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1st International qPCR Symposium & Application Workshop

Determination of real-time PCR efficiency - an overview



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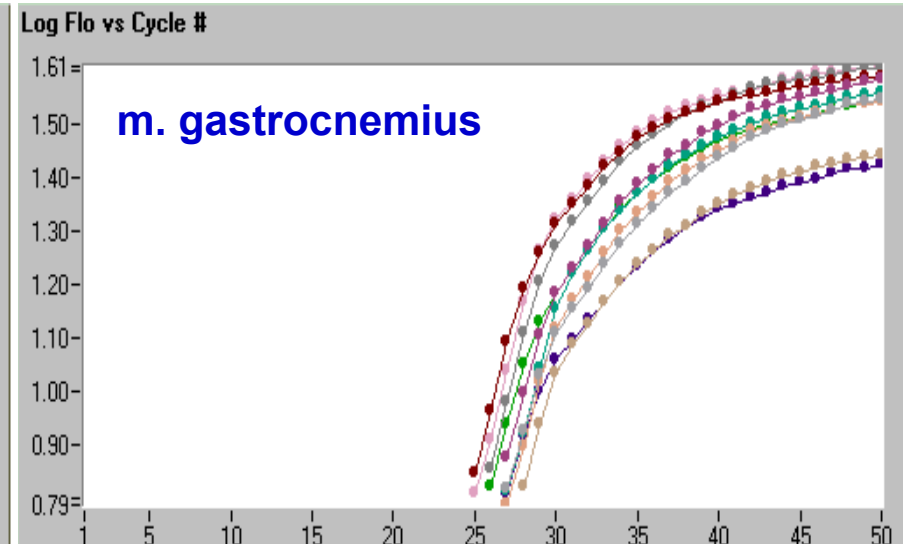
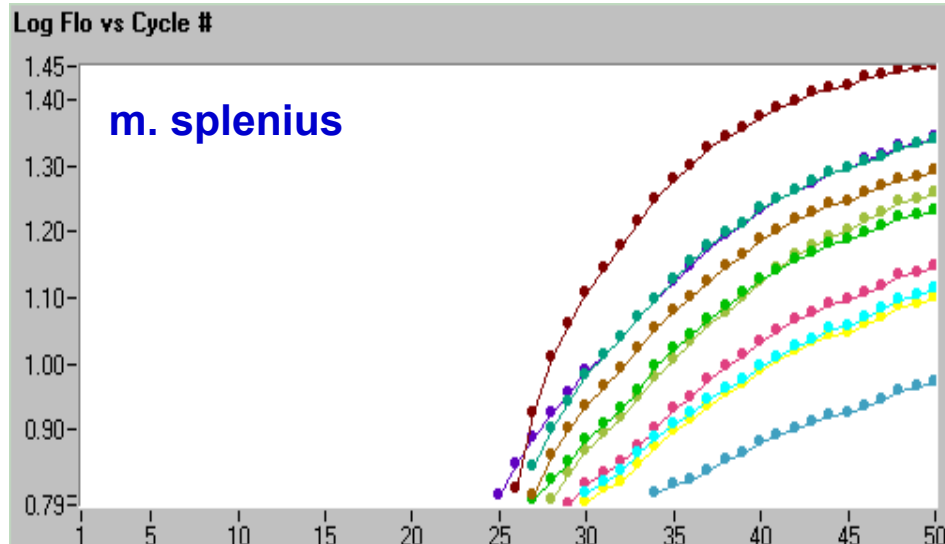
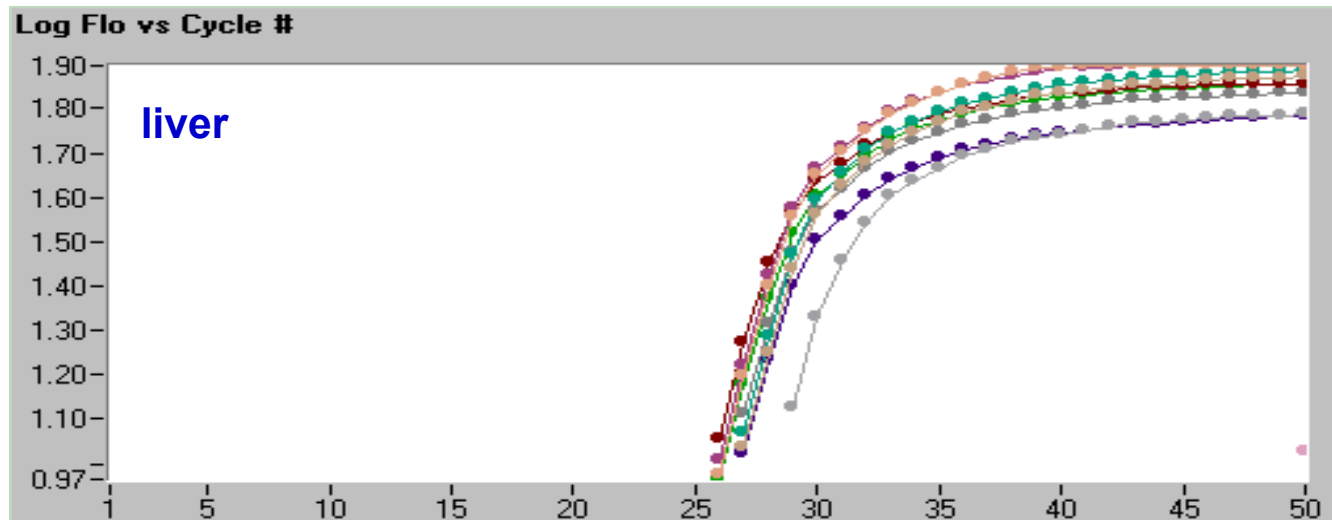
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„Tissue matrix” interference with real-time PCR efficiency and amplification fidelity

