Real-Time Quantitative PCR
Assay Data Analysis, Evaluation and Optimization

A Tutorial on
Quantification Assay Analysis and Evaluation and
Trouble-Shooting Sub-Optimal Real-Time QPCR Experiments

by
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Content IV: A&E Class

- **Introduction:**
  - Real-time QPCR & Amplification Efficiency,
  - Mathematics of QPCR

- **Data Analysis and Evaluation:**
  - Quantification Strategies in QPCR
    - Absolute Quantification
    - Relative Quantification:
      - Standard curve method
      - Comparative CT method
  - Fidelity in QPCR
    - Specificity, Sensitivity, Accuracy, Reproducibility
    - Experimental Variations, Replicates,
    - Standard Deviation Calculations

- **Optimizing QPCR experiments**
  - Primer and probe optimization
  - Multiplex assay optimization
Essentials - One More Time

• Target Reporter Fluorescence...
  - is determined from the fractional cycle at which a threshold amount of amplicon DNA is reached:
    • \( R_{CT} = R_0 \cdot (1 + E_T)^{CT} \)
  - Amplification Efficiency (@ threshold T): \( E_T = 10^{-1/s} - 1 \)
    • slope (s) of linear regression of \( C_T \) values vs. \( \log[\text{cDNA}] \)
  - Fluorescence increase \( I \) is proportional to the amount of target DNA: \( I = k \cdot R_{CT} \)
# Mathematics of QPCR

**• Basic Equations:**

- \( R_{CT} = R_0 \cdot (1+E_T)^{CT} \)
  
  - Taking the logarithm yields: \( \log(R_{CT}) = \log(R_0) + \log(1+E) \cdot CT \)
  
  - rearrangement: \( CT = \frac{\log(R_{CT})}{\log(1+E)} - \frac{\log(R_0)}{\log(1+E)} \), or:
    \[
    CT = -1 / \log(1+E) \cdot \log(R_0) + \log(R_{CT}) / \log(1+E)
    \]

  - Comparison with \( y = sx + b \) indicates that plotting \( CT \) versus \( \log(R_0) \) produces a line with the slope \( s \), therefore:
    \[
    s = -1 / \log(1+E), \quad \text{or: } \log(1+E) = -1/s
    \]

  - Solving the logarithm then yields the amplification efficiency:
    \[
    1+E = 10^{-1/s}, \quad E = 10^{(-1/s)} - 1
    \]
    
    [for \( E=1: \ 2 = 10^{-1/s}, \text{ or } \log2 = -1/s, \text{ or: } s = -1 / \log2 = -3.32 \]

- Because we aim at obtaining the initial numbers of target molecules, it is appropriate to now substitute reporter fluorescence \( R \) with numbers \( N \):

  - \( N_0 = N_{CT}/(1+E)^{CT} \) (I) and \( I = k N_{CT} \)
Quantification Strategies in QPCR

• Absolute Quantification
  - Absolute Standard Curve Method > requires standards of known quantities
    • STND1/2/…/6, UNKN, NTC

• Relative Quantification
  A comparative method: requires a reference, which is also a target (2\textsuperscript{nd} amlicon), = active reference.
  - Relative Standard Curve Method: relative target quantity in relation to standard curves of standard and reference
    • STND\textsubscript{1, 2, …, 6}, REF\textsubscript{1, 2, …, 6}, UNKN, NTC
  - Comparative $C_T$ Method ($\Delta\Delta C_T$): relative target quantity in relation to an endogenous control only (no standards)
    • REF, UNKN, NTC
Absolute Quantification: AQ

• A Calibration Curve Method
  - Known amounts of external targets are amplified in a parallel group of reactions run under identical conditions to that of the unknown samples.
  - Standards: recRNA, recDNA, gDNA
  - The absolute quantities of the standards must first be determined by some other independent means.
  - SDS determines $N_0$ for each Unknown based on linear regression calculations of the standards.
AQ ... continued

- No Data Munching
  - Quantities exported
    - to Excel
    - to text only calculated on the basis of a calibration curve (standard curve).

