miRNA microarrays

Using a miRNA microarray can be broken down into 5 steps:

1. Post-Processing (Can be done ahead of time)
2. Isolation of Small RNAs from sample (Can be done ahead of time)
3. Labeling of RNA (Done the day of hybridization)
4. Hybridization
5. Washing and Scanning (Done the day after hybridization)

Note: You will be applying RNA directly to the array, always use gloves when handling the arrays. Arrays should be stored desiccated at room temperature.

1. Post-Processing—After post-processing arrays can be used for up to several weeks

Materials

Metal slide racks
Re-hydration trays (Sigma H6644)
Centrifuge with slide rack adaptors
Succinic anhydride
1-methyl-2-pyrrolidinone
1 M Sodium Borate Solution, pH 8.0. (adjust pH with NaOH)
Diamond-tipped glass etching pen (VWR 52865-005)
StrataLinker for UV cross-linking

Protocol

a) Pour 100 ml 0.5x SSC into hydration tray and warm on slide warmer. Set the warmer to 37°C
b) Pre-heat a heating block at max (>100°C)
c) After processing, the arrays will not be visible, so their boundaries need to be marked. Holding the diamond pen perpendicular to the slide, mark the boundaries of the array on **back** side of the slide.
d) Set slide array side down on the hydration tray and observe spots until full hydration is achieved (this will look like a light layer of condensation covering the array). Do not rehydrate more than 1 min.
e) Upon reaching full hydration, dry the slides by flipping one at a time onto the heating block with the **array face up.** Do this in one smooth motion, with one hand, pinching the array at one end and flipping it over as you move it to the hot plate. Having a good bright light at the right angle will help you see the slide drying. The array should dry within 1-2 seconds. Remove the slide and place into a metal slide rack.
f) UV cross link the arrays at 60mJ (if you are using a stratalinker, push the energy button, lighting-up the indicator for ujoulesx100, enter 600 and then press start).
g) Measure 335 ml of 1-methyl-2-pyrrolidinone into a clean, dry 500mL beaker. Dissolve 5.5 g of succinic anhydride in the 1-methyl-2-pyrrolidinone using a stirbar. Note that the stock bottle of solid succinic anhydride should be stored

under desiccation and vacuum. Do not use if exposed to moisture, avoid using clumps.

h) **IMMEDIATELY** after succinic anhydride dissolves, mix in 15 ml of 1M sodium borate **pH 8.0.**

i) Quickly pour the buffered blocking solution into a clean, dry glass slide dish. Plunge the slides rapidly into blocking solution and vigorously shake, keeping the tops of the slides under the level of solution. After 30 seconds of plunge-mixing, put a lid on the glass box, and let shake gently on a rotator for 15 minutes.

j) Transfer slide rack to a glass jar filled with room temp distilled water. Plunge the slides up and down a few times in the water.

k) Transfer the rack to a glass dish of 95% EtOH and plunge several times to rinse. Make sure the EtOH is crystal clear. Do not use if it appears to have particulates or appears cloudy.

l) Spin slide rack in a benchtop centrifuge for 1 minute at 550 rpm.

m) After spinning, the slides should be clean and dry (if not dry you can try spinning again, if not clean you can try re-washing in ethanol followed by another spin).

2. Isolation of Small RNAs from sample—it is usually advisable to check for degradation of your RNA either after extraction or before this step. This can be done on a gel or on an Agilent Bioanalyzer.

There are two options for small RNA isolation. 1) flashPage, an Ambion product, which is quick but not efficient for running many samples in parallel, or 2) acrylamide gel size fractionation—slow, but many samples can be run in parallel.

1) Purify RNAs of $\leq$ 40 nt for use on the array using Ambion's flashPAGE electrophoresis unit according to their protocol:
http://www.ambion.com/techlib/prot/bp_13100.pdf.
RNAs can then be recovered by the flashPage (http://www.ambion.com/techlib/prot/bp_12200.pdf) clean-up kit or by ethanol precipitation (http://www.ambion.com/techlib/misc/acetate_precip.pdf) (**Note: Do not use a glycogen carrier)**.

or

2) Purifying small RNA on a denaturing acrylimide gel

Materials
10% Denaturing Polyacrylamide Gel
Trizol Purified RNA Samples (50-500 ug)
Gel Loading Buffer (with bromophenol blue and xylene cyanol)
Ethanol
3 M Sodium Acetate
15 mL Falcon Tubes

