Amplified RNA Microarray Protocol(from BRI lab)
2/11/04

Spec purified RNA; want 260/280 ratio >1.8.

CDNA synthesis from total RNA

Use cDNA Synthesis Kit, Roche cat#1 117 831.

Total RNA	1ug
T7 Oligo (dT)24 100 pmol/uL	1 uL
Rnase-free H2O	
	10.5 uL

Mix.

Melt 70°C 10m.

Anneal on ice 2m.

Add on ice:	uL
RNA-primer mix	10.5
5X RT buffer	4
0.1 M DTT	2
AMV RT 25 U/uL	1
Rnase Inhibitor 25 U/uL	0.5
10 mM dNTP Mix	2
	20

Mix.

Incubate 42°C 2h.

Ice.

Prepare on ice for second strand synthesis:

	uL
cDNA from RT	20
5X 2nd strand buffer	15
10 mM dNTP Mix	0.75
2nd strand enzyme mix	3.25
Rnase-free water	36
	75

Mix.

Incubate 16°C 2h.

Ice.

Add 10 uL (10 U)T4 DNA polymerase.

Mix.

Incubate 16°C 5m.

Add 8.5 ul 0.2 M EDTA pH 8.0 to stop second strand rxn.

Mix.

Add 0.75 uL (7.5 U) Rnase I to degrade RNA.

Mix.

Incubate 37°C 30m.

Add 2.5 uL (1.5U) proteinase K to degrade Rnase.

Mix.

Incubate 37°C 30m.

Purification of dsDNA

Use Qiaquick PCR purification kit cat 28104.

Add 3.75 uL 3 M NaOAc pH 5.2 to dsDNA.

Add 325 uL PB buffer.

Mix.

Apply to coloumn.

Spin 15s 13Krpm.

Wash 3X 600 uL PE buffer.

Spin same.

Spin 1m 13Krpm to dry tube.

Transfer to new column.

Add 40 uL Elution Buffer to center of column to elute.

Incubate RT 1m.

Spin 1m 13Krpm.

Nanodrop.

Preparation of amino-allyl labeled aRNA

Use MegaScript T7 Ambion cat 1334.

Keep 10 X Buffer at RT while assembling the rxn.

Prepare on ice:	uL
dscDNA	100 ng
Rnase-free water	
10 X Buffer	2
ATP 75 mM	2
CTP 75 mM	2
GTP 75 mM	2

UTP 75 mM	0.3
aa-UTP 50 mM	2.5
Enzyme Mix 10X	2
	20

Mix.

Incubate 37°C 16h.

Run 1uL on 1% agarose gel.

Expect 0.5-3 Kb smear.

Purification of aRNA

Use Rneasy, Qiagen cat 74104.

Add 350 uL RLT buffer to aRNA sample.

Mix.

Add 250 uL 95% EtOH.

Apply to column.

Spin 10Krpm 15s.

Re-apply flowthru to column.

Wash 2X 500uL RPE buffer.

Same spin.

Spin dry 1m 10K rpm.

Clean tube.

Add 50 uL 65°C Rnase-free H2O to center of column to elute.

Incubate 4m 65°C.

Spin 1K rpm 1m.

Spin 10 Krpm 1m.

Repeat elution.

Spectrophotometer.

Optional storage at -80°C.

Coupling *in dark*

Use Amersham Biosciences cat RPN5661.

Dry 10 ug aRNA in speedvac ~1 h. NO HEAT.

Resuspend aRNA in 9 uL 0.05 M NaHCO3.

Resuspend desired cy-dye with aRNA soln.

Incubate 2h RT.

Add 9 uL 1 M Tris ph 7.5-8.0 to quench remaining Cy-dyes.

Incubate 15m RT.

Fragmentation *in dark*

Add 4.5 uL 50 mM ZnCl2; vortex before use.

Incubate 60°C 30m.

Purification Cy-aRNA

Add 2.25 uL 3M NaAce pH 5.2.

Add 56.25 uL 100% EtOH (-20°C).

Vortex.

Precipitate on dry ice 30m.

Spin 14Krpm 30m 4°C.

Wash pellet 4X 1 mL 70%ETOH (-20°C) to clear supernatent.

Dry pellet 5m speed vac.

Resolve pellet in 6 uL H20.

Nanodrop.

Hybridization *in dark*

Prepare prehybrization buffer	n=1(uL)
20XSSC	25
10% SDS	1
10%BSA	10
H20	64
	100

Filter 0.2 micron.

Preheat 42°C.

Pipet 20 uL 3X onto coverslip.

Bring microarray down to coverslip.

Incubate 42°C 1h.

Remove coverslip with 0.1XSSC.

Dry under clean air stream.

Add 15 uL DIG Easy Hyb solution to each probe.

Mix cy3 and cy5 aRNA.

Melt 95°C 5m.

Cool on ice 1m.

Preheat 42°C.

Pipet 3X 20 uL aRNA onto cover slip.

Bring microarray down to cover slip.

Incubate 42°C ON.

Pre-warm 1XSSC/0.2%SDS at 37°C and 42°C.

Remove cover slip by dipping 42°C 1XSSC/0.2%SDS.

Wash slide 10 m rocking in 42°C 1XSSC/0.2%SDS.

Wash slide 5 m rocking in 37°C 1XSSC/0.2%SDS.

Wash slide 5 m rocking in 1XSSC/0.2%SDS.

Wash slide 2X 5 m rocking in 0.1XSSC.

Dry under clean air stream.

Scan.