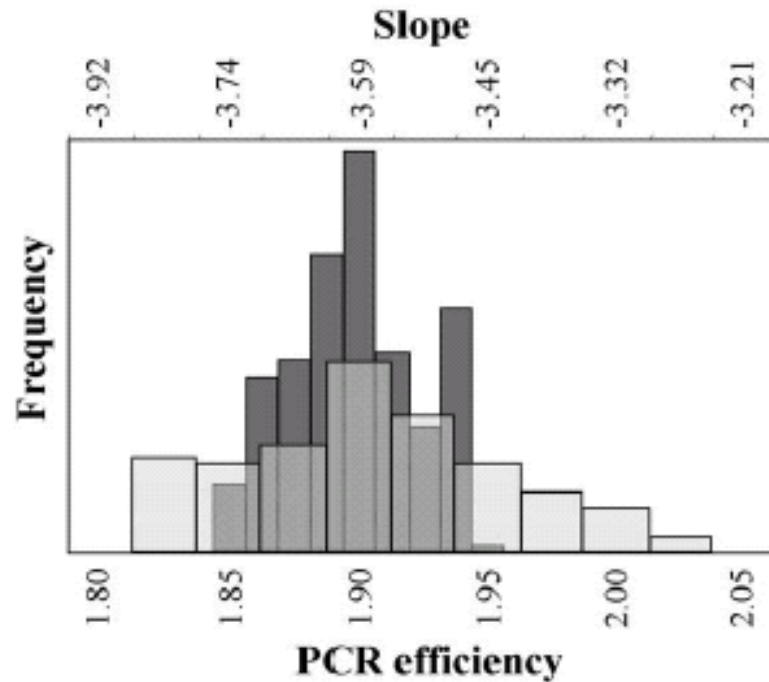
(IGF-1 mRNA amplification in three cattle tissues)



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, Ca²⁺
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

