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COMPETITIVE PCR FOR QUANTITATION OF mRNA

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PCR has proved useful in amplifying specific mRNAs, especially those present in low copy number. For example, it has been possible to amplify, subclone, and characterize low-abundance mRNA (Frohman *et al.* 1988) and to detect unique mRNA transcripts from abnormal cells in a background of normal cells (Chelly *et al.* 1988; Kawasaki *et al.* 1988; Lee *et al.* 1987).

Although it has been possible to detect and amplify large amounts of rare mRNA transcripts, it has been more difficult to quantitate the amount of mRNA present in the starting material. This has precluded, for example, the use of PCR in the analysis of induction of mRNA in response to exogenous stimuli. The main constraint in obtaining quantitative data is inherent in the amplification process. Because amplification is (at least initially) an exponential process, small differences in any of the variables that control the reaction rate will dramatically affect the yield of PCR product. Variables that influence the rate of the PCR include the concentrations of polymerase, dNTPs, Mg, DNA, and primers; annealing, extension, and denaturing temperatures; cycle length and cycle number; ramping times; rate of "primer–dimer" formation; and presence of contami-

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nating DNA (see Chapter 1). Even when these parameters are controlled precisely, there is sometimes a tube-to-tube variation that precludes accurate quantitation. For example, significant differences in yield occur in PCR samples that are prepared as a pool, aliquoted into separate tubes, and amplified in the same run. The basis for this variation is not certain; it may be related to events that occur during the first few cycles or small temperature variances across the thermal cycler block.

We describe here a technique that obviates these problems and allows the precise quantitation of specific mRNA species. The strategy involves co-amplification of a competitive template that uses the same primers as those of the target cDNA but can be distinguished from the target cDNA after amplification.

Ideally, the competitive template is a mutant cDNA containing a new restriction site. These mutants can be prepared in most cases by a single base-pair change and are easily synthesized by using PCR for site-directed mutagenesis (Higuchi *et al.* 1988; Chapter 22). The mutant template can be distinguished from the target cDNA by restriction enzyme digestion following PCR. Alternatively, genomic plasmid DNA can be used as a competitive template provided oligonucleotide primers are in separate exons and flank a small intron (100 to 200 bp). The amplified competitive template can then be distinguished from the target cDNA by size. A disadvantage in using genomic DNA is the possibility that it may not be amplified as efficiently as target cDNA is, either because of increased size or increased duplex melting temperature.

Target cDNA is co-amplified with a dilution series of competitor DNA of known concentration. Since a change in any of the variables previously listed will affect the yield of competitive template and target cDNA equally, relative ratios of the two should be preserved with amplification. The relative amounts of target cDNA versus competitor can be measured by direct scanning of ethidium-stained gels or by incorporation of radiolabeled dNTPs. Because the starting concentration of the competitive template is known, the initial concentration of the target cDNA can be determined.

This method can be used to accurately quantitate less than 1 pg of target cDNA from 1 ng of total mRNA and can distinguish twofold differences in mRNA concentration. The technique can be applied to quantitation of mRNA from as few as 10 cells and is thus useful in screening cultured colonies of cells or flow-sorted cells for specific mRNA production under various conditions.

Protocols

Reagents

1. *Choice of Primers.* Primers should be chosen according to the general guidelines outlined elsewhere in this book. We usually use 30-mers with noncomplementary 3′ ends, 50 to 60% G + C content, and minimal overlap with known sequences by GenBank analysis. Primers are chosen to flank an intron so that the amplified product is readily distinguished from contaminating genomic DNA that may be present. We generally choose primers that will give fragments between 200 and 600 bp.
2. *Preparation of Competitive Templates.* We prefer to use mutant competitive templates that are identical to the target cDNA sequence except that they either contain a single new restriction site or lack an existing restriction site. We have found the PCR-based method of Higuchi *et al.* (1988) (Chapter 22) to be a rapid and accurate means of generating site-specific mutants. Mutant template should be characterized by digestion with appropriate restriction enzymes and by accurate spectrophotometric determination of concentration. A precise dilution series should be prepared, ranging from 10 ng to 1 fg of template, in relatively large volumes (e.g. 1 ml) so that the same dilution series can be used for multiple concentration determinations.
3. dNTP stock, 2.5 m*M* of each nucleoside triphosphate. Concentrations of each nucleotide should be determined spectrophotometrically before mixing.
4. 10× PCR buffer [500 m*M* KCl, 100 m*M* Tris–HCl (pH 8.3), 15 m*M* $MgCl_2$, 0.1% w/v gelatin]
5. *Taq* polymerase, 5 units/μl
6. AMV or MuLV reverse transcriptase
7. RNase inhibitor
8. Dithiothreitol, 20 m*M* stock

