Complex cDNA Probes

Leslie Vosshall/Axel Lab/11-1999

1. Make polyA+ mRNA from the tissue of your choice. I use Stratagene mRNA purification kit (Cat. #200347). Quantify mRNA yield by spectrophotometry or, if quantities are expected to be small, DNA Dipstick (Invitrogen). Store RNA at -80 degrees C in either RNase Free dH2O supplemented with RNasin or as an ethanol precipitate.

2. Reverse transcribe mRNA (0.1 ug to 5 ug). I use Promega's Kit (Reverse Transcription System, Cat. #A3500) and scale their suggested reaction up to 100ul, independent of amount of RNA supplied in the reaction. Also good is Stratagene's RT-PCR kit (Cat. #200420).

These are suggested volumes, check your kit for specifics:
20ul 25mM MgCl2
10ul Reverse Transcription Buffer
10ul 10mM dNTP mixture
2.5ul rRNasin
3.25ul AMV Reverse Transcriptase
2.5ul Oligo-dT primer
0.1-5ul mRNA in RNase free dH2O

Adjust final volume to 100ul with RNase free dH2O and incubate 1 to 2 hours at 42 degrees C.

3. Destroy mRNA

To reaction add:
10ul 0.5M EDTA
10ul 10% SDS
15ul 3N NaOH
Mix well after each addition and incubate 30 minutes at 68 degrees C.

Remove from high temperature incubation, allow to cool for one minute at room temperature, then add:
50ul 1M Tris-HCl pH 7.5
15ul 2N HCl
Mix well.

Extract with phenol:chloroform (250ul each).

Purify aqueous phase by G50 Sephadex chromatography (Pharmacia ProbeQuant G50 columns). You may need to split up sample and use several columns, depending on the capacity of the column you use (Pharmacia's allow a maximum sample volume of 50ul).

Ethanol precipitate the purified cDNA and store overnight at -80 degrees. Spin out cDNA, wash pellet with 70% ethanol, resuspend in sterile, nuclease free TE.

4. Quantify cDNA yield by spectrophotometry or DNA Dipstick. Store cDNA in small aliquots at -80 degrees C. This stock of cDNA will make high quality complex probes for 12-24 months from the date of synthesis if stored at –80 degrees C.

5. Random Prime label 25 ng first strand cDNA for each probe. I use PrimeIt II kits from Stratagene:

  25ng cDNA
  10 ul primers
  dH20 to 34 ul

Boil in water bath 1 minute.
Quick chill on ice.
Add:

  10ul 5X dCTP* Buffer
  5 ul 32P-dCTP (we use Amersham, Redivue Cat. # AA0005)
  1 ul Klenow.

Incubate at 37 degrees 30'-60'

Purify probe by G50 Sephadex chromatography (Pharmacia ProbeQuant G50 columns). Boil 10 minutes and chill on ice before adding to hybridization mix.

6. Count 1 ul of purified probe in scintillation fluid and estimate total yield. Set up hybridization according to these parameters:
3 to 5 million cpm/ml hybridization solution. Use 2-2.5 ml hybridization solution per 137mm filter. I have done as many as 20 filters per round tupperware dish with no problem. We hybridize in Buck Buffer (0.5M Sodium Phosphate pH7.3, 1%BSA, 4%SDS) plus 500 ug/ml denatured salmon sperm DNA

7. Hybridize complex probes to target for 36 hours at 65 degrees. Wash 3X30 minutes at 65 degrees C, 0.5XSSC/0.5%SDS. Expose to film: 3-5 days for phage lifts, 8-24 hours for reverse northerns.