

Amplification efficiency of TaqMan® Gene Expression Assays

Assays tested extensively for qPCR efficiency

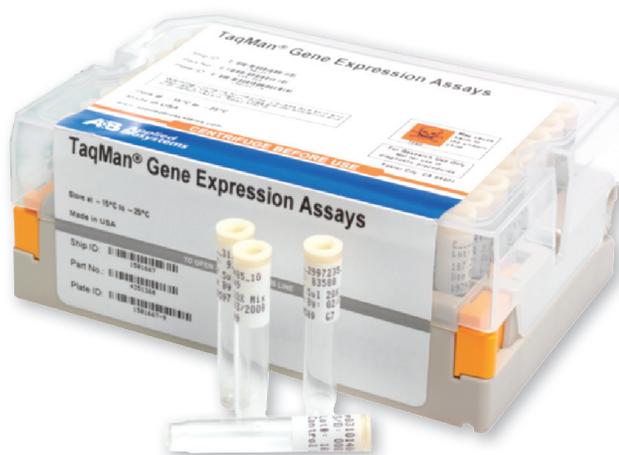
Key factors that affect efficiency

- Efficiency estimates can vary significantly if a rigorous measurement protocol is not followed
- Measurements must be obtained over a broad dilution range (5–6 orders of magnitude) and include replicates to decrease the effects of laboratory errors (e.g., pipetting accuracy)
- PCR efficiency can be affected by substances known to inhibit PCR, such as heme compounds and detergents; users must ensure that cDNA templates are free of inhibitors

Introduction

We have designed and manufactured over 680,000 real-time PCR assays for measuring the expression of human, mouse, rat, *Arabidopsis*, *Drosophila*, and *Caenorhabditis elegans* genes. TaqMan® Gene Expression Assays each consist of a fluorescently labeled TaqMan® probe and forward and reverse amplification primers in a single-tube formulation.

One of users' major concerns regarding any real-time PCR assay is the amplification efficiency of the PCR reaction. We have 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 of assays designed using our bioinformatics pipeline is essentially 100%. Additionally, our results show that the $\Delta\Delta C_t$ method for relative quantitation gives better correlation between expected and calculated fold changes in gene expression than methods using predetermined amplification efficiency values for individual assays.

A key attribute of any real-time PCR reaction is the amplification efficiency. Specifically, there should

be a doubling of PCR product in every cycle [1,2]. The equation that describes the exponential amplification of PCR is Equation 1:

$$X_n = X_o * (1 + E_x)^n$$

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 PCR using TaqMan[®] probes, X_n is proportional to the reporter fluorescence R, so Equation 1 can be rewritten as Equation 2:

$$R_n = R_o * (1 + E_x)^n$$

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 (detailed for the ABI PRISM[®] 7700 Sequence Detection System in [3]), the efficiency (E_x) of amplification of the target and of the endogenous control must be approximately equal (see [1] for derivation of the $\Delta\Delta C_t$ equations).

The results described in this application note show that all of the TaqMan[®] Gene Expression Assays have equivalent amplification efficiencies, because they all have an efficiency (E_x) of 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 for each

dilution. A plot of C_t vs. 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 our genome-aided probe and primer design pipeline. Design parameters such as %GC content, melting temperature (T_m), and amplicon length were optimized to ensure that all TaqMan[®] Gene Expression Assays have high amplification efficiency. Additionally, genome-aided QC of all assays assures specificity for the target gene. This minimizes nonspecific 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 with random primers using the High-Capacity cDNA Reverse Transcription Kit to generate cDNA. For each assay tested, 100 ng of cDNA was amplified with TaqMan[®] Universal PCR Master Mix and the TaqMan[®] Gene Expression Assay in a 50 μ L reaction volume for 40 cycles using universal cycling conditions

(95°C for 15 seconds, 60°C for 1 minute, for 40 cycles) on the Applied Biosystems[®] 7000 Real-Time PCR 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 μ L of each PCR product was run on a 4% NuSieve[®]/SeaKem[®] agarose gel with size and quantitation standards (25 bp ladder) to estimate the amount of product generated. The remaining 45 μ L of each PCR product was loaded on a purification column (QIAquick[®] PCR purification kit, Qiagen[®]) and purified according to the manufacturer's directions. Bound DNA was eluted in 100 μ L of elution buffer. Then, 10 μ L of the eluate was used to estimate cDNA concentration using the PicoGreen[®] dsDNA Quantitation Kit.

