Quantitative Competitive PCR* (QC-PCR) Protocol
( Human FAS Ligand, Cat. #: FAS-7011 )

INSTRUCTION MANUAL

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This product is intended for research use only and not for diagnostic purposes.

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I. Introduction

Quantitative PCR
The use of PCR to examine levels of gene transcripts, often referred to as reverse transcriptase PCR (RT-PCR), has become very popular because it is sensitive and rapid (1,2). However, quantitation of mRNA levels or changes in mRNA levels can be problematic due to the exponential nature of PCR, where small variations in amplification efficiency can lead to dramatic changes in product yields. This obscures differences in levels of the target mRNA during amplification. Several methods have been described to address this problem. They all involve the use of an internal standard to compare the efficiency of the PCR in different reactions.

One method uses an endogenous internal standard in a multiplex PCR, in which two sets of primers are used in the same PCR reaction—one set specific to the target gene cDNA and the other set specific to a gene transcript invariant in the experiment, such as a “housekeeping” gene (3, 4). However, expression of a putative “stable” housekeeping gene (GAPDH or b-actin) can be actually varied as much as that of the target gene (5, 6). It may be necessary to use several house-keeping genes for this approach.

Another method utilizes an exogenous internal standard in competitive PCR, during which one set of primers is used to amplify both the target cDNA and another DNA fragment (the internal standard)—in essence the second DNA fragment competes with the target DNA for the same primers and thus acts as an internal standard. The method was first described by Gilliland (7) and Becker-Andre (8). The internal standard can be a homologous DNA fragment that has the same primer templates as the target DNA but is designed to generate a PCR product of a different size than the target DNA. Alternatively, a non-homologous DNA fragment of a desired size may be engineered to contain primer templates (9, 10).

Perhaps the greatest advantage of using competitive PCR is that the labor-intensive step of determining the exponential phase of the amplification is eliminated (7, 8). Since it is only during the exponential phase of the amplification that the amounts of products are proportional to the amount of starting target DNA, knowing when the amplification is proceeding exponentially is otherwise imperative but often difficult to predict and can necessitate pilot experiments. In some cases, amplification may start to plateau shortly after bands are first detected on the electrophoretic gel (4). Even under optimal conditions, products from each amplification tube must be assayed after several different numbers of cycles—a laborious process.

PCR COMPETITORS
Human Fas Ligand PCR Competitor is a DNA fragment derived from the Human Fas Ligand gene with a 44bp deletion. The PCR Competitors must be used in conjunction with Maxim’s corresponding RT-PCR primer sets. A 250 bp PCR fragment can be generated from amplifying target FasL gene and a 206bp PCR fragment can be generated from amplifying Human Fas Ligand PCR COMPETITOR when using Maxim’s PCR primer FAS-1005/1006.

Competitive PCR using PCR Competitors is outlined in Figure 1. Typically, serial dilutions of PCR Competitors are added to PCR amplification reactions containing constant amounts of the experimental cDNA sample. The PCR Competitor and target templates thus compete for the same primers in the same reaction. By knowing the amount of PCR Competitor added to the reactions, one can determine the amount of target template, thus the initial mRNA levels.

Note for customers who wish to precisely determine the relative changes in mRNA by number of target mRNA molecules (not just “fold” changes in mRNA levels): Determining the efficiency of reverse transcription of the target RNA population may be desirable. For this, the PCR COMPETITOR protocol can be modified to generate RNA COMPETITORS by incorporating an RNA polymerase promoter and poly-A tail into the secondary PCR product. In this case, the composite primers should contain a promoter sequence on one primer and a poly-T tail on the other. In vitro transcription of the PCR product will generate synthetic RNAs that contain the target primer sequences and a poly-A tail. RNA samples can then be titrated with RNA COMPETITOR during the reverse transcriptase step. Transcriptional promoters have been successfully incorporated into PCR products via primer sequences (11), and competitive RNA fragments have been generated by this method (12).
I. Introduction continued

**Prepare Target Sequences**
- Isolate RNA

**Conduct Competitive PCR**
- Make ten-fold serial dilutions of PCR Competitor
- Add portion of each PCR Competitor dilution to 1st-strand cDNA samples
- Perform PCR & electrophorese products on an agarose gel
- Analyse above data. Make proper two-fold serial dilution of PCR Competitor with selected 10-fold dilution
- Add dilution to cDNA samples, perform PCR, and electrophorese on an agarose gel