- Easy, but ...
  - Standards
    - DNA: appropriate?
    - RNA: different RT
  - Expensive
  - Least accurate method
    - quantitative accuracy = f(standards, RT, standard curve)
Relative Quantification: RQ

• An Active Reference
  - ...is used to determine changes in the amount of a given sample relative to another -internal - control sample.
    • a different amplicon in the same PCR reaction as the amplification of the amplicon for the GOI
  - Does not require standards with known concentrations

• Calculation Methods for Relative Quantitations
  - Standard Curve method ($\Delta C_T$)
    • Two 'standard' curves (relative control & GOI)
    • May include a 2nd normalization with an arbitrarily chosen calibrator
  - Comparative $C_T$ method ($\Delta\Delta C_T$)
    • no standards, but with amplification of a reference
    • contingent upon similar amplification efficiencies of the amplicons for GOI and reference
    • Always relative to a calibrator sample
RQ: Intuitively

- $\Delta C_T = \text{const}$ because $E = \text{const}$ (note: $E_A \neq E_B$ is allowed)

![Graph showing fluorescence saturation over cycle number with different RNA levels]

- Same amplicon:
  - $E_A = E_B \Rightarrow N_A / N_B = 2^{-\Delta C_T}$
  
  For example: if $\Delta C_T$ between A and B is 5 cycles, then there is $2^{-5} = 1/32$ as much A than B.

- Different amplicons:
  
  For example: GOI (x) and endogenous control (c):
  
  - $E_x \neq E_c \Rightarrow N_x / N_c = K (1+E_c)^{C_{Tc}} / (1+E_x)^{C_{Tx}}$
RQ: Mathematically

- \( N_{CT} = N_0 (1+E)^{CT} \) and \( I = k N_{CT} \)

- The relative Intensities of samples A and B is:
  - \( I_A = k_A \cdot N_{CTA} = k_A \cdot N_{0A} (1+E_A)^{CTA} \) and
  - \( I_B = k_B \cdot N_{CTB} = k_B \cdot N_{0B} (1+E_B)^{CTB} \)

- at threshold: \( I_A = I_B \) thus: \( k_A \cdot N_{CTA} = k_B \cdot N_{CTB} \)

- Solving for constants yields: \( K = k_B/k_A = N_{CTA}/N_{CTB} \)
  - inserting \( N_{CTA} = N_{0A} (1+E_A)^{CTA} \) and \( N_{CTB} = N_{0B} (1+E_B)^{CTB} \)
  - rearranging we get:

- \( N_{0A}/N_{0B} = K \cdot (1+E_B)^{CTB} / (1+E_A)^{CTA} \) (II)
  - The fractions of A and B expressed as percentages are:
    - \( A = 100 \cdot \frac{K \cdot (1+E_B)^{CTB}/(1+E_A)^{CTA}}{1+K \cdot [(1+E_B)^{CTB}/(1+E_A)^{CTA}]} \)
    - \( B = 100 \cdot \frac{1}{1+K \cdot [(1+E_B)^{CTB}/(1+E_A)^{CTA}]} \)

- Relative Standards:
  - For example: the ratio of treatment (†) vs. control (c):

\[
\frac{(N_A/N_B)}{(N_A/N_B)} = K \frac{(1 + E_B^†)^{CTB^†}}{(1 + E_A^†)^{CTA^†}} \\
\frac{(N_A/N_B)}{(N_A/N_B)} = \frac{(1 + E_B^c)^{CTB^c}}{(1 + E_A^c)^{CTA^c}}
\]
Relative Standard Method, Example A

- Two serial dilutions: one for GOI (c-myc), another one for the endogenous control (GAPDH)
- Expression profiling in brain, kidney, liver, lung
RQ: Data Munching in Excel

- Average replicates, then divide the average c-myc (GOI) value by the average GAPDH reference value of the corresponding samples.
- For example:

\[ \frac{\text{GOI}}{\text{Ref}} \]

2nd normalization: Calibrator = Brain

see slide 30 for error handling
... continued

- Relative Quantification with Absolute Values: involves the division by a calibrator value:
  - normalize using an endogenous control, then
  - divide the normalized values by an arbitrarily chosen calibrator value (e.g. kidney in this example)

