer – 5µl per reaction
- DMSO - 2µl per reaction
- Hela RNA (control RNA)

The following materials are supplied in the Low Elution cDNA Pur. Module (Cat.#46-6346)
- Low Elution columns pre-inserted in clear collection tubes
- Amber collection tube(s)
- Loading Buffer with isopropanol added
- Wash Buffer with ethanol added

The following materials are supplied in the Alexa Fluor® 555 and Alexa Four® 647 Reactive Dye DecaPacks(Cat. #46-6556)
- Alexa Fluor® 555
- Alexa Fluor® 647
PROCEDURE
This protocol was adapted from protocols provided with the Invitrogen SuperScript™ Plus Indirect cDNA Labeling System, Corning GAPS II Coated Slides, and Sambrook’s DNA Microarray Manual.

This is a three day process. One can store cDNA after either cDNA purification if needed.

**Day 1**: First-Strand cDNA Synthesis with 3-hr. incubation, Hydrolysis and Neutralization, Purification of cDNA (Store @ -20°C), Labeling with Fluorescent Dye (Overnight Reaction)

**Day 2**: Purify cDNA (Store @ 4°C), Q.C., Pre-hyb., Hybridization (Overnight Reaction)

**Day 3**: Post-Hybridization Washes and Scans

Preparations

- Aliquot the dNTPs and Anchored Oligo(dT)₂₀ primer into 200uL PCR tubes. I suggest 2 - 4 reaction aliquots per tube. Aliquoting prevents degradation due to freezing and thawing.

- Make sure the Loading Buffer and the Wash Buffer for cDNA column purifications have been prepared correctly. The following table illustrates what needs to be added to the two buffers.

<table>
<thead>
<tr>
<th></th>
<th>10-Rxn Kit</th>
<th>30-Rxn Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading Buffer</td>
<td>5.5 ml</td>
<td>18.0 ml</td>
</tr>
<tr>
<td>100% Isopropanol</td>
<td>2.0 ml</td>
<td>6.5 ml</td>
</tr>
<tr>
<td>Final Volume</td>
<td>7.5 ml</td>
<td>24.5 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>10-Rxn Kit</th>
<th>30-Rxn Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Buffer</td>
<td>2 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>8 ml</td>
<td>20 ml</td>
</tr>
<tr>
<td>Final Volume</td>
<td>10 ml</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

- Hybridization Buffer Stock Solution: Store at -20°C.

<table>
<thead>
<tr>
<th></th>
<th>100% Formamide</th>
<th>500 uL</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>250 uL</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 uL</td>
<td></td>
</tr>
<tr>
<td>Water UltraPure</td>
<td>240 uL</td>
<td></td>
</tr>
</tbody>
</table>

- Pre-hyb. should mimic hyb. conditions. Post-hyb. should be more stringent/ higher temp.

<table>
<thead>
<tr>
<th>Examples</th>
<th>Pre-hyb.</th>
<th>Hyb.</th>
<th>Post-hyb. Washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGRC</td>
<td>42°C 50% Formamide</td>
<td>42°C 50% Formamide</td>
<td>50°C</td>
</tr>
<tr>
<td>Brian</td>
<td>42°C-50°C 50% Formamide</td>
<td>42°C-50°C ~38% Formamide</td>
<td>50°C - 65°C</td>
</tr>
<tr>
<td>Elizabeth</td>
<td>42°C 50% Formamide</td>
<td>47°C ~38% Formamide</td>
<td>52°C</td>
</tr>
</tbody>
</table>
Day 1

- Make sure the vacuum centrifuge refrigerator is on.
- Leave DEPC-H2O out during 3-hr. incubation.
- After the First-Strand cDNA Synthesis 3-hr. incubation, thaw DMSO and 2x Coupling Buffer.

First-Strand cDNA Synthesis
The following reaction is designed to convert 20µg of RNA to first strand cDNA.

Note: RNaseOUT Recombinant RNase Inhibitor has been included in the system to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.