*Figure 1. Outline for using a PCR COMPETITOR in competitive PCR to quantify mRNA levels.* The target sequence is prepared by reverse transcription of RNA. To quantitate your target sequence, decreasing amounts of PCR COMPETITORs are added to PCR reactions containing a constant amount of target cDNA. Following PCR, the products derived from the COMPETITOR and target are resolved and amounts compared on an agarose gel.
II. List of Components

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Kit Component</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASL-QOB0</td>
<td>10X QPCR Buffers</td>
<td>500 ul</td>
<td>-20°C</td>
</tr>
<tr>
<td>FAS-4011C</td>
<td>FasL PCR Competitor</td>
<td>25 ul (100 attomoles/ul</td>
<td>-20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or 6x 10^7 molecules/ul</td>
<td></td>
</tr>
<tr>
<td>FAS-4011</td>
<td>FasL PCR Control</td>
<td>25 ul (100 attomoles/ul</td>
<td>-20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or 6x 10^7 molecules/ul</td>
<td></td>
</tr>
<tr>
<td>FAS-1005/6</td>
<td>10X FasL PCR Primers</td>
<td>500 ul</td>
<td>-20°C</td>
</tr>
<tr>
<td>NTP-0001</td>
<td>12.5X dNTPs</td>
<td>400 ul</td>
<td>-20°C</td>
</tr>
<tr>
<td></td>
<td>ddH2O (DNase free)</td>
<td>1.2 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td></td>
<td>Dilution solution</td>
<td>2x 1 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td></td>
<td>(10 ug/ml of tRNA in H2O)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

III. Additional Materials Required

- AmpliTaq DNA Polymerase
- Sterile H2O
- PCR Thermocycler

**PCR Master mix**

Note: We recommend using the following PCR Master mix in competitive PCR to help ensure tube-to-tube consistency. However, you may need to design your own mix depending on the enzyme, primers, and magnesium cation concentration used. Prepare enough PCR Master mix for each experiment plus an extra sample. (e.g., if 10 tubes, make mix for 11)

<table>
<thead>
<tr>
<th></th>
<th>Per 50 ul rxn</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X QPCR Buffer</td>
<td>5 ul</td>
<td>1X</td>
</tr>
<tr>
<td>Sterile H2O</td>
<td>31.6 ul</td>
<td>N/A</td>
</tr>
<tr>
<td>12.5X dNTP mix</td>
<td>4 ul</td>
<td>1X</td>
</tr>
<tr>
<td>10X PCR Primers</td>
<td>5 ul</td>
<td>1X</td>
</tr>
<tr>
<td>AmpliTaq DNA</td>
<td>0.4 ul</td>
<td>2.0 units/rxn</td>
</tr>
<tr>
<td>Total volume</td>
<td>46 ul</td>
<td></td>
</tr>
</tbody>
</table>

Add 2 ul PCR COMPETITOR

2 ul PCR POSITIVE or SAMPLES

Just before starting PCR.
IV. General Considerations

PLEASE READ THROUGH THE ENTIRE PROCEDURE BEFORE STARTING.

1. All dilutions of the COMPETITOR should be made in TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA) containing 1 ug/ml tRNA, nucleic acid grade. The COMPETITOR stock solution and dilutions should be stored at –20°C in a constant temperature freezer. They are stable for at least one year.

2. When possible, prepare master mixes for the PCR reactions which are common to all tubes such as the 10X buffer, nucleotide mix, primers, and enzyme. Add the cDNA and PCR COMPETITOR last. Then immediately start the thermocycling.

3. The molar quantities of PCR COMPETITOR you will be using are extremely small, and it is convenient to use the term attomole (amol), which is equal to $1 \times 10^{-18}$ moles.

4. Although the COMPETITOR dilutions are stable at –20°C, avoid multiple freeze-thaw cycles. After the third use, discard and start with a fresh dilution series.

5. Because of the small volumes used in PCR experiments, careful pipeting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer.

