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1st International qPCR Symposium & Application Workshop

# Determination of real-time PCR efficiency - an overview

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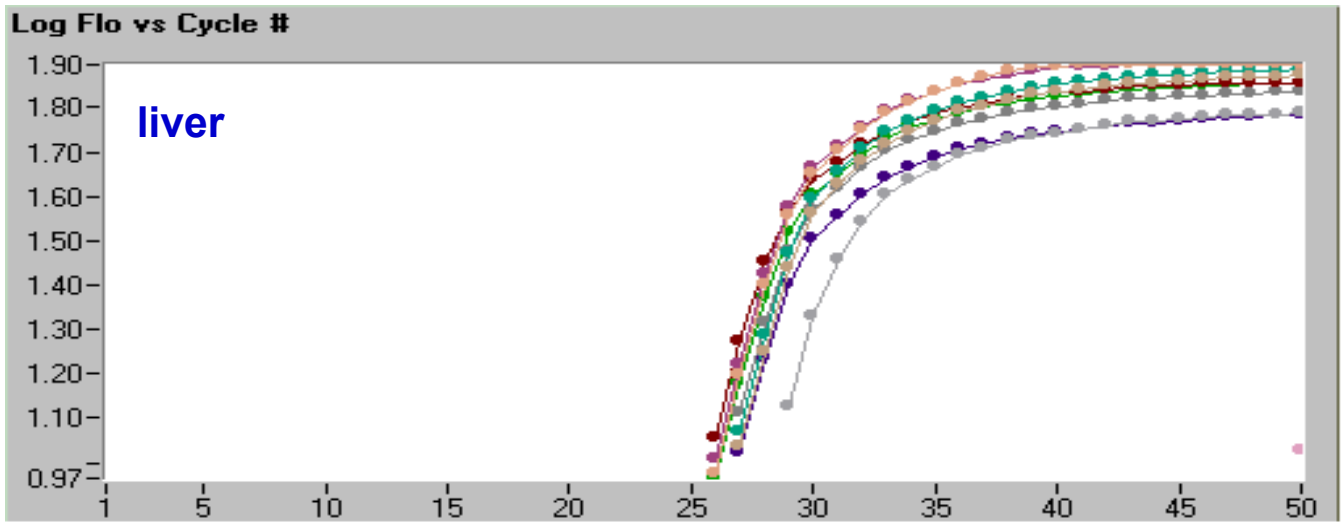
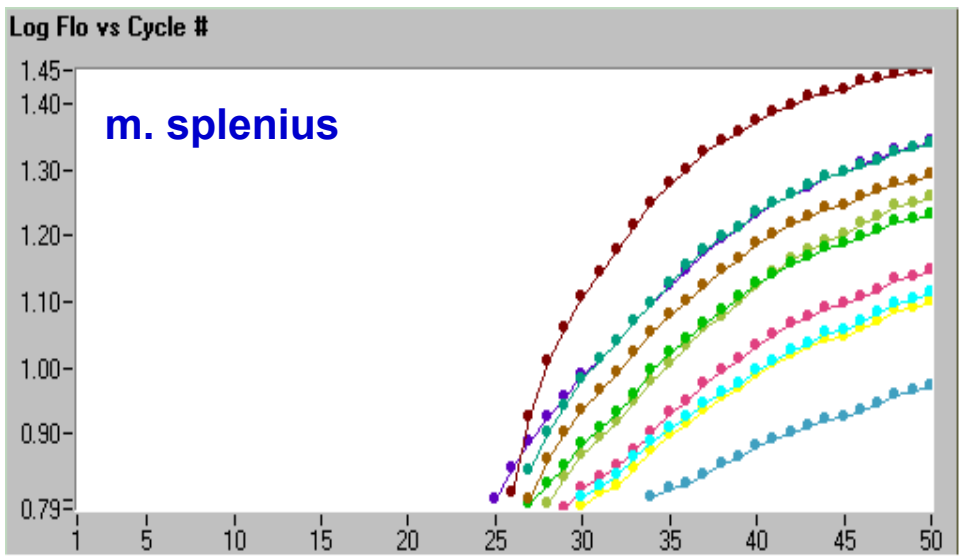
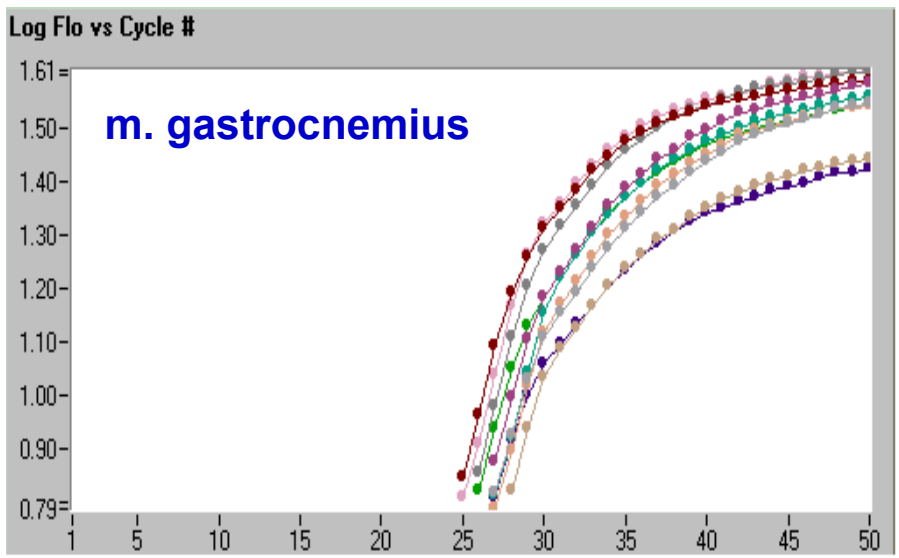
# *„Tissue matrix"* interference with real-time PCR efficiency and amplification fidelity

(IGF-1 mRNA amplification in three cattle tissues)



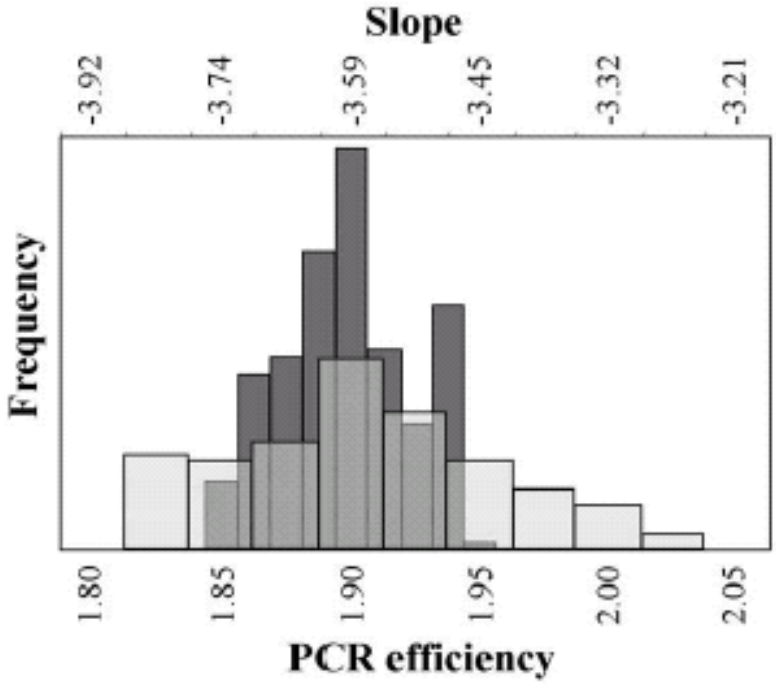



# Determination of RT real-time PCR efficiencies

**273 bp GAPDH amplicon**

**calculated from 729 slope of the standard curve;**

**six different total RNA concentrations**




**E = 1.81 – 2.02**

**E = 1.86 – 1.94 triplicate**

**Assumption-free analysis of quantitative real-time PCR data**

Ramakers et al. (2003) Neurosci Lett **339(1):** 62-66

## PCR inhibitors:

Hemoglobin, Urea, Heparin
Organic or phenolic compounds
Glycogen, Fats, $Ca^{2+}$
**Tissue matrix effects**
Laboratory items, powder, etc.

