

Maxim Biotech, Inc.

DUAL QUANTITATIVE RT-PCR* KIT

GENERAL INSTRUCTION MANUAL

*These products are designed and sold for use in the Polymerase Chain Reaction (PCR) process 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 otherwise negotiating a license with Perkin-Elmer.

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

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Dual Quantitative RT-PCR Amplification

I. Introduction to Dual Quantitative RT-PCR

With the advent of RT-PCR, the characteristics of any gene, regardless of the quality or quantity of starting material, can be revealed. Prior to RT-PCR, scientists studying genes could only use RNA as the raw material. RNA is a difficult molecule to work with because of its instability and sensitivity to nuclease activity. Northern blotting, nuclease protection assays, and in situ hybridization experiments are able to measure the steady state level of individual RNAs. However, these techniques are all very time consuming, labor intensive, and most importantly, have limited sensitivity. Under the most perfect conditions, a minimum of 10,000 copies of an RNA transcript must be present to be detected by the most sensitive of all the above assays, the ribonuclease protection assay. RT-PCR overcomes all these obstacles and furthermore, the end product of RT-PCR reaction can be directly quantified, cloned, used as a probe for blotting purposes, used as a template for in vitro transcription, or sequencing.

Now that RT-PCR has become the norm for most molecular biologists, many time-saving variations of the basic RT-PCR reaction have been made. These variations are targeted to aid the scientists in his/her particular application. One such variation is called relative RT-PCR. The premise of relative RT-PCR is quite simple: start with equal quantities of RNA from different sources, use identical RT-PCR conditions on all samples, amplify the same target from each sample. In the end, the amount of PCR product from each reaction should be proportional (i.e. "relative") to the abundance of RNA transcripts in the original samples. The disadvantage of relative RT-PCR is reliability and accuracy because the results are based solely on the quality and quantity of the starting RNA samples. If quantification of the original RNA samples is in any way "off", PCR will amplify this discrepancy. If the RNA is not "pure" enough, PCR could amplify a contaminant (or, a contaminant could interfere with binding of primers to template) thus confounding the results.

Maxim Biotech developed the Dual Quantitative RT-PCR, one target specific gene plus one well defined housekeeping gene (Dual), kit to overcome this hurdle of standard relative RT-PCR. Maxim's Dual Quantitative RT-PCR is developed and based on our propriety and patented technologies multiplex PCR (MPCR). The Dual Quantitative RT-PCR is a powerful but easy method for relative quantitative analysis of gene expression. The kit contains all the materials needed to get reliable and accurate information about the relative abundance of different RNA species in different RNA samples. The general considerations of relative quantitative RT-PCR and advantages of Maxim's Dual Quantitative RT-PCR are addressed as follows.

II. General Considerations of Relative Quantitative RT-PCR

a) Sample to sample variability

Generally, there are two sources of sample-to-sample variability in relative RT-PCR experiments: differences caused by variations in the quantity or quality of the samples (e.g. partial degradation or the presence of contaminants), and random sample-to-sample variation.

Unfortunately, random variability is a fact in PCR. The best way to minimize variability is to run duplicate samples and average the data. Making cocktails of reagents can minimize random variability caused by operator error.

b) Multiplex RT-PCR with an endogenous standard

To compensate for variations in RNA quality, initial quantitation errors, and random tube-to-tube variations, "multiplex" RT-PCR can be performed. The premise of this variation of RT-PCR is to use two or more sets of primers in a single PCR reaction. One set of primers to amplify the gene of interest and another set to amplify a constant endogenous control gene. The amount of unknown product from the gene of interest is normalized against the product from the constant endogenous control gene.

c) Ribosomal RNA as an internal standard

Transcript RNA is measured as a percentage of the total RNA in a sample. As mRNA, which also contributes to the total RNA sample, can differ between different tissues, the percentage of transcript RNA can also be perceived as varying between these samples regardless of whether it actually changes or remains constant. Dual quantitative PCR requires the use of an endogenous standard that will remain constant regardless of the type of cell, cell cycling, or administered treatment. Actin is one of the most commonly used standards for this purpose. However, results from Northern analysis reveal that actin levels vary with different tissue samples.

rRNA would also be a logical choice as an endogenous control as it remains fairly constant between tissue samples. The drawback to using rRNA lies in its natural abundance in a total RNA sample. One of the requirements for an ideal control in addition to its invariability is that it must be positioned in the same linear range as the RNA to be quantified while undergoing PCR, which is difficult as different RNA samples are unique by nature and will therefore exhibit different expression levels. As a result, no single endogenous standard can be used universally for all RNA samples.