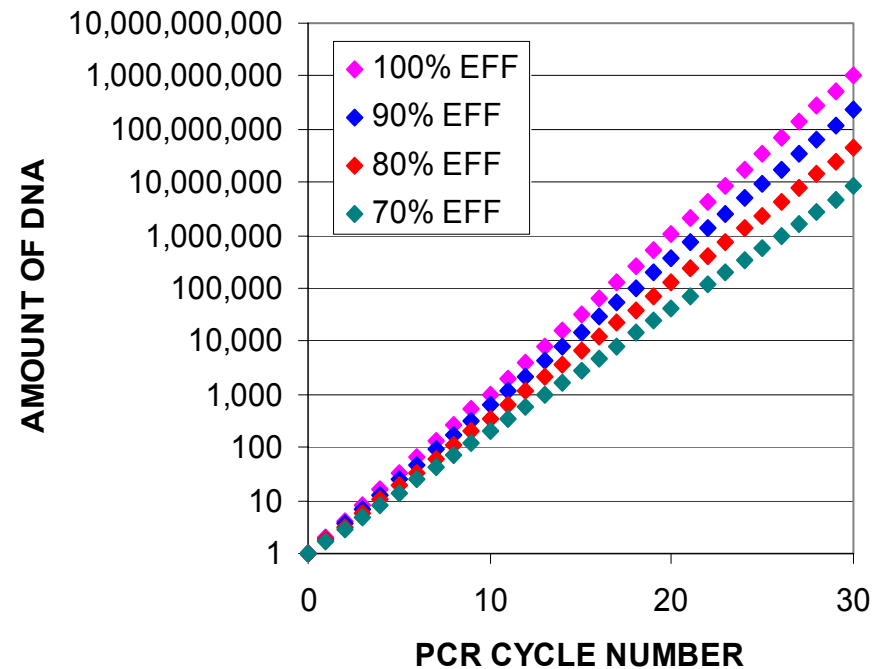
Cycle conditions

DNA
concentration

PCR reaction
components

Hardware:
PCR platform & cups

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



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 %

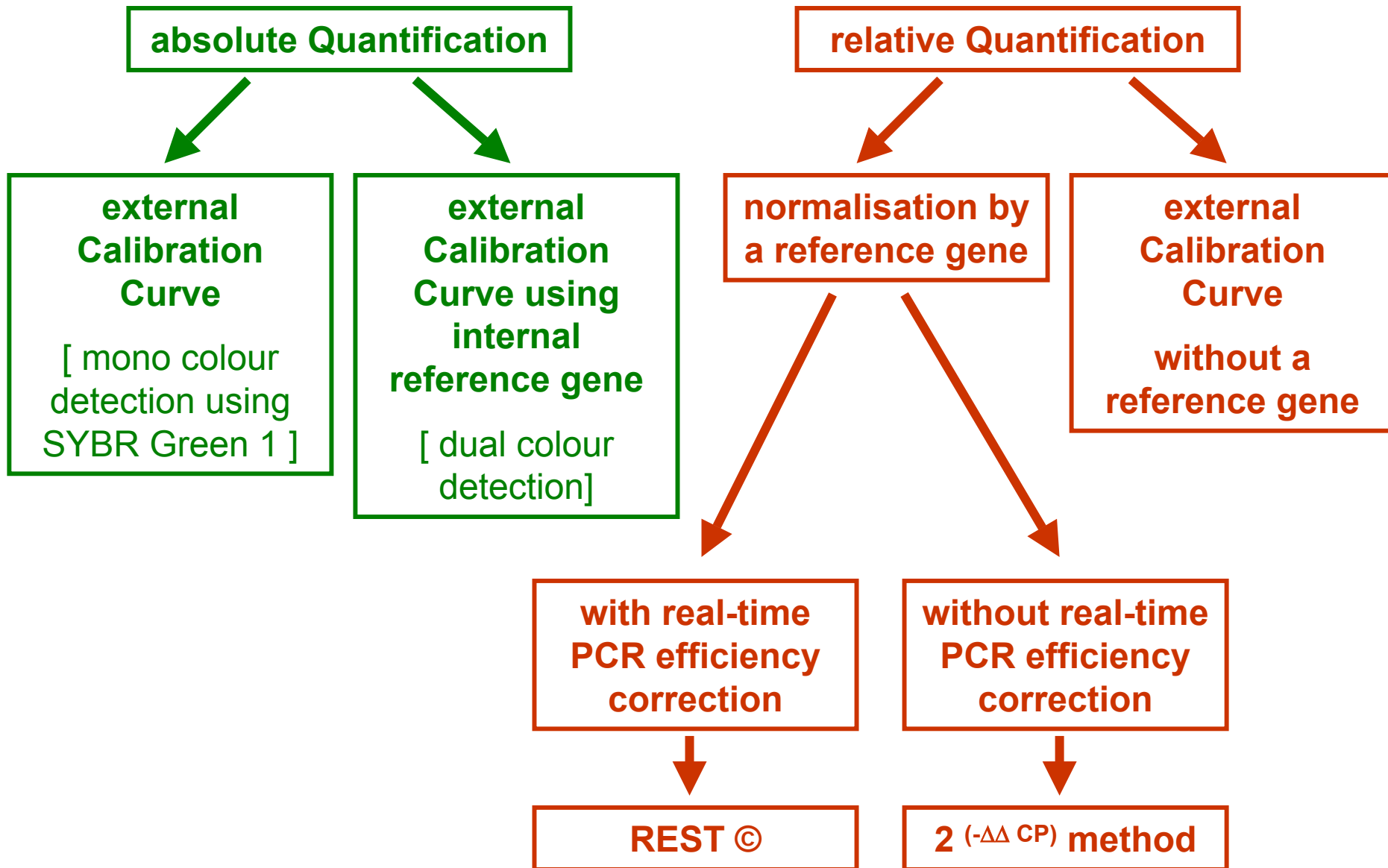
Table 1: Errors of quantification relative to a theoretical PCR efficiency of 2.00

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.