## PCR enhancers:

DMSO, Glycerol, BSA
Formamide, PEG, TMANO, TMAC etc.
**Special commercial enhancers:**
Gene 32 protein, Perfect Match, Taq Extender,
E.Coli ss DNA binding

# real-time PCR efficiency

DNA degradation

Tissue degradation

unspecific PCR products

Lab management

DNA dyes

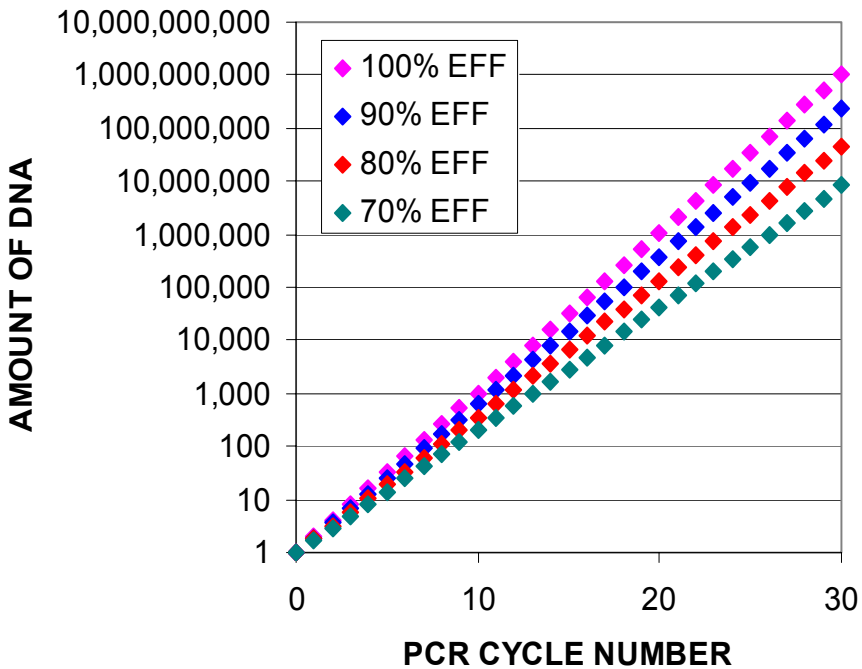
Cycle conditions

Hardware: PCR platform & cups

PCR reaction components

DNA concentration

| CYCLE | AMOUNT OF DNA 100% EFFICIENCY | AMOUNT OF DNA 90% EFFICIENCY | AMOUNT OF DNA 80% EFFICIENCY | AMOUNT OF DNA 70% EFFICIENCY |
|---|---|---|---|---|
| 0 | 1 | 1 | 1 | 1 |
| 1 | 2 | 2 | 2 | 2 |
| 2 | 4 | 4 | 3 | 3 |
| 3 | 8 | 7 | 6 | 5 |
| 4 | 16 | 13 | 10 | 8 |
| 5 | 32 | 25 | 19 | 14 |
| 6 | 64 | 47 | 34 | 24 |
| 7 | 128 | 89 | 61 | 41 |
| 8 | 256 | 170 | 110 | 70 |
| 9 | 512 | 323 | 198 | 119 |
| 10 | 1,024 | 613 | 357 | 202 |
| 11 | 2,048 | 1,165 | 643 | 343 |
| 12 | 4,096 | 2,213 | 1,157 | 583 |
| 13 | 8,192 | 4,205 | 2,082 | 990 |
| 14 | 16,384 | 7,990 | 3,748 | 1,684 |
| 15 | 32,768 | 15,181 | 6,747 | 2,862 |
| 16 | 65,536 | 28,844 | 12,144 | 4,866 |
| 17 | 131,072 | 54,804 | 21,859 | 8,272 |
| 18 | 262,144 | 104,127 | 39,346 | 14,063 |
| 19 | 524,288 | 197,842 | 70,824 | 23,907 |
| 20 | 1,048,576 | 375,900 | 127,482 | 40,642 |
| 21 | 2,097,152 | 714,209 | 229,468 | 69,092 |
| 22 | 4,194,304 | 1,356,998 | 413,043 | 117,456 |
| 23 | 8,388,608 | 2,578,296 | 743,477 | 199,676 |
| 24 | 16,777,216 | 4,898,763 | 1,338,259 | 339,449 |
| 25 | 33,554,432 | 9,307,650 | 2,408,866 | 577,063 |
| 26 | 67,108,864 | 17,684,534 | 4,335,959 | 981,007 |
| 27 | 134,217,728 | 33,600,615 | 7,804,726 | 1,667,711 |
| 28 | 268,435,456 | 63,841,168 | 14,048,506 | 2,835,109 |
| 29 | 536,870,912 | 121,298,220 | 25,287,311 | 4,819,686 |
| 30 | 1,073,741,824 | 230,466,618 | 45,517,160 | 8,193,466 |




M. Hunt et al., Department of Pathology and Microbiology, University of South Carolina

http://www.med.sc.edu:85/pcr/realtime-home.htm

# Efficiency variation in real-time RT-PCR

| Detection Cycle (n) PCR efficiency | 10 | 15 | 20 | 25 | 30 | 35 |
|---|---|---|---|---|---|---|
| 2.00 | - | - | - | - | - | - |
| 1.97 | 16 % | 25 % | 35 % | 46 % | 57 % | 70 % |
| 1.95 | 29 % | 46 % | 66 % | 88 % | 113 % | 142 % |
| 1.90 | 67 % | 116 % | 179 % | 260 % | 365 % | 500 % |
| 1.80 | 187 % | 385 % | 722 % | 1 290 % | 2 260 % | 3 900 % |
| 1.70 | 408 % | 1 045 % | 2 480 % | 5 710 % | 13 000 % | 29 500 % |
| 1.60 | 920 % | 2 740 % | 8 570 % | 26 400 % | 80 700 % | 246 400 % |

**Table 1:** Errors of quantification relative to a theoretical PCR efficiency of 2.00

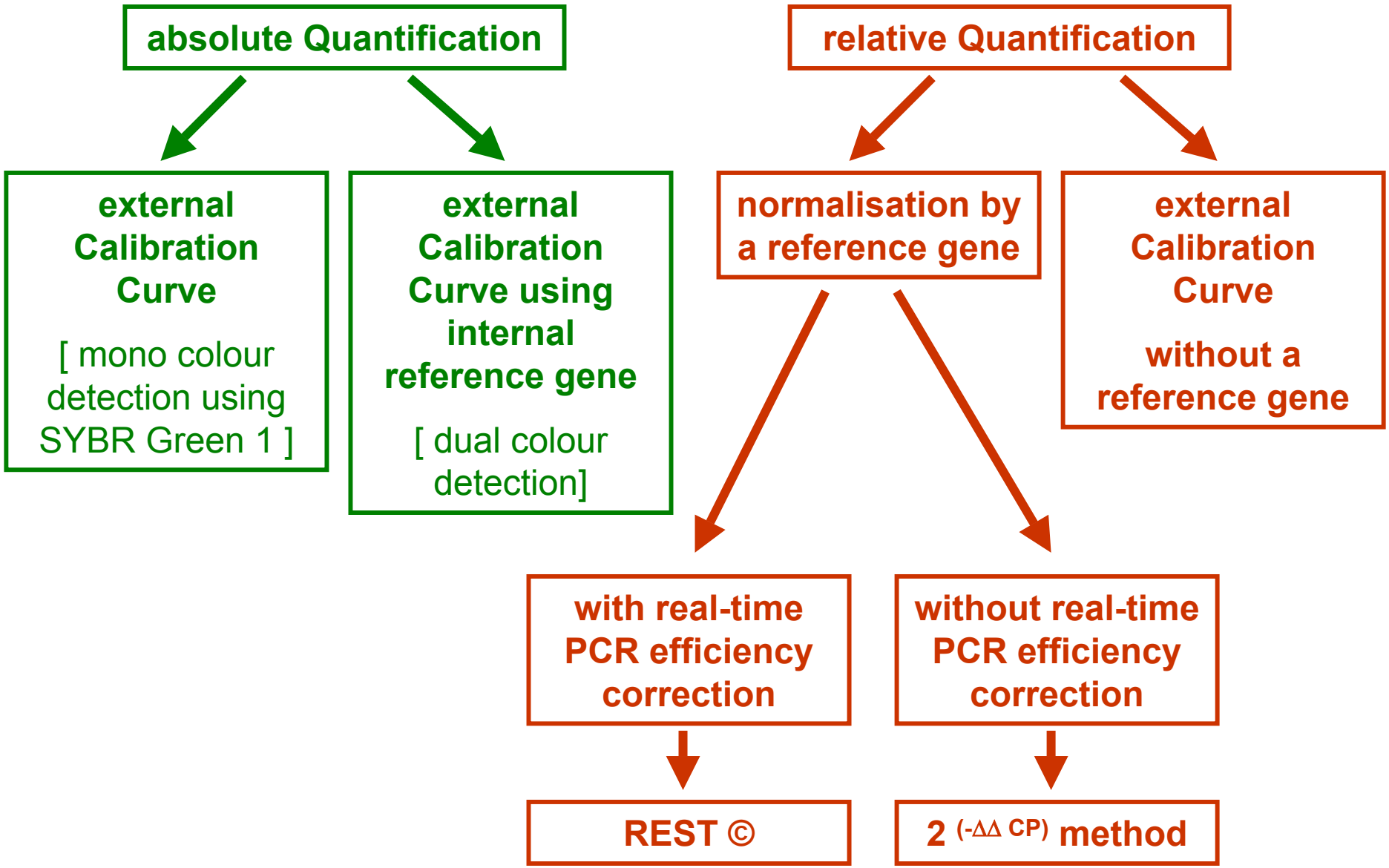
Error calculation: $(2^n/E^n-1) \times 100$

**Example:**

When the difference in PCR efficiency is 0.2 between two samples in the same run and both samples are detected in cycle 25 a more than 10-fold difference in the amount of the samples will be calculated.

