

Coupling Protocol V1-010703

Annealing anchored oligo dT to RNA

Reagents
Anchored oligo dT @ 1ug/ul (can use 3ug/ul for dilute RNA)
5 to 10ug of RNA in a volume of 12ul

- Add 6ul oligo to RNA (total volume 18ul)
- Incubate 5 minutes at 70°C.
- Incubate 10 minutes at RT; spin briefly in microfuge.
- Incubate 10 minutes on ice.

Reverse transcription incorporating amino allyl labeled dNTP's

Reagents
Superscript RT (GIBCO #18064-014)
DTT (comes with superscript)
Superscript 5X buffer(comes with RT)
30XdNTP's (3:1 modified dNTP:dNTP); 1X = 200uM)
Sigma A0410:AAAdUTP (1mg/17.2ul = 100mM; 5mg/86ul = 100mM)

30x dNTPs:			
(100mM Stock)	Volume	30x [Final]	1x [Final]
dATP	9µl	6mM	200µM
dGTP	9µl	6mM	200µM
dCTP	9µl	6mM	200µM
dTTP	2.28µl	1.52mM	51µM
AAAdUTP	6.72µl	4.48mM	149µM
H ₂ O	114µl		
Final Volume	150µl		

RT Mix (per sample):

5X Buffer	6µl
30X dNTP	1µl
DTT	3µl
Superscript	2µl
Total:	12µl

- Add these 12 µl to each reaction; mix well.
- Incubate at 42°C for 2 hours.

Hydrolysis of RNA template and cleanup of cDNA

Reagents
1.0 N NaOH (make fresh)
0.5M EDTA
1.0 N HCl
Qiagen QiaQuick PCR cleanup kit (catalog #28104)

- ❑ Add 10 μ l 0.5M EDTA and 10 μ l 1.0 N NaOH to each reaction.
- ❑ Incubate at 70°C for 10 minutes.
- ❑ Cool to RT for 2 minutes.
- ❑ Add 10 ul 1.0M HCl to neutralize; **mix well**.
- ❑ Add 300ul buffer PB-5X volume (Qiaquick kit) and transfer to column.
- ❑ Wash 2 times with 750 μ l 80% ETOH (1 minute spins; discard washes.) Do not use PE buffer--Tris interferes with coupling.
- ❑ Spin 1 minute to dry column.
- ❑ Elute 2 times with 30 μ l nuclease-free H₂O (**Pipet directly onto membrane and let sit 1 minute prior to 30 second elution spin.**) Note: check pH of water--it must be neutral/slightly basic to elute effectively--modify pH with .05M NaBicarb, pH 9
- ❑ Take OD @ 260/280. Note: ssDNA may be stored at -20°C overnight at this point.
- ❑ Dry down cDNA for 35 minutes at medium heat in speed vac.

Coupling of ester-linked cy-dyes to ssDNA

NOTE: the following should be done in low-light.

Reagents
Cy3/Cy5 dyes:(Amersham #PA23001, #PA25001) Resuspend in 30 μ l DMSO (Aldrich ACS 99.9% catalog #47,230-1). Aliquot 5 μ l into a .5ml tube; dry in speed vac 35 minutes at medium heat; store @-20°C
0.1 M NaBicarbonate buffer, pH 9.0
4 M Hydroxylamine (Sigma-Aldrich#43,136-2)

- ❑ Resuspend cDNA in 7 μ l 0.1M NaBicarbonate Buffer, pH 9.0.
- ❑ Let sit at RT for 5-10 minutes to ensure resuspension.
- ❑ Transfer the cDNA/Bicarb solution to an aliquot of dye. **Do not mix with pipet!** Flick tube to mix; microfuge briefly to pull coupling to bottom of tube.
- ❑ Incubate for 60 minutes at RT in the dark on a rocking platform.
- ❑ Add 1/2 volume of 4M Hydroxylamine. Mix well by flicking tube—**do not mix with pipet!**
- ❑ Incubate 15 minutes at RT in the dark on rocking platform.