Quantification strategies in real-time RT-PCR



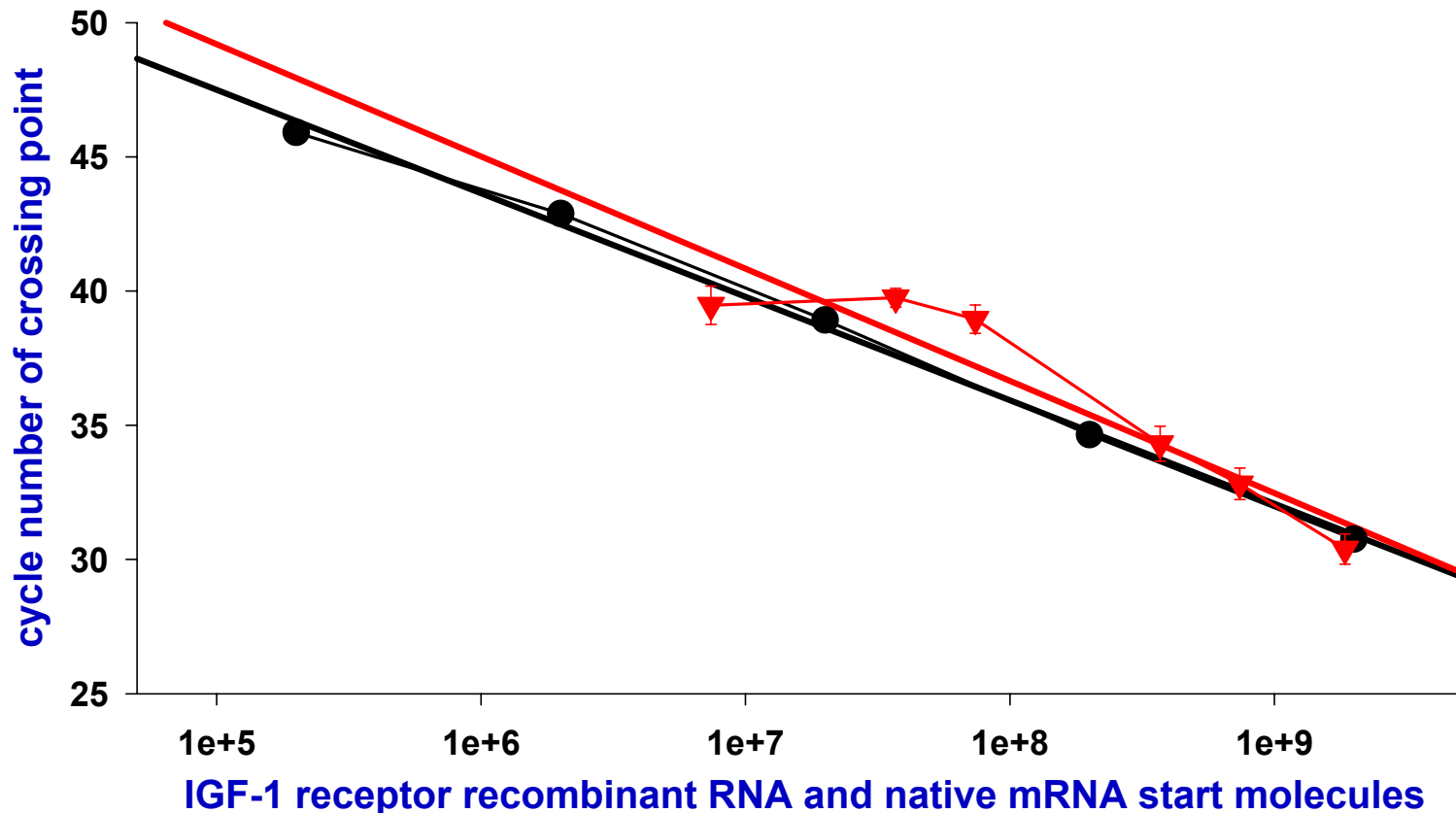
Absolute quantification of IGF-1 receptor

two step qRT-PCR efficiency (recombinant 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)