*Roche Diagnostics, **LIghtCycler rel. Quantification software**, March 2001*

# Quantification strategies in real-time RT-PCR




Gene.Quantifiaction@wzw.tum.de

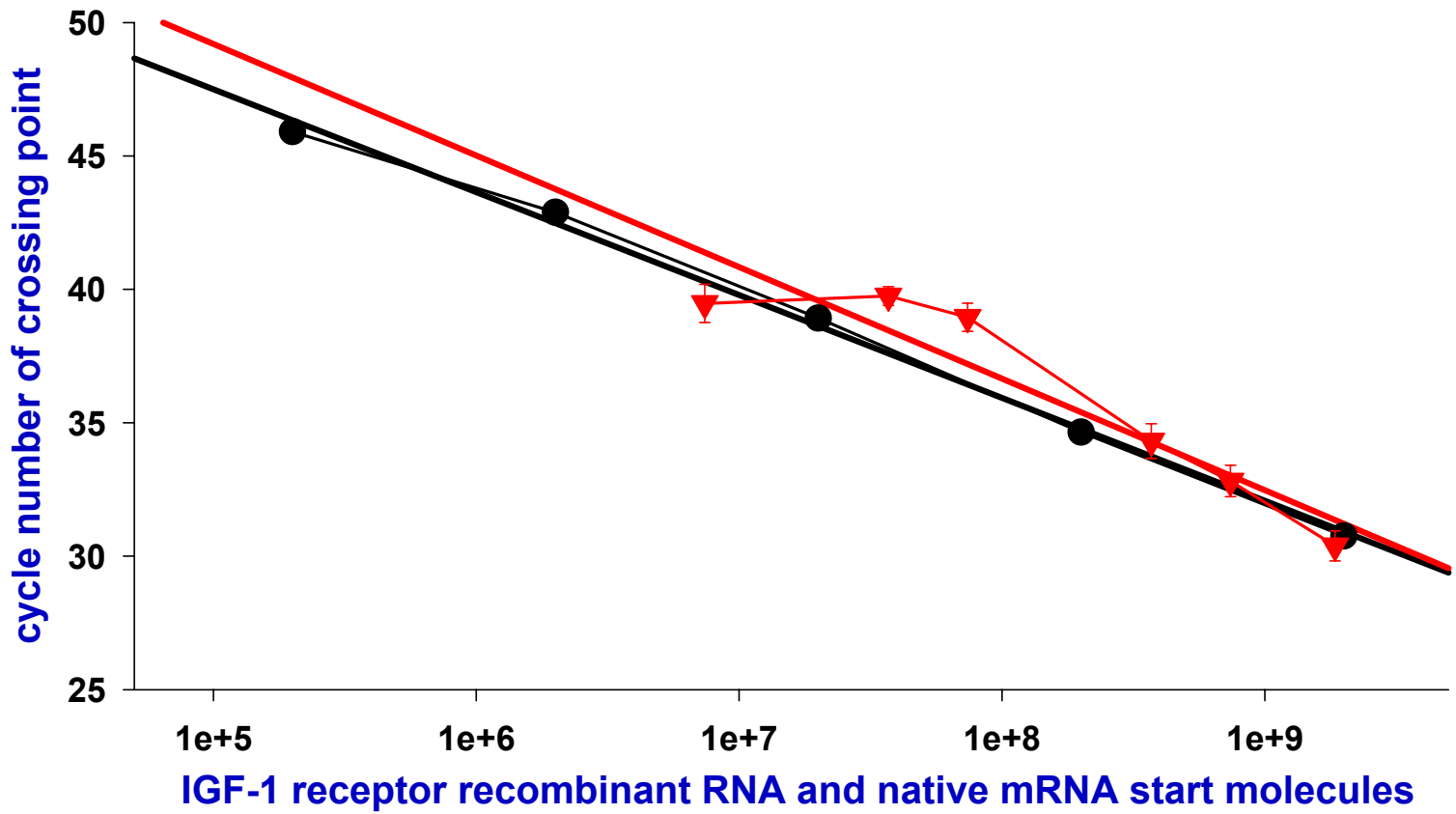
# Absolute quantification of IGF-1 receptor

**two step qRT-PCR efficiency (recombinat RNA) = 1.81**

(n = 4; r = 0.998; $2 * 10^5$ - $2 * 10^9$ recRNA standard molecules)

**two step qRT-PCR efficiency (native mRNA molecules) = 1.78**

(n = 4; r = 0.939; 0.1 - 25.0 ng total muscle RNA)




*Pfaffl et al., 1998*

## *Relative quantification of a target gene versus a reference gene (housekeeping gene)*

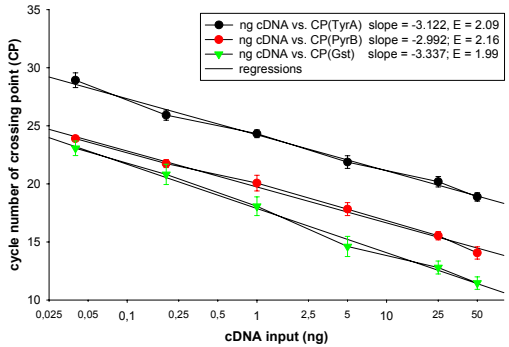
$$\text{relative expression} = \frac{E_{target}^{\Delta CP_{target}\,(control\,-\,sample)}}{E_{reference}^{\Delta CP_{ref}\,(control\,-\,sample)}}$$

*Pfaffl, Nucleic Acids Research 2001*

$$\text{relative expression} = \frac{(E_{ref})^{CP_{Sample}}}{(E_{target})^{CP_{Sample}}} \div \frac{(E_{ref})^{CP_{Calibrator}}}{(E_{target})^{CP_{Calibrator}}}$$

*Roche Diagnostics, LC relative Quantification software, March 2001*

# Determination principles of real-time PCR efficiency



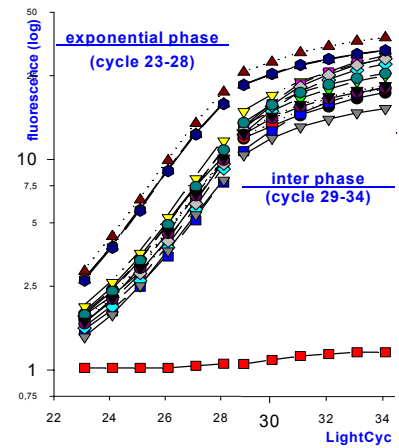

## Direct methods:

- Dilution series

  *(Rasmussen 2001, Peirson et al. 2003, etc.)*

- Determination of absolute increase in fluorescence

  *(Rasmusen 2001; Peccoud & Jacob 1998; Pfaffl 2001)*




## Indirect methods: Fit of mathematical models

- Sigmoidal model

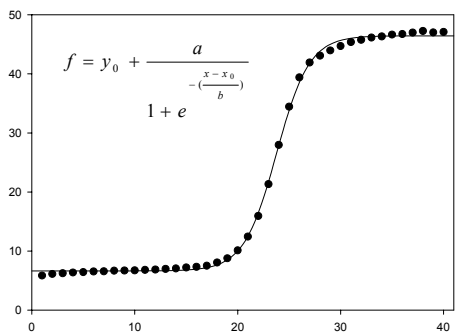
  *(Lui & Saint 2002; Tichopad et al. 2002 & 2004)*

