ARISA Protocol – 377 with degassing, paper comb
Updated November 15, 2011, CC

I. Amplifying the ARISA samples

Quantify DNA samples with Picogreen (Invitrogen); see separate protocol.
*Use the same amount of DNA for each PCR reaction (usually 2-5ng depending on the sample type).
*So Cal Bight/Ocean samples are typically 2ng DNA/PCR reaction.
*It's recommended to prepare a small volume "working stock" of diluted DNA (@2ng/µl) to reduce freeze/thaw cycles and contamination of original DNA stocks.

The final concentrations of the reagents per PCR reaction are as follows:

PCR buffer – 1X.
Magnesium Chloride – 2.5mM.
dNTPs – 0.2mM.
BSA – 0.2mg/mL.
Primers – 0.4-0.8µM. (0.2-0.4µM each is ok)
AmpliTaq Gold – 5units (2.5units is better, but 5 is ok for problematic samples)

The PCR reaction has a final volume of 50µL.
DNA (same ng amount to each reaction) + PCR Water (may vary per sample)+ Master Mix = 50µL.

*It is not necessary to use 5 units of Taq (2.5 units works fine) and the primer concentration listed is relatively high. 0.2uM is also fine.
*BSA and $MgCl_2$ concentration can be increased for "tricky" samples – like sediments.

ARISA primers: #78 Forward – 5'-GYACACACCGCCCGT-3'
#79 Reverse – 5'-[TET]GGGTTBCCCCATTCRG-3'

Amplification cycles:
95°C – 5min
95°C – 40sec]
56°C – 40sec] 30 cycles
72°C – 1min30sec]
72°C – 7min
4ºC – hold, (to save wear and tear on equipment, best not to leave more than overnight in machine).

II. PCR Clean up

Zymo Clean and Concentrate Kit -5. Use as directed, except elute with two rounds of 6ul of PCR water. When adding any solution to the spin columns, make sure it goes in the center of the column. After adding PCR water for elution, let sit 5-10 minutes before final spin.

Quantify PCR concentrates with Picogreen. This time include a 20ng/µL standard. When reading on the Stratagene, remember to lower the gain to 4x or the high standard

will not read correctly. Please remember to put the gain settings back to 8x when finished.

Dilute PCR concentrates to between 5-10ng/µL (you might overload the CCD at 10ng/ul though) to run on slab gel.

III. ABI 377 protocol - Running Slab Gel
03/09/2007
Matrix standards: tet, fam, rox & tamra

- Prepare fresh 5X TBE (preferably the day of running the gel). It's crucial that there's no precipitate and the pH is at 8.3. [It should be 0.45um filtered. – *If prepared that day, there is no need to filter it, as long as all salts are in solution. I usually let it sit on the stir plate for 10 minutes at least*]
- Make sure 36cm plates have no lint or imperfections on them. You can use MilliQ water and a compressed air can. (DO NOT USE ORGANIC SOLVENTS).
    - Run in dishwasher with hot water wash at 90-95degrees, if plates have been sitting out for a while.
- Place separators on bottom plate using a little water to stick them to plate, one each side.
- Prepare and pour gel using a Lonza Long Ranger Single Pack for 36 cm plates or for 48 cm plates.

    Degassing the gel: If you're getting red rain or if you know that your fragments will be upwards of ~800bp you should degas the gel.

    1. Take a thick wall glass bottle (the 125ml Wheaton ones work just fine).
    2. Attach a 25mm filter apparatus through a two hole silicone stopper with a rinsed 25mm polycarbonate (do not use a glass filter) filter. Typically 0.8um pore size is used but the pore size doesn't matter so much. Through the other hole attach a hose going to the vacuum side of a vacuum pump.
    3. Remove the black clip from the Lonza gel pack and mix by hand for one minute. Then cut the pack making sure to leave the area between the white and red clips intact. Pour the contents of the pack into the filter apparatus. Apply a hard vacuum ~25mm/Hg.
    4. Swirl vigorously for 10 minutes making sure to visually form bubbles. The idea is to cause any gas in solution to rise to the surface and be removed from solution by the vacuum. This should considerably reduce the chances of bubbles forming in the read region as a result of heat stress by the laser.

    (Note: You will see bubbles forming on the surface – this is a good thing. It means that gas is being removed from the gel solution).

    5. Remove filter apparatus and vacuum.
    6. Cut open the part of the gel pack that is between the red and white clips making sure not to lose any of the solution.

7. With a 5ml pipette remove all of the liquid and gently pipette into the degassed glass bottle. There should be roughly 5ml's of solution in the area between the red and white clips.
8. Cover and swirl by hand for 1 minute.
9. Pour the gel as usual making sure to pack the bottom of the gel with whatever leftover gel solution that you have.