As indicated earlier, there are several ways to determine gene expression of a gene. However a PCR based method provides a gold standard now, including real time RT-PCR, quantitative comparative RT-PCR and dual quantitative RT-PCR. The following is a summary of the various PCR based methods:

	<u>Real Time RT-PCR</u>	<u>QCRT-PCR</u>	<u>DQRT-PCR</u>
Economical	Expensive	Moderate	Yes
Ease of Use	Yes	Moderate	Excellent
Reliability	Yes	Yes	Yes
Sensitivity	Excellent	Excellent	Excellent
Specificity	Excellent	Very Good	Very Good
Hybridization	Probe	No	No

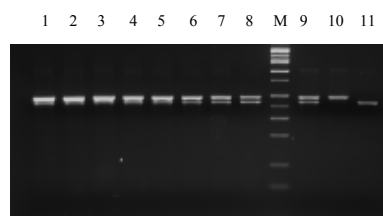


Fig 1. Competitor vs. Dual Quantitative RT-PCR. Competitor RT-PCR amplification with 1: 2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9 ration of competitor. Lane 1-8: amplicons of 1:2 to 1:9 competitor
M: 100 bp DNA M.W. Marker
9: Maxim's Dual Quantitative RT-PCR
10: Amplicon of single housekeeping gene
11: Amplicon of single target specific gene

Various internal control strategies, such as the use of housekeeping genes, similar size amplicons with same primer sequences, and competitors are commonly used to determine the expression of genes of interest (Fig. 1). Each strategy has its advantages and disadvantages. Maxim has recently developed a dual quantitative PCR technique to determine the relative target gene expression level by applying proprietary and patented technologies (Fig 2). The optimized dual quantitative PCR technique is as easy as standard PCR. The high expression level of housekeeping genes will not interfere the low expression level of target genes or vice versa in our system (Fig 3). The gel picture on the left clearly indicated that in the range of 10^2 to 10^6 copy number, the internal control did not interfere with the expression of the target gene. This observation demonstrates that Maxim's proprietary and patented technologies provide major advantages for researchers who desire high throughput in an assay as simple as regular RT-PCR.

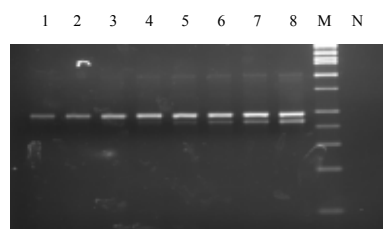


Fig 2. Amplicons of various cycle numbers. Dual RT-PCR amplification with 10^6 housekeeping gene and 10^2 target specific gene were analyzed every two cycles. Lane 1-8: amplicons of cycle 16-30
M: 100 bp DNA M.W. Marker
N: Negative control

Additionally, we have compared our unique dual quantitative RT-PCR to a system-using competitor. The competitor system uses a specific competitor to suppress the amplification of an over-expressed housekeeping gene in order to normalize the rarely expressed target gene. Users need to optimize the ratio between the competitor and the housekeeping gene primers in order to suppress the housekeeping gene sufficiently. Maxim's dual quantitative RT-PCR system does not require optimization. The user needs only to select a proper housekeeping gene whose expression level does not change in response to treatment. Maxim provides several housekeeping genes including GAPDH (G), 18S (S), T-Transferin Receptor (T), P-Phospholipase A2 (P), L32 (L) and β -Actin (B) in a [MPCR kit \(HKG-M052\)](#) format for users to identify the appropriate housekeeping gene that did not change gene expression level before and after treatments. Additionally, we provide the following Dual Housekeeping Genes Quantitative Exam kit ([Human: DP-K0001, Mouse: DP-K0002, Rat: DP-K0003](#)) for users to identify the housekeeping genes in a standard RT-PCR format instead of MPCR.

