# Amplification Efficiency of TaqMan® Gene Expression Assays

TaqMan® Gene Expression Assays average 100% efficiency (+/- 10%)

Key Factors That Affect Efficiency

- Efficiency estimates can vary significantly if a rigorous measurement protocol is not followed

- Measurements must be obtained over a broad dilution range (5–6 logs) and include replicates to decrease the effects of laboratory errors (i.e., pipetting accuracy)

- Efficiency of a PCR reaction can be affected by substances known to inhibit PCR, such as heme and detergents; users must ensure that cDNA templates are free of PCR inhibitors
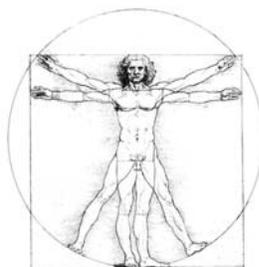
Abstract

Applied Biosystems has designed and manufactured over 680,000 real-time PCR assays for measuring the expression of human, mouse, rat, *Arabidopsis*, *Drosophila*, and *C. elegans* genes. TaqMan® Gene Expression Assays each consist of a fluorescent-labeled TaqMan® probe and amplification primers (forward and reverse) formulated into a single tube.

One of the major user concerns regarding any real-time PCR-based assay product (TaqMan® probe and primers) is the amplification efficiency of the PCR reaction. Applied Biosystems has done extensive R&D validation of our TaqMan Gene Expression Assays to address this concern.

Two types of experiments have been performed to evaluate the amplification efficiency of TaqMan Gene Expression Assays. First, we determined the amplification efficiency of a large sampling of assays using statistically relevant methods. Second, we evaluated the effect of amplification efficiency on the ability to determine a difference in the relative quantity of a transcript expressed in two different samples. Our results indicate that the amplification efficiency associated with assays designed using our informatics pipeline is 100%. Additionally, our results show that the $\Delta\Delta C_T$ method for relative quantitation is better correlated with expected fold change in gene expression than methods using pre-determined amplification efficiency values for individual assays.

Introduction

A key attribute of any real-time PCR reaction is the amplification efficiency of the PCR. Specifically, is there a doubling of PCR product at every PCR cycle[1,2]? The equation that describes the exponential amplification of PCR is:

$$X_n = X_O * (1+E_X)^n$$

Equation 1 where:

X_n = number of target molecules at cycle n

X_O = initial number of target molecules

E_X = efficiency of target amplification

n = number of cycles

For real-time amplification using TaqMan probes, X_n is proportional to the reporter fluorescence R, so that Equation 1 can be re-written as:

$$R_n = R_O * (1+E_X)^n$$

Equation 2 where:

R_n = reporter fluorescence at cycle n

R_O = initial reporter fluorescence

E_X = efficiency of target amplification

n = number of cycles

In order to use the $\Delta\Delta C_T$ method for relative quantitation as outlined in Applied Biosystems User Bulletin #2[3], the efficiency (E_X) of the target amplification and the efficiency of the endogenous control amplification must be approximately equal (refer to references 1 and 3 for derivation of the $\Delta\Delta C_T$ equations).

The results in this application note show that all of the TaqMan Gene Expression Assays have amplification efficiencies that are equivalent, because they all have an efficiency of one (E_X = 1.0).

Methods

Efficiency Measurements

In this study, efficiency values were measured using the C_T slope method. This method involves generating a dilution series of the target template and determining the C_T value for each dilution. A plot of C_T versus log cDNA concentration is constructed. With this method, the expected slope for a 10-fold dilution series of template DNA is -3.32, when E_X = 1.0.

Assay Designs

All TaqMan Gene Expression Assays have been designed by the Applied Biosystems genome-aided probe and primer design pipeline. Design parameters such as %GC content, T_m, and amplicon length were optimized to ensure that all of the TaqMan Gene Expression Assays have high amplification efficiency. Additionally, genome aided QC of all of the assays assures specificity for the target gene. This eliminates non-specific amplification—a factor that could contribute to decreased amplification efficiency of the true target.

