Preparation of Fluorescent cDNA Probe from Human mRNA (alternate protocol)

1. To anneal primer, mix 2µg of mRNA with 2µg of a regular or anchored (5'-TTT TTT TTT TTT TTT TTT TTV N-3') oligo-dT primer in a total volume of 15µl:

	Cy3	Cy5
mRNA	2 µg	2 µg
Oligo-dT	2 µg	2 µg
Total volume:	15 µL	15 µL

2. Heat to 70°C for 10 min and cool on ice.

3. Add 15 µL of reaction mixture each to Cy3 and Cy5 reactions:

Reaction mixture		Unlabeled dNTPs	Vol.	Final conc.
5X first-strand buffer*	6.0	dATP (100 mM)	25 µL	25 mM
0.1M DTT	3.0	dCTP (100 mM)	25 µL	25 mM
Unlabeled dNTPs	0.6	dGTP (100 mM)	25 µL	25 mM
Cy3 or Cy5 (1 mM, Amersham)	3.0	dTTP (100 mM)	15 µL	15 mM
Superscript II (200 U/µL, Gibco BRL)	2 µL	ddH2O	10 µL	
	------		-------	
Total volume:	15 µL	Total volume:	100 µL	

4. 5X first-strand buffer: 250 mM Tris-HCL (pH 8.3), 375mM KCl, 15mM MgCl2)

5. Incubate at 42°C for 1.5-2hrs.

6. Degrade RNA by addition 15µl of 0.1N NaOH, and incubation at 70°C for 10 min.

7. Neutralize by addition of 15µl of 0.1N HCL, and bring the volume to 500µl with TE (10mM Tris, 1mM EDTA).

8. Add 20 µg of Cot1 human DNA (Gibco-BRL).

9. Purify probe by centrifuging in a Centricon-30 micro-concentrator (Amicon). Look for nice concentration of the "colored probe" in the centricon.

10. Combine the separate concentrated probes (Cy3 and Cy5) into a fresh centricon, bring to a volume of 500µl with TE, and concentrate again to a volume of less than 7µl.

11. Add 1µL of 10µg/ul polyA RNA (Sigma, #P9403) and 1 µl of 10µg/µl tRNA (Gibco-BRL, #15401-011).

12. Adjust volume to 9.5µl with distilled water.

13. For final probe preparation add 2.1 µl 20XSSC (1.5M NaCl, 150mM NaCitrate (pH8.0)) and 0.35 µl 10%SDS. Final probe volume can be adjusted to between 12 µl and 15 µl according to preference (the volume will also need to be increased for larger, 22mm x 40 mm coverslips). Be sure to adjust salt concentration proportionately.

14. Denature probe by heating for 2 min at 100°C, and incubate at 37°C for 20-30 min.

15. **Place on the array under a glass cover slip.**

16. **Hybridize at 65°C for 14 to 18 hours in a custom slide chamber with humidity maintained by a small reservoir of 3XSSC.**

17. **Wash arrays by submersion and agitation for 2-5 min in 2X SSC with 0.1%SDS, followed by 1X SSC, and 0.1X SSC.**

18. **"Spin dry" by centrifugation for 2 min in a slide rack in a Beckman GS-6 tabletop centrifuge in Microplus carriers at 650 RPM for 2 min.**