- Logistic model

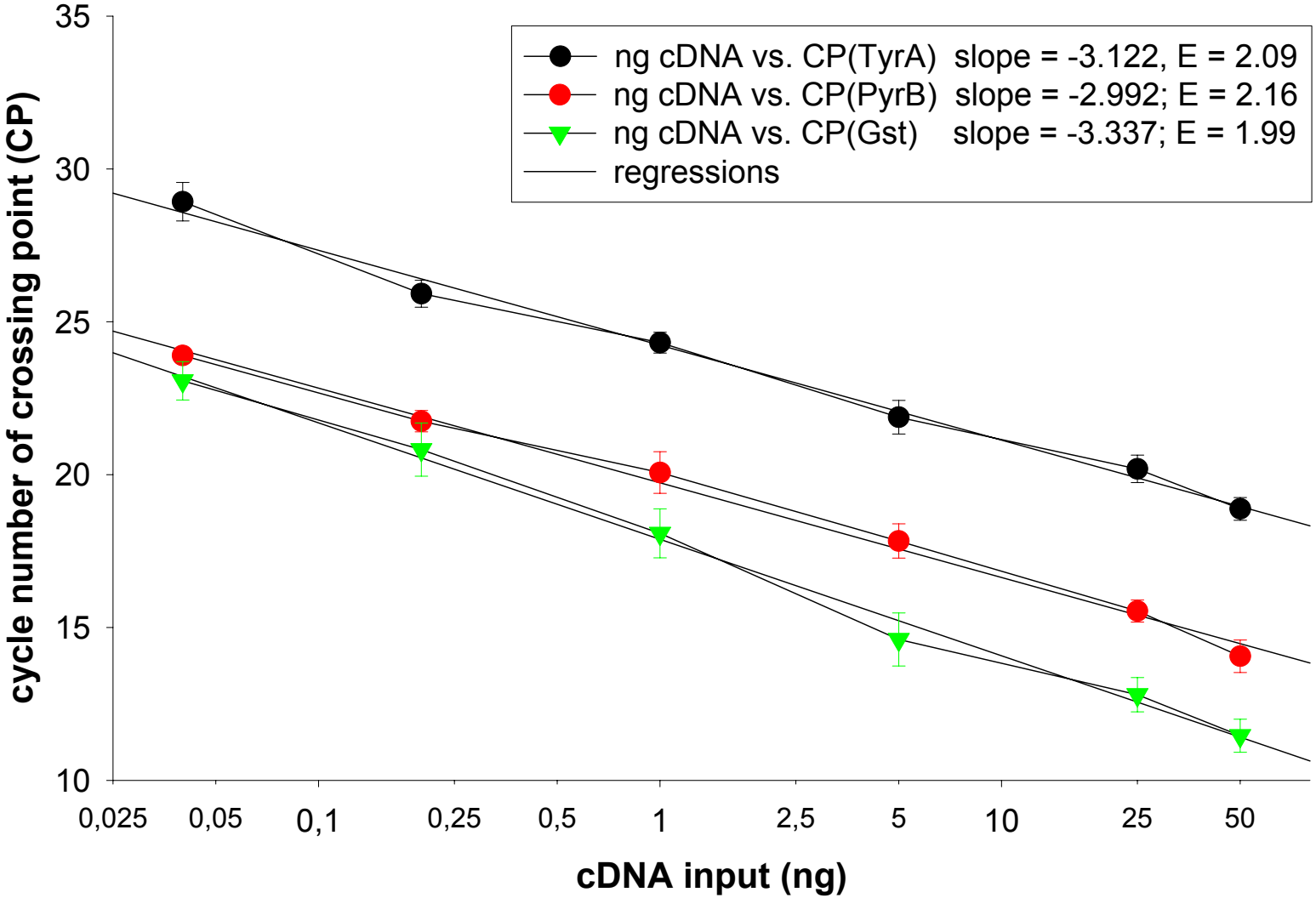
  *(Wittwer et al. 2000; Tichopad et al. 2003)*

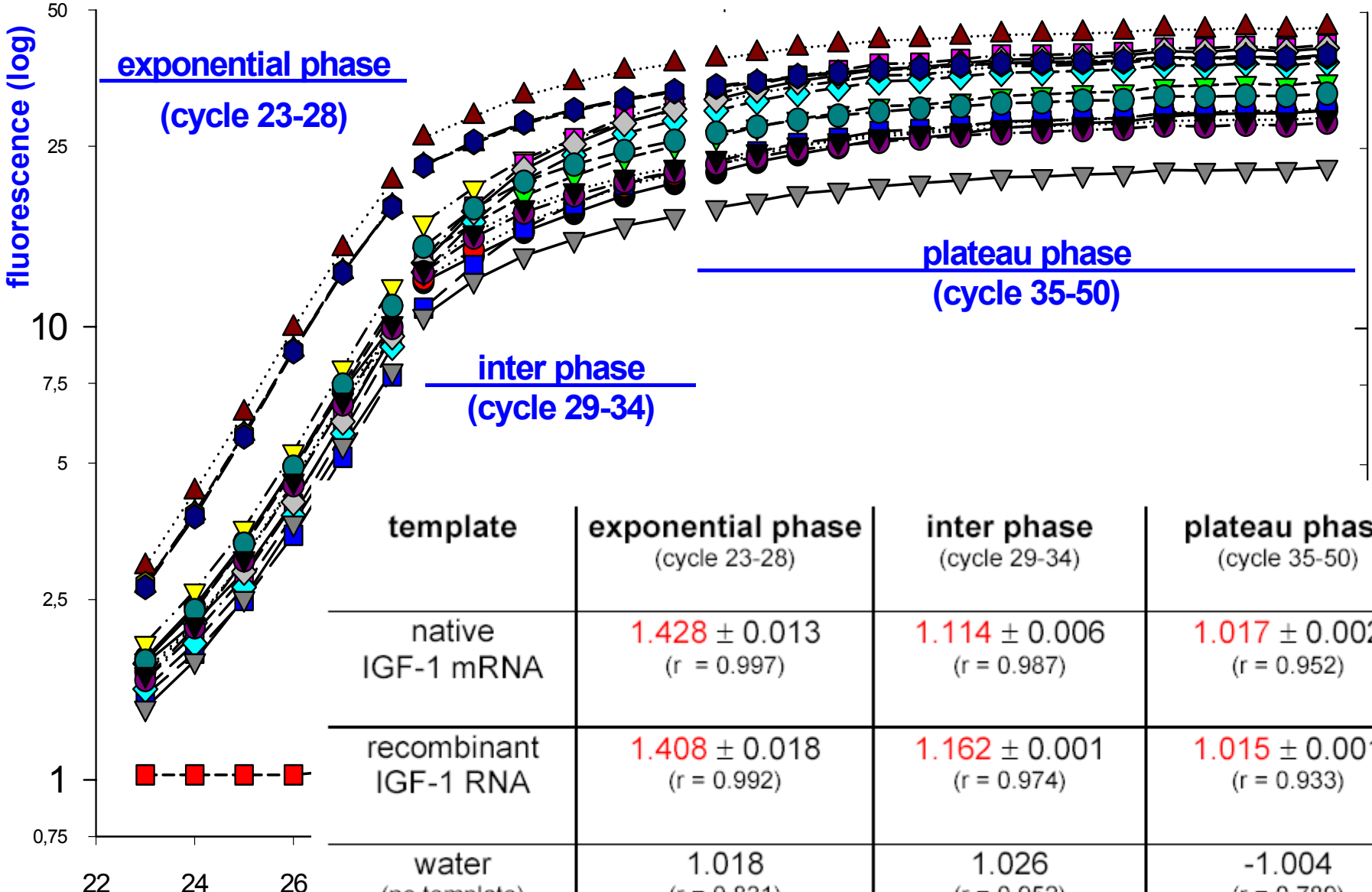
- Comparative Quantitation Analysis

  Rotor-Gene 3000 software



# Determination principles of real-time PCR efficiency:
# **Dilution series**






| template | exponential phase (cycle 23-28) | inter phase (cycle 29-34) | plateau phase (cycle 35-50) |
|---|---|---|---|
| native IGF-1 mRNA | 1.428 ± 0.013 (r = 0.997) | 1.114 ± 0.006 (r = 0.987) | 1.017 ± 0.002 (r = 0.952) |
| recombinant IGF-1 RNA | 1.408 ± 0.018 (r = 0.992) | 1.162 ± 0.001 (r = 0.974) | 1.015 ± 0.001 (r = 0.933) |
| water (no template) | 1.018 (r = 0.831) | 1.026 (r = 0.952) | -1.004 (r = 0.789) |

## Statistical estimations of PCR amplification rates

Peccoud J & Jacob C (1998) in *Gene Quantification (eds. Francois Ferre)*

We have previously proposed and characterized the estimator $\hat{m}_n$ (Jacob and Peccoud, 1996a; Jacob and Peccoud, 1996b) which fits the 3 requirements aforementioned:

$$\hat{m}_n = \frac{X_{n-2} + X_{n-1} + X_n}{X_{n-3} + X_{n-2} + X_{n-1}} \qquad (4)$$

## A new quantitative method of real time RT-PCR assay based on simulation of polymerase chain reaction kinetics

Liu W & Saint DA, *Anal Biochem.* 2002 **302(1):** 52-59

$$\left(\frac{R_{n,A}}{R_{n,B}}\right)^{\frac{1}{C_{t,A}-C_{t,B}}} = (1+E)$$

or

$$E = \left(\frac{R_{n,A}}{R_{n,B}}\right)^{\frac{1}{C_{t,A}-C_{t,B}}} - 1 \;..$$