1. Vortex and centrifuge each reagent tube (at 4000rcf for 30sec.) before use, except for the RNaseOUT™ and SuperScript™ III RT. Mix those by slowly pipetting up and down without bubbles.
2. Mix (by pipetting up-and-down) and centrifuge each component in 200µl RNase-free tubes for individual reactions, or 1.5ul RNase-free tubes for larger reactions.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µg total RNA</td>
<td>Xµl</td>
</tr>
<tr>
<td>Anchored Oligo(dT)20 Primer (2.5µg/µl)</td>
<td>2µl</td>
</tr>
<tr>
<td>DEPC treated H₂O</td>
<td>To 18µl</td>
</tr>
</tbody>
</table>

3. Incubate at 70°C for 5 min, and then chill to 4°C for 1 min.
4. Add the following to each tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First-Strand Buffer</td>
<td>6µl</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>1.5µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1.5µl</td>
</tr>
<tr>
<td>RNaseOUT™ (40U/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>SuperScript™ III RT (400U/µl)</td>
<td>2µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>30µl</td>
</tr>
</tbody>
</table>

Note: If making a master mix, add .5µl to each volume for each additional reaction. Also, add the RT last and directly before adding the above mix to the RNA samples.

5. Mix by pipetting up-and-down, and briefly centrifugation. Incubate at 46°C for 3 hours. We use a thermocycler at this step programed to run forever at 46°C.
6. Proceed directly to Hydrolysis and Neutralization.

Note: You do not want the reaction to sit a temperature lower than 46°C for an extended period of time, because the enzyme may continue to synthesis cDNA (at a slow rate) that are not as specific to the template.
Hydrolysis and Neutralization
This step is taken to degrade the original RNA

1. Add 15µl of 1N NaOH (1M NaOH) to each reaction tube from the First Strand cDNA synthesis reaction. Mix Thoroughly.
2. Incubate tube at 70°C for 10 min.
3. Add 15µl of 1N HCl (1M HCl) immediately after the 10min incubation to neutralize the pH and mix gently.
4. Proceed directly to Purifying First-Strand cDNA.

Purifying First-Strand cDNA
This step is used to purify the sample by removing any contaminants from the amplified cDNA.

Note: Make sure the isopropanol/ethanol has been added to the buffers. (See Preparations.) Thaw DMSO, Coupling Buffer, and DEPC-treated Water.

1. Add 700 uL of Binding Buffer to a 1.5 mL DNase free tube. Pipet reaction product to tube of binding buffer.
   - Rinse 200µl tube with the binding buffer to transfer residual reaction product.
   - Vortex and centrifuge briefly.
2. Load binding buffer/cDNA to Low-Elution Spin Column (preinserted into collection tube).
3. Centrifuge at 3,300 x g for 1 minute. Discard flow-through.
4. Add 600 uL Wash Buffer to Spin Column inserted in same collection tube.
5. Centrifuge at maximum speed (16100 x g) for 30 sec. Discard flow-through.
6. Centrifuge at maximum speed (16100 x g) for 30 sec, using same collection tube.
8. Add 20 uL of DEPC-treated water to center of the Spin Cartridge.
9. Incubate for 1 minute at RT.
10. Centrifuge at maximum speed (16100 x g) for 1 minute. The eluate contains your purified cDNA.
11. Measure the cDNA concentration on the Nanodrop using the Nucleic Acid module.
Labeling with Fluorescent Dye
This step will label the amino-modified cDNA with the Alexa Fluor® dyes.

1. Remove the appropriate Alexa Fluor® dye vials and DMSO from -20°C storage.
   
   Note: DMSO is hygroscopic and will absorb moisture from the air, which react with the dyes to reduce the coupling efficiency. So, warm DMSO to room temperature before use and keep the cap closed on the vial when not in use.

2. Add 2µl of DMSO directly to each dye vial, vortex slowly/briefly and centrifuge. Leave the sample at RT for several minutes to allow the sample to resuspend.

3. Dry purified first-strand cDNA to 3 uL in a speed vac at medium heat for 10 min.

4. Centrifuge AlexaFluor dye tubes and buffer tube.

5. Add 5 uL of 2x Coupling Buffer to the cDNA tubes.

6. Add 2uL of dye/ DMSO solution to each tube. After adding the dye to a tube, aspirate the contents of the tube, without changing the tip. If contents are less than 10uL, add 1uL of DEPC-treated water at a time until the final volume is 10uL. Mix by pipetting up and down slowly 3 times.

7. Mix samples by vortexing slowly and centrifuge briefly.

8. Incubate at R.T. for 1hr. and then at 4°C in the dark overnight.

   Note: Invitrogen Protocol states to incubate at RT for 1-2 hours, and that it can be stored overnight.

9. Go to Purification of Labeled cDNA after the dark incubation.
Day 2
This is a good spot to make the agarose gel and start pre-hybridizing the microarray slides. Go to Quality Control and Pre-Hybridization, respectively, for instructions.

