Comparing Algorithms for Calculating Amplification Efficiencies of Real-Time PCR  
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Background
- Quantitative real-time RT-PCR is the most sensitive and widely used method for the measurement of gene expression. In real-time PCR, a fluorescent dye is used to monitor the amplification of target genes by a thermostable DNA polymerase.
- The three principles of real-time PCR quantification are:
  1. The accumulation of fluorescence is proportional to accumulation of PCR product.
  2. The amplification efficiencies of all samples must be comparable.
  3. The amplification threshold used for analysis must be set within the exponential phase of the PCR to ensure that the amount of amplicons generated at the threshold cycle (C_t) truly reflects the initial template amount.
- The analysis of real-time PCR data has become the focus of mathematical modeling to increase quantification precision and accuracy.
- Various strategies have been proposed and practiced to estimate the amplification efficiency of PCR. These include:
  1. The slope-derived efficiency calculation from the standard curve method using threshold cycle (C_t) or crossing point (CP) values determined either from
     a. The fit-point method OR
     b. The second derivative maximum (SDM) of the four-parameter logistic model
  2. The single amplification plot methods to compute the efficiency values from individual PCR kinetic curves using comprehensive algorithms such as
     a. The mid-value point regression (Data Analysis for Real-Time PCR or DART-PCR) OR
     b. The window-of-linearity algorithm (LinRegPCR) OR
     c. The noise-resistant iterative nonlinear regression (Real-Time PCR Miner^®)

Experimental Design
- Mouse Inflammatory Cytokines & Receptors RT 2 Profiler™ PCR Arrays (Cat # PAMM-011, SuperArray Bioscience, Hercules, CA), Stratagene Mx3005P™ (Cedar Creek, TX) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each dilution series was run with RT 2 SYBR Green iPCR Master Mix (Cat # PA-012, SuperArray) for the Cycler I or with RT SYBR Green®/Rox iPCR Master Mix (Cat # PA-011, SuperArray) for the Mx3005P and ABI 7500. Each method used a different series separate PCR arrays with each well containing 1% of the standard PCR Arrays were run in triplicates for each standard. The amplification efficiency for each assay on the PCR Array was computed with the five methods mentioned above.

Results
- Why it is important to know the amplification efficiency of the PCR for each gene target?
- The two methods above may potentially introduce biases in quantification, because both of them are based on the assumption of identical reactions in mice among different samples. The latter comparative C method also assumes a constant efficiency of 100% for all PCR assays (Figure 1).

Methods for Estimation of PCR Amplification Efficiencies
- Various strategies have been proposed and practised to estimate the amplification efficiency of PCR. These include:
  1. The slope-derived efficiency calculation from the standard curve method using threshold cycle (C_t) or crossing point (CP) values determined either from
     a. The fit-point method OR
     b. The second derivative maximum (SDM) of the four-parameter logistic model
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Study Aim
- To directly compare various standard curve and amplification plot methods to obtain the amplification efficiency values for a panel of PCR assays performed on different real-time PCR instruments.

Conclusions
- This study demonstrates that the Real-time PCR Miner provides the best precision in efficiency estimation independent of the PCR instrument, while the precisions for other methods are platform-dependent. Hence, the Real-time PCR Miner, a completely objective and noise-resistant algorithm, is the ideal tool for estimating PCR amplification efficiencies.

References:

Table 1. Comparisons Between Different Methods for the Estimation of Amplification Efficiencies of 99 SYBR Green Real-Time PCR Assays

<table>
<thead>
<tr>
<th>Method</th>
<th>RT SYBR Green iPCR Master Mix</th>
<th>RT SYBR Green®/Rox iPCR Master Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fit-Point</td>
<td>85.7% 94.2% 96.8% 96.0%</td>
<td>86.0% 90.1% 91.0%</td>
</tr>
<tr>
<td>SDM</td>
<td>83.3% 94.2% 95.5% 90.1%</td>
<td>85.7% 90.1% 91.0%</td>
</tr>
<tr>
<td>iCycler iQ™</td>
<td>85.7% 94.2% 96.8% 96.0%</td>
<td>86.0% 90.1% 91.0%</td>
</tr>
<tr>
<td>Real-Time PCR Miner</td>
<td>102.6% 103.9% 99.4% 94.3%</td>
<td>101.5% 101.3% 99.4%</td>
</tr>
<tr>
<td>Real-Time PCR</td>
<td>91.0% 90.1% 86.0% 85.7%</td>
<td>90.1% 90.1% 91.0%</td>
</tr>
<tr>
<td>Average Efficiency</td>
<td>91.0% 90.1% 86.0% 85.7%</td>
<td>90.1% 90.1% 91.0%</td>
</tr>
<tr>
<td>Average Difference vs Std Curve Method</td>
<td>91.0% 90.1% 86.0% 85.7%</td>
<td>90.1% 90.1% 91.0%</td>
</tr>
</tbody>
</table>

*Note: Results from the RT SYBR Green and DART-PCR methods are compared with the fit-point method, as these methods use automatic fluorescence detection. Unmatched, Real-Time PCR Miner values are compared with those from the SDM method, at both of their usable ranges.*