- Make sure there are no air bubbles. Place flat side of a plastic 96 well comb in the space at top of gel. Put at least 4 clamps over the comb and about three on each side.
- After ~ 30 minutes place a damp paper towel over the bottom of the top plate making sure not to touch the gel itself or allow water to touch the gel. Cover with a cut Ziploc bag and seal with saran wrap. Take care so that the bag does not contact the bottom of the gel.
- Allow gel to polymerize for another 1.5 hours.
- Prepare master mix as follows:

  Per reaction: Deionized formamide: 2.5uL
  Marker 1 (map marker 1000): 0.25uL
  Marker 2 (custom marker): 0.25uL
  Loading Dye: 0.5uL

  Make sure that the deionized formamide is fresh.

  The dye used is a home-made dye from the Hedgecock lab which is heavier and darker. **{OR use the Bioventures loading dye – which works fine, but is more faint. Hedgecock dye had been giving us haze issues with their last batch}**

  Previous protocols asked for 0.5ul of each dye, but 0.25ul of each is sufficient and more cost-effective!

  You will add 1uL of 5-10ng/uL of DNA to each sample which will then be run in duplicate. 10ng/ul is standard for ARISA, otherwise maintain consistency in your samples/project for TRFLP runs, etc.
- Keep the samples on ice and take over in bucket.
- Take off all the clamps, the napkin and the saran wrap.
- Pull out the comb and wash comb with DI water. Wipe off any acrylamide pieces that might be stuck to the comb.
- Wash the well area of the gel with water. This will cause any loose acrylamide pieces to come out.
- Make 1X TBE from 5X TBE stock.
- Rinse the well with 1X TBE and leave some in the well for the paper comb to absorb.
- Put the paper comb in. It's easiest to hold the gel upright on a lab bench so you can see where the acrylamide is. Comb sits so that the teeth are just into the gel. Once the paper comb has contacted the gel interface hold it for 30 seconds to expand.
- Put the gel in the rack
- Use MilliQ to thoroughly clean the area when the gel is scanned and blow away any lint with compressed air. Clamp the scanner.

- Open ABI prism 96 collection program. Go to file -- new – genescan run. Select the following modules: Plate Check A. Prerun PR-36A-2400, Run 36A-2400. Other selections, 96 lanes, dummy sample sheet (on the desktop), use the most newest matrix file in the list and run for 5.5 hours. Put in your **name AND acrylamide gel Lot #** as the operator and save the file name with the date. This makes it easier to locate and track the files. We are using filter set A for our matrix standards.
- Attach the bottom tray and then secure the gel to the ABI 377.
- Start plate check. It takes a few minutes. Keep an ear out for a huge clunking sound. It means its doing its thing. You might have to cancel and start again.
- Cancel the plate check after a few scans. If you see large peaks on the gel image, take out the gel and clean the read area of the plate again till the scan lines are flat.
- Attach the heat exchange and secure. Attach the upper buffer tray and secure as well. Make sure everything is plugged in.
- Add 1X TBE to upper and lower tray.
- Use a syringe to get rid of bubbles in the wells. Use gentle pressure only.
- Start prerun (it will take ~20 minutes). Again, you might have to stop and restart. Click on window and then status. Keep an eye on the temperature. The main point of the prerun is to get the temperature up close to the run temp (51 C), but you can pause it once it reaches 40 C and start loading samples (see below).
- While the pre-run in on, denature your samples at 95ºC for 5 minutes. Spin them in the plate spinner after you are done. Make sure to place them back on ice immediately.
- Pause the prerun anytime after it gets to about 40ºC. Load samples in duplicate. Load replicate set 1 in lanes 1-48 and replicate set 2 in lanes 49-96 .Add 1-1.5uL per well.
- Continue prerun for 2 minutes to gently run samples into the gel. If at any point the samples appear to be diffusing out of the wells you can run them into the gel and then continue loading.
- Terminate prerun.
- REMOVE PAPER COMB
- Hit Run. You can monitor the run in progress through the gel image window.
- Save

To Clean up:

- Remove heat exchange. Have a large Kimwipe ready to absorb any leaking buffer.
- Remove gel with its rack and the upper buffer tray. Take it over to a sink and remove the upper tray there (the buffer will spill easily).
- Use a plastic plate separate (DO NOT USE METAL) to pry open the two plates. Be careful not to break the razor blade or scratch the plates.
- Use large Kimwipes or paper towels to pull up the acrylamide and dispose of it.
- Rinse plates with DI water and take care to remove any stuck on acrylamide.
- Place plates in dishwasher and set the temperatures of both cycles at 95C. Do not use any detergents.

- Wash buffer trays, comb and separators with DI water.

Notes on Running ABI 377:
If you do not choose a sample sheet you will not be able to run.
Old deionized formamide will not do its job!
Make sure TBE buffer is filtered, the right pH, and has no crystals. (if made the same day, filtering the buffer is not crucial. Just make sure it is well mixed).