Protocol

a) Run the gel at constant 65 mA until the gel reaches about 50°C.
b) Mix the sample RNA with equal volume of gel loading buffer and heat at 70°C for 2 min, and put on ice. Rinse out urea with reservoir buffer.
c) Run the gel until there is about 1.5 cm separating the xylene cyanol and bromophenol blue bands.
d) Cut out the slice between the bands and cut apart separate samples. Use a syringe plunger to crush the gel. Place in a 15 mL falcon tube and add 1 mL of 0.3M sodium acetate for every cm in width of lane used to run sample.
e) Rock overnight at 4°C.
f) Centrifuge tube for 5 minutes (2000 xg), collect supernatant and put at -20°C.
g) Add to the gel 1/5 the volume of sodium acetate used for the first elution. Rock for 1 hour at room temperature.
h) Centrifuge as before, collect the supernatant and add to the supernatant from the first elution.
i) Add 4 volumes of 100% ethanol to the collected supernatant. Vortex.
j) Incubate at -20°C overnight.
k) Centrifuge at 16,000 xg at 4°C.
l) Discard supernatant.
m) Wash the pellet with 500 μL 75% ice cold ethanol. Centrifuge at 16,000 xg for 10 minutes at 4°C. Discard the supernatant and air dry the pellet.

3. Labeling of RNA—this is best done just before hybridization, although there is one possible stopping point in the protocol, after which the RNA can be stored for 1-2 days.

Label small RNAs using Ambion's *mir*VANA labeling kit according to their protocol: http://www.ambion.com/techlib/prot/fm_1562.pdf.
Note: The Cy3 and Cy5 fluorophores will photo bleach, it is best to minimize their exposure to light as much as possible—this includes the hybridized array.

4. Hybridization

The amount of RNA to use for hybridization will vary depending on the concentration of the miRNAs that you're trying to visualize. Hybridizing with the small RNAs isolated from 20 μg of total RNA might be a good starting point.

Materials
Labeled RNA
Hybrization chamber
Waterbath at 42°C
Heat Block at 100°C
Ambion 3x hybridization buffer (Cat # 1567 *mir*Vana™ miRNA Bioarray Essentials Kit)
22x25 mm lifter slips (Erie 22x25I-M-5226)

Protocol

a) Heat 3x hyb buffer at 65°C and vortex to resuspend
b) Add 5 to 10 100 μL drops of 5x SSC in the bottom of the hybridization chamber to prevent the arrays from drying out.
c) Place the slides in the hybridization chamber, array side up. Add the lifter slip over the arrays (this is where the diamond pen marking comes in handy). A flat metal spatula can be helpful to align one side of the lifter slip, so that you can gently drop the other side down. Use the spatula to align the lifter slip over the array. **Be careful not to scratch the array surface.**
d) Determine the volume of eluted RNA (should be around 20 μL) and add half this volume in hyb buffer (ie. for a 20 μl sample, add 10 μL).
e) Heat denature samples in a 95°C heat block for 2 minutes. Microfuge to cool.
f) Immediately apply sample.
g) Close hybridization chamber, carefully transfer immediately to a 42°C waterbath for 12-16 hours.

5. Washing and Scanning

Materials
Ambion Salt Concentrate (Cat # 1567 *mir*Vana™ miRNA Bioarray Essentials Kit)
Ambion Detergent Concentrate (Cat # 1567 *mir*Vana™ miRNA Bioarray Essentials Kit)
Metal slide rack
4 Glass slide boxes with covers (in which the slide racks fit)
Centrifuge rotor adaptors for metal slide racks

Protocols
a) Prepare 4 slide boxes with washing solutions. The first two are low stringency (4 mL detergent concentrate, 20 mL salt concentrate, and 376 mL H2O) and the second two are high stringency (20 mL salt concentrate and 380 mL H2O) .
b) Carefully remove hybridization chamber from the water bath.
c) Remove slides one at a time from the chamber, tip off lifter slip in first low stringency bath and immediately place the slides in the metal rack sitting in the 2nd low stringency bath.
d) Wash slides in the low stringency bath, by plunging them up in down in the solution for1 minute (do not hit the bottom of the box too hard or you will damage your slides).
e) Wash in first high stringency bath for 1 minute.
f) Wash in second high stringency bath for 1 minute.
g) Spin slide rack in a benchtop centrifuge for 3 minutes at 800 rpm.
h) Place slides in a plastic slide box and scan immediately.

Note: Much of this protocol has only seen minor adaptations from protocols of Michael McManus and Caroline Mrejen, many thanks for their assistance.