Reverse Transcription

Several methods may be used to obtain mRNA suitable for reverse transcription. In general, highly purified mRNA gives the best re-

sults for quantitation (e.g., guanidinium isothiocyanate lysis followed by centrifugation over $CsCl_2$, with at least two precipitation steps). It is not necessary to use poly(A)-purified mRNA. However, in many circumstances it is desirable to use mRNA from very few cells, which effectively precludes standard techniques for mRNA purification. Adequate amounts of mRNA can be obtained from small numbers of cells by lysing in 0.5% NP-40, 10 m*M* Tris (pH 8.0), 10 m*M* NaCl, 3 m*M* $MgCl_2$ for 5 minutes on ice, microfuging for 2 minutes to remove nuclei and cell debris, and performing reverse transcription on the supernatant.

Reverse transcription may be primed by using specific antisense primer for the gene of interest, oligo(dT), or random hexanucleotide primers. Using oligo(dT) permits amplification of more than one gene from the same reverse transcriptase (RT) mixture, but the target sequence needs to be relatively close to the poly(A) tail. Random hexanucleotide primers will prime all species of RNA present and allow the amplification of any desired target sequence from the RT mix.

1. Prepare RT mixture containing
   mRNA to be amplified (10 to 100 ng of total RNA, or supernatant from 10 or more cells)
   Primer (20 to 50 pmol of specific antisense primer or oligo(dT), 100 pmol of hexanucleotide primer)
   dNTP (500 μM in each nucleotide)
   1× PCR buffer
   Reverse transcriptase (use number of units recommended by manufacturer)
   Dithiothreitol, 1 m*M* final concentration
   RNase inhibitor, 2 to 5 units
   Diethylpyrocarbonate-treated water to a final volume of 20 μl
2. Incubate at 37°C for 1 hour.

   NOTE: When multiple samples are to be analyzed for content of a given mRNA, a master mix may be prepared containing all the components except the mRNA source.

Polymerase Chain Reaction

The accuracy of this method is improved through the use of master mixes. We usually titrate against a broad range of dilutions in log in-

crements for the first quantitation to obtain a rough estimate of the amount of cDNA present. We then perform a second titration over a narrower (100-fold) range for precise quantitation. In most cases, only the second series of titrations is necessary once the range is known for a given set of experiments. To quantitate the amount of PCR product, we usually add [α-^{32}P]dCTP to a final concentration of 50 μCi/ml. It is important not to adjust the concentration of individual dNTPs in the dNTP stock (e.g., lowering the dCTP concentration to increase specific activity), as this will interfere substantially with the amplification. Alternatively, a densitometer may be used to scan ethidium-stained gels to quantitate the amount of each PCR product present.

Prepare a master mix containing oligonucleotide primers (10 to 20 pmol each), dNTPs (200 μM in each as final concentration), 10× PCR buffer, *Taq* polymerase (0.5 units), and an appropriate amount of cDNA. (We generally add 10 μl of the RT mixture as the source of cDNA.) Add 90 μl of this mixture to 10 μl of previously prepared competitive template of known concentration in a dilution series. An example of a reaction mixture that we typically use follows. Because this technique is not dependent on variables previously noted, any conditions (annealing temperature, cycle length, cycle number, etc.) that give good amplification of template DNA can be used for quantitation.

After amplification, an aliquot of each sample is digested with an appropriate restriction enzyme. Triplicate cut and uncut samples are run in parallel on a NuSieve/agarose gel. When amplification is performed using a genomic intron-containing competing template, as in the example that follows, samples may be run directly on gels and cut out for counting without restriction digestion.