The basic technical information of human, Dual Housekeeping Gene Quantitative Exam kit ([DP-K0001](#)), including GAPDH (G), 18S (S), Transferin Receptor (T), Phospholipase A2 (P), L32 (L) and β -Actin (B) is provided as follows:

Target	Cat. No.	Amplicon Size	Ta	GenBank Accession No.
18S (S)	DP-00001	488 bp	58°C	X03205
β -Actin (B)	DP-00002	474 bp	58°C	X00351
GAPDH (G)	DP-00003	496 bp	58°C	M33197
L32 (L)	DP-00004	143 bp	58°C	X03342
Phospholipase A2 (P)	DP-00005	483 bp	58°C	M86400
Transferin Receptor (T)	DP-00006	484 bp	58°C	M11507

This Dual Quantitative RT-PCR kit provides many advantages:

Advantages:

- Cost Effective
- Quantitative
- Co-amplification with a housekeeping gene
- Normalized Sample-to-sample variation
- Minimize Contamination
- Maximize Outcome in a single tube

Kit Order Information

Housekeeping Gene List:

GAPDH (G), 18S (S), T-Transferin Receptor (T), P-Phospholipase A2(P), L32 (L) and β -Actin (B)

Please replace the housekeeping gene code, -G-S-T-P-L-B to the end (-X) of each catalog number from the target RT-PCR gene list. For example, DP-10009-G refers to the Human Bcl2 target gene and GAPDH housekeeping gene. DP-10009-S refers to the Human Bcl2 target gene and 18S housekeeping gene. **Note:** If no housekeeping gene is selected, a standard 18S housekeeping gene will be supplied in the kit.

Kit Components:

- Premixed target specific primers
- Premixed internal HKG primers
- Positive control of target and housekeeping genes
- Optimized Dual PCR buffer (including chemicals, enhancer, stabilizer and dNTPs)
- ddH₂O
- PCR DNA M.W. marker
- Manual

Components needed BUT not provided in the kit:

- Taq DNA polymerase

How does it work

Each target gene is co-amplified with a specific housekeeping gene resulting in quantitative PCR amplification in a single tube. Maxim has integrated Multiplex PCR proprietary and patented technology into this Dual Quantitative PCR amplification kit. The optimized conditions make the Dual quantitative PCR amplification as simple as a standard PCR reaction. The users first select a housekeeping gene that does not change expression level in response to treatment. Co-amplification of the desired target gene with such housekeeping gene provides a quantitative result. Changes in gene expression level may be monitored, while avoiding sample-to-sample loading variation. This dual quantitative PCR amplification kit provides a complete solution for RT-PCR gene expression and identification needs. For RT-PCR applications, it is usually not necessary to isolate the polyadenylated fraction to use as template in the reverse transcription step, even for rare messages. This is because PCR can efficiently amplify targets even when they comprise only a very small proportion of the total cDNA produced in the RT. Usually it makes little or no difference whether random hexamers, or oligo dT is used as primer in a reverse transcription reaction. However, ***in order to use the 18S as the internal control, random primers must be used in the RT reaction.***

I. Sample 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.. The molecular cloning manual by Sambrook, et al. 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 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 approximately twice as intense as the 18S band.

Isolated RNA may 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 isolation of undegraded, intact RNA is essential for successful first strand synthesis and PCR amplification. Care should be taken to avoid RNase contamination of buffers and containers used for RNA work by pretreating with DEPC, autoclaving, and baking. Always wear sterile gloves when handling all reagents. Approximately 0.5-1.0 μg of cDNA, may be obtained from total RNA extracted from 10^5 cells, will be applied to each Dual RT-PCR reaction.