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

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

Pfaffl, Nucleic Acids Research 2001

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

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

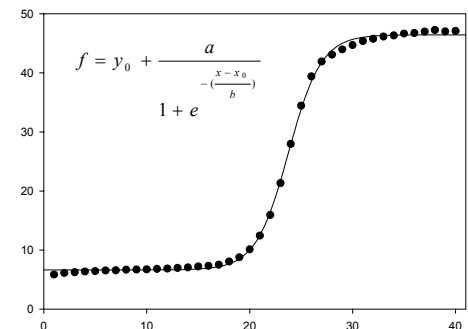
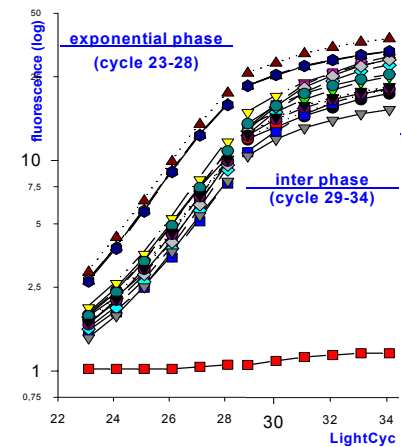
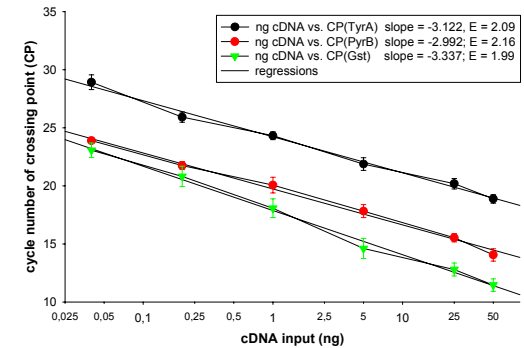
➤ Sigmoidal model