Example

The quantitation of granulocyte macrophage colony stimulating factor (GM-CSF) cDNA (cGM) using a genomic GM-CSF (gGM) competitive template is demonstrated in Figs. 1–3. Primers have been chosen from exon 1 and exon 2 that give a 197-bp fragment when cDNA is amplified and a 290-bp fragment when genomic DNA is amplified (Fig. 1).

Tube	gGM (competitive template)		
1.	10.0 ng/μl	10 μl	
2.	1.0 ng/μl	"	
3.	0.8 ng/μl	"	
4.	0.6 ng/μl	"	
5.	0.4 ng/μl	"	
6.	0.2 ng/μl	"	
7.	0.1 ng/μl	"	
8.	0.08 ng/μl	"	
9.	0.06 ng/μl	"	
10.	0.04 ng/μl	"	
11.	0.02 ng/μl	"	
12.	0.01 ng/μl	"	
13.	0.001 ng/μl	"	
Master mix	GM-CSF sense primer (30-mer, 5 nmol/ml)		48 μl
	GM-CSF antisense primer		48 μl
	dNTP stock (2.5 m*M* in each)		96 μl
	cGM (known concentration of 0.6 ng/μl)		12 μl
	10× PCR buffer 120		μl
	Water 756		μl
	Taq (5.0 units/μl)		1.2 μl

Add 90 μl of master mix to each tube 1–11.

40 cycles (e.g., 94°C 1 minute / 62°C 1 minute / 72°C 2 minutes).

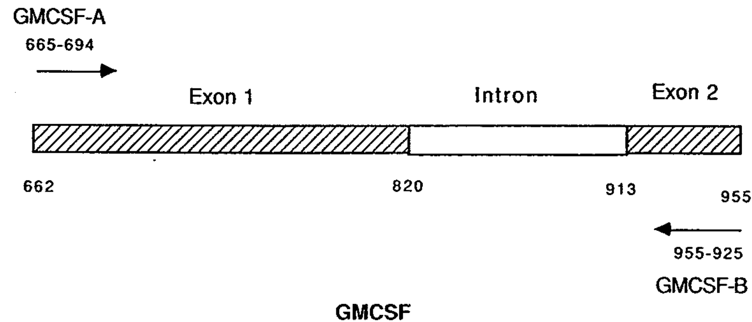

Figure 1 GM-CSF map with position of oligonucleotide primers.

When using mutant templates containing altered restriction sites, heat to 94°C for 4 minutes after completion of thermal cycling. (As discussed as follows, this will normalize for heteroduplex formation between mutant and wild-type strands.)

Run 20 μl/well in triplicate on a 2.5% NuSieve, 1% agarose gel containing ethidium bromide.

Analysis

Determine the amount of gGM and cGM present in each lane. The gel can be photographed and analyzed by densitometry (see Fig. 2), or, if bands contain [^{32}P]dCTP, the labeled bands can be cut out and counted. The amount of gGM is multiplied by the ratio of cGM bp per gGM bp to correct for increased label/ethidium staining per mole by the larger fragment. gGM per cGM can then be plotted as a function of the amount of known competitive gGM (see Fig. 3). The point of equivalence (i.e., where there is a 1 : 1 ratio) is where cGM equals gGM and represents the concentration of cDNA in the unknown.

When mutant template containing a new restriction site is used as a competitor, it is not necessary to correct for differences in molecular weight, since competitive and wild-type templates are identical in size. However, because wild-type and mutant fragments only differ by a single base pair, heteroduplex annealing can occur when the primer is not present in excess. Since heteroduplexes will not be cleaved by the restriction enzyme, the amount of wild-type template is overestimated. We have found this to be a minimal problem with runs of less than 40 cycles.

Figure 2 GM-CSF gDNA versus cDNA. Lanes 1–13 contain various amounts of genomic GM-CSF (0.01 ng to 100 ng) competed against a fixed concentration of GM-CSF cDNA (0.6 ng) corresponding to tubes 1–13 in text.

