

AMPLIFYING RNA TO GENERATE MICROARRAY PROBES

Starting with total RNA, this protocol requires 2 days to generate Cy-coupled aRNA probes for microarray use. The first day requires roughly 6 hours (with 2 x 2 hour enzymatic reactions, during which you can do other things), ending with an overnight in vitro transcription reaction. The second day requires roughly 2 hours, by the end of which you will have Cy-labeled probe.

A shopping list follows this protocol.

Step 1: Prepare your total RNA for the amplification process

a) Starting with total RNA prepared by your method of choice (we use the Ambion RNAqueous Kit, which we use to isolate roughly 10 µg of total RNA from a 60 mm dish of bone marrow macrophages, which have been preserved with Ambion RNAlater, in a process that takes about 2 hours) proceed to concentrate 1 - 5 µg of RNA using the Zymo Research RNA Clean Up Kit – 5, according to the manufacturer's instructions. If using less RNA input, be advised that your eluted RNA needs to be at a concentration not less than 100 ng/µl. If your RNA from the Ambion RNAqueous Kit is already of sufficient concentration (~ 150 ng/µl) you can skip the concentration step.

b) Quantify your RNA eluate (we use the NanoDrop spectrophotometer, which requires only 1 µl of sample) and place your samples on ice. If possible, proceed immediately to Step 2; otherwise, put your samples at -80 °C.

Step 2: First and Second Strand cDNA Synthesis

This step follows exactly the protocol supplied with the Ambion Amino Allyl MessageAmp II aRNA Amplification kit, with the following 2 exceptions:

a) All of your reactions will be halved – half as much input RNA, half as much of each reagent, half the elution volumes. This will allow you to get twice as many reactions out of this kit.

b) When the “cDNA Filter Cartridges” provided by Ambion (catalog # 10081G) run out, you will then use the “Labeled aRNA Filter Cartridges” that they also provide with the kit (catalog # 10081G4) in order to purify your ds cDNA. Follow the instructions as before. Also, note the following changes:

1) After the initial centrifugation step to load your sample onto the column, take the flow-through and load again. This ensures complete binding of your material to the column.

2) After column-purifying the cDNA after second strand synthesis, elute in 8 µl nuclease-free water. Take the eluate flow-through and load it onto the column again, repeating the elution with the same 8 µl (do not add an additional

8 µl). You will need to recover 7 µl for the next step. There is no utility in quantifying the cDNA at this step.

3) After the last wash and before the elution, dump the flow-through and spin the columns an extra time.

If possible, proceed immediately to Step 3; otherwise, put your samples at –80 °C.

Step 3: *In vitro* Transcription

This step follows exactly the protocol supplied with the Ambion Amino Allyl MessageAmp II aRNA Amplification kit, with the following 2 exceptions:

a) All of your reactions will be halved – half the volume of input cDNA (7 µl), half as much of each reagent. This will allow you to get twice as many reactions out of this kit.

b) You will need to have ordered extra aRNA Filter Cartridges from Ambion (catalog # 10051G) because the Ambion kit only comes with enough for 20 purifications. Note the following changes:

1) When preparing to column purify the aRNA after the overnight *in vitro* transcription, know that the aRNA will start to precipitate as soon as you add the RNA Binding Buffer. Have your columns ready, add the buffer and mix 2-3 times with a pipet – no vortexing, no centrifuging – and immediately put the material on the Zymo column.

2) After the initial centrifugation step to load your sample onto the column, take the flow-through and load again. This ensures complete binding of your material to the column.

3) After the last wash and before the elution, dump the flow-through and spin the columns an extra time.

4) After column purifying the aRNA, elute in 25 µl nuclease-free water that has been pre-warmed to 65 °C. Repeat the elution with an additional 25 µl pre-warmed water, combining the two eluates.

Quantify your aRNA eluate (we use the NanoDrop spectrophotometer, which requires only 1 µl of sample) and place your samples on ice. You should get 25 – 50 µg aRNA. If possible, proceed immediately to Step 4; otherwise, put your samples at –80 °C.

Step 4: Coupling your aRNA to Cy Dyes

See the “Hybing with MEEBO” protocol.