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

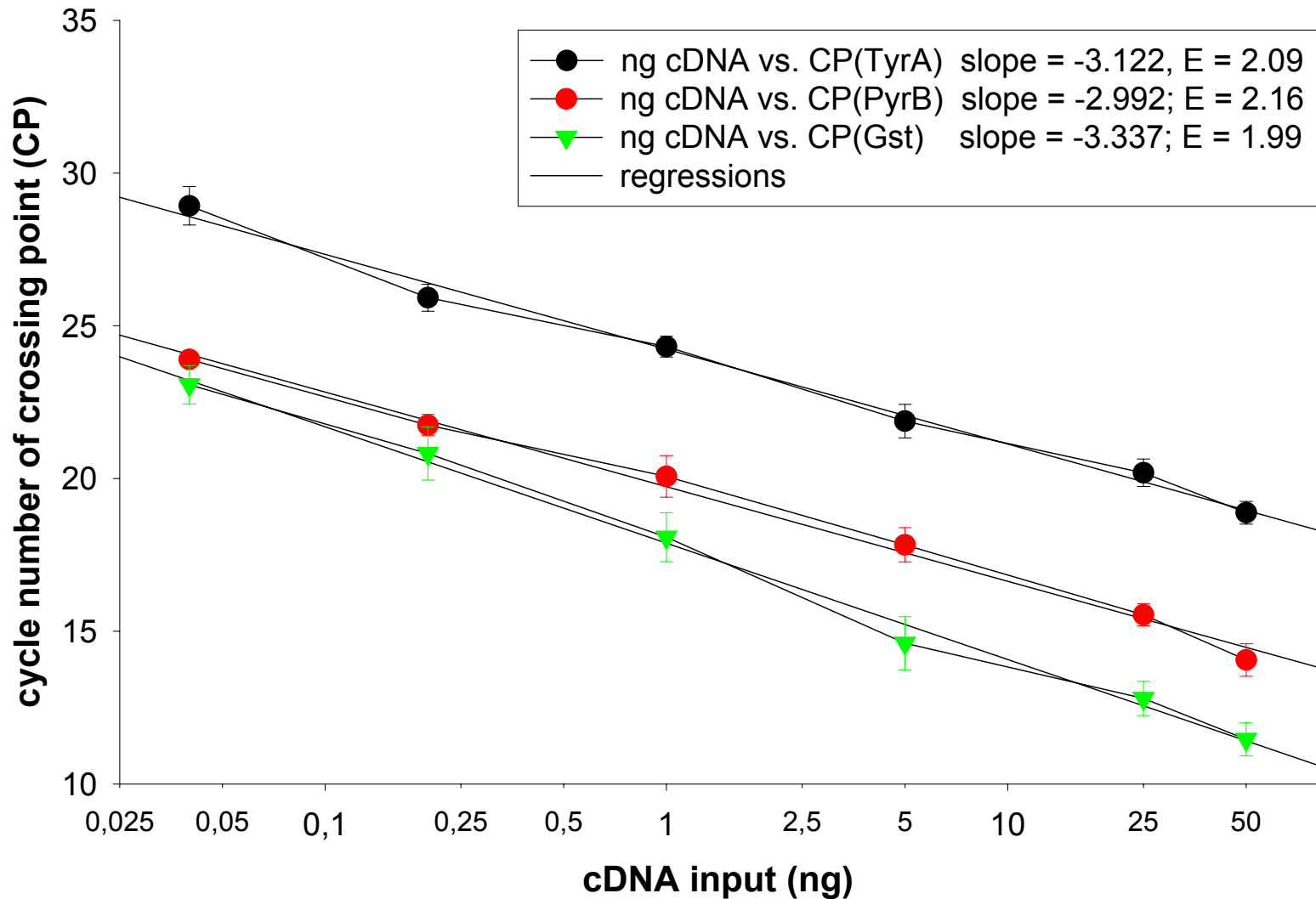
➤ Logistic model

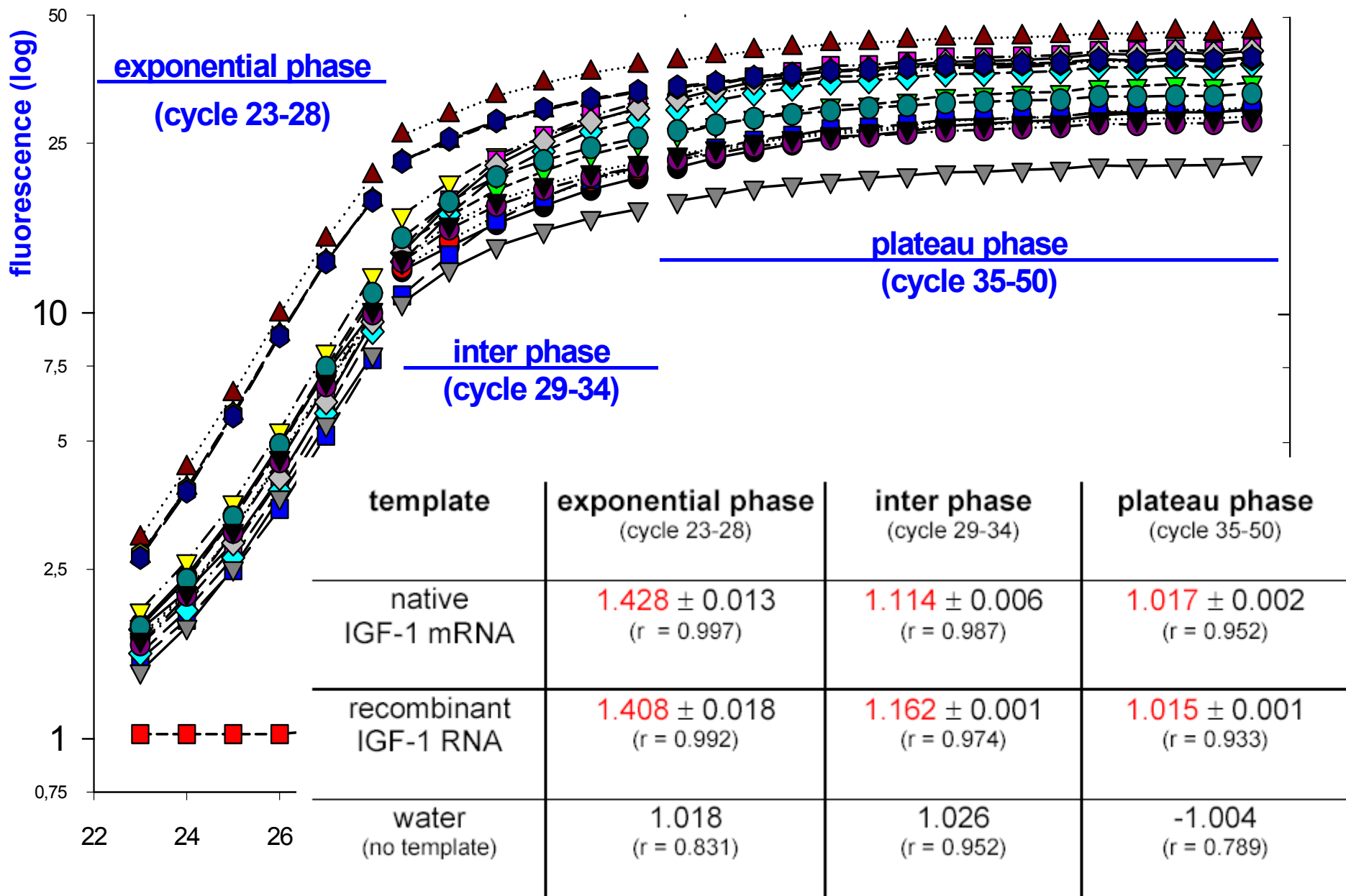
(Wittwer et al. 2000; Tichopad et al. 2003)