6. When adding solution to a tube, immerse the tip into the solution, deliver the solution, and rinse the pipet tip by pipeting up and down several times.

7. Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can result in undesirable nonspecific amplification, producing DNA bands even in the absence of template DNA. We recommend using a dedicated lab bench area equipped with dedicated pipettors, tips, and solutions to set up PCR reaction mixtures. If possible, perform post-PCR analysis in a separate laboratory area with separate sets of pipettors.

8. It is important to use tubes of even thickness for uniform heat conductance during the reaction.

9. The cycling parameters for PCR COMPETITOR construction have been optimized using a Perkin-Elmer DNA Thermal Cycler 2400. The optimal parameters may vary with different polymerase mixes, and thermal cyclers.

10. Number of Amplification Cycles. It is not necessary to assay PCR products exclusively during the exponential phase of the amplification for competitive PCR. However, too few or too many cycles may make analysis of product yields more difficult. Too few cycles may lead to products which are hard to visualize. Too many cycles may cause the agarose gel to be overloaded, obscuring the separation of the PCR COMPETITOR and target.

11. Use some form of hot start in PCR to avoid an unacceptable level of nonspecific amplification (18).

12. The PCR COMPETITOR has been constructed to provide an amplification efficiency similar to the target gene when using Maxim’s RT-PCR Primers.
V. Target Preparation

A. RNA Isolation

The use of high-quality RNA is critical for the success of RT-PCR analysis. The RNA must not be degraded by ribonucleases, as determined by the intactness of ribosomal (rRNA) bands. Contaminating genomic DNA must also be removed. The most common and consistently successful methods for isolating pure, intact total RNA are modifications of the original guanidinium thiocyanate method of Chirgwin, et al. (13). The molecular cloning manual by Sambrook, et al. (14) also contains useful information on how to isolate and handle RNA properly. Additionally, several companies offer kits for RNA isolation, including Maxim’s Total RNA Isolation Kit (EXT-0003).

When isolating RNA from small amounts of tissue or cells, a carrier nucleic acid such as polyinosinic acid (15) should be added at the beginning of the extraction to facilitate handling of the RNA and to improve yields. To ensure optimal RT-PCR, all RNA preparations should be examined by denaturing agarose gel electrophoresis. If the RNA is intact, it will exhibit sharp 28S and 18S rRNA bands, with the 28S band about twice as intense as the 18S band. Isolated RNA can be stored conveniently as an ethanol precipitate at –20°C or in aqueous solution at –70°C for up to one year without appreciable deterioration. Repeated freeze and thaw cycles should be avoided.

B. cDNA Synthesis

The cDNA template for RT-PCR is synthesized from RNA by reverse transcription. We have successfully used both avian myoblastosis virus (AMV) and Moloney murine leukemia virus (MMLV) reverse transcriptases. A discussion of cDNA synthesis is provided in the manual by Sambrook, et al. (14). Maxim offers the 1st-strand cDNA synthesis kit (RTK-0010 & RTK-0050), specifically designed for RT-PCR. A brief protocol is as follow:

1. **On ice**, pipet 1-2 µg mRNA or 10 ug total RNA (from 10^6 cells) dissolved in pure water or 2 µl control GAPDH RNA into a RNAse free reaction vial. We strongly recommend including a positive control reaction when setting up an RT-PCR reaction for the first time.
2. Add sterile water to a final volume of 14.5 µl.
3. Add 4 µl random hexamer (50 µM) or Oligo(dT) (50 µM).
   **NOTE**: The hexamer and Oligo(dT) RT reactions may be run simultaneously.
4. Incubate tube(s) at 70°C for 5 minutes and quickly chill on ice.
5. Begin your RT reaction by adding the following reagents to your hexamer or Oligo mixture:

<table>
<thead>
<tr>
<th>Reagent (add in order)</th>
<th>Description</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase Inhibitor</td>
<td>130 U/µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>5 X RT buffer</td>
<td>250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15mM MgCl₂, 50mM DTT</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1mM each</td>
<td>20 µl</td>
</tr>
<tr>
<td>MMLV RT</td>
<td>250 U/µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