Step 5: Preparing the Cy-coupled aRNA probes for application to the array

See the “Hybing with MEEBO” protocol.

Following this protocol, I apply the probes to arrays that have been post-processed according to:

http://derisilab.ucsf.edu/pdfs/Malaria_post_process.pdf.

Step 6: Washing the Arrays

See the “Hybing with MEEBO” protocol.

Shopping List

Ambion RNAlater (Ambion cat. # 7020)

Ambion RNAqueous Kit (Ambion cat. # 1912)

Ambion Amino Allyl MessageAmp II aRNA Amplification kit (Ambion cat. # 1753)

Zymo Research RNA Clean and Concentrate-5 (Zymo cat. # R1023)

Zymo Research RNA Clean and Concentrate-25 (Zymo cat. # R1025)

Amersham Cy dyes (Amersham cat. # RPN5661)

DMSO (Sigma-Aldrich cat. # D2438-50ML)

HYBING WITH THE MEEBO ARRAYS

*There are 3 parts to this procedure: preparing the MEEBO chips (aka post-processing), labeling the aRNA probes and applying them to the array, and post-hybridization washes prior to scanning. This can all be done in roughly 3 days (i.e. applying the probes to the post-processed arrays on day 1, and scanning the arrays on day 3). All glassware should be clean and completely dry. All water should be MilliQ. All ethanol should be 95% reagent grade. This protocol is not meant to be exhaustive, and assumes that you have general array knowledge – it would be good to first be very familiar with the “regular” protocols, such as those developed by the DeRisi Lab for their malaria arrays:
http://derisilab.ucsf.edu/pdfs/Malaria_cDNA_label_hyb_wash.pdf
http://derisilab.ucsf.edu/pdfs/Malaria_post_process.pdf*

Part 1: Post-processing the MEEBO arrays

Advance preparation:

The night before pre-warm to 60 °C approximately 500 ml of Array Shampoo (recipe follows at end) and one slide chamber without a rack.

Immediately before starting:

Have ready in a fume hood: a stir plate holding a 1000 ml glass beaker with a stir bar, a 500 ml glass cylinder, pyrrolidinone, and 1 M sodium borate pH 8.0. On a rocking or shaking platform, have ready one slide chamber without a rack. Have access to succinic anhydride being stored in a vacuum dessicator at 4 °C. In one area on your bench, have two slide chambers without racks filled with water, and one slide chamber without a rack filled with ethanol. In another area on your bench have one 4 L glass beaker filled with water, one slide chamber without a rack filled with water, and one slide chamber without a rack filled with ethanol.

a) Etch the arrays

Using a diamond etching pen, lightly mark the boundaries of the array on the underside of the array.

b) Cross-link the oligos to the slide

Place your arrays, spot side facing up, in a UV cross-linker. Cross-link using 600 x 100 µJ. When done, carefully place the slides in a clean, dry slide rack. In the rack, space the slides as far apart as possible to prevent accumulation of SDS bubbles later on. Place the rack in an empty slide chamber for protection, or in some other safe, dust-free place.

c) Shampoo your arrays

Place an empty styrofoam rack (e.g. from 50 ml centrifuge tubes) on your bench, and onto this place your pre-warmed slide chamber. Fill the slide chamber with

your pre-warmed Array Shampoo. Immediately, and without hesitation, immerse your cross-linked arrays into the Shampoo. Let sit *without any agitation* for 5 minutes. Without hesitation or draining, transfer the entire rack to a fresh slide chamber containing water. Let sit for 10 seconds without agitation, and then plunge gently for 30 seconds. Drain the rack for 5 seconds, and then transfer to the second water-filled slide chamber. Plunge gently for 30 seconds. Drain the rack for 5 seconds, and then transfer to the ethanol-filled slide chamber. Plunge gently for 30 seconds. Walk the chamber over the table-top centrifuge – you want to expose your semi-wet arrays to air for as little time as possible (you want them either submerged, or completely dry). Set up your balance. Drain your array-holding rack for 5 seconds, and transfer to a slide rack holder padded with a paper towel, to spin for 5 minutes at 500 RPM. Transfer the entire rack to the empty slide chamber on the rocking platform and store it there while you make your pyrrolidinone solution.