Testing Across a Broad Template Dilution Range

Total RNA from Stratagene Universal Human Reference RNA was reverse transcribed using random primers (Applied Biosystems High Capacity cDNA Reverse Transcription Kit) to generate cDNA. For each assay tested, 100 ng of cDNA was amplified with Applied Biosystems TaqMan® Universal PCR Master Mix and the TaqMan Gene Expression Assay in a 50 µL volume for 40 cycles using universal cycling conditions (40 cycles at 95ºC for 15 seconds; 60ºC for 1 minute) on an ABI Prism® 7000 Sequence Detection System. This initial amplification was performed to produce sufficient template in the form of PCR product to enable the generation of a broad template dilution range.

A total of 5 µL of PCR product was run on a 4% agarose gel (NuSieve®/ SeaKem®) with size and quantitation standards (25 bp ladder from Invitrogen) to estimate the amount of product generated. The remaining 45 µL of PCR product was loaded on purification columns (QIAquick PCR purification kit; Qiagen) and purified according to the manufacturer's directions. Bound DNA was eluted in a 100 µL volume of elution buffer. Then, 10 µL of the elute was used for picogreen staining (PicoGreen® dsDNA quantitation kit, Molecular Probes) to estimate cDNA concentration.

A 6-log dilution range was generated using 10-fold serial dilutions of the target PCR product. Each of these dilutions was subjected to real-time PCR amplification as previously described, using TaqMan® Universal PCR Master Mix without AmpErase® UNG on an Applied Biosystems 7900HT Fast Real-Time PCR System. The C_T values obtained over this 6-log dilution range were plotted against cDNA concentration (Figure 1). Dilutions with a C_T higher than 35 were not included in the results because C_T values greater than 35 approach the sensitivity limits

Efficiency Hs00171135_m1

y = -3.3386x + 39.574
R^2 = 0.9933
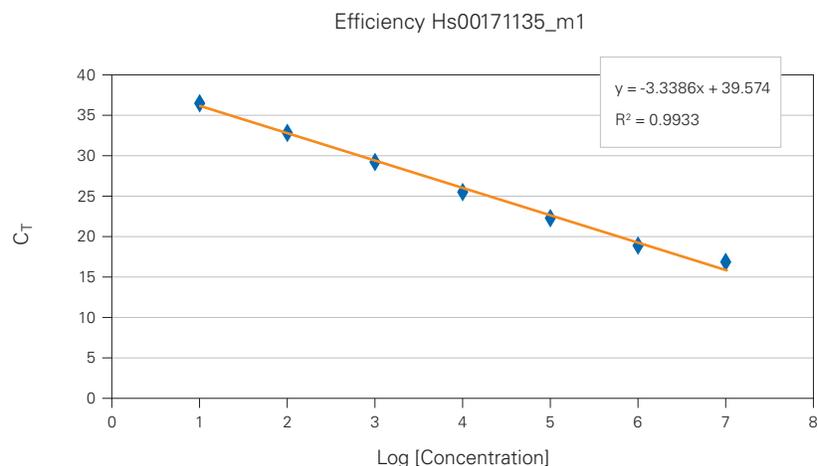
Figure 1. Measured efficiency (E_X) of assay Hs00171135_m1 (to the exon 3–4 boundary of human CXCL14 mRNA) using the C_T slope method with 7 data points (concentrations) covering a 6-log dilution range. The calculated efficiency for this assay is 99.3%.

of the real-time PCR detection system. Amplification efficiency was calculated from the slope of this graph using the equation:

$$E_X = 10^{(-1/slope)} - 1$$

Equation 3

For example, if the slope of the line is -3.33, then the amplification efficiency is:

$$E_X = 10^{(-1/-3.33)} - 1$$
$$= 10^{0.30} - 1$$
$$= 1.995 - 1$$
$$= 0.995 \text{ or } 99.5\%$$

Use of the Comparative C_T Method for Determination of Fold Change in Gene Expression