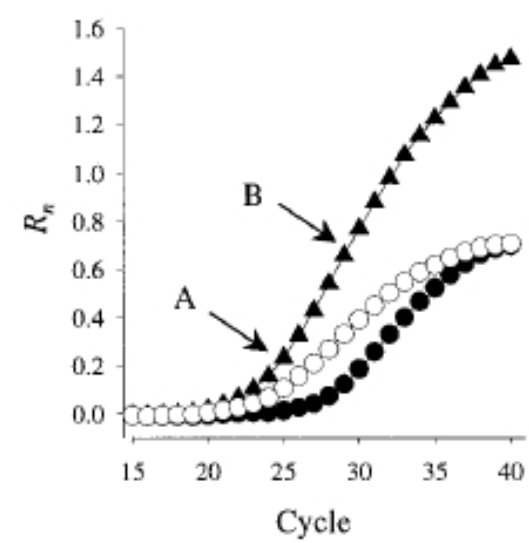
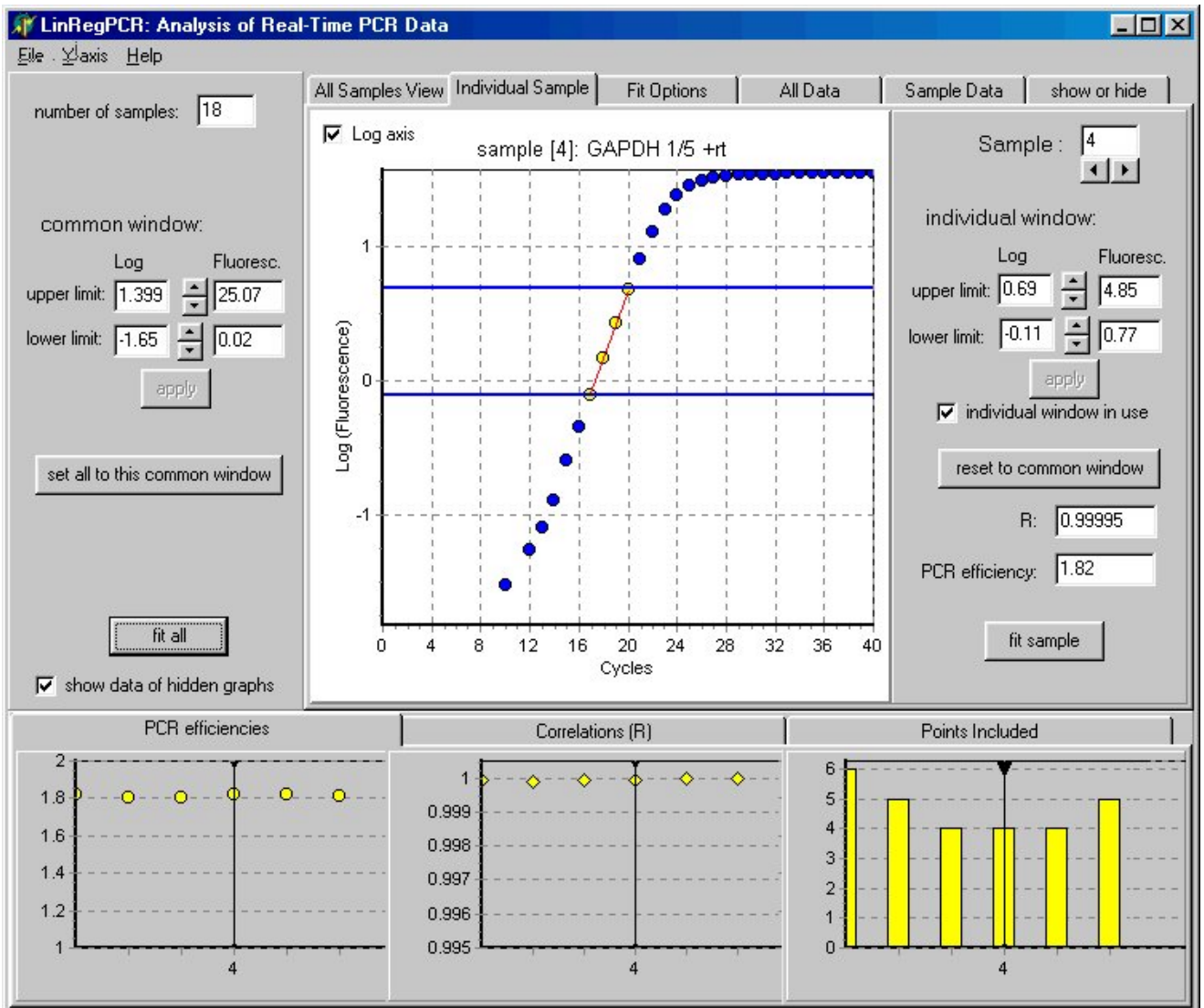



FIG. 1. Calculation of amplification efficiency from the kinetic PCR curve. Three curves were chosen representing *GAPDH* (▲) and two different target genes (○ and ●). $R_n$ is measured at two thresholds along the exponential phase as shown at A and B. Amplification efficiency is calculated using Eq. [3].

# Calculation of real-time PCR efficiency: **LinRegPCR Interface**

Ramakers et al., Neurosci Lett 2003 Mar 13;339(1): 62-66

1. 4-6 data points in exponential phase
2. Data input from LightCycler and ABI software



# Kinetic Outlier Detection (KOD) in real-time PCR

Tzachi Bar et al., Nucleic Acids Research, 2003, **31(17)** e105

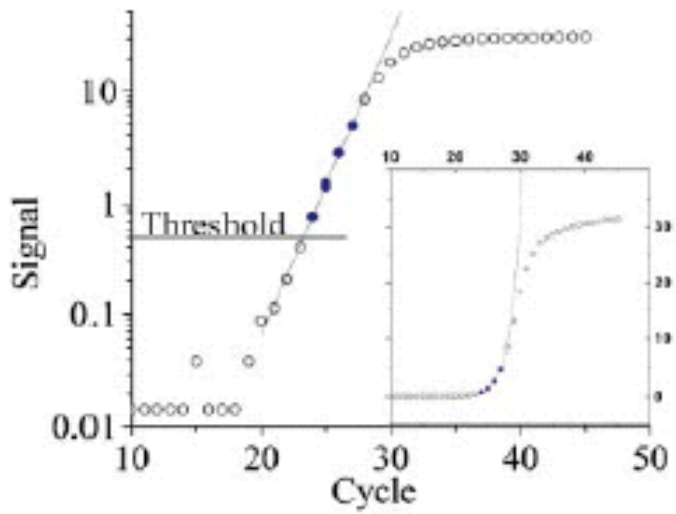



**Figure 1.** Estimation of PCR efficiency by exponential fit. Three to five data points (filled circles) above threshold level are fitted by an exponential equation **1** to estimate PCR efficiency. Main frame: semi-logarithmic scale, inset: linear scale.