d) Measure out 5.5 g succinic anhydride. Try to avoid any clumps (I shake the bottle vigorously first, to break up any clumps). Set aside the succinic anhydride within easy reach.

e) In the hood, use the glass cylinder to measure out approximately 335 ml pyrrolidinone. Transfer to the 1000 ml beaker on the stir plate. Do not splash the pyrrolidinone, but stir vigorously. Add the succinic anhydride, and immediately get ready (but do not yet add) 15 ml sodium borate. As soon as the succinic anhydride is in solution (you may have to turn down the stirring to observe this, and there may be small bits that fail to go into solution) add the sodium borate and continue to stir for 10 seconds. Wrap a paper towel around the beaker to help catch drips and carry it to your rocking platform.

f) Remove the array-containing slide rack from the slide chamber, and set it to the side on the lid to the slide chamber. Carefully pour the pyrrolidinone into the slide chamber. Without hesitation, immerse the rack into the solution and plunge for 30 seconds. Cover, and rock gently for 5 minutes, making sure that no part of any slide is ever above the surface of the solution.

g) Drain for 5 seconds, and then immerse in the 4 L beaker of water. Plunge for 60 seconds.

h) Drain for 5 seconds, and then immerse back in the pyrrolidinone. Plunge for 30 seconds. Rock gently for 5 minutes, making sure that no part of any slide is ever above the surface of the solution.

i) Drain for 5 seconds, and then immerse in a fresh 4 L beaker of water. Plunge for 60 seconds.

j) Drain for 5 seconds, and then immerse in an ethanol-filled slide chamber. Plunge for 60 seconds.

k) Walk the chamber over the table-top centrifuge – you want to expose your semi-wet arrays to air for as little time as possible (you want them either submerged, or completely dry). Set up your balance. Drain your array-holding rack for 5 seconds, and transfer to a slide rack holder padded with a paper towel, to spin for 5 minutes at 500 RPM. Transfer the slides to a plastic slide box.

Part 2: Preparing your aRNA probes and applying them to the array

Advance preparation:

For every sample, you will need at least 10 µg aRNA, at a minimum concentration of 555 ng/µl. You will need very high quality DMSO, Cy dyes, 1M sodium bicarbonate pH 9.0, and Zymo RNA Clean-Up 5 and -25 columns. You need to have arrays, coverslips, and hyb chambers. It is also a good idea to make 1 L Array Wash Solution 1 and put it to warm at 60 °C, along with two slide chambers (one without a rack, and one with a rack).

Immediately before starting:

Place your arrays in the slide chamber, first tapping them lightly on their side or use a keyboard duster (the mechanical kind, not compressed air) to remove any large dust particles. Clean your coverslips with kimwipes and MilliQ water, ethanol (VWR cat. #MK-701910), then MilliQ water again. Finally, blow them off with a house air with an inline filter (again, no compressed air). Carefully place the coverslips on the arrays *with the lifter stripe facing down*. No part of the coverslip should be resting on the chip label. Cover the hyb chamber and leave it where you can later add your samples and get it into the water bath quickly. Have nearby a screwdriver to help seal the hyb chamber.

a) For each aRNA sample, transfer 5 µg to an eppendorf tube and bring to 9 µl with water. Add 1 µl 1 M sodium bicarbonate to each sample. Additionally, you will need to make a reference pool. For each sample, transfer an equivalent volume of sample to a reference pool tube; for all of your samples, contribute 5 µg to this tube. Add to this reference tube a volume of water equally the sum of the volume of water you added individually for each sample, and an equal sum of sodium bicarbonate.

b) For every 3 samples, resuspend one vial each of Cy 3 and Cy 5 dyes in 33 µl DMSO. For instance, if you have 9 samples, add 100 µl DMSO to one Cy 5 tube and vortex thoroughly; transfer the dye/DMSO to the next Cy 5 tube and repeat; repeat until you have all of your Cy 5 in one tube in 100 µl DMSO. Do the same in a separate tube with the Cy 3 dye packet(s).