7. Incubate the RT mixture at 42°C or 37°C for 60 minutes.
8. Then, heat RT mixture at 95°C for 10 minutes and quickly chill on ice.
   **NOTE**: 1-2ul of this RT mixture may be used in PCR after this step.
9. Perform one phenol (pH 8.0) extraction once & phenol/chloroform extraction once.
10. Add 10% of the total volume of 4M potassium acetate (pH 7.0) and an equal volume of ethanol. Mix well.
11. Chill for 1 hour at -70°C or overnight at -20°C.
12. Pellet DNA by spinning at maximum speed for 10 minutes and resuspend pellet in 100 µl water or TE buffer. 1-2 ul cDNA may be used in QC-PCR. However, more or less DNA may be needed in PCR depending on the copy number of the specific gene.
VI. Competitive PCR Protocol

A. Preliminary Competitive PCR Amplification
You will first titrate a constant amount of your experimental target DNA (or a positive control target cDNA provided with Maxim’s kit) against serial dilutions (ten-fold) of your PCR COMPETITOR. Based on the results, you will set up a fine-tuned COMPETITOR serial dilution (two-fold) for the quantitative PCR.

1. Label eight 0.5-ml microcentrifuge tubes C₁-C₈. Add 9 ul of COMPETITOR dilution solution to each tube.

2. Prepare the following ten-fold serial dilution stock solutions:

<table>
<thead>
<tr>
<th>Concentration (attomole/ul)</th>
<th>Tube label</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>C₀</td>
</tr>
<tr>
<td>10</td>
<td>C₁</td>
</tr>
<tr>
<td>1</td>
<td>C₂</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>C₃</td>
</tr>
<tr>
<td>10⁻²</td>
<td>C₄</td>
</tr>
<tr>
<td>10⁻³</td>
<td>C₅</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>C₆</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>C₇</td>
</tr>
</tbody>
</table>

Note: For very abundant gene transcripts, it may be necessary to use dilution C₁. For very rare gene transcripts, it may be necessary to use dilution C₈.

The dilution series can be stored at –20°C.

3. Set up six new tubes for PCR.

4. Add to a tube for each dilution:
   - 2 ul cDNA from reverse transcription reaction
   - 2 ul one dilution (C₂ through C₇)
   - 46 ul PCR Master mix (See Section III)

   50 ul final reaction volume

   Note: For very abundant gene transcripts, it may be necessary to use dilution C₁. For very rare gene transcripts, it may be necessary to use dilution C₈.

5. Begin PCR using standard cycling parameter for the primers in use. An example of a time-temperature profile for Human FasL Primers’ PCR optimized for Perkin Elmer machine types 480 and 9600 is provided below:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>1'</td>
<td>1X</td>
</tr>
<tr>
<td>94°C</td>
<td>1'</td>
<td>30X</td>
</tr>
<tr>
<td>55°C</td>
<td>1.5'</td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>10'</td>
<td>1X</td>
</tr>
<tr>
<td>20°C</td>
<td>Soak</td>
<td></td>
</tr>
</tbody>
</table>

6. Electrophorese 10 ul per sample on a 2 % agarose gel.
B. Fine-Tuned Competitive PCR Amplification

1. Determine which ten-fold COMPETITOR dilution produces PCR COMPETITOR and target cDNA template bands of equal intensity. Then use the COMPETITOR dilution tube (from Section VI.A) ten-fold less dilute to start making your two-fold serial dilutions.
For example, if you determine that the C₃ dilution gives PCR COMPETITOR to target bands of equal intensity, begin the two-fold serial dilution series with C₂.
2. Label six 0.5-ml microcentrifuge tubes 2C₁–2C₆.
3. To make the two-fold serial dilution series, place 5 ul of the selected COMPETITOR dilution solution in each tube. Then,

<table>
<thead>
<tr>
<th>Concentration (attomole/ul)</th>
<th>Tube label</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>C₂</td>
</tr>
<tr>
<td>5.0 x 10⁻¹</td>
<td>2C₁</td>
</tr>
<tr>
<td>2.5 x 10⁻¹</td>
<td>2C₂</td>
</tr>
<tr>
<td>1.25 x 10⁻¹</td>
<td>2C₃</td>
</tr>
<tr>
<td>6.25 x 10⁻²</td>
<td>2C₄</td>
</tr>
<tr>
<td>3.125 x 10⁻²</td>
<td>2C₅</td>
</tr>
<tr>
<td>1.56 x 10⁻²</td>
<td>2C₆</td>
</tr>
</tbody>
</table>

Note: If several experiments are to be performed, the volume of the two-fold dilutions can be increased.