<table>
<thead>
<tr>
<th></th>
<th>GOI raw</th>
<th>18S raw</th>
<th>Normalized GOI/18S</th>
<th>Relative Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>kidney</td>
<td>82</td>
<td>3592</td>
<td>0.023</td>
<td>1.0</td>
</tr>
<tr>
<td>liver</td>
<td>18351</td>
<td>8996</td>
<td>2.05</td>
<td>90</td>
</tr>
<tr>
<td>ovary</td>
<td>44</td>
<td>1669</td>
<td>0.03</td>
<td>1.3</td>
</tr>
<tr>
<td>spleen</td>
<td>1</td>
<td>8</td>
<td>0.13</td>
<td>5.6</td>
</tr>
</tbody>
</table>

- Quality of quantification using the relative standard curve method:
  - quantitative accuracy = f (standard curve)
  - More accurate than the absolute standard method
Relative Standard Method, Example B

- e.g. c-myc Expression Analysis in Liver, Kidney Tissues
  - GOI is c-myc, endogenous control is GAPDH,
  - reference sample is RNA isolated from lung tissue
  - 2 Standard curves: serial dilutions of a cDNA sample generated from lung tissue tRNA - one series is analyzed for c-myc, the other for GAPDH.

From: Applied Biosystems Documentation PN 4376785 Rev D
SDSv2 Does the Analysis For You
Relative Standard Method, Example C

- Relative to endogenous control AND treatment(s)
- For example: +/- TNFa induced TNFAIP3 and GAPDH

\[
\frac{(N_A / N_B)_t}{(N_A / N_B)_c} = K \frac{(1 + E_{Bt})^{CTB_t}}{(1 + E_{At})^{CTA_t}} \times \frac{(1 + E_{Bc})^{CTB_c}}{(1 + E_{Ac})^{CTA_c}}
\]

\[
K = \frac{(NA / NB)_c}{(NA / NB)_t}
\]

SuperArray Bioscience Corporation Newsletter 1

TNFa untreated: \(C_t(TNFAIP3) = 24.25\) \(C_t(GAPD) = 16.49\)
TNFa treated: \(C_t(TNFAIP3) = 19.17\) \(C_t(GAPD) = 16.36\)

\[
\frac{(TNFAIP3/GAPD)_{treated}}{(TNFAIP3/GAPD)_{untreated}} = \frac{0.17 / 0.14}{0.0048 / 0.13} = 32.9
\]
The Comparative $C_T$ Method

- **Derivation of the $\Delta\Delta C_T$ Method**
  - Targets at threshold cycle $C_T$: $N_{CT} = N_0 \cdot (1+E)^{CT}$
    - For $X_T$: number of target GOI molecules at threshold
    - and $R_T$: number of reference molecules at threshold
    - $X_T/R_T = X_0 \cdot (1+E_X)^{CTX} / R_0 \cdot (1+E_R)^{CTR} = K_x/K_R = K$
  - If $E_X \approx E_R =: E \Rightarrow K = X_0/R_0 \cdot (1+E)^{CTX-CTR} = X_N \cdot (1+E)^{\Delta CT}$
    - Whereby $\Delta C_T = CT_X-CT_R$, and $X_N = X_0/R_0$
    - Rearranged: $X_N = K/(1+E)^{\Delta CT}$, or $X_N = K \cdot (1+E)^{-\Delta CT}$ (III)
  - Another normalization of each normalized sample $X_N$ by the $X_N$ of a calibrator (cb) yields:
    - $X_{N,cb} = K (1+E)^{-\Delta CT}/K (1+E)^{-\Delta CT,cb} = (1+E)^{-\Delta \Delta CT}$
  - $E = \text{const.}$, and with $N = X_N/X_{N,cb}$: $N = 2^{-\Delta \Delta CT}$ (IV)
- **Quality of quantification:**
  - quantitative accuracy = $f$(amplification efficiency)
  - Accurate and most efficient QPCR data analysis method.
  - (don’t use the $\Delta\Delta CT$ method if $CV > 4\%$, see later)
ΔΔCT Method continued

- SDS v2 does it for you! Otherwise, use Excel
- Normalize GOI signals to signals of an endogenous reference (e.g. 18S): \( CT_{GOI} - CT_{18S} \Rightarrow \Delta CT_r \)
- Normalize each \( \Delta CT_r \) value to a particular \( \Delta CT_c \) value of an assay calibrator (cb): \( \Delta CT_r - \Delta CT_{cb} \Rightarrow \Delta\Delta CT_r \) and one \( \Delta\Delta CT_{cb} \).
  - This is a second subtraction, and \( \Delta\Delta CT_{cb} = 0 \)
  - Calibrator cb may be a control treatment, or the sample with the highest \( \Delta C_{TR} \) value
- The relative target number \( N \) then is \( 2^{-\Delta\Delta CT} \)