➤ Comparative Quantitation Analysis Rotor-Gene 3000 software



Determination principles of real-time PCR efficiency: Dilution series





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:

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

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

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

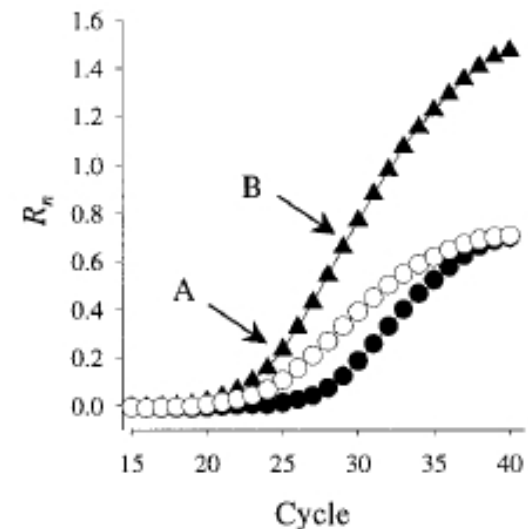
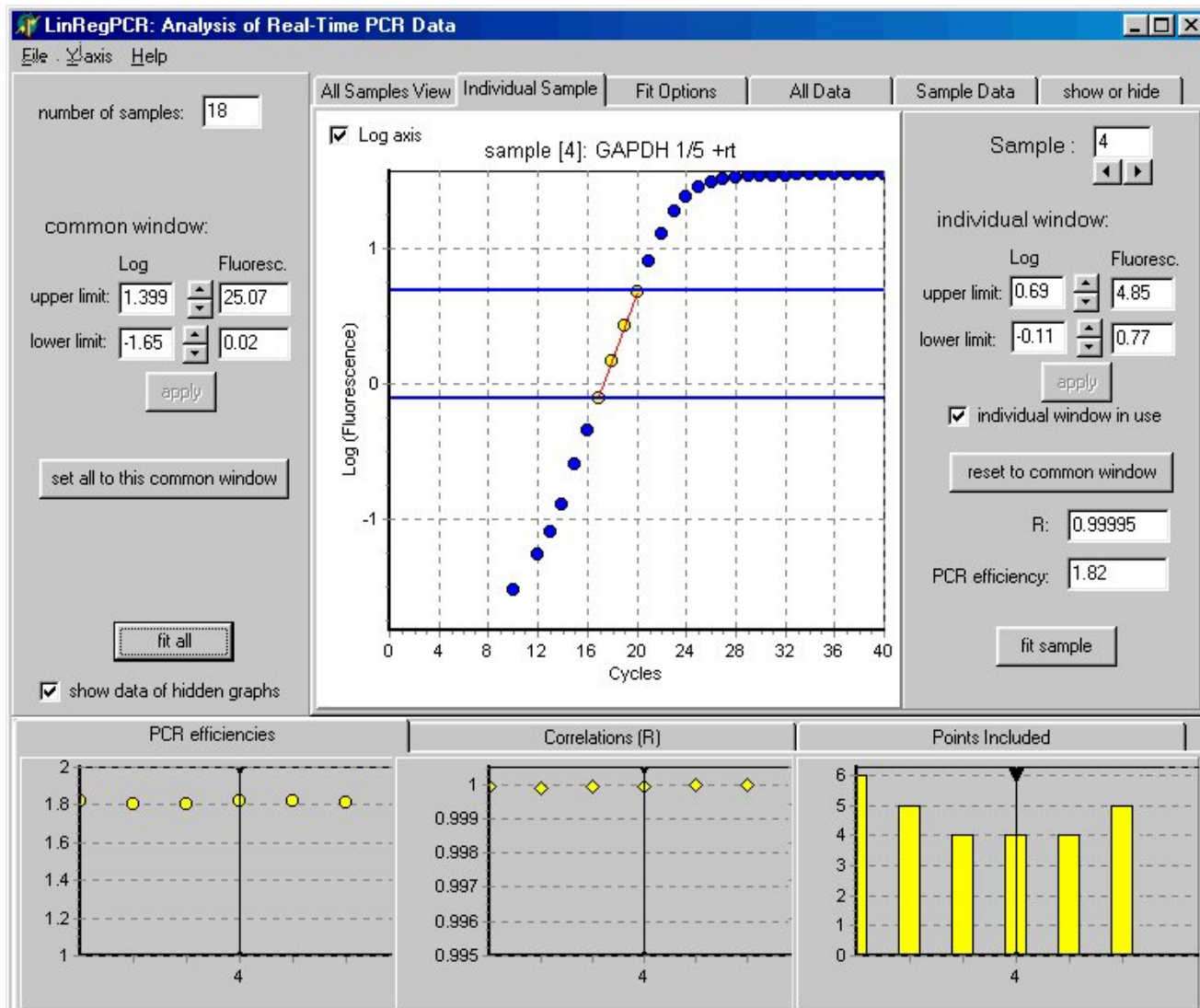