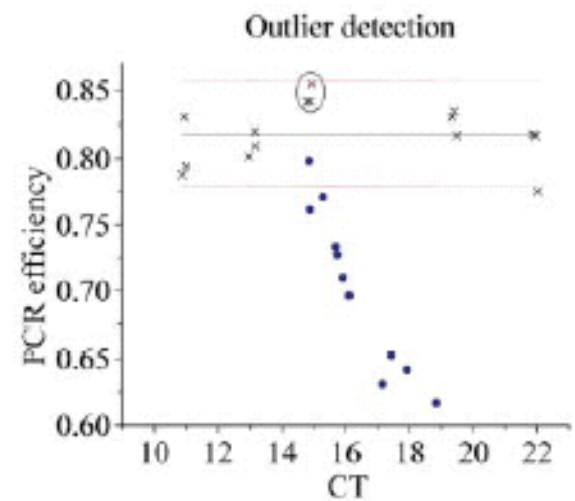
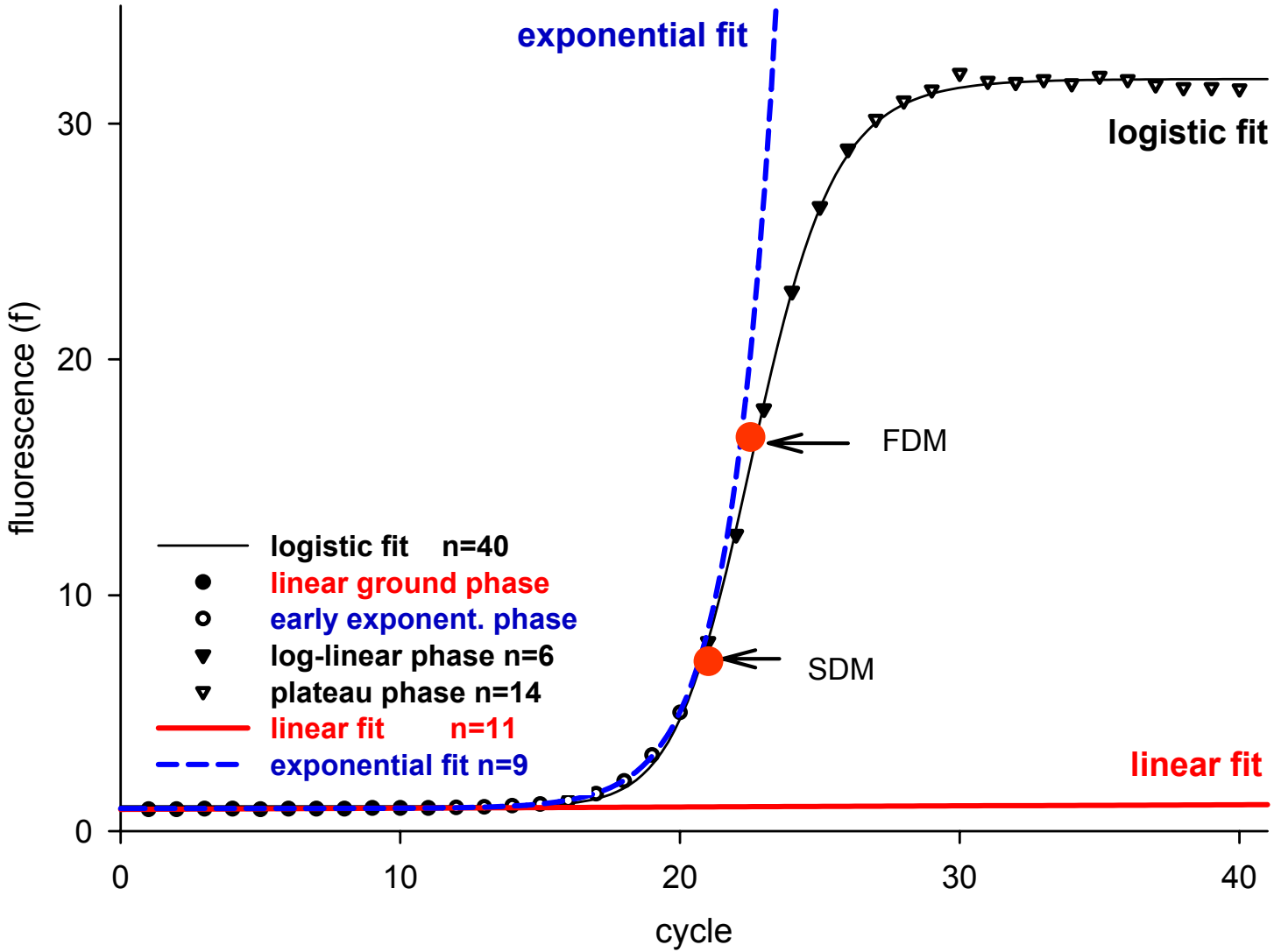


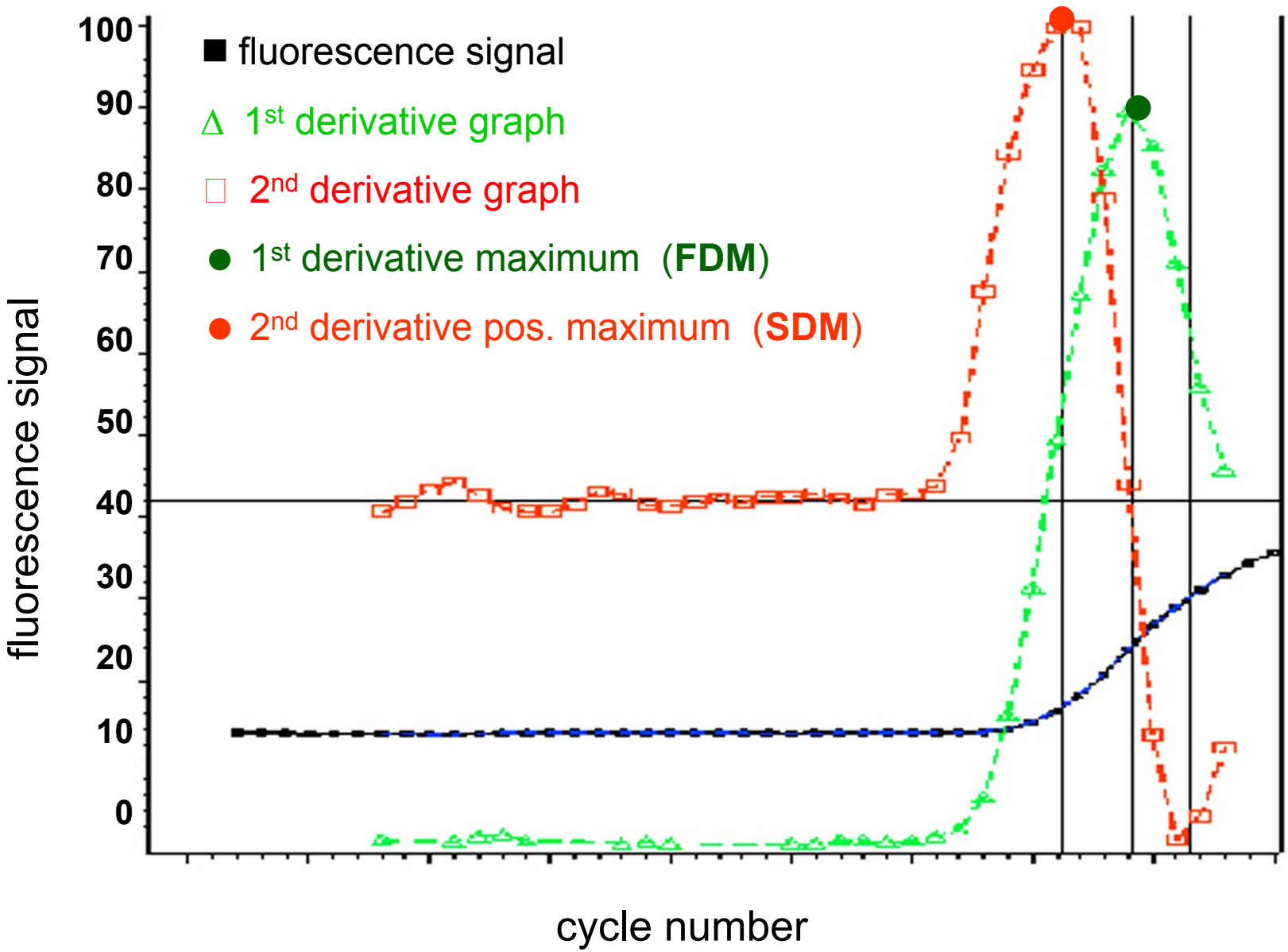

**Figure 6.** Dilution series of purified PCR product was used as a training set (crosses) for outlier detection in test samples (circles) containing equal starting numbers of template molecules as the encircled concentration in the dilution series, but with elevated concentrations of dNTP as inhibitor. PCR efficiency was estimated by exponential fit in the optimal range of setting and plotted versus CT. The central line is the mean and the dotted lines indicate 95% confidence interval of the efficiency.