<table>
<thead>
<tr>
<th></th>
<th>GOI CT</th>
<th>18S CT</th>
<th>Norm. I ΔCT</th>
<th>Norm. II ΔΔCT</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>24</td>
<td>14</td>
<td>10</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>P</td>
<td>20</td>
<td>11</td>
<td>9</td>
<td>-2</td>
<td>4</td>
</tr>
<tr>
<td>E+P</td>
<td>21</td>
<td>11</td>
<td>10</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>DMSO</td>
<td>27</td>
<td>16</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Comparative $C_T$ Method ($\Delta \Delta C_T$) Example B

- e.g. p53 Expression in Liver, Kidney, Brain Tissues
  - GOI is TP53, endogenous control is GAPDH
  - Assumption: similar amplification efficiencies ($E_{TP53} = E_{GAPDH}$) ($\Delta \Delta C_T$ validation experiment, see later)

For comparison:
Relative standard method: 48 wells

From: Applied Biosystems Documentation PN 4376785 Rev D
SDSv2 Does the Analysis For You
ΔΔCT Method, Example C

- siRNA Transfection
  - Quantitation of % Knock-down and remaining gene expression:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amplicon</th>
<th>CT</th>
<th>ΔCT</th>
<th>ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA</td>
<td>Primer/Probe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Target</td>
<td>Target</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOI</td>
<td>GOI</td>
<td>26.98</td>
<td>15.23</td>
<td>4.89</td>
</tr>
<tr>
<td>GOI</td>
<td>18S rRNA</td>
<td>11.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>GOI</td>
<td>22.87</td>
<td>10.34</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>18S rRNA</td>
<td>12.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent remaining gene expression:</td>
<td>2^{exp-ΔΔCT}</td>
<td>2^{-4.89} = 3.37%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent knockdown:</td>
<td>100 - 3.37%</td>
<td>96.63%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Validation Experiment

- **ΔΔCT Method** is contingent upon $E_{GOI} \approx E_{Ref}$
  - The absolute value ($|s|$) of the slope $s$ of log input amount (or dilutions) vs. $ΔC_T$ should be less than 0.1

- Comparing important linear regression plots for qPCR:

  - $E_{X}$ vs. $E_{R}$
    - Efficiencies: $|s| < 0.1$
    - $E_{max}$ amplification efficiency: $s = -3.32$

Livak and Schmittgen, 2001, Methods 25, 402-408
What If $E_{GOI} \neq E_{ref}$?

- **Use Efficiency Correction**
  - Note: Rainer does NOT recommend this method of QPCR data analysis (if you had followed all the recommendations thus far, you most likely would not have this problem now)

  $$\text{Relative } N = \frac{(E_X)^{\Delta CT_x(\text{control-sample})}}{(E_R)^{\Delta CT_R(\text{control-sample})}}$$

  $$= \frac{(E_R)^{CT_{\text{sample}}}}{(E_X)^{CT_{\text{sample}}}} + \frac{(E_R)^{CT_{\text{calibrator}}}}{(E_X)^{CT_{\text{calibrator}}}}$$

- **Use REST Software**
  - REST (Relative Expression Software Tool)
    - [http://www.gene-quantification.info/](http://www.gene-quantification.info/) then go to 'Data Analysis', 'qPCR software applications', 'REST versions', then scroll down to 'New REST software application are available:'

Rainer B. Lanz, M.S., Ph.D.
Fidelity in QPCR

✓ Specificity
  - Assay design and project integration: a prerequisite
  - Determining the amplification efficiency: a prerequisite
  - Melting curve analysis: maybe (for spotting primer-dimers)

✓ Sensitivity
  - TaqMan® or SYBR®: comparable dynamic range, sensitivity

✓ Efficiency
  - $E_{exp} = 10^{(-1/s)} - 1$ over a wide range of input material
  - Pearson correlation coefficient $r \geq 0.95$