Stratagene Universal Human Reference RNA (100 ng/µL) was reverse transcribed as previously described to obtain cDNA. The cDNA was diluted in a 100-fold dilution range to obtain a dilution series with known amounts of cDNA. A constant amount of cDNA reverse transcribed from a non-human transcript (not detected in the Stratagene RNA pool) was used as a control to measure fold change. This transcript was made by T7 transcription of a 227 bp fragment from the Hepatitis C virus 5' UTR inserted into a TOPO-TA plasmid. Each of the Universal Human Reference cDNA dilutions was subjected to real-time PCR amplification as described above, using TaqMan Universal PCR Master Mix without AmpErase® UNG on an Applied Biosystems 7900HT Fast Real-Time PCR System. The relative quantities of diluted target cDNA vs. Hepatitis C cDNA were determined by either the $\Delta\Delta C_T$ method (Applied Biosystems User Bulletin #2[3]) or by inserting the pre-determined efficiency values for target and endogenous control into the equation:

$$R_{0,T} / R_{0,R} = \frac{(1 + E_R)^{C_{T,R}}}{(1 + E_T)^{C_{T,T}}}$$

Equation 4

For derivation of the above equation, see Liu and Saint, 2002[2]. The calculated fold change from each method was plotted versus the expected fold change, and the correlation between calculated and expected results was determined.
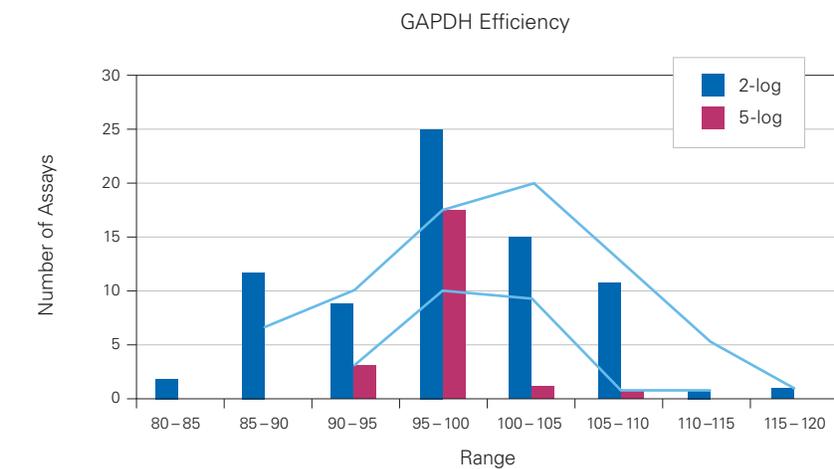
Results

Determination of Amplification Efficiencies of TaqMan Gene Expression Assays

Initial testing of 30 assays using a 1-log dilution range (with three concentration points at 10, 5, and 1 ng/µL) and a 3-log dilution range (with four concentration points at 10, 1, 0.1, and 0.01 ng/µL) indicated there were statistical problems with these narrow dilution ranges when measuring amplification efficiencies. Narrow dilution ranges are often used because of the limited amount of cDNA available to do the experiment, especially when quantitating low-copy number transcripts within a cDNA sample.

The use of a broad dynamic range and more data points significantly altered the distribution of amplification efficiency values. The use of a broader log dilution range, however, requires the generation of PCR product or other high concentration template such as a cDNA clone. Therefore, the best protocol for determining amplification efficiency is more difficult to implement.

The initial data indicated that the *accuracy* of efficiency measurements is extremely sensitive to both the number of data points and the dilution range used in a single experiment. Replicate experiments indicated that the *precision* of an amplification efficiency measurement increases when the experiment is performed over a broader range of template concentrations, or dilutions.

To further define the variability associated with measuring amplification efficiencies, we tested one assay targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript using either a 2-log (cDNA template) or 5-log (PCR product) dilution series with 5 and 6 concentration points, respectively. Multiple measurements were performed and the results are depicted in Figure 2. The 2-log measurements exhibited a much broader range of efficiency values, from 82% to 112% (avg. = 97.6%). The 5-log measurements exhibited much less variation, ranging from 92% to 105% efficiency (avg. = 98.4%). Note that the *average* efficiency values were not significantly different between the two dilution ranges, and were close to 100%.