- ❑ Combine Cy3 and Cy5 targets.
- ❑ Add 5X volume PB Buffer (Qiaquick kit) and transfer to column.
- ❑ Wash column 3X with 750 μ l 80% ETOH as before.
- ❑ Elute 2 times with 30 μ l EB buffer as before.
- ❑ Take spec readings at OD 260, OD 649, and OD 550 to assess coupling.

Equation	Measurement	Wavelength	Ext. Coeff./ Conc. Factor
$\text{pmols} = \frac{(\text{OD} * 60\mu\text{l})}{\text{ext coeff}}$	pmols of Cy5	649	0.25
	pmols of Cy3	550	0.15
$\mu\text{g/ml} = (\text{OD} * \text{conc. factor})$	μ g/ml of ssDNA	260	33

- ❑ Dry down cDNA for 35 minutes at medium heat in speed vac. Start pre-hyb of slide while target is drying down.

Prehybridization

- ❑ While samples are drying, slides must be prehybridized to eliminate non-specific interactions (from Hedge et al (2000) Biotechniques 29:548-562)
- ❑ Prepare a solution of 0.2% SDS at RT. Put slides in a slide rack and vigorously plunge the slides into the solution. (This vigorous washing rapidly removes any unbound material and prevents "comet tails" from forming on the slides.)
- ❑ Move slides immediately into Prehyb solution prewarmed to 55°C: 1%BSA, 5xSSC, 0.1%SDS
- ❑ Prehyb slides at 55°C for 45 minutes.
- ❑ Rinse 5x with MilliQ water taking care to rinse away all prehyb solution.
- ❑ Rinse 1x with isopropanol and spin or air dry slides.
- ❑ Slides should be perfectly clean after prehyb. Any streaking indicates incomplete rinsing.

NOTE: Slides should be hybridized immediately after prehyb. A loss of hybridization signal is reported if slides are left to dry more than 1 hour.

Hybridization

Make 2X hyb buffer fresh daily:

50% formamide
10X SSC
0.2% SDS

- ❑ Clean Lifterslip with 70% EtOH prior to use
- ❑ Depending on the array and size of coverslip, resuspend target material as follows:

Coverslip Size (cat #)	Volume Water	Volume 2x Hyb Sol	Vol 10mg/ml PolyA RNA	Total Hyb Vol
24x40mm (2-4959)	40ul	40ul	1ul	81ul
24x60mm (2-4733)	50ul	50ul	1ul	101ul

- Incubate 2 minutes at 100°C. Spin briefly to bring contents to bottom of tube
- Apply to array by carefully pipetting the hyb solution onto the slide. Carefully place the Lifterslip onto the slide and make sure the hyb solution is evenly distributed under the Lifterslip.
- Incubate O/N at 42°C (14-16 hours) in a humidified hyb chamber
- (Humidity in the chamber is best maintained with 1X hyb buffer. Choice of hyb chamber has little effect on array results. While the Corning hyb chambers are recommended, a carefully placed 50ml tube works very well).

Wash

Heat Wash Solution 1 to 55°C before use.

After hyb, remove slide from chamber:

<u>Wash</u>		
1	2	3
2x SSC, .1% SDS	1x SSC	.1x SSC
40ml 20x SSC	20 ml 20x SSC	2ml 20x SSC
2ml 20% SDS		
358 ml H ₂ O	380 ml H ₂ O	398 ml H ₂ O

- Wash in each solution for 5 minutes with gentle stirring/agitation taking care to avoid SDS carryover from Solution 1.
- Place washed slides in slide rack and spin 5 minutes at 50xg to dry.
- Scan immediately. (While immediate scanning is recommended, slides may be stored dry in the dark for extended times (approx 2 weeks) with minimal loss of signal.