- Make agarose gel w/o EtBr.

Purifying Labeled cDNA
This step will purify the labeled cDNA by removing any non-bound dyes.

1. Add 700 uL binding buffer to 1.5 mL amber tube with labeled product.
   - Vortex and centrifuge briefly.
2. Load cDNA/ buffer to Low-Elution Spin Column (preinserted into collection tube).
3. Centrifuge at 3,300 x g for 1 minute. Discard flow-through.
4. Add 600 uL Wash Buffer to Spin Column inserted in same collection tube.
5. Centrifuge at maximum speed (16100 x g) for 30 sec. Discard flow-through.
6. Centrifuge at maximum speed (16100 x g) for 30 sec, using same collection tube.
8. Add 20 uL of DEPC-treated water to center of the Spin Cartridge.
9. Incubate for 1 minute.
10. Centrifuge at maximum speed (16100 x g) for 1 minute. The eluate contains your purified cDNA.
11. Measure cDNA concentration and dye incorporation using the Nanodrop Microarray module.
Quality Control of Labelled cDNA Samples (Q.C.)

Agarose Gel Electrophoresis

1. Prepare a 1% w/v agarose gel in 1X TBE without EtBr or Sybr Green Stain.
2. Use 1 g agarose in 100 mL buffer.
3. Combine 1 uL labeled cDNA and 1 uL 10% ficoll (and 3 uL water if you like).

*Note: Do not use normal loading buffer containing Bromophenol Blue or Xylene Cyanol, which will interfere with the detection of the dye fluorescence.*

4. Dry load samples into gel and an aliquot of loading buffer containing Bromophenol Blue into a side well. (It is important to dry load since there is no dye in 10% ficoll.)

5. Separate the diluted samples on an agarose gel in mini-gel chamber in 1X TBE for ~50 minutes at 105V.

- Protect the samples from light during Electrophoresis. (Cover with aluminum foil.)
- Let electrophoresis proceed until the top band of the Bromophenol Blue dye has proceeded to about ¼ - ⅓ of the gel length and the bottom band has proceeded to just past half-way.
Agarose Gel Electrophoresis

6. Clean Typhoon Variable Mode Imager with ddH2O before and after scanning.
7. Scan the gel with a Typhoon Variable Mode Imager.
   - Detect Alexa555 by excitation with 532 nm laser and using emission filter 555BP20.
   - Detect Alexa647 by excitation with 633nm laser and using emission filter 670BP30.
   - Set PMT to 500V, focal plane to +3mm, 200 pixels, and use normal sensitivity.
8. Optional: Estimate the success of purification step. Take samples before and after purification of the labeled cDNA and separate them on the same gel.
9. Used “Image Quant” to measure incorporation in banding-region. I did a “Volume” measurement. There is no need to “separate” the images.
   - View: Gray Scale, Side by side
   - Draw rectangle around all of the major bands--use the same rectangle for all gel-image quantifications to make comparisons across labeling rxns.
   - Select “Volume Report” under Analysis menu. Click Display, Okay. Double click on spreadsheet, which exports data to Excel and save data in Excel.

NanoDrop Reading

Note: Determine the labeling efficiency and sample purity of a 1.5uL sample of labeled cDNA.

1. Select the microarray module.
   - Sample type: Other
   - Constant: 33 (Extinction Coefficient for single-stranded cDNA)
   - Dye1: Alexa Fluor® 555
   - Dye2: Alexa Fluor® 647
2. Verify blank by measuring a water sample. The graph should follow baseline almost exactly.
3. Clean Nanodrop with UltraPure water and by buffing with Kimwipes.
4. Choose “Print Screen” to print the graph of the absorbance spectrum.
5. Clean Nanodrop after use to prevent caking of salts and other reagents on the spec. surface.

Note: The sample can be stored at -20°C for up to one week prior to hybridization. Avoid freeze\thawing.
Pre-Hybridization
This step will prepare the microarrays for hybridization and hopefully reduce background.
Note: SSPE can be substituted with SSC, as long as the same buffer is used throughout.