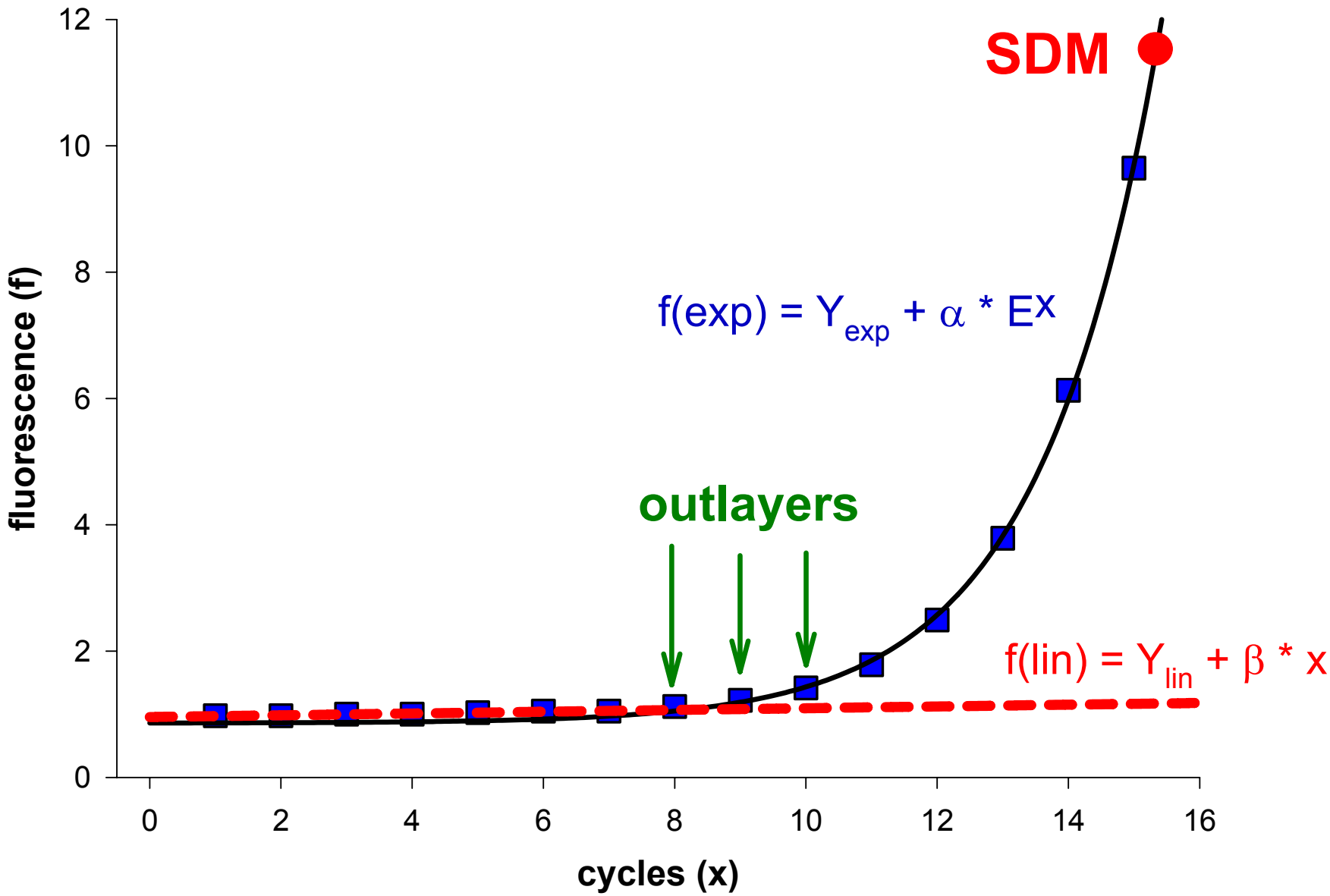
# Standardized determination of real–time PCR efficiency from a single reaction setup

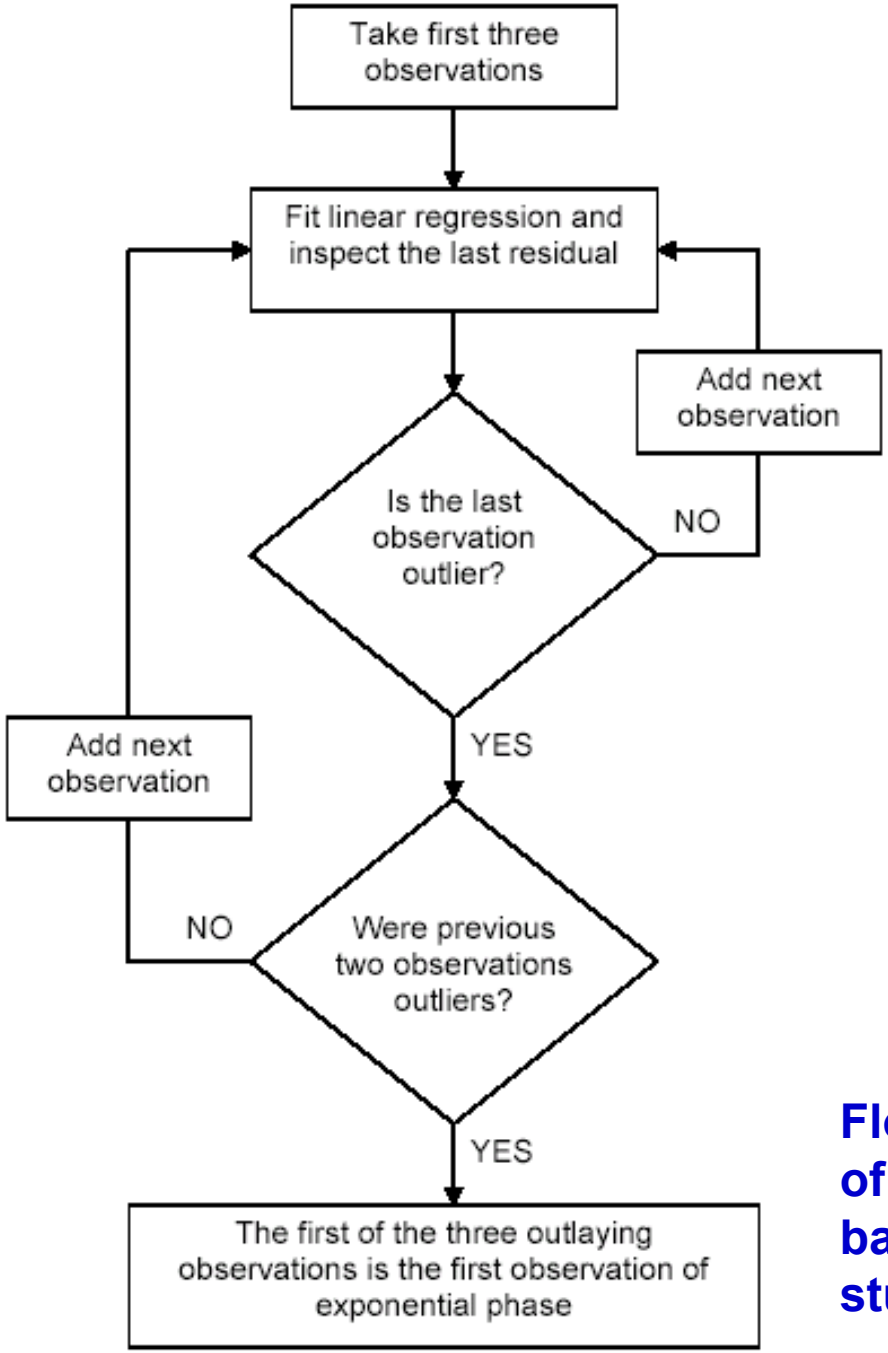
Tichopad et al., 2003 Nucleic Acids Research **31(20):** e122



# Principal of *"Second Derivative Maximum"* method




SDM
f(exp) = Y_exp + α * E^x
outlayers
f(lin) = Y_lin + β * x
fluorescence (f)
cycles (x)
0
2
4
6
8
10
12
14
16




**Flowchart of statistical estimation of the exponential phase beginning based on inspection of externally studentised residuals.**

# Comparison of different methods

# for optimal CP and real-time PCR efficiency determination

| | | | | $E1_{fit\ point}$ | | | $E1_{SDM}$ | | | $E2_{FDM}$ | | | | $E2_{SDM}$ | | | | $E_{new}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conc. | n | $CP_{fp}$ | $CP_{sdm}$ | $E_{all}$ | Y | CV% [Y] | $E_{all}$ | Y | CV% [Y] | E | CV % [E] | Y | CV% [Y] | E | CV% [E] | Y | CV% [Y] | E | CV% [E] | Y | CV% [Y] |
| **2.65E+07** | 3 | 11.02 | 14.10 | | 8.58E+10 | 138.40 | | 2.67E+11 | 5.40 | 1.37 | 0.23 | 2.59E+09 | 5.49 | 1.47 | 0.19 | 1.04E+09 | 1.47 | 1.84 | 0.40 | 1.43E+11 | 7.46 |
| **2.65E+06** | 3 | 15.93 | 17.20 | | 1.10E+11 | 28.62 | | 2.03E+11 | 0.38 | 1.37 | 0.16 | 6.74E+08 | 1.99 | 1.47 | 0.17 | 1.35E+08 | 0.42 | 1.85 | 0.67 | 1.04E+11 | 11.96 |
| **2.65E+05** | 3 | 18.47 | 20.53 | | 5.82E+10 | 16.70 | | 1.79E+11 | 5.12 | 1.37 | 0.22 | 2.02E+08 | 7.92 | 1.48 | 0.25 | 1.72E+07 | 1.59 | 1.85 | 0.28 | 7.88E+10 | 5.64 |
| **2.65E+04** | 3 | 21.45 | 24.88 | | 4.24E+10 | 15.15 | | 3.09E+11 | 13.33 | 1.37 | 0.37 | 7.25E+07 | 7.52 | 1.47 | 0.14 | 2.20E+06 | 1.33 | 1.86 | 1.59 | 1.36E+11 | 30.54 |
| **2.65E+03** | 3 | 26.08 | 28.18 | | 1.25E+11 | 69.40 | | 2.67E+11 | 14.56 | 1.36 | 0.48 | 1.83E+07 | 7.45 | 1.46 | 0.81 | 2.55E+05 | 1.21 | 1.84 | 1.34 | 7.71E+10 | 24.79 |
| **2.65E+02** | 3 | 30.31 | 32.66 | | 1.74E+11 | 65.65 | | 5.09E+11 | 24.13 | 1.36 | 0.38 | 6.28E+06 | 7.91 | 1.46 | 0.58 | 3.04E+04 | 1.09 | 1.83 | 0.15 | 9.25E+10 | 24.72 |
| **summary for n=18** | | | | **1.95** | 9.91E+10 | **79.7** | **1.92** | 2.89E+11 | **41.5** | **1.37** | **0.46** | 5.93E+08 | **159.8** | **1.47** | **0.71** | 1.99E+08 | **195.9** | **1.84** | **0.62** | 1.05E+11 | **30.8** |

**Conc.** – input concentration of nucleic acid in sample.
**n.** - repeats
**$CP_{fp}$** – Crossing point based on Fit–point method.
**$CP_{sdm}$** – Crossing point based on second derivative maximum – *SDM* computing method by LightCycler software 3.3 (Roche Diagnostics).