FIG. 1. Calculation of amplification efficiency from the kinetic PCR curve. Three curves were chosen representing *GAPDH* (▲) and two different target genes (○ and ●). R_n is measured at two thresholds along the exponential phase as shown at A and B. Amplification efficiency is calculated using Eq. [3].

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

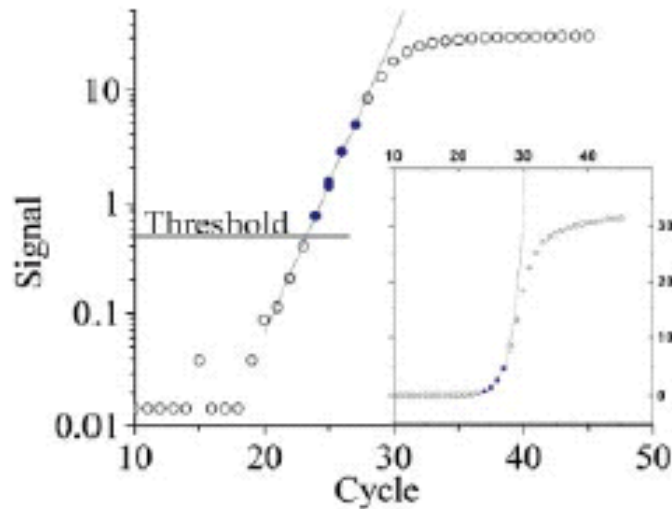


Figure 1. Estimation of PCR efficiency by exponential fit. Three to five data points (filled circles) above threshold level are fitted by an exponential equation 1 to estimate PCR efficiency. Main frame: semi-logarithmic scale, inset: linear scale.

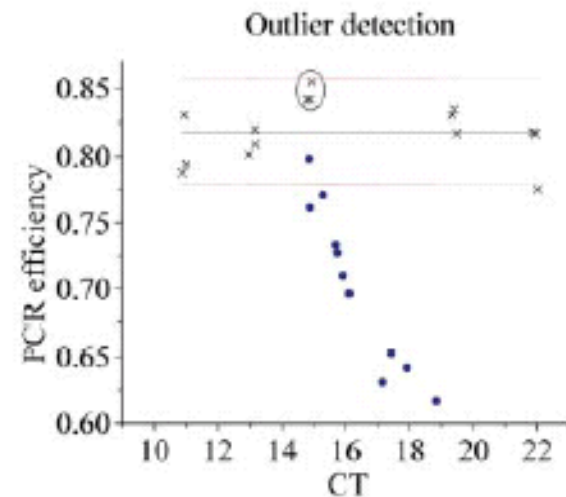