1. Scan slide before Pre-Hybridization, record any significant background.
2. Incubate arrays at 42°C (42°C-50°C) for at least 1.5 hours in a solution of the following:
   - 50% formamide
   - 5X SSPE
   - 0.1% SDS
   - 0.1 mg/ml BSA (I-Block)

<table>
<thead>
<tr>
<th>[Stock]</th>
<th>[Final]</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Formamide</td>
<td>50% Formamide</td>
<td>25ml</td>
</tr>
<tr>
<td>20X SSPE</td>
<td>5X SSPE</td>
<td>12.5ml</td>
</tr>
<tr>
<td>10%SDS</td>
<td>0.1%SDS</td>
<td>0.5ml</td>
</tr>
<tr>
<td>BSA (I-Block)</td>
<td>0.1 mg/mL BSA (I-Block)</td>
<td>100mg</td>
</tr>
<tr>
<td>Total Volume</td>
<td>Fill to 50 ml with Water</td>
<td></td>
</tr>
</tbody>
</table>

Note: Pre-Hybridizing the slides can take place for more than an hour without harming the slides.


<table>
<thead>
<tr>
<th>[Final]</th>
<th>Recipe</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>0.1 x SSPE</td>
<td>250uL 20xSSPE</td>
<td>Room Temp.</td>
</tr>
<tr>
<td>Tube 2</td>
<td>0.1 x SSPE</td>
<td>250uL 20xSSPE</td>
<td>Room Temp.</td>
</tr>
<tr>
<td>Tube 3</td>
<td>Water</td>
<td>Water</td>
<td>Room Temp.</td>
</tr>
<tr>
<td>Tube 4</td>
<td>Water</td>
<td>Water</td>
<td>Room Temp.</td>
</tr>
<tr>
<td>Tube 5</td>
<td>Isopropanol</td>
<td>Isopropanol</td>
<td>Room Temp.</td>
</tr>
</tbody>
</table>

Note: It is important to make certain all SDS has been removed during wash.

- Tubes 1 & 2: Invert tubes for 1 min. at the beginning and end of 5min. Place on shaker during invervening time.
- Tubes 3 & 4: Invert tubes constantly.

4. Immediately, dry the slides by centrifuging at 500 x g for 3-5 min.
Note: Slides should be hybridized within 1 hour of drying.
5. Scan slide (Prescan) and record background reading.
Hybridization
This step will hybridize the labeled cDNA to the microarray

1. Pool equal masses (ng) of the two labeled cDNA samples, and bring the volume up to 50μL using UltraPure H2O. (Use as much of the product as possible while standardizing [DNA].)
2. Create the hybridization buffer solution by diluting a stock solution of 50% formamide, 5X SSPE, and 0.1% SDS as follows:

<table>
<thead>
<tr>
<th>Stock</th>
<th>Final</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Formamide</td>
<td>38% Formamide</td>
<td>170μL of stock solution (See Preparations.)</td>
</tr>
<tr>
<td>20X SSPE</td>
<td>&lt; 5X SSPE</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>&lt; 0.1% SDS</td>
<td></td>
</tr>
<tr>
<td>COT-1 DNA (1μg/μL)</td>
<td>COT-1 DNA (0.009μg/μL)</td>
<td></td>
</tr>
<tr>
<td>Poly(A) (1μg/μL)</td>
<td>Poly(A) (0.1μg/μL)</td>
<td></td>
</tr>
<tr>
<td>Labelled Target + DEPC</td>
<td>Labelled Target</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td></td>
<td>222 μl</td>
</tr>
</tbody>
</table>

Note: The Hyb solution components should be run through a 0.22μm filter before assembled (i.e. mixed with other components), but I did NOT do that.

3. Incubate the buffer/cDNA solution at 95°C for 2 min.
   - Leave metal slide in chamber until inserting real microarray slide for hyb.
   - Change the hybridization temperature by double-clicking on Step 19 HEAT To 47°C.
   - Click Run Button (►). Follow instructions.
     - 1st Section: Flush system before hybridization. (Steps 1-10)
     - 2nd Section: Prime to remove bubbles from lines. (Steps 11-16)
     - 3rd Section: Insert slide and start hybridization. (Steps 17-23)
       - Rinse syringe 5 times with Ultra Pure H2O.
       - Inject heated probe when prompted (w/o centrifugation) and continue hyb.
     - Rinse syringe 15 times with Ultra Pure H2O.
     - Stop Protocol (■) after the hybridization has run 14-20 hours.
   - Wash the slides manually. (See Post Hybridization Washes.)
   - Flush the system after hybridization. (4th Section)
     - Insert metal slide.
     - Under main menu: Run > Run Selected Steps ✓
     - Highlight steps under last section: Steps 24 - 36 [END]
     - Click Run Button (►).
5. Hybridize at 47°C (42°C-50°C) for 14-20 hours. Do not rush the hyb.
Day 3
- Make Post-hybridization solutions.
- Set rcf speed on centrifuge.