• Accuracy and Reproducibility
  - Replicates for intra-assay precision
  - Strategy: RT = main source of variability $\Rightarrow$ single cDNA pool, RT assay optimization
  - Repetitions for inter-assay precision (Reproducibility)
    • not necessary ($\times$ peer reviewer’s thinking)
    • Use a calibrator for inter-plate-normalizations
  - Optimizing sub-optimal experiments: always E, RT rxn
Experimental Variation

- **Biological Variations**
  - \( = f\{\text{population being studied}\}\),
  - Large CV (e.g. gene expression: CV 20 to 100%)

- **Process Variations**
  - Random variations: common-cause errors, not affecting all samples, \( = f\{\text{accuracy, standard operating procedure}\}\)
    - e.g. pipetting errors
  - Systemic variations: biasing all samples, \( = f\{\text{calibration, standard operating procedure}\}\)
    - e.g. software settings in sequence detection systems

- **System Variations**
  - System constant, affecting all samples equally, \( = f\{\text{instrument accuracy}\}\)
    - Fluorescence increase \( I \) is proportional to the amount of target DNA: \( I = k \cdot R_{CT} \)
Accuracy versus Precision

- **Accuracy**
  - How close a measurement is to the true or actual value

- **Precision**
  - How close the measured values are to each other,
  - $= f\{\text{variability of the data}\}$

- **Example: 4 Populations**
  - A, B: small system and population variability, large fold difference between the means (30-fold, ~3% CV)
  - C, D: larger dispersion around the means, small fold difference between the means (1.3-fold, ~30% CV)
Replicates

• **Biological Replicates**
  - Separate biological samples, same treatment, > variability of the biology + variability of the quantitation process
    - e.g. different RNA extractions from multiple animals, ...

• **Technical (Systematic) Replicates**
  - Aliquots from the same source run through the quantitation process independently, > variability of the process
    - e.g. triplicates for PCR from cDNA from one RT reaction

• **How Many Replicates?**
  - The greater the fold changes between the means of different populations, the fewer replicates are needed.
  - The more dispersed the population variability, the more biological replicates are needed:

<table>
<thead>
<tr>
<th>Fold Difference</th>
<th>10%</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>4</td>
<td>7</td>
<td>18</td>
<td>38</td>
<td>136</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>16</td>
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<tr>
<td>3</td>
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<td>6</td>
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<td>3</td>
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<td>11</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

one-tailed t-test
PCR Reproducibility

- **Standard Deviation and Coefficient of Variation**
  - Expressed as the Standard Deviation (SD) in $C_T$, as the square root of the variance. The variance is
    \[
    SD^2 = \frac{\sum_{i=1}^{n} (C_{T_i} - \langle C_T \rangle)^2}{n - 1}
    \]
    where $\langle C_T \rangle$ is the mean of the measured $C_T$

- Use “=STDEV(number1, number2, number3, ...)” in Excel

- The relative uncertainty in the number of DNA molecules is expressed by the CV, the **Coefficient of Variation**, which is the ratio of the standard deviation of a distribution to its arithmetic mean ($\langle X \rangle$):
  \[
  CV = \frac{SD}{\langle X \rangle}, \text{ or for QPCR: } CV = \frac{SD}{\langle C_T \rangle}, \text{ or in } \%
  \]
  \[
  CV\% = 100 \frac{SD}{\langle (1+E)^{-CT} \rangle}
  \]
  where $\langle (1+E)^{-CT} \rangle$ is the mean of $(1+E)^{-CT}$
Coefficient of Variation: Example

\[ CV\% = 100 \frac{SD}{\langle (1+E)^{-CT} \rangle} \]

0.039 / 14.561 x 100 = 0.267%

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Detector</th>
<th>Reporter</th>
<th>Task</th>
<th>Ct</th>
<th>Ct mean</th>
<th>St dev</th>
<th>CV on Ct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dil. 1:10</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>14.589</td>
<td>14.561</td>
<td>0.039</td>
<td>0.267</td>
</tr>
<tr>
<td>Dil. 1:10</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>14.577</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:10</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>14.517</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:100</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>18.115</td>
<td>18.148</td>
<td>0.092</td>
<td>0.508</td>
</tr>
<tr>
<td>Dil. 1:100</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>18.252</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>18.077</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:1000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>22.051</td>
<td>21.973</td>
<td>0.085</td>
<td>0.387</td>
</tr>
<tr>
<td>Dil. 1:1000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>21.882</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:1000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>21.882</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:10000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>25.462</td>
<td>25.365</td>
<td>0.088</td>
<td>0.348</td>
</tr>
<tr>
<td>Dil. 1:10000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>25.291</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:10000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>25.341</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. 1:10000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
<td>29.261</td>
<td>29.244</td>
<td>0.024</td>
<td>0.083</td>
</tr>
<tr>
<td>Dil. 1:10000</td>
<td>18S</td>
<td>VIC</td>
<td>Std</td>
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<td>VIC</td>
<td>Std</td>
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</table>
Calculating Standard Deviations