1. Equilibrate 3 water baths at 42°C , 70°C , and 95°C .
2. On ice, pipet 1-2 μg mRNA or 10 μg total RNA (from 10^6 cells) dissolved in pure water and 2 μl control GAPDH RNA, from Maxim's RT kit, into a RNase free reaction vial. We strongly recommend including a positive control reaction when setting up a RT-PCR reaction for the first time.
3. Add sterile water to a final volume of 14.5 μl .
4. Add 4 μl random hexamer (50 μM) or Oligo(dT) (50 μM). Incubate at 70°C for 5 minutes and quickly chill on ice. Both the hexamer and Oligo(dT) RT reactions may be run simultaneously. (*Note: if using 18S as the internal control, random primers must be used in the RT reaction*)
5. Set up RT by adding the following reagents in order:

Reagent	Per reaction
RNase Inhibitor (130 u/ μl)	0.5 μl
5 X RT Buffer (250 mM Tris-HCl, pH8.3, 375 mM KCl, 15 mM MgCl ₂ , 50 mM DTT)	10.0 μl
dNTPs (1 mM each)	20.0 μl
MMLV RT (250 u/ μl)	1.0 μl

6. Incubate the RT mixture at 42°C or 37°C for 60 minutes. Heat the RT mixture at 95°C for 10 minutes and quickly chill on ice.
7. Add another 50.0 μl 0.1X TE buffer. 2-5 μl cDNA is sufficient for most genes in a standard RT-PCR reaction. However, more or less cDNA may be needed in PCR depending on copy number of target specific gene. It is highly recommended that this cDNA to be used should be no more than 20% of final volume of any PCR, QPCR, Dual PCR or MPCR reaction.

II. PCR Protocol:

Taq DNA polymerase from Perkin-Elmer or its derivatives are highly recommended. *AmpliTaq* Gold, however, is **NOT** recommended because its own optimal buffer system is required.

1. Master Mixture Preparation:

A. Set up Dual Quantitative RT-PCR reactions with test samples and Optimized Dual Quantitative RT-PCR buffers provided in the Dual Quantitative RT-PCR kit according to the table below:

- a. Spin all tubes briefly before doing any experiments
- b. Mix 250 μl of premixed target specific primers and 250 μl of premixed internal HKG primers to one tube of optimized dual PCR buffer, total two tubes. Label tubes as **Master Mixture**.
- c. This **“Master Mixture”** should be aliquot and stored at -20°C for one year (long term storage) and **“Ready To Use Master Mixture”** at 4°C for one-day.

Volume (Per assay)	Reagent (Add in order)
35.0 μl	Prepared Master Mixture
0.3 μl	Taq DNA Polymerase (5 units/ μl)
2.0-5.0 μl	Specimen cDNA or Control cDNA from kit
X μl	Distilled water bring the final volume to 50 μl

*: ^{32}P dNTPs may be used here to achieve higher sensitivity and better quantitation. 5-10 uCi [$a\text{-}^{32}\text{P}$]dCTP (3000 Ci/mmmole) should be used per Dual PCR.

B. Due to the minimal volume of Taq enzyme used, **“Ready-to-use Master Mixture”**, including Taq DNA polymerase and all other components except positive control or testing DNA/cDNA, should be prepared to cover daily usage. The **“Ready-to-use Master Mixture”** is stable at 4°C for one-day.

C. The Optimized Dual RT-PCR buffer has been optimized for each system. User should not exchange the buffer from different lots or different kits to avoid sub-optimized amplification. Factors like EDTA concentration in the test sample must not exceed 0.5 mM because Mg^{++} concentration in RT-PCR buffers is limited to certain ranges. Additional Mg^{++} may be added to the PCR mixture to compensate for EDTA. We strongly recommend running an RT-PCR reaction with the positive control provided in the kit. Since the RT-PCR DNA polymerase volume needed in each reaction is very small, it is recommended that all PCR components be premixed in a sufficient quantity and dispensed into individual reaction vials. This will help to achieve more accurate measurements. “Hot start” PCR is highly recommended but is not necessary in our optimized Dual Quantitative RT-PCR kits.