Post Hybridization Washes
This step is used to remove any non-hybridized cDNA from the arrays.

Note: Post-hyb temp. should be higher than hyb. temp. up to 55°C to remove non-specific binding. You can increase the temperature more, but a higher temp. may cause comet-tailing.

1. Make up the following wash solutions in 50ml Falcon tubes.

Note: The Hyb solution components should be run through a 0.22µm filter, but I don't.

<table>
<thead>
<tr>
<th>Tube</th>
<th>[Final]</th>
<th>Recipe</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 x SSC, 0.01% SDS</td>
<td>1.25mL 20xSSPE, 50uL 10% SDS</td>
<td>52°C</td>
<td>Invert ~20x</td>
</tr>
<tr>
<td>2</td>
<td>0.5 x SSC, 0.01% SDS</td>
<td>1.25mL 20xSSPE, 50uL 10% SDS</td>
<td>52°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>3</td>
<td>0.5 x SSC</td>
<td>1.25mL 20xSSPE</td>
<td>52°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>4</td>
<td>0.1x SSC</td>
<td>250uL 20xSSPE</td>
<td>Room Temp.</td>
<td>Invert for 45sec.</td>
</tr>
<tr>
<td>5</td>
<td>0.01x SSC</td>
<td>25uL 20xSSPE</td>
<td>Room Temp.</td>
<td>Invert for 45sec.</td>
</tr>
<tr>
<td>6</td>
<td>Isopropanol</td>
<td>Isopropanol</td>
<td>Room Temp.</td>
<td>Invert Twice</td>
</tr>
</tbody>
</table>

Fill tubes to a 50mL total volume with Millipore H₂O. Measure 1.25mL as 2X 625uL.

2. When all tubes are prepared and heated, transfer slide from hybridization chamber to Tube#1 for a quick rinse with about 20 inversions.

Note: Keep the slide protected from light as much as possible. Do not let the slide dry out at any time during the washing procedure.

3. Transfer to Tube 2, submerge in the 52°C water bath for 5 min. Invert ~20 times at the beginning and end of 5 minutes.

Note: Wrap the Falcon tube in aluminum foil to protect the array from light.

4. Transfer to Tube 3 submerge in the 52°C water bath for 5 min. Invert ~20 times at the beginning and end of 5 minutes.

Note: Make sure the centrifuge is set up for drying the array(s)

5. Transfer to Tube 4 at RT. Invert for about 45 seconds.

6. Transfer to Tube 5 at RT. Invert for about 45 seconds.

7. Transfer to Tube 6 (Isopropanol) at RT. Invert twice.

8. Immediately and as quick as possible, centrifuge the slide at 500 x g for 5 min to dry.

9. For best results, scan immediately after the array is dry.
Scanning Hybridized Slides

PreScan

1. PreScan at about 400PMT for both the red and green channels.

   *Note: Always scan the entire slide in both channels to avoid unequal photo-bleaching.*

2. Increase or decrease the PMT until one of the channels has a few saturated spots, and the other channel has few to no saturated spots.

3. Having established the optimal PMT for one of the channels, adjust the PMT on the other channel until the signal is balanced:
   - The count ratio 635/532 equals 1 -- (0.9-1.1 is acceptable).
     - Set the minimum just beyond the background where the red and green channels intercept.
     - Set the maximum to 65530.
     - Zoom in such that only the region of the slide that you want to measure is viewable on the screen.
   - The red and green intensity distributions are roughly the same in height and shape. Unfortunately, the red channel is often stronger below ~1000 pixels and the green channel is stronger above ~30,000 pixels.
   - Check that positive controls are yellow--that the ratio is close to one. I often notice a slight tendency toward the green channel, because positives are high intensity spots, and high intensity spots tend to be biased toward the green channel.
   - If used, check that the spiking controls are yellow--that the ratio is close to one.

Scan

4. Scan at the optimized settings.

5. View the histogram and count ratio as before.

   - Fine tune the PMT settings. Sometimes the count ratio can change dramatically between PreScan and Scan.
   - Verify that there are 2-3 saturated spots, and only 2-3 saturated spots.

   *Note: For Generation 2 Daphnia microarrays, even 15 saturated spots would be reasonable, because of the redundant nature of the microarrays. Consistently, the top 15+ most highly expressed spots Blast aligned to a putative serine/threonine protein kinase.*

6. Save final scan as a Multi-Image Tiff file.