A set of six 10-fold serial dilutions of the PCR product was generated. Each of these dilutions was subjected to real-time PCR as previously described, using TaqMan[®] Universal PCR Master Mix without AmpErase[®] UNG on the 7900HT Fast Real-Time PCR System. The threshold cycle (C_t) values obtained over this 6-fold dilution range were

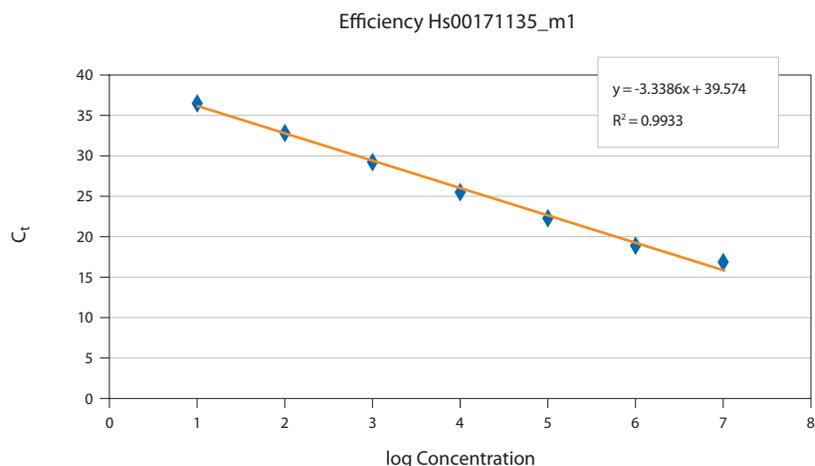


Figure 1. Determination of efficiency (E_x) of assay Hs00171135_m1 (covers the exon 3–4 boundary of human *CXCL14* mRNA) using the C_t slope method with 7 concentrations covering a range of 6 orders of magnitude. The calculated efficiency for this assay is 99.3%.

plotted against cDNA concentration (Figure 1). Reactions with a C_t higher than 35 were not included in the results because such values approach the sensitivity limits of the real-time PCR system. Amplification efficiency was calculated from the slope of this graph using Equation 3:

$$E_x = 10^{(-1/\text{slope})} - 1$$

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

$$\begin{aligned} E_x &= 10^{(-1/(-3.33))} - 1 \\ &= 10^{0.30} - 1 \\ &= 1.995 - 1 \\ &= 0.995, \text{ or } 99.5\% \end{aligned}$$

Use of the comparative C_t method for determination of fold change in gene expression

Stratagene Universal Human Reference RNA (100 ng/ μ L) was reverse transcribed as previously described to obtain cDNA. The cDNA was diluted in a 100-fold range to obtain a dilution series with known amounts of cDNA. A constant amount of cDNA reverse transcribed from a nonhuman transcript (not detected in the Stratagene RNA pool) was used as a control to measure fold change. This transcript was made by T7 RNA polymerase transcription of a 227 bp fragment from the hepatitis C virus 5'-untranslated region 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 the 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 or by inserting the predetermined efficiency values for target and endogenous control into Equation 4:

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

For derivation of this equation, see Reference 2. The calculated fold change from each method was plotted against 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 10-fold dilution range (with three concentrations: 10, 5, and 1 ng/ μ L) and a 1,000-fold dilution range (with four concentrations: 10, 1, 0.1, and 0.01 ng/ μ L) 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 dilution range and more data points significantly altered the distribution of amplification efficiency values. The use of a broader 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 accurate determination of efficiency 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.