- SD = f{QPCR Data Analysis Method}

- For the Standard Curve Method:
  - The SDQ for the normalized (GOI/Ref) quotient Q is calculated using: \( SD_Q = CV_Q \cdot \langle X \rangle \), with \( CV_Q = (CV_{GOI}^2 + CV_{Ref}^2)^{1/2} \)

- For the Comparative Method:
  - The SDS for the difference (of \( \Delta C_T \) values) is based on the SD of the GOI AND SD of the reference values: \( SD_S = (SD_{GOI}^2 + SD_{Ref}^2)^{1/2} \)
  - The SD of the \( \Delta \Delta C_T \) is the same as the SDS.

OK, now let’s put everything together - Error Handling for the relative quantification in practice:
- a) Standard curve method, b) Comparative method
### a) Error Handling for the Standard Curve Method

- \[ N = \left( \frac{N_{GOI}}{N_{Ref}} \right) \times \left( CV_{GOI}^2 + CV_{Ref}^2 \right)^{1/2} \]

  - The average values of the GOI replicates is divided by the average values of the reference samples \((N_{GOI}/N_{Ref} =: Q)\). The SD_Q of the quotient is calculated using:

  \[ CV_Q = \frac{SD_Q}{\langle X \rangle} = \left( CV_{GOI}^2 + CV_{Ref}^2 \right)^{1/2} \]

  i.e., calculate the SDs for the replicates of GOI and Ref first, then their individual CVs. Use these CVs to calculate the CV for the normalized (GOI/Ref) using (V). Obtain the SD_Q of the quotient using \(SD_Q = CV_Q \cdot \langle X \rangle\)

<table>
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<tr>
<th></th>
<th>GOI mean</th>
<th>GOI SD</th>
<th>GOI CV</th>
<th>Ref mean</th>
<th>Ref SD</th>
<th>Ref CV</th>
<th>GOI/Ref</th>
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<td>0.051</td>
<td>0.402</td>
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*: \( SQRT[0.1026^2 + 0.063^2] = 0.12 \)

#: \( SQRT[0.039^2 + 0.051^2] = 0.06 \)

\( SD = CV \langle X \rangle = 0.12 \times 0.072 = 0.0087 \)

\( SD = CV \langle X \rangle = 0.06 \times 0.402 = 0.0258 \)

* #: samples from Table 1, slide 13

\[ SD = CV \langle X \rangle = 0.12 \times 0.072 = 0.0087 \]
b) Error Handling for the Comparative C_T Method

- N = 2^-\Delta \Delta C_T (2^-\Delta \Delta C_T-SDs - 2^-\Delta \Delta C_T+SDs )
  - Calculate mean, SD and CV for replicate C_T values of GOI and Ref, reject >4%CV.
  - Determine \Delta C_T_r = \langle C_T_{GOI} \rangle - \langle C_T_{18S} \rangle. The SD of the difference (SD_S) is based on the SD of the GOI and the SD of the reference values: SD_S = (SD_{GOI}^2 + SD_{Ref}^2)^{1/2}
  - Normalize each \Delta C_T_r value to a particular \Delta C_T_c value of an assay calibrator (cb): \Delta \Delta C_T_r = \Delta C_T_r - \Delta C_T_{cb}. The SD of the \Delta \Delta C_T_r is the same as the SD_S (SD_{\Delta \Delta C_T_r} = SD_{\Delta C_T_r}).
  - The final relative values (fold induction) are 2^-\Delta \Delta C_T with \Delta \Delta C_T_r - SD_S and \Delta \Delta C_T_r + SD_S

\[
\begin{align*}
\text{a, b: } & \sqrt{0.15^2 + 0.09^2} = 0.175, & \text{c: } & 2^{0.0+0.175} = 1.1, 2^{0.0-0.175} = 0.88 \\
a, b: & \sqrt{0.06^2 + 0.08^2} = 0.100, & \text{c: } & 2^{2.5+0.100} = 6.06, 2^{2.5-0.100} = 5.28
\end{align*}
\]
Remarks to Quantitative Precision

