Amplified RNA Microarray Protocol

cDNA Synthesis from total RNA:

Use cDNA Synthesis Kit, Roche Cat #1117831

1. Anneal primer:

Total RNA	1ug
(6)T7 Oligo (dT)24, 100 pmol/ul	1 ul
Final Volume:	10.5 ul

2. Denature at 70˚C for 10 minutes.
3. Anneal on ice for 2 minutes.
4. Set up first strand synthesis on ice:

RNA-primer mix	10.5 ul
(1)5X RT Buffer	4 ul
(3)0.1 M DTT	2 ul
(2)AMV RT 25 U/ul	1 ul
(4)RNase Inhibitor	0.5 ul
(7)10 mM dNTP Mix	2 ul
Final Volume:	20 ul

5. Mix, and incubate at 42˚ for 2 hours.
6. On ice, prepare for second strand synthesis:

cDNA from RT	20 ul
5X 2 Strand Buffer	15 ul
10 mM dNTP Mix	0.75 ul
2 Strand Enzyme Blend	3.25 ul
Rnase-free Water	36 ul
Final Volume:	75 ul

7. Mix and incubate at 16˚C for 2 hours.
8. Place on ice, and add 10 ul (10 U) T4 DNA Polymerase.
9. Mix, and incubate at 16˚ C for 5 minutes.
10. Add 8.5 ul 0.2 M EDTA pH 8.0 to stop second strand reaction.
11. Add 0.75 ul (7.5 U) RNase I to degrade RNA.
12. Incubate 37˚C for 30 minutes.
13. Add 2.5 ul (1.5 U) Proteinase K to degrade RNase.
14. Incubate at 37˚C for 30 minutes.

Purification of dsDNA

Use Qiaquick PCR purification kit Cat. # 28104

1. Add 3.75 ul 3 M NaOAc pH 5.2 to dsDNA.
2. Add 325 ul PB Buffer.
3. Mix, and apply to column.
4. Spin 15 seconds at 13,000 rpm.
5. Wash 3X with 600 ul PE Buffer.
6. After discarding last flow-through, spin 1 minute at 13.000 rpm to dry column.

7. Transfer column to new tube.
8. Add 40 ul Elution Buffer (EB) directly to center of column.
9. Incubate at room temperature for 1 minute.
10. Spin 1 minute at 13.000 RPM.
11. Analyze sample with Nanodrop.

Preparation of amino-allyl labeled aRNA
Use MegaScript T7 Ambion Cat # 1334.

Keep 10 X Buffer at room temperature with assembling reaction, and assemble reaction at room temperature.

1. Mix:

dscDNA	(100 ng)
RNase-free water	
10 X Buffer	2 ul
ATP (75 mM)	2 ul
CTP (75 mM)	2 ul
GTP (75 mM)	2 ul
UTP (75 mM)	0.3 ul
aa-UTP (50 mM)	2.5 ul
Enzyme Mix	2 ul
Final Volume:	20 ul

2. Incubate at 37˚C for 16 hours.
3. Run 1 ul on agarose gel, expect 0.5-3 kb smear.

Purification of aRNA
Use RNeasy, Qiagen # 74104

1. Mix RLT Buffer and B-mercaptoethanol.
2. Add 350 ul RLT buffer to aRNA sample and mix.
3. Add 250 ul 95% EtOH and mix.
4. Apply to columb.
5. Spin 10K for 15 seconds.
6. Re-apply flow-through to column, and spin as above.
7. Wash 2 times with 500 ul RPE Buffer, spin 10 K for 15 seconds
8. After second wash, discard flow-through and spin column 1 minute at full speed.
9. Transfer column to a clean tube.
10. Add 50 ul of 65˚C RNase-free water directly to center of column.
11. Incubate column 4 minutes at 65˚.
12. Spin 1 minute at 1K RPM.
13. Spin 1 minute at 10K RPM.
14. Repeat elution.
15. Check concentration on nanodrop.

    *Optional: Store at -*80˚.

Coupling
All further reactions should be carried out in the dark.

1. Dry 10 ug aRNA in speedvac ~1 hour.
2. Resuspend aRNA in 9 ul 0.05 M $NaHCO_3$.
3. Resuspend desired Cy-Dye (Amersham Biosciences Catalog #RPN5661) with aRNA solution.
4. Incubate at room temperature for 2 hours.
5. Add 9 ul 1M Tris pH 7.5-8.0 to quench remaining Cy-Dyes.
6. Incubate at room temperature 15 minutes.

Fragmentation
1. Add 4.5 ul 50 mM $ZnCl_2$ (vortex before use).
2. Incubate 30 minutes at 60˚C.

Purification of Cy-aRNA
1. Add 2.25 ul 3 M NaOAc pH 5.2.
2. Add 56.25 ul 100% EtOH (-20˚C).
3. Vortex.
4. Precipitate on dry ice for 30 minutes.
5. Spin full speed for 30 minutes at 4˚C.
6. Wash pellet 4 times with 1 ml 70% EtOH (-20˚C) to clear supernatant.
7. Dry pellet in speed vac.
8. Resolve pellet in 6 ul H_2O.
9. Nanodrop.

Hybridization
1. Prepare prehybridization buffer:

20 X SSC	25 ul
10% SDS	1 ul
10% BSA	10 ul
H_2O	64 ul
Final Volume:	100 ul

Filter with 0.2 um filter.
2. Preheat to 42˚C.
3. Place LifterSlip on microarray slide.
4. Carefully pipet 60 ul between slide and lifterslip.
5. Incubate at 42˚C for 1 hour.
6. Remove coverslip by placing slide in 0.1 X SSC until LifterSlip floats away.
7. Dry under clean air stream or by centrifugation.
8. Add 30 ul DIG Easy Hyb slution to each probe.
9. Mix Cy3 and Cy5 aRNA.
10. Melt 95˚C for 5 minutes.
11. Cool on ice for 1 minutes.

12. Preheat to 42˚C.
13. Place a new LifterSlip onto the microarray slide.
14. Pipet all of Hyb/Cy-aRNA solution onto slide.
15. Incubate at 42˚C overnight.

Wash

1. Pre-warm 1 X SSC/0.2% SDS at 37˚C and 42˚C.
2. Remove LifterSlip by dipping slide in 42˚C 1 X SSC/0.2% SDS.
3. Wash slide 10 minutes rocking in 42˚C 1 X SSC/0.2% SDS.
4. Wash slide 5 minutes rocking in 37˚C 1 X SSC/0.2% SDS.
5. Wash slide 5 minutes rocking in 1 X SSC/0.2% SDS at room temperature.
6. Wash slide twice in room temperature 0.1 X SSC for 5 minutes.
7. Dry slide by centrifugation.
8. Scan.