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 100-fold dilution series with 5 concentration points (cDNA template) or a 10⁵-fold (PCR product) dilution series with 6 concentration points. Multiple measurements were performed, and the results are shown in Figure 2. The narrower dilution series exhibited a much broader range of efficiency values, from 82% to 115% (avg. = 97.6%), whereas the broader series exhibited much less

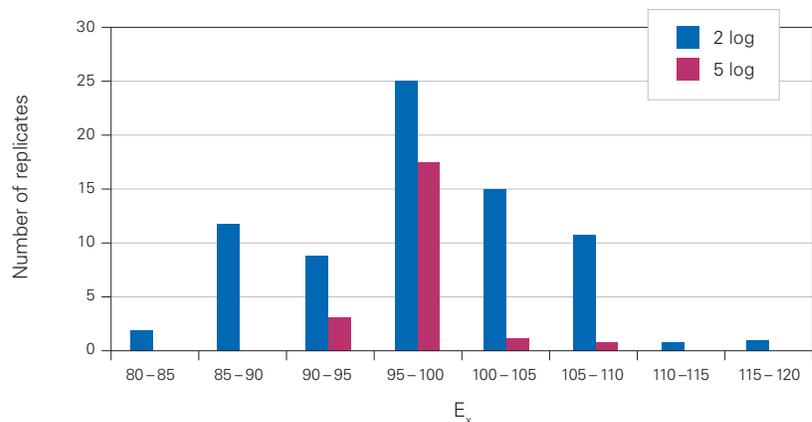


Figure 2. Assay efficiency ranges were measured for the GAPDH assay over a 100-fold (blue) and 10⁵-fold (red) dilution series. The 100-fold range = 82%–115% (avg. = 97.6%, n = 76), and the 10⁵-fold range = 92%–105% (avg. = 98.4%, n = 22).

variability, 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 show how dramatically the variability in efficiency measurement can increase (possibly giving very misleading results) over a small dilution range (2–3 orders of magnitude) vs. a larger dilution range (5–6 orders of magnitude). The increase is because 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 (53–186 bp), and secondary structural features (free energy 0 to –19.36 kcal/mol). 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 and approximates a normal distribution with a mean of 98.73%.

Determination of fold change

Fold changes in transcript levels for target templates present in the

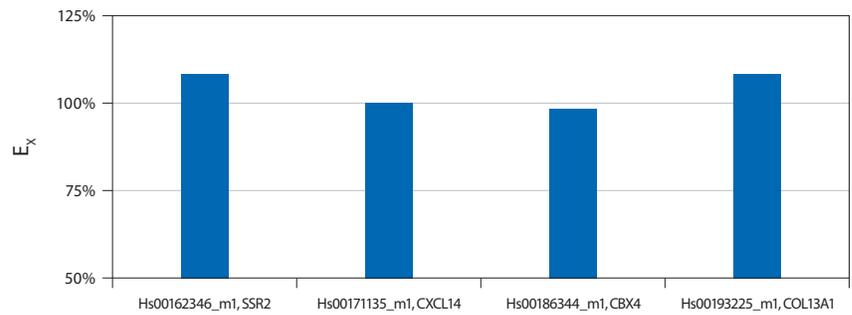


Figure 3. Measured efficiency of four representative TaqMan® Gene Expression Assays. Assays are for the genes *SSR2* [signal sequence receptor, beta], *CXCL14* [chemokine [C-X-C motif] ligand 14], *CBX4* [chromobox homolog 4 [Pc class homolog, *Drosophila*]], and *COL13A1* [collagen, type XIII, alpha 1].