4. Set up six new tubes for PCR.
5. Add to a tube for each dilution:
   2 ul cDNA from reverse transcription reaction (Section V.B)
   2 ul one dilution (2C₁ through 2C₆)
   46 ul PCR Master mix (See Section III)

   50 ul final reaction volume


7. Electrophorese 10 ul of each sample on a 2 % EtBr-agarose gel. There should be a 250 bp PCR product from Human Fas Ligand gene and a 206 bp PCR product from PCR COMPETITOR.

8. To quantitate the amount of target in the PCR sample, determine which two-fold serial dilution gives target and COMPETITOR bands of equal intensity and proceed to Section C.
C. Quantitation of PCR Product Yields

In our experience, visual inspection of an EtBr-stained agarose gel is sensitive and precise enough to detect changes as low as two-fold. If greater discrimination is necessary, several methods are available. The simplest procedure is to add a radioactively labeled dNTP to the PCR reaction. After gel analysis, the band may be excised and counted in a scintillation counter. Alternatively the gel may be dried and an autoradio-gram may be generated which can be scanned in a densitometer. Another method is to label the 5’ end of one or both of the primers with $^{32}$P, which is incorporated into the PCR products and then assayed for radioactivity (16).

Southern blot hybridization with synthetic DNA probes may also be performed to verify and quantitate PCR generated products, either by densitometry of an autoradiogram or by excising and counting the signal from a hybridization membrane. This method also quantitates only the target product without interference from nontarget products or primer-generated artifacts.

Nonradioactive quantitation methods include the use of biotinylated or digoxigenin-labeled primers in conjunction with the appropriate detection methods (17) or HPLC analysis. For an in-depth discussion of the various methods of PCR product quantitation, refer to the review article by Bloch (1).

In addition to the above methods, several companies now offer gel video systems which can scan and quantitate EtBr-stained gel bands in much the same way a densitometer does.
VII. Quantitative PCR Example

To examine the ability of competitive PCR to accurately measure relatively minor changes in the levels of a specific mRNA, we constructed a Human CCR5 PCR COMPETITOR for our Human CCR5 Primer Set. To imitate a defined induction in CCR5 mRNA, we synthesized cDNA from 0.5 ug of human total RNA and then performed competitive PCR. Four identical experiments, each starting with the reverse transcription, were performed to determine the reproducibility of the overall method.

To determine the appropriate amount of CCR5 COMPETITOR to use in the PCR amplification, we performed a preliminary experiment in which CCR5 from cDNA derived from 0.5 ug of total RNA was amplified in the presence of ten-fold serial dilutions of the CCR5 COMPETITOR. After data analysis, we performed a QC-PCR in the presence of two-fold serial dilutions of the CCR5 COMPETITOR. The EtBr-staining pattern obtained from one of the four independent experiments is shown in Figure 2. The sizes of the CCR5 target gene and CCR5 COMPETITOR PCR products were 246bp and 183bp, respectively.

The amount of change in CCR5 mRNA can be estimated by visually noting how much more of the COMPETITOR must be added to achieve an equimolar amount of products on a regular agarose gel. Because the molar quantity of the competitive PCR COMPETITORs is known, the actual number of target DNA molecules added to the PCR reaction can be calculated. In turn, the number of mRNA molecules can be calculated in the RNA sample used for reverse transcription if it is assumed that the efficiency of cDNA synthesis is 100%. Of course, the actual efficiency must be less than this value. Thus, such a calculation would give the minimum number of mRNA molecules. While the number of mRNA molecules can only be estimated, it is possible to determine relative changes in mRNA levels. In our experience, visual inspection of an EtBr-stained agarose gel is sensitive and precise enough to detect changes as low as two-fold.

For a more accurate determination of the number of mRNA molecules, one can generate RNA COMPETITORs using Maxim's PCR COMPETITOR to determine the efficiency of reverse transcription and factor this into the calculation. Please contact Maxim's technical service for more detail.
VIII. References