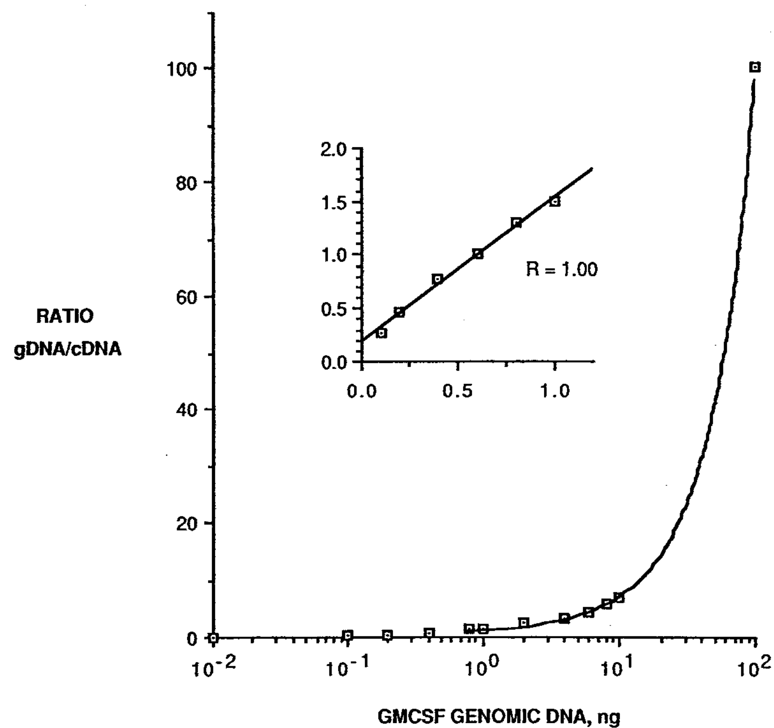

Figure 3 Plot of the ratio of genomic GM-CSF/cDNA after amplification versus genomic DNA added to the original mixture. Inset shows an expansion of the 0.1–1.0 ng range.

Discussion

Competitive PCR provides a rapid and reliable way to quantitate the amount of cDNA in a sample prepared from as few as 10 cells. The advantages of this technique are that (1) quantitation is independent of the many variables that affect amplification and (2) it is more sensitive than Northern blotting or ribonuclease protection assays are for quantitating specific mRNAs. For example, the technique may be readily applied to screening colonies of cells for specific mRNA. Several other methods have been described for quantitating cDNA species by using PCR, usually by co-amplifying a second, unrelated

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template (Rappolee *et al.* 1988). These methods are critically dependent on several variables, including cycle number and the amount of starting mRNA of each species. Even when these variables are adequately controlled, it is unlikely that the unrelated control template will be amplified at precisely the same rate as that of the unknown template. Small differences in the rate of amplification of the two templates are magnified during PCR and may grossly over- or underestimate the amount of the unknown template present.

As would be predicted, the ratio of competitive template per unknown template plotted against competitive template is a hyperbolic relationship that approaches an asymptote when one species is present in vast excess. For this reason, the most accurate results are obtained when competitive template and unknown template are amplified at nearly equivalent concentrations. As previously noted here, we generally perform an initial titration in log increments to determine the approximate concentration of the unknown cDNA. We then perform a finer titration (as in the example cited in this chapter) to obtain the most accurate results. Accordingly, twofold differences in cDNA concentrations can be accurately determined.

Competitive PCR should be readily applied to the assay of reporter gene mRNAs such as human growth hormone. This would provide a more accurate way for determining the activity of putative regulatory sequences, since, in contrast to the standard HGH reporter assay, it measures the transcription product directly and does not rely on synthesis and/or secretion of HGH protein by a transfected cell.

Competitive PCR has several limitations. First, it quantitates the amount of cDNA present in a given sample, but, if efficiency of reverse transcription is less than 100%, the method will underestimate the actual amount of mRNA present. To obtain an internal mRNA control, we have used random hexanucleotides to prime the RT reaction and have used competitive PCR to assay the amount of cDNA of a "housekeeping" gene, β-actin. Alternatively, a known amount of mutant cRNA template prepared by using T7 polymerase can be added to the RT mixture as an internal standard.

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