

Reagents:

Anc-Oligo dT: Anchored oligo-dT with the sequence 5'-T₍₂₀₎VN-3' (V=any nucleotide except T, N=any nucleotide).

Reverse Transcriptase: SuperScript from Gibco/Life Technologies (includes 5x buffer and DTT).

Purification kit: Qiagen PCR purification kit (Cat. Number 28104).

30x dNTP Mix: equal dye:dCTP

Unlabeled dNTPs (100mM Stock)	Volume	30x [Final]	1x [Final]
dATP	6 μ l	6mM	200 μ M
dGTP	6 μ l	6mM	200 μ M
dTTP	6 μ l	6mM	200 μ M
dCTP	1.8 μ l	1.8mM	60 μ M
H ₂ O	80.2 μ l		
Final Volume	100μl		

Above dilutions are for the use of Cy-dye labeled dCTP

RT Reaction

	Concentration	Volume
Oligo dT	1 μ g/ μ l	3 μ l
Total RNA (30μg)	Variable (>2.63ng/ μ l)	Up to 11.4 μ l
H₂O	Variable	Final volume = 14.4 μ l

1.2 μ g mRNA may be used in place of total RNA

Incubate RNA and primers for 5 minutes at 70°C.

Incubate at RT for 10min.

Chill to 4°C. Spin down tubes before adding RT Master mix.

All subsequent reactions should be done in low-light conditions to prevent degradation of the Cy-dyes.

Add	Volume (μ l) per reaction
5x Buffer	6
30x dNTPs	1
DTT (0.1M)	3
SuperScript	2
Cy-Dye	1.8 (60 μ M final)
H₂O	1.8
	30μl total

5x Buffer: 250mM Tris-HCl (pH 8.3), 375 mM KCl, 15mM MgCl₂

Mix well and incubate 42°C for 2 hours

Hydrolysis

Add 20µl 0.5M NaOH/0.25M EDTA

Incubate 10 minutes at 70°C

Bring to room temperature

Add 10µl 1M HCl; pH must be between 5-7 to bind to QiaQuick column efficiently. (*may be neutralized with HEPES instead of acid*)

Probe Clean-up

QIAGEN PCR purification kit (Cat. Number 28104)

To each column, add 5 Vol. (600 µL) Buffer PB

Combine the two targets together in a single tube (120µl total)

Apply to columns containing PB buffer.

Spin 30 sec., discard flow-through; replace column

Wash 2X with 750 µL 80%EtOH, spin 30 sec and discard flow-through

Spin column dry 1 min after final wash

Elute DNA into clean tube with 30 µL of Buffer EB

Wait 1 minute after adding elution buffer before spinning.

Repeat elution step 1x

Final volume will be ~60µl

Take spec readings at 649 and 550λ.

$\text{pmols} = \frac{(\text{OD} * 60)}{\text{ext coeff}}$	Measurement	Wavelength	Ext. Coeff.
	pmols of Cy5	649	0.25
	pmols of Cy3	550	0.15

Dry in speedvac (30 min at medium heat) to reduce volume to <30µl.
Should see a purple pellet when dry (*intensity of purple color will vary*).

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 preheated 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:

Wash		
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).