Quantitative Competitive PCR Kit for Human GAPDH Gene
Cat No. QP-10045

INSTRUCTION MANUAL

*These products are designed and sold for use in the Multiplex PCR (MPCR) covered by patent # 5,582,989. Use of the MPCR process requires a license. A limited, non-automated research field license under the patent to use only this amount of the product to practice the MPCR process is conveyed to the purchaser by the purchase of this product.

The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license. A license for diagnostic purposes may be obtained from Roche Molecular System. A license for research may be obtained by the purchase and the use of authorized reagents and DNA thermocyclers from the Perkin-Elmer Corporation or by negotiating a license with Perkin-Elmer.

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

The human GAPDH PCR Competitor is a DNA fragment derived from the Human GAPDH gene with a 50 bp deletion. The PCR Competitors must be used in conjunction with Maxim’s corresponding RT-PCR primer sets. A **500 bp** PCR fragment can be generated from amplifying target gene GAPDH and a **450 bp** PCR fragment can be generated from amplifying human GAPDH PCR COMPETITOR when using Maxim’s PCR primer GAP-1001/1004.

Competitive PCR using PCR Competitors is outlined in Figure 1 on the next page. 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).
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.
To examine the ability of competitive PCR to accurately measure relatively minor changes in the levels of a specific mRNA, we constructed a Human GAPDH PCR COMPETITOR for our Human GAPDH Primer Set. To imitate a defined induction in Human GAPDH mRNA, we synthesized cDNA from 0.5 µg 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 GAPDH COMPETITOR to use in the PCR amplification, we performed a preliminary experiment in which GAPDH from cDNA derived from 0.5 µg of total RNA was amplified in the presence of ten-fold serial dilutions of the GAPDH COMPETITOR. After data analysis, we performed a QC-PCR in the presence of two-fold serial dilutions of the GAPDH COMPETITOR. The EtBr-staining pattern obtained from one of the four independent experiments is shown in Figure 2. The sizes of the GAPDH target gene and GAPDH COMPETITOR PCR products were 500 bp and 450 bp, respectively.

The amount of change in GAPDH 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 at (650) 871-1919.

Figure 2. Quantitative analysis of Human GAPDH gene by QC-PCR.

Lane N: PCR without template (Negative)
Lane 1: PCR with 10^6 copies of GAPDH gene plus 10^8 copies of Competitor.
Lane 2: PCR with 10^6 copies of GAPDH gene plus 5x10^7 copies of Competitor.
Lane 3: PCR with 10^6 copies of GAPDH gene plus 2.5x10^7 copies of Competitor.
Lane 4: PCR with 10^6 copies of GAPDH gene plus 1.3x10^7 copies of Competitor.
Lane 5: PCR with 10^6 copies of GAPDH gene plus 6.3x10^6 copies of Competitor.
Lane 6: PCR with 10^6 copies of GAPDH gene plus 3.2x10^6 copies of Competitor.
Lane 7: PCR with 10^6 copies of GAPDH gene plus 1.6x10^5 copies of Competitor.
Lane 8: PCR with 10^6 copies of GAPDH gene plus 8x10^3 copies of Competitor.
KIT COMPONENTS

<table>
<thead>
<tr>
<th>Product Code</th>
<th>Kit Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP-QOB0</td>
<td>2X GAPDH QC-PCR Buffers</td>
<td>1250 µl X 2</td>
</tr>
<tr>
<td>GAP-4001C</td>
<td>GAPDH PCR Competitor (100 attomoles/µl or 6x 10^7 molecules/µl)</td>
<td>25 µl</td>
</tr>
<tr>
<td>GAP-4001</td>
<td>GAPDH PCR Control (100 attomoles/µl or 6x 10^7 molecules/µl)</td>
<td>25 µl</td>
</tr>
<tr>
<td>GAP-1001/1004</td>
<td>10X GAPDH PCR Primers</td>
<td>500 µl</td>
</tr>
<tr>
<td>MRB-0011P</td>
<td>ddH₂O (DNase free)</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>MRB-0014</td>
<td>DNA M.W. Marker (100bp Ladder)</td>
<td>100 µl</td>
</tr>
<tr>
<td>QCP-D001</td>
<td>Dilution solution (10X) (10 µg/ml of tRNA in H₂O)</td>
<td>1.8 ml</td>
</tr>
</tbody>
</table>

QP-10045
Store all reagents at -20°C

PCR PRODUCT QUANTITATION

I: Radioactive Quantitation

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 ³²P, 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.

II: Non-Radioactive Quantitation

Nonradioactive quantitation methods include the use of biotinylated or digoxigenin-labeled primers in conjunction with the appropriate detection methods (17), use of a bioanalyzer or WAVE. 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. Lab-on-a-chip (BioAnalyzer), CE, HPLC, and WAVE may also be used to analyze MPCR products and quantitate simultaneously.
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 µg/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 x 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.

NOTE: SPIN ALL TUBES BEFORE USING AND VORTEX ALL REAGENTS FOR AT LEAST 15 SECONDS BEFORE USING!!
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.

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 myeloblastosis 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 follows:

1. **On ice**, pipet 1-2 µg mRNA or 10 µg total RNA (from 10⁶ cells) dissolved in pure water or 2 µl control GAPDH RNA into a RNAase 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</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 (pH8.3)</td>
<td>10 µl</td>
</tr>
<tr>
<td></td>
<td>375 mM KCl, 15 mM MgCl₂, 50 mM DTT</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 mM each</td>
<td>20 µl</td>
</tr>
<tr>
<td>MMLV RT</td>
<td>250 U/µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

6. Incubate the RT mixture at 37°C for 60 minutes.
7. Then, heat RT mixture at 95°C for 10 minutes and quickly chill on ice.
8. Add another 50 µl water or 0.1X TE buffer.
9. **2-5 µl** of above cDNA is sufficient for most genes in a standard MPCR reaction. However, more or less DNA may be needed in PCR depending on the copy number of the specific gene.
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 µl of COMPETITOR dilution solution to each tube.

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

<table>
<thead>
<tr>
<th>Concentration (attomole/µl):</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>10⁻¹</td>
<td>C2</td>
</tr>
<tr>
<td>10⁻²</td>
<td>C3</td>
</tr>
<tr>
<td>10⁻³</td>
<td>C4</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>C5</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>C6</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>C7</td>
</tr>
<tr>
<td></td>
<td>C8</td>
</tr>
</tbody>
</table>

COMPETITOR stock solution provided
Add 1 µl C0, mix, and change pipet tip.
Add 1 µl C1, mix, and change pipet tip.
Add 1 µl C2, mix, and change pipet tip.
Add 1 µl C3, mix, and change pipet tip.
Add 1 µl C4, mix, and change pipet tip.
Add 1 µl C5, mix, and change pipet tip.
Add 1 µl C6, mix, and change pipet tip.
Add 1 µl C7, and mix.

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

3. Set up six new tubes for PCR. Prepare PCR Master Mixture according to table below:

<table>
<thead>
<tr>
<th>Volume (Per 50 µl rxn)</th>
<th>Reagent (Add in order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0 µl</td>
<td>2X QC-PCR Buffer Mixture</td>
</tr>
<tr>
<td>5.0 µl</td>
<td>10X PCR Primers</td>
</tr>
<tr>
<td>0.4 µl</td>
<td>AmpliTaq DNA Polymerase</td>
</tr>
<tr>
<td></td>
<td>(5 units/µl)</td>
</tr>
<tr>
<td>15.6 µl</td>
<td>Sterile H₂O</td>
</tr>
<tr>
<td>46.0 µl</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

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

50 µl final reaction volume

Note: For very abundant gene transcripts, it may be necessary to use dilution C 1. For very rare gene transcripts, it may be necessary to use dilution C 8.

5. Begin PCR using standard cycling parameter for the primers in use. An example of a time-temperature profile for Human GAPDH Primers' PCR optimized for Perkin Elmer machine types 480 or 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>3 min</td>
<td>1X</td>
</tr>
<tr>
<td>94°C</td>
<td>1 min</td>
<td>27-30X</td>
</tr>
<tr>
<td>55-58°C</td>
<td>1.5 min</td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>10 min</td>
<td>1X</td>
</tr>
<tr>
<td>25°C</td>
<td>soak</td>
<td></td>
</tr>
</tbody>
</table>

6. Electrophorese 10 µl 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 Protocol Part 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 µl of the selected COMPETITOR dilution solution in each tube. Then,

<table>
<thead>
<tr>
<th>Concentration (attomole/µl)</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. Prepare PCR Master Mixture according to table below:

<table>
<thead>
<tr>
<th>Volume (Per 50 µl rxn)</th>
<th>Reagent (Add in order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0 µl</td>
<td>2X QC-PCR Buffer mixture</td>
</tr>
<tr>
<td>5.0 µl</td>
<td>10X PCR Primers</td>
</tr>
<tr>
<td>0.4 µl</td>
<td>(5 units/µl)</td>
</tr>
<tr>
<td>15.6 µl</td>
<td>Sterile H₂O</td>
</tr>
<tr>
<td>46.0 µl</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

5. Add to a tube for each dilution:

   2.0 µl cDNA from reverse transcription reaction (Target Preparation Part B.)
   2.0 µl one dilution (2C1 through 2C6)
   46.0 µl PCR Master mix (See Above)

50.0 µl final reaction volume

6. Begin PCR amplification using cycling parameters optimized for the gene-specific primers in use. See previous page for example.

7. Electrophorese 10 µl of each sample on a 2% EtBr-agarose gel. There should be a 500 bp PCR product from Human GAPDH gene and a 450 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.
# TROUBLESHOOTING

## QC-PCR AMPLIFICATION

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible Cause</th>
<th>Recommended Action</th>
</tr>
</thead>
</table>
| **1.1. No signal or missing some bands during amplification even using positive control provided in kit.** | 1.1a. The annealing temperature in thermocycler is too high.  
1.1b. Dominant primer dimers. | 1.1a. Decrease PCR annealing temperature 3-5°C gradually.  
1.1b. Use any one of "Hot Start" PCR procedures. |
| **1.2. Too many nonspecific bands.** | 1.2a. The annealing temperature in the thermocycler is too low.  
1.2b. Pre-PCR mispriming.  
1.2c. cDNA is interfering with QC-PCR | 1.2a. Increase PCR annealing temperature 3-5°C gradually.  
1.2b. Use any one of "Hot Start" PCR procedures.  
1.2c. Clean cDNA with Phenol/Chloroform.  
1.2d. Use Maxim's 3M™-MPCR Kit. |
| **1.3. No difference in gene expression among treatments** | 1.3a. PCR amplification of this specific gene has passed the exponential phase.  
1.3b. Variation in sample preparation, RT reaction and amounts of input cDNA. | 1.3a. Decrease PCR cycle number or decrease the input cDNA.  
1.3b. Run a parallel PCR with a house-keeping gene to eliminate variables. |
**PRECAUTIONS AND STORAGE**

**Storage**

1. Store all QC-PCR Kit components at -20°C. Under these conditions components of the kit are stable for 1 year.
2. Isolate the kits from any sources of contaminating DNA, especially amplified PCR product.
3. Do not mix QC-PCR kit components that are from different lots. Each lot is optimized individually.

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**REFERENCES**