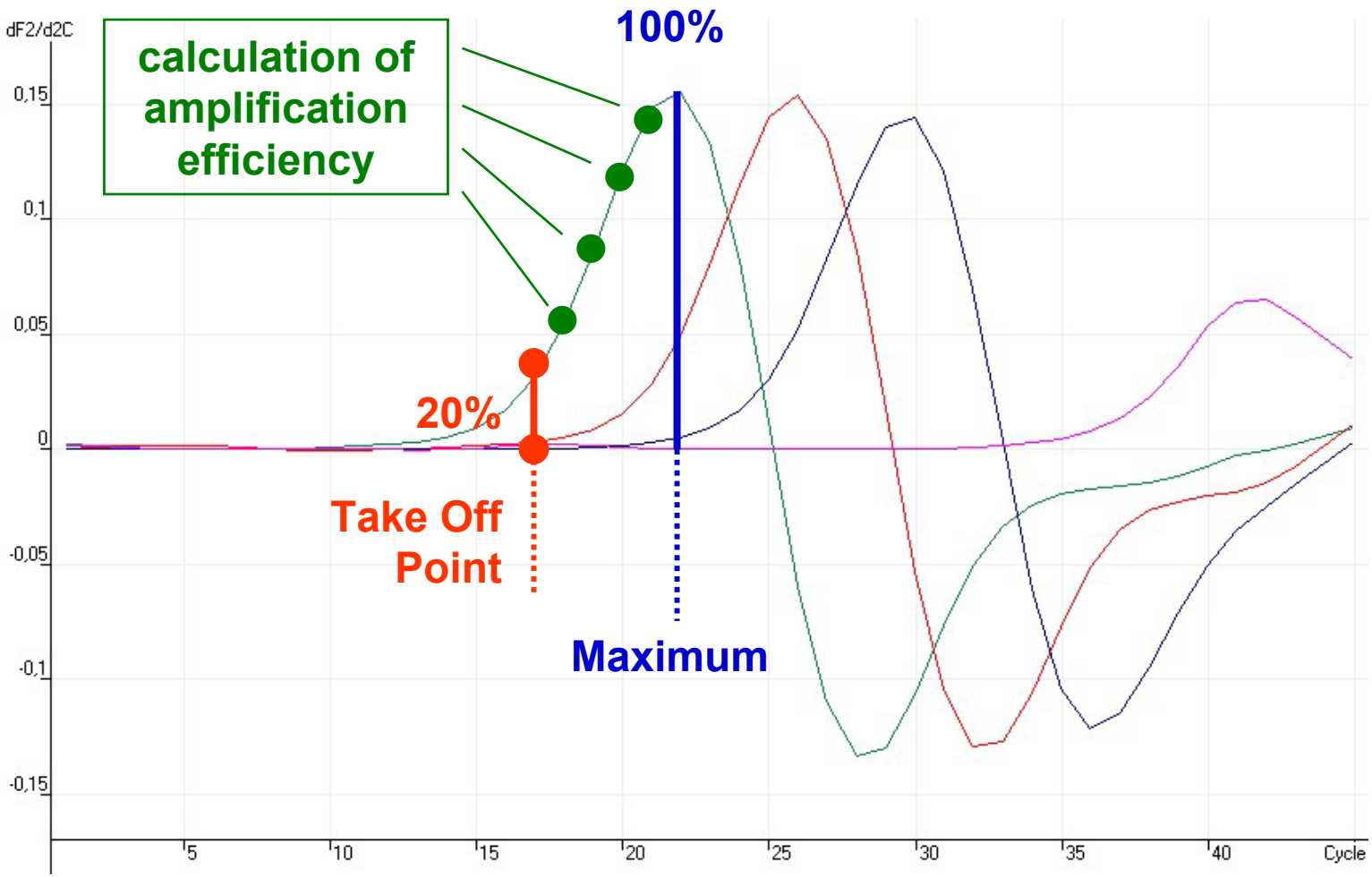
***$E1_{fit\ point}$*** – Amplification efficiency computed from calibration curve[11] where crossing points are obtained as Fit–points.
***$E1_{sdm}$*** – Amplification efficiency computed from calibration curve where crossing points are computed as *SDM*.
***$E2_{fdm}$*** – Amplification efficiency computed from absolute fluorescence increment in point of inflexion (first derivative maximum) of amplification trajectory (22).
***$E2_{sdm}$*** – Amplification efficiency computed from absolute fluorescence increment in *SDM* of amplification trajectory model.
***$E_{new}$*** – Amplification efficiency computed according to the method suggested here. ***E*** – The mean value(s) of efficiency for n=3. **Y** – Fluorescence product computed from equation (10) for respective ***E*** for n=3. **CV** – Coefficient of variation for n=3.

**summary** – either the overall mean or overall CV for n=18.

*Tichopad et al., 2003 Nucleic Acids Research **31(20):** e122*

# *„Take Off point“* and *„Amplification efficiency“*

determined by Rotor-Gene 3000 *Comparative Quantitation Analysis*



| Summary | Sample individual determination | Overestimation + Intermediate ∅ Underestimation - | Combination of Efficiency & CP determination |
|---|---|---|---|
| **Dilution series** (fit point or SDM) *Rasmussen, 2001* | **no** | + n = 3-5 | |
| **Fluorescence increase** *various authors* | + | - n = 3-6 | |
| **Fluorescence increase** *Peccoud & Jacob, 1998* | + | - n = 3 | |
| **Sigmoidal model** *Lui & Saint, 2002* *Tichopad et al., 2002 & 2004* | + | - n = 1 | |
| **LinRegPCR** *Ramakers et al., 2003* | + | ∅ n = 4-6 | |
| **KOD** *Bar et al., 2003* | + | ∅ n = 3-5 | |
| **Logistic model** *Tichopad et al., 2003* | + | ∅ n > 7 | + |
| **Rotor-Gene 3000** Comparative Quantitation Analysis | + | ∅ n = 4 | + |

# Determination of PCR Efficiency

## Determination of real-time PCR efficiency

- **Estimation via classical "*calibration dilution curve and slope calculation*"**
  - *on each individual cDNA (Rasmussen 2001)*
  - *on each individual cDNA (Stahlberg et al. 2002)*
  - *on a mixture of multiple cDNA (cDNA pool, Pfaffl 2001)*
  - *Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis (Peirson, 2003)*
- **Estimation via increase in "*absolute fluorescence*" method 1** (linear regression, Pfaffl 2001)
- **Estimation via increase in "*absolute fluorescence*" method 2** (Liu & Saint 2002)
- **Estimation via increase in "*absolute fluorescence*" method 3** (3 data points, Peccoud & Jacob 1998)
- **Estimation via increase in "*absolute fluorescence*" method 4** (window-of-linearity, Ramakers et al. 2003)
- **KOD (Kinetic Outlier Detection) in real-time PCR** (Tzachi Bar et al., 2003)
- **Estimation via "*theoretical sigmoidal fit*"** (all fluorescence data points, Liu & Saint 2002)
- **Estimation via "*experimental four parametric sigmoidal model fit*"** (all fluorescence data points, Tichopad et al. 2002)
- **Estimation via "*experimental four parametric logistic model fit*"** (all fluorescence data points, Tichopad et al. 2003)
- **ERRATUM: correction of Figure 2 Tichopad et al. 2003** - Standardized determination of real-time PCR efficiency from a single reaction set-up
- **PCR Efficiency estimation via "*experimental four parametric sigmoidal model fit*"** (including all fluorescence data points, Tichopad et al. MCP 2003 - Inhibition of real-time RT–PCR quantification due to tissue-specific contaminants)

# *Thank you for your attention !*