c) Add 10 µl of Cy 5 in DMSO to each of your samples. Vortex, centrifuge briefly, and put in the dark for 30 minutes. For your reference pool, add 10 µl of Cy 3 in DMSO for each sample (e.g. if you have 9 samples for which you will be using your reference pool, add 90 µl of Cy 3 in DMSO to the reference pool). Vortex and centrifuge briefly. If necessary, split the reference pool at this point into multiple tubes so that no tube has more than 25 µg aRNA (e.g. for a reference pool used for 9 samples, split the labeling reaction into 2 separate 90 µl reactions), and put in the dark for 30 minutes.

To illustrate:

3/23/05									
MF infection with WT, hly- Lm: aRNA to generate Cy-labeled probes									
#	Sample	ng/uL	µg to use	µl to use	water	1M NaBicarb	total dye in DMSO		
1	uninfected A	619	4	6.5	2.5	1	10	10	10
2	WT Lm t = 30 A	614	4	6.5	2.5	1	10	10	10
3	WT Lm t = 60 A	579	4	6.9	2.1	1	10	10	10
4	WT Lm t = 120 A	451	4	8.9	0.1	1	10	10	10
5	WT Lm t = 180 A	521	4	7.7	1.3	1	10	10	10
6	hly- Lm t = 30 A	414	4	9.0	0.0	1	10	10	10
7	hly- Lm t = 60 A	508	4	7.9	1.1	1	10	10	10
8	hly- Lm t = 120 A	496	4	8.1	0.9	1	10	10	10
9	hly- Lm t = 180 A	524	4	7.6	1.4	1	10	10	10
reference samples:									
combine same volume of each probe listed above									
	add water	12.0							
	add 1M NaBicarb	9							
	add Cy3 dye in DMSO	90							

4) While the samples are coupling to dye, prepare your Zymo columns. For each sample, prepare one RNA Clean-Up 5 column. For every reference pool tube, prepare one RNA Clean-Up 25 column (e.g. for 9 samples, prepare 9 Clean-Up 5 columns, and 2 Clean-Up 25 columns). Process according to the normal Zymo protocol, and elute your samples in 2 sequential 11 µl volumes of water, and your reference samples in 2 sequential 80 µl of water. I often find a strange white precipitate in my samples, so immediately after the last eluting centrifugation, pipet off the dye-coupled samples into fresh tubes, leaving the white precipitate behind. Sometimes you can't even see the precipitate, but it's still worth doing this extra step. By doing this, I have drastically reduced the strange high background that sometime occurs at the end of the array closest to where the probe was added.

5) Measure the Zymo-purified dye-coupled aRNA on a NanoDrop (if possible). Note the amount of recovered aRNA and the dye incorporation. (Jess gets 90% recovery of his aRNA, and between 5 and 10 pmol dye incorporated per ul {usually between 9 and 10 pmol} This corresponds to approximately 42pmol dye/ug aRNA).

6) Assemble your microarray probes. The 2 sequential 11 µl elutions of your samples will yield 20 µl sample – just leave your samples in their tubes and add the other components. To each tube you will add 30 µl Cy 3 labeled reference pool. Briefly mix, and then add, in order: 2 µl PolyA @ 10 mg/ml, 2 µl yeast tRNA @ 10 mg/ml, 2.0 µl 1 M HEPES pH 7.4, and 12 µl 20X SSC. Vortex your samples and centrifuge briefly. Add 1.8 µl 10% SDS to each sample, and gently flick to mix, avoiding bubbles. Boil your samples for 2 minutes and then allow to cool to room temperature.

Alternatively: You can make cocktail mixes to add components. After combining the dye-coupled sample and reference aRNAs (in 50 ul final volume)

- Add 4 ul PolyA/tRNA mix (this can be made just prior to use, and nothing crashes out of solution; also, I have anecdotal evidence that too many freeze-thaw cycles of the PolyA and tRNA is bad -- aliquot it out into small volumes and treat these as you would any RNA samples)*
- Add 14 ul SSC/HEPES mix (again, the cocktail is stable)*
- Add the 1.8 ul 10% SDS. Your final probe volume should be just under 70 ul*

7) Add roughly 18 evenly spaced 10 μ l 3X SSC spots to the floor of your array-containing hyb chamber.

