Amplification Efficiency:
linking baseline and bias
in qPCR data analysis

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qPCR Data

RNA

RT

cDNA

etc.

\[ N_n = N_0 E^n \]

PCR product after n cycles

number of PCR cycles

Efficiency (1-2; 2=100%)

start concentration

\( n \) cycles

\( 0 \)  1  2  3

\( 1 \)  2  4  8  N

\( 2^0 \)  \( 2^1 \)  \( 2^2 \)  \( 2^3 \)
Observed (=raw) Data

\[ N_{n,\text{raw}} = \text{baseline} + N_0 E^n \]
qPCR Analysis Principle

\[ N_n = N_0 E^n \]

\[ N_0 = \frac{N_q}{E^{C_q}} \]
Target / Reference Ratio

\[ \frac{N_n}{N_t} = \frac{E_{R}^{C_q,R}}{E_{T}^{C_q,T}} \]

\[ \frac{N_n}{N_t} = \left( \frac{N_{q,T}}{E_{T}^{C_q,T}} \right) / \left( \frac{N_{q,R}}{E_{R}^{C_q,R}} \right) \]

Ratio \[ = \frac{N_{0,T}}{N_{0,R}} \]
Definition of PCR efficiency

\[ \text{Eff} = \frac{N_{c+1}}{N_c} \]
Efficiency and Baseline

\[ \text{Eff} = \frac{N_{c+1}}{N_c} \]

baseline too low:

\[ \text{Eff}_L = \frac{N_{c+1} + A}{N_c + A} \]
Efficiency and Baseline

\[ Eff = \frac{N_{c+1}}{N_c} \]

baseline too low:

\[ Eff_L = \frac{N_{c+1} + A}{N_c + A} < Eff \]
Efficiency and Baseline

\[ Eff = \frac{N_{c+1}}{N_c} \]

baseline too high:

\[ Eff_H = \frac{N_{c+1} - A}{N_c - A} > Eff \]
Effect of Baseline Error

Target / Reference ratio: >1000%

$N_0 \pm 200\%$

Eff. $\pm 2\%$

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Heart Failure Research Centre
System Baseline Correction

baseline = regression line through X ground phase cycles

raw data = not baseline corrected

system baseline trends are extrapolated ground phase noise
Recognizing Baseline Error

Baseline error
- ▲ 5% \{ over-estimated \}
- ▲ 1% \{ over-estimated \}
- □ 0% \{ correct \}
- ◦ 1% \{ under-estimated \}
- ● 5% \{ under-estimated \}

Log(Fluorescence)
Baseline Estimation Principle

$S_{\text{top}} > S_{\text{bottom}}$
baseline too low

$S_{\text{top}} < S_{\text{bottom}}$
baseline too high

$S_{\text{top}} = S_{\text{bottom}}$
baseline OK

Log(Fluorescence)

10^{-2} - 10^{+2}

Cycle

10 - 40

SDM-cycle
Baseline Estimation Algorithm

Baseline Estimate

Slope

Stop criterion:

\[ |S_{\text{top}} - S_{\text{bottom}}| < 0.00001 \]
LinRegPCR Baseline Correction

raw data

LinRegPCR

BL 3-5
Baseline too high
Baseline too low

BL 3-10

BL 3-15

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qPCR Analysis per Sample

\[ N_0 = \frac{N_q}{E_{\text{individual}}^C} \]
\[ N_n = N_0 E^n \]

LinRegPCR (v7.4)
(Ramakers et al. 2003)
qPCR Analysis per Sample

\[ N_n = N_0 E^n \]

- \( E_{\text{individual}} \)
- \( E_{\text{amplicon}} \)
- \( N_0 \)
Efficiency derived from Standard Curve

\[ C_q \]

\[ \log(N_0) \]
Efficiency derived from Standard Curve

5 dilutions
5 replicates per dilution \} 3125 standard curves
Efficiencies derived from Standard Curve

5 dilutions
5 replicates per dilution \{ 3125 standard curves

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Efficiency derived from Standard Curve

Window-of-Linearity

5 dilutions
5 replicates per dilution \{ \text{25 individual efficiency values} \}
Efficiencies derived from Standard Curve

W-o-L-derived PCR Eff. vs. Frequency

PCR Efficiency

E_{\text{mean}}
Variation of individual PCR Efficiencies

GAPDH: 20 biological samples, 10 replicates/sample
qPCR Analysis per Sample

\[ N_n = N_0 E^n \]

- \( E_{\text{individual}} \)
- \( E_{\text{mean, amplicon}} \)

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e.g. Karlen et al. 2007
Cikos et al. 2008
Ruijter et al. 2009
Efficiency and Bias

PCR efficiency

- Reference
- Gene of Interest

*individual windows*

*common window*

*amplicon windows*

$E_{\text{mean}}$

$E_{\text{common}}$

$E_{\text{mean}}$
Expression Ratio

$N_0$ ratio

$Bias = \left( \frac{E\text{ Amplicon}}{E\text{ common}} \right)^{C_{t,B}+C_{t,A}}$

$$Bias = \left( \frac{1.91}{1.87} \right)^{(20+24)} = 3$$

3 or 7% interest
Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data

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program (latest version) and manual:
http://LinRegPCR.HFRC.nl  (direct download)

Frequently Asked Questions:
http://LinRegPCR.nl
Efficiency derived from Standard Curve

\[ \log(N_t) = \log(N_0) + C_t \cdot \log(E) \]

\[
C_t = \frac{\log(N_t)}{\log(E)} - \frac{1}{\log(E)} \log(N_0)
\]
Efficiency derived from Standard Curve

\[ \log(N_t) = \log(N_0) + C_t \cdot \log(E) \]

Ct = \frac{\log(N_t)}{\log(E)} - \frac{1}{\log(E)} \log(N_0)
Efficiency derived from Standard Curve

5 dilutions
5 replicates per dilution \{ 3125 standard curves
Efficiencies derived from Standard Curve

5 dilutions
5 replicates per dilution \Rightarrow 3125 standard curves

\[ E_{\text{mean}} \]
Bias from using the Wrong Efficiency

- Max. SC-derived efficiency
- Min. SC-derived efficiency

N₀ vs. dilution graph.
Bias from using the Wrong Efficiency

- N0
- max. SC-derived efficiency
- min. SC-derived efficiency

100x

10x

dilution
<table>
<thead>
<tr>
<th><strong>Input</strong></th>
<th><strong>Hybridisation</strong></th>
<th><strong>Amplification</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA primers</td>
<td>ds-cDNA primer - cDNA</td>
<td>specific amplicon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-specific</td>
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<td>(expon)</td>
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<td></td>
<td>a-specific</td>
</tr>
<tr>
<td></td>
<td>(linear)</td>
</tr>
</tbody>
</table>
Baseline Model?

Input
- cDNA
- primers

Hybrids
- ds-cDNA
- primer - cDNA
- primer - amplicon
- amplicon - cDNA
- ds-amplicon

Baseline Fluorescence
- changing concentrations
- competition for Sybr Green

Specific Fluorescence

Fluorescence values in first cycles cannot be used to estimate the baseline