

COMPETITIVE/QUANTITATIVE PCR (TOM)

REVERSE TRANSCRIPTION

Modified procedure from BRL Superscript and Gilliland, G., et al.

Competitive PCR for quantitation of mRNA. In *PCR Protocols. A Guide to methods and applications* (ed. M.A. Innes, D.H. Gelfand, J.J. Sninsky, and T.J. White) pp. 60-69.

DNAase Treatment

This step may not be necessary if your primers span an intron but you will probably amplify DNA sequences since the LiCl precipitation used during RNA extraction does bring down some DNA. I usually DNAase treat half of my RNA sample (in case something goes wrong) using Boehringer RNAase-free DNAase in the presence of RNAase inhibitor.

1. Resuspend RNA in 100 μ l DEPC dH₂O. Run 5 μ l on test gel to check the integrity of RNA. (Just run a regular 0.8% agarose gel but make sure the gel box is clean and the buffer is fresh otherwise your RNA will go away).
2. Set up DNAase digestion:

50 μ l RNA
10 μ l Tab II salts
0.5 μ l RNA inhibitor
1.0 μ l DNAase I
39 μ l DEPC dH₂O

Incubate at 37°C for 30' to 1 h and extract with an equal volume phenol:chloroform. Add 1/10 volume 3M NaOAc pH 4.5 and precipitate with 2 x volume 95% EtOH. Freeze at -70°C, spin 10', wash in 70% EtOH and dry pellet. Resuspend in 20 to 80 μ l DEPC dH₂O depending on amount of RNA. I shoot for a final concentration of 1-2 μ gml⁻¹ total RNA to use in RT. Run 5 μ l on a test gel and watch the DNA disappear.

Reverse Transcription

It is a good idea to run a no RNA sample as a negative control to measure incorporation. Work at the hot bench.

1. Set up RT reaction as follows:

- a. Make a cocktail of DEPC treated water and oligo dT and dispense to tubes. Add 2 μ l RNA and heat at 70°C for 10' and then place on ice.
- b. Make up a reaction mix containing RT buffer, DTT, dNTP's, RNA inhibitor, ^{32}P dATP and reverse transcriptase.
- c. Dispense reaction mix into tubes containing RNA and incubate at 42°C for 2 h.

2 μ l	total RNA (1 to 4 μ g)
1 μ l	BRL Superscript
4 μ l	5 x RT buffer (Superscript Kit)
2 μ l	DTT (Superscript Kit)
0.2 μ l	oligo dT12-15 (0.5 μ g μ l $^{-1}$)
1 μ l	10 mM dATP
1 μ l	10 mM dTTP
1 μ l	10 mM dCTP
1 μ l	10 mM dGTP
1 μ l	$\alpha^{32}\text{P}$ dATP (1:10 dil = 1 μ Ci μ l $^{-1}$)
6 μ l	dH ₂ O
0.1 μ l	RNA Guard (Promega)

2. Terminate reaction with 4 μ l 0.5 M EDTA.

QUANTITATING TRANSCRIPT LEVELS

1. Add 76 μ l DEPC dH₂O to tubes and run 95 μ l through Sepharose CL6B column to get rid of unincorporated nucleotides. Keep 5 μ l to measure efficiency of cDNA synthesis.
2. Spot 10 μ l onto a whatman GFC filter and allow to dry completely (about 10 min.). Wash filters 3 x 10' in 5% ice cold TCA and rinse with 80% EtOH. Wash in a large volume i.e. 10 ml.

3. Allow the filters to dry and place in scintillation vials with enough scintillant to cover the filters. With a scintillation counter calculate cpm per μ l.

COMPETITIVE PCR REACTIONS

keep everything on ice

may need to play with $MgCl_2$ concentrations

1. Make 10 fold dilutions of competitor plasmids. To look at *Ac* levels, I used 3 plasmid concentrations of 0.1, 0.01 and 0.001 μ g μ l $^{-1}$ to find the range where the cDNA could effectively compete with the competitor.
2. Determine the cpm per reaction if between 2 and 4 μ l of cDNA is to be used e.g. usually adding 1000 cpm per reaction requires 2 to 4 μ l of hot cDNA.
3. Make up reaction mix containing water, buffer, $MgCl_2$, dNTP's and Taq. Dispense 138 μ l (four reactions per cDNA) to eppendorf tubes and add amount of cDNA required for three reactions. Divide the total volume by three and dispense into microfuge tubes,

2-4 μ l	hot cDNA
33 μ l	dH ₂ O
5 μ l	10 x buffer (Promega)
4 μ l	25 mM $MgCl_2$ (Promega)
1 μ l	10 mM dATP
1 μ l	10 mM dTTP
1 μ l	10 mM dCTP
1 μ l	10 mM dGTP
0.2 μ l	5 μ g μ l $^{-1}$ Taq (Promega)

4. Add 1 μ l competitor to each tube on ice (Whenever possible add competitor to reaction mixes to avoid pipetting errors).
5. Add 1 μ l 25 μ M primer 1 to each tube

6. Add 1 μ l 25 μ M primer 2 to each tube

7. Run PCR reaction:

95°C 2' 30"

95°C 45"

55°C 1'

72°C 1'

4°C

8. Run out products on 1.4 % agarose gel (5 μ l).

Once the range has been determined, make dilutions within a ten-fold range to accurately quantitate the amount of cDNA e.g. if mostly competitor DNA is present in the 0.5 and mostly cDNA in the 0.05 reaction, a dilution series of 0.5, 0.4, 0.3,0.05 $\text{ng}\mu\text{l}^{-1}$ of competitor should then be used in another set of PCR reactions to get an accurate cDNA value. The products can then be run out on an agarose gel and photographed. I usually use 8 different competitor concentrations and take the four closest to the mid point (i.e. where competitor and cDNA concentrations are closest to each other) to calculate cDNA values. The negative can then be scanned with a densitometer to calculate relative cDNA amounts. Plot % cDNA versus ng competitor to obtain ng of competitor that results in 50% cDNA. See M. Clementi, S. Menzo, P. Bagnarelli, A. Manzin, A. Valenza and P.E. Varaldo. 1993. Quantitative PCR and RT-PCR in Virology. *PCR Methods Applic.* 2: 191-196. for a better description of calculating cDNA values.