8) One at a time, pipet your samples under the coverslip on an array. Do not hesitate, and load the samples as quickly as is reasonable. Do not worry too much about any air bubbles; while bubbles are to be avoided, they seem to often go away during the hybridization, and there's nothing you can do about them at this point anyway. If you do see lots of bubbles, in the future reexamine your array post-processing procedure and your coverslip washing. As soon as you have pipeted out the entire volume of probe, immediately proceed to the next sample – you do not need to wait for the sample to flow completely across the array surface.

9) Once you have loaded all the arrays in the chamber, immediately secure the lid. If you choose to use a screwdriver, take care not to tighten so hard that you either strip the chamber threads or make it too difficult to remove them later. Put on an extra pair of gloves for insulation, and transfer the chamber to a 63 °C water bath, taking care to keep it level. Let hybridize for 36 – 48 hours.

Part 3: Washing Your Arrays

Advance preparation:

Make sure to have 1 L Array Wash 1 preheated to 60 °C, along with two slide chambers (one without a rack, and one with a rack). At room temperature, have one slide chamber without a rack filled with Array Wash 2, and one slide chamber without a rack filled with Array Wash 3.

Immediately before starting:

Have ready a pair of forceps to help retrieve the arrays from the hyb chamber, a large supply of paper towels, and your screwdriver. Place two styrofoam blocks on your bench to help insulate your pre-warmed chambers. On these blocks place your pre-warmed chambers and fill with pre-warmed Array Wash 1. Make sure that they're not too hot, as this can cause the poly-L-lysine coating to de-adhere. If necessary, just let them cool down for a few minutes to 60 °C.

a) Remove your arrays from the water bath and quickly dry the outside of the chamber with paper towels. Remove the screws and the chamber lid. If you've been using two pairs of gloves for insulation, remove the dirty outer pair now.

b) Using forceps, pry up the edge of one array, and then carefully grasp by the slides both slide and coverslip with your fingers. Carefully flip the whole array upside-down. Transfer the array to the first Wash 1 chamber, and let the coverslip drop off (if you use your other hand to help here, make sure that you don't touch any of the spots). Quickly transfer the array to the rack in the second Wash 1-containing chamber. Repeat for all arrays.

c) Plunge-mix gently in Wash 1 for 60 seconds. Without hesitation or any draining, transfer the whole rack to Wash 2.

d) Plunge-mix for 60 seconds. Drain the rack for 5 seconds, and then transfer the whole rack to Wash 3.

e) Plunge-mix for 60 seconds.

f) Walk the chamber over the table-top centrifuge – you want to expose your semi-wet arrays to air for as little time as possible (you want them either submerged, or completely dry). Set up your balance. Drain your array-holding rack for 5 seconds, and transfer to a slide rack holder padded with a paper towel, to spin for 5 minutes at 500 RPM. Transfer the slides to a plastic slide box. Scan as you would normally, but perhaps allowing for a few more saturated pixels, as the MEEBO array has so many bright positive control spots (I typically have the 635 PMT set at 720, and the 532 PMT set at 600).

Recipes:

Array Shampoo

3X SSC	20X SSC	75mL
0.2% SDS	10% SDS	10mL
filtered	water	415mL

Array Wash 1

2X SSC	20X SSC	100mL
0.03% SDS	10% SDS	3mL
filtered	water	897mL

Array Wash 2

1X SSC	20X SSC	25mL
filtered	water	475mL

Array Wash 3

0.2X SSC	20X SSC	5mL
filtered	water	495mL

Ordering Info:

Poly A (Sigma, P-9403)

tRNA (Invitrogen, 15401-011)

Lifter Slips (Fisher, 22-037-223)

Ethanol (Fisher, MK-701910)

DMSO (Sigma, D2438-50ML)

Cy dyes (Amersham, RPN5661)

RNA Clean-Up 5 (Zymo Research, R-1023)

RNA Clean-Up 25 (Zymo Research, R-1025)