The data shows how dramatically the range in efficiency measurement can increase (possibly giving very misleading results) over a small dilution range (2–3 log) versus a larger dilution range


Figure 2. Assay efficiency ranges were measured for the GAPDH assay over a 2-log (in blue) and 5-log (in red) dilution series. The 2-log range = 82%–112% (avg. = 97.6%; n = 76), and 5-log range = 92%–105% (avg. = 98.4%, n = 22).
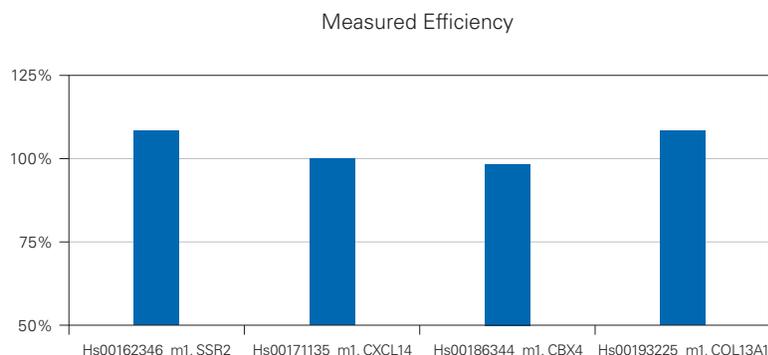
Figure 3. Measured efficiency (E_X) of four representative TaqMan Gene Expression Assays for the genes: SSR2 (signal sequence receptor, beta); CXCL14 (chemokine [C-X-C motif] ligand 14); CBX4 (chromobox homolog 4 [Pc class homolog, *Drosophila*]); and COL13A1 (collagen, type XIII, alpha 1).
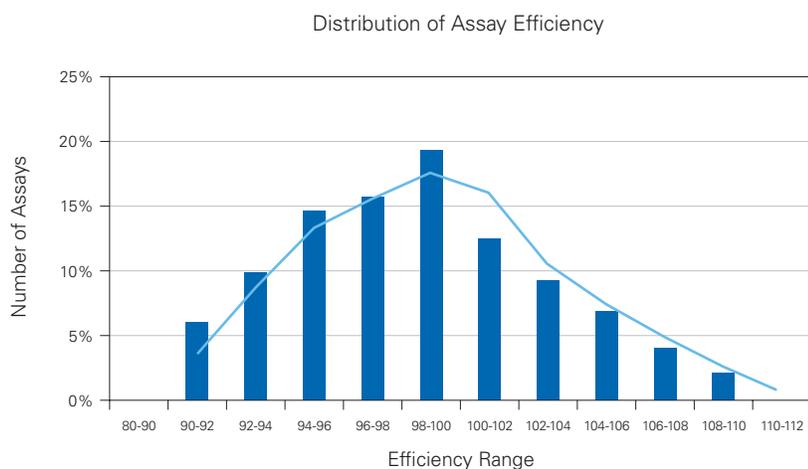
Figure 4. This data shows the distribution range obtained when a single GAPDH assay was tested multiple times.
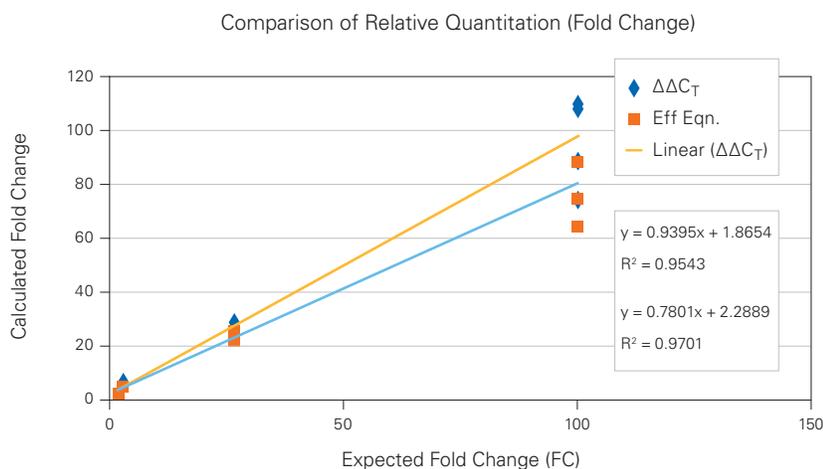
Figure 5. Comparison of fold change (FC) values obtained using the $\Delta\Delta C_T$ method versus using pre-determined efficiency values in the equation (see Equation 4). The graph plots the expected fold change versus the calculated fold change determined by the two different calculation methods.