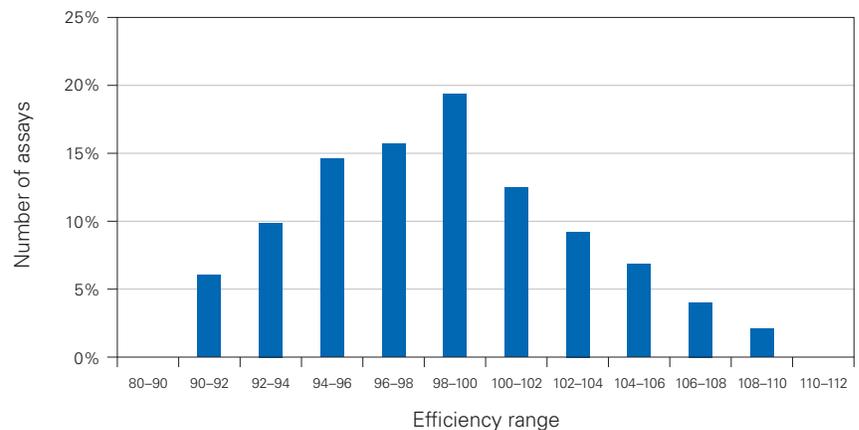


Figure 4. Distribution of measured amplification efficiency (%) of over 750 TaqMan® Gene Expression Assays.

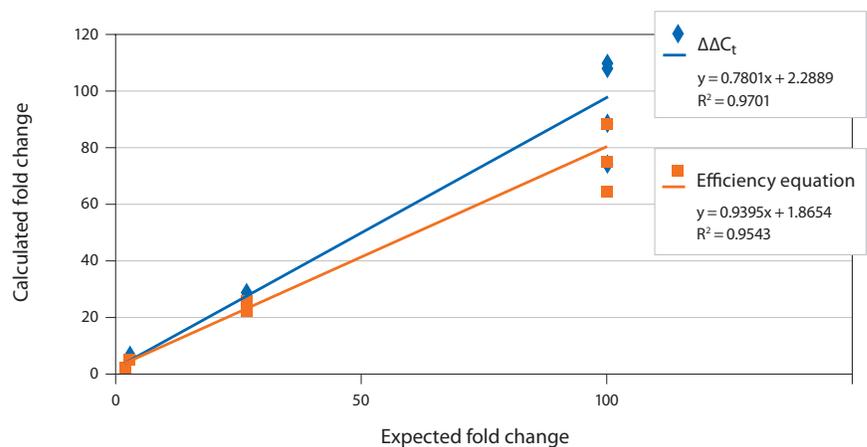


Figure 5. Comparison of relative quantitation fold change values obtained using the $\Delta\Delta C_t$ method and using predetermined efficiency values in the equation (see Equation 4). The plot shows the calculated fold change against the expected fold change determined by the two different calculation methods. The $\Delta\Delta C_t$ method gives closer correlation of expected vs. calculated fold change.

Stratagene Universal Reference RNA dilution series were determined using a spiked nonhuman 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 better 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 point to the potential hazard of using "predetermined" efficiency values to do relative quantitation calculations. If the efficiency determinations used in the equations are inaccurate, then the error is propagated to 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 every TaqMan[®] Gene Expression Assays is equivalent to any other target assay because they all have amplification efficiencies very close to 1.

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 ($\pm 10\%$) when measured over 6 orders of magnitude 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 (10^5 to 10^6 -fold dilution range of template DNA.
- Use at least 5 points in this dilution range to generate the C_t vs. log concentration plot.
- Use a DNA template with high enough target template concentration to create a 10^5 to 10^6 -fold dilution series that will generate C_t values of less than 36 on a 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); or
- 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 KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ $\Delta\Delta C_t$ method. *Methods* 25:402–408.
2. Liu W, Saint DA (2002) A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal Biochem* 302:52–59.
3. Relative quantitation of gene expression: ABI PRISM[®] 7700 Sequence Detection System. User Bulletin #2, Rev. B; part number 777802-002.

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