- **Implications**
  - The calculations of precision given above have been questioned in some peer-reviewed publications.
  - Replicate standard curves may produce potentially large inter-curve variations.
  - In general, the intra-assay variation of 10-20% and a mean inter-assay variation of 15-30% on molecule basis is realistic over the wide dynamic range (of over a billion fold range).
  - Variability is highest at $>10^7$ and $<10^2$ template copy ranges
    - Cut-off value: cycle 35, i.e. disregard $C_T$ values for cycle numbers 36 and higher.
  - For the threshold methods, the precision is dependent on the proper setting of the threshold, which itself is dependent on proper base line settings.
## A Recent User Submission

<table>
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<th>GOI</th>
<th>REF</th>
<th>AV GOI</th>
<th>AV Ref</th>
<th>STDEV GOI</th>
<th>STDEV REF</th>
<th>CV on CT GOI</th>
<th>CV on CT ref</th>
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<th>SD ΔCT</th>
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Integrated Genomics - The Future?

• Real-Time StatMiner™
Optimizing Primer Concentrations

- **Primer Optimization Matrix**
  - Maximize $\Delta R_n$:

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<th>Reverse Primer [nM]</th>
<th>Forward Primer [nM]</th>
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<tr>
<td></td>
<td>300/50</td>
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<tr>
<td></td>
<td>900/50</td>
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<td>300/900</td>
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<tr>
<td></td>
<td>900/900</td>
</tr>
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</table>

- Suggested conc.:
  - 900nM for TaqMan
  - 50nM for SYBR Green

*Applied Biosystems SDS Chemistry Guide (PN 4348358)*
Optimizing Probe Concentrations

- Secondary to Primer Optimization
  - Maximize $\Delta R_n$

<table>
<thead>
<tr>
<th>Primer [nM]</th>
<th>Probe [nM]</th>
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<tbody>
<tr>
<td>100/900</td>
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<tr>
<td>100/900</td>
<td>125</td>
</tr>
<tr>
<td>100/900</td>
<td>250</td>
</tr>
<tr>
<td>100/900</td>
<td>500</td>
</tr>
</tbody>
</table>

- Suggested conc.: 250nM
Optimizing Genotyping Experiments

- Scattering of Data Points / Diffuse Clusters
  - Low DNA concentrations
  - Suggested: > 1ng (relatively high)

Applied Biosystems SDS Chemistry Guide (PN 4348358)
Multiplexing

- **Primer-Limited Assays**
  - ABI Vic® reporter dyes are primer limited, allowing multiplexing of TaqMan® endogenous controls with GOI quantitation.
  - Extensive assay optimization
  - Normal probe levels: 250nM
  - Suggested primer conc.:
    - 50nM or less
  - Determine plateau region:
    - CT values are constant

*Applied Biosystems SDS Chemistry Guide (PN 4348358)*
Revisiting the Goals

• Questions a PI should ask when presented with QPCR data:
  - How does this assay integrate with the project?
    • 1 primer pair per question! (1pppq)
  - Did you use a 'One-step' kit?
    • If “Yes” -> deny the assay!
  - What assay was used? commercial or custom design?
  - What chemistry was used? Why?
    • If TaqMan: MGB or conventional probe?
  - What is the amplification efficiency (E) for this amplicon?
    • Show me the ‘Primer validation’ experiment!
  - How do the amplification plots look like?
    • How did you adjust the baseline, the threshold?
  - How many times did you measure this result? How many runs were necessary to get to this result?
  - What method of data evaluation did you use?
    • If ΔΔC_T: show me the validation experiment.
  - How many replicates were used for the measurements?
  - Are any C_T values larger than 35?
  - What did you do for error handling?
Selected References

- Bookout A. and Mangelsdorf D. (2003), Nuclear Receptor Signaling 1, e012,
- Livak, K.J. et al. (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl.4:357-62