(5–6 log). The increase is due to the fact that the effect of C_T variation (from pipetting error, for example) is minimized when using larger dilution ranges.

The initial data from the first 30 assays and GAPDH allowed us to design the optimal protocol for measuring amplification efficiency of a statistically significant sample of over 750 TaqMan Gene Expression Assays.

The tested assays represented a wide range of oligonucleotide compositions, for example: base composition (26%–79% GC), amplicon length (53 bp–186 bp), and secondary structural features (free energy 0 to -19.36 Kcal/mole). Results from sample assays are shown in Figure 3. Our assay design pipeline ensures that the amplicon sizes are the shortest possible, and avoids designs that have larger amplicon sizes.

The assays tested had amplification efficiency values ranging from 90% to 110% with very few (four) outliers. Repeat testing of these outliers resulted in values in the 90% to 110% range. The distribution of efficiency is shown in Figure 4. The distribution approximates a normal distribution with a mean of 98.73%.

Determination of Fold Change

Fold change in transcript levels for target templates present in the Stratagene Universal Reference RNA dilution series were determined using a spiked non-human transcript (NHT) as an invariant endogenous control. The results obtained for GAPDH are shown in Figure 5. The fold change results obtained using the $\Delta\Delta C_T$ method showed the best correlation to the expected fold change (slope = 0.94; expected slope = 1.0).

The $\Delta\Delta C_T$ method assumes that the amplification efficiency for both assays (GAPDH and NHT) is very similar and close to 100%. The fold change calculated using measured efficiency values (GAPDH assay efficiency = 97.5% and NHT = 94%) had a poor correlation to the expected fold change (slope = 0.78; expected slope = 1.0). The data

points to the potential hazard of using "pre-determined" efficiency values to do relative quantitation calculations. If the efficiency determinations used in the equations are inaccurate, then the error is propagated into the relative quantitation calculations, i.e., the more biologically relevant results.

Conclusions and Recommendations

Our results show that when you use TaqMan Gene Expression Assays, calculated fold change values correlate better to expected fold change values when the $\Delta\Delta C_T$ method for relative quantitation is used. This is because all of our assay products meet the assumptions necessary to use this simplified equation. In other words, the amplification efficiency of all TaqMan Gene Expression Assays are equivalent to any other target assay because they all have amplification efficiencies very close to one.

Therefore, when using TaqMan Gene Expression Assays, it is not necessary to measure efficiency. We have extensively tested our design parameters and are confident that the resulting assays will have 100% efficiency (+/-10%) when measured over a 6-log dilution range, in samples that are free of PCR inhibitors. Determination of amplification efficiency (E_X) using a template dilution series can generate highly variable results depending on the conditions employed. Small differences in measured C_T values can translate into significant changes in calculated efficiency, especially when few points over a narrow dilution range are used to generate the C_T vs. log concentration plot, and thus calculate a C_T slope.

When using custom designed TaqMan® probe and primer sets that are not a part of the TaqMan Gene Expression Assays product line, we recommend:

- To obtain reliable efficiency values, use a 5–6 log (10^5–10^6 fold) dilution range of template DNA

- Use at least 5 data points in this dilution range to generate the C_T versus log concentration plot

- Use a DNA template with high enough target template concentration to create a 5–6 log dilution series that will generate C_T values of less than 36 on an Applied Biosystems Real-Time PCR System

We suggest you use:

– PCR product (be very careful to avoid contamination of the laboratory space with amplified DNA);

– A cDNA clone at a high template concentration; or

– Genomic DNA (if your TaqMan probes and primers are designed within an exon)

References

1. Livak and Schmittgen, (2001) *Methods* 25:402–408

2. Liu & Saint, (2002) *Analytical Biochemistry*, 302:52–59

3. "Relative Quantitation of Gene Expression: ABI PRISM® 7700 Sequence Detection System" Livak (1997), Applied Biosystems User Bulletin #2 (part number 4303859)

Additional Recommended Reading

"Essentials of Real-Time PCR" Applied Biosystems Application Note (part number 105622)

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