Example 1: For one positive and one negative control with 10 testing samples, the following steps provide a solution to prepare the reaction. (Assuming a 2.0 μl cDNA per sample will be used)

1. Prepare a **“Ready to Use Master Mixture”**, including the following as 10 testing samples plus 2 controls:

<u>Reagent</u>	<u>Per assay</u>	<u>Master Mixture (12 samples)</u>
Master Mixture (prepared as above)	35.0 μl	35.0 μl x 12= 420.0 μl
Taq DNA Polymerase	0.3 μl	0.3 μl x 12=3.6 μl
ddH ₂ O	13.0 μl	13.0 μl x 12=156.0 μl

(Note: use ddH₂O to adjust final volume to 50.0 μl after positive control or testing cDNA)

2. Mix thoroughly the above **“Ready to Use Master Mixture”**.
3. Quick spin.
4. Aliquot **48.0 μl “Ready to Use Master Mixture”** into each tube.
5. Add **2.0 μl testing cDNA or positive control** to each corresponding labeled tube.
6. Start the preset and standard Dual RT-PCR cycling program. (See PCR thermocycle profile section)
7. At the end of reaction, mix 10.0 μl of amplified PCR products with 2.0 μl , 6X DNA gel loading dye.
8. Load and run on a 1% agarose gel, 100 volts for 30-45 minutes.
9. Exam under UV transilluminator and take a picture as records.

Example 2: For one positive and one negative control with 10 testing samples, the following steps provide a solution to prepare the reaction. (Assuming a 5.0 µl cDNA per sample will be used)

1. Prepare a “Ready to Use Master Mixture”, including the following as 10 testing samples plus 2 controls:

Reagent	Per assay	Master Mixture (12 samples)
Master Mixture (prepared as above)	35.0 µl	35.0 µl x 12= 420.0 µl
Taq DNA Polymerase	0.3 µl	0.3 µl x 12=3.6 µl
ddH ₂ O	0.0 µl	0.0 µl x 12=0.0 µl

(Note: use ddH₂O to adjust final volume to 50.0 µl after positive control or testing cDNA)

2. Mix thoroughly the above “Ready to Use Master Mixture”.
3. Quick spin.
4. Aliquot **45.0 µl “Ready to Use Master Mixture”** into each tube.
5. Add **5.0 µl testing cDNA or positive control** to each corresponding labeled tube.
6. Start the preset and standard Dual RT-PCR cycling program. (See PCR thermocycle profile section)
7. At the end of reaction, mix 10.0 µl of amplified PCR products with 2.0 µl, 6X DNA gel loading dye.
8. Load and run on a 1% agarose gel, 100 volts for 30-45 minutes.
9. Exam under UV transilluminator and take a picture as records.

2. PCR thermocycle profile:

Reaction profiles require optimization according to the machine type and user need. An example of a time-temperature profile for the positive control PCR reaction optimized for Perkin Elmer machine types 2400 and 9600 is provided below:

Temperature	Time	Cycles
96°C	60 sec.	1X
94°C	60 sec.	
58°C	90 sec.	28-32X*
72°C	10 min.	1X
20°C	Soak	

28-32 X*: Use higher number of cycles for low copy genes and lower number of cycles for high copy genes. For some very rare copy number of genes, 40-45 cycles may be used and for some very high copy genes, 20-25 cycles may be used instead.

III. PCR Product Detection:

PCR products (Amplicons) can be analyzed by various technologies, agarose gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), Southern/dot hybridization, high pressure liquid chromatograph (HPLC), capillary electrophoresis (CE) and micro-fluid BioAnalyzer. Two methods, agarose gel electrophoresis and micro-fluid BioAnalyzer, are illustrated in the following as examples:

1. Agarose Gel Electrophoresis:

5 µl of the amplified samples are mixed with 1µ, 6X gel loading buffer (cat. no. MRB-0004, 6X gel loading buffer, MRB-0005, 10X gel loading buffer) and subjected to electrophoresis in a 1% agarose (cat. No. MRB-0018, 100g, MRB-0019, 500g) gel containing 0.5 µg/ml ethidium bromide (cat. No. MRB-0006) in 0.5-1.0 X TBE (cat. No. MRB-0003, 10X TBE) buffer. A 100-150 constant voltage is applied for 30-45 minutes or until the dye front reached two-third of the gel before visualized by UV transilluminator. The following is a typical example:

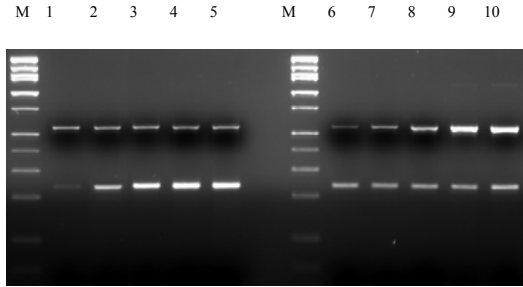
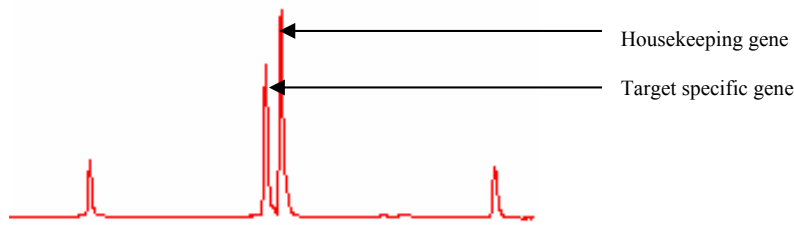


Fig 3. Housekeeping gene and target specific gene are amplified independently in a single tube.
 M: 100 bp DNA M.W. Marker
 Lane 1-5: 10^3 housekeeping gene plus $10^2 - 10^6$ target specific gene
 Lane 6-10: 10^3 target specific gene plus $10^2 - 10^6$ housekeeping gene

2. **BioAnalyzer:**

1 μ l of the amplified samples is subjected to electrophoresis in a Lab-On-A-Chip micro-fluid device. The device is prepared according to the manual of manufacturer, Agilent Technologies. The following is a typical example:



IV. Gene Expression Quantitative:

The amplicons can be quantitative after analysis by various techniques mentioned above. The gel image can be digitized and gene expression quantitative can be calculated accordingly or the chromatogram and gene expression quantitative can be calculated directly. The internal housekeeping gene is served as a normalizer for gene expression quantitative. Under predefined optimization condition, all gene expressions are in a linear range, the relative percentage of target specific gene expression can be calculated as follows:

$$\frac{X}{Y} \times 100\% = \text{Normalized Target Specific Gene Expression \%}$$

Where X = target specific gene expression level, Y = housekeeping gene expression level. Assuming the housekeeping gene selected did not change its expression level before and after treatments and loading the same initial quantity of samples, all Y value from different samples should be the same or very close number.

V. Troubleshooting Guideline:

Q: How should I determine which housekeeping gene to use?

A: The basic guideline to select a housekeeping gene as the internal control should be the one that does not change its gene expression level before and after treatments. Additionally, users should select the housekeeping gene that has similar expression level as the target specific gene. Maxim provides several housekeeping genes including GAPDH (G), 18S (S), T-Transferin Receptor (T), P-Phospholipase A2 (P), L32 (L) and β -Actin (B) in a MPCR kit (HKG-M052) format for users to identify the appropriate housekeeping gene that did not change gene expression level in a single tube format. Additionally, we provide the following Dual Housekeeping Genes Quantitative Exam kit (Human: DP-K0001, Mouse: DP-K0002, Rat: DP-K0003) for users to identify the housekeeping genes in a standard RT-PCR format instead of MPCR. We usually recommend the 18S as the internal housekeeping gene control due to its abundance compared to other genes. However, *in order to use the 18S as the internal control, random primers must be used in the RT reaction.*

Q: Is it possible that target specific gene amplification is expressed over the housekeeping gene control?

A: Yes, it is possible that target specific gene expression level is higher than selected housekeeping gene. This will not change the results.

Q: How should I calculate the relative expression level of target specific gene when the expression of housekeeping gene changed from sample to sample?

A: User should first identify what's the cause of such changes. There are several possibilities that may cause the expression level of housekeeping gene changed from sample to sample:

1. Improper selection of housekeeping gene – Some expression level of housekeeping gene changed before and after sample treatment. Users should select another housekeeping gene as the internal control.
2. Different initial concentration of RNA – Use the same concentration for all RNA samples
3. Poor reverse transcription of some samples – Use same lot of reagents
4. Inhibitors in certain samples – In such cases, the expression level of housekeeping gene should also reduce as target specific gene does. Users should first normalize their expression levels of housekeeping gene; on the samples that shown reduced gene expression, against the gene expression of samples that did not show inhibition. A multiplying factor then should be determined for the housekeeping gene and then use that multiplying factor for the target specific gene before comparing the relative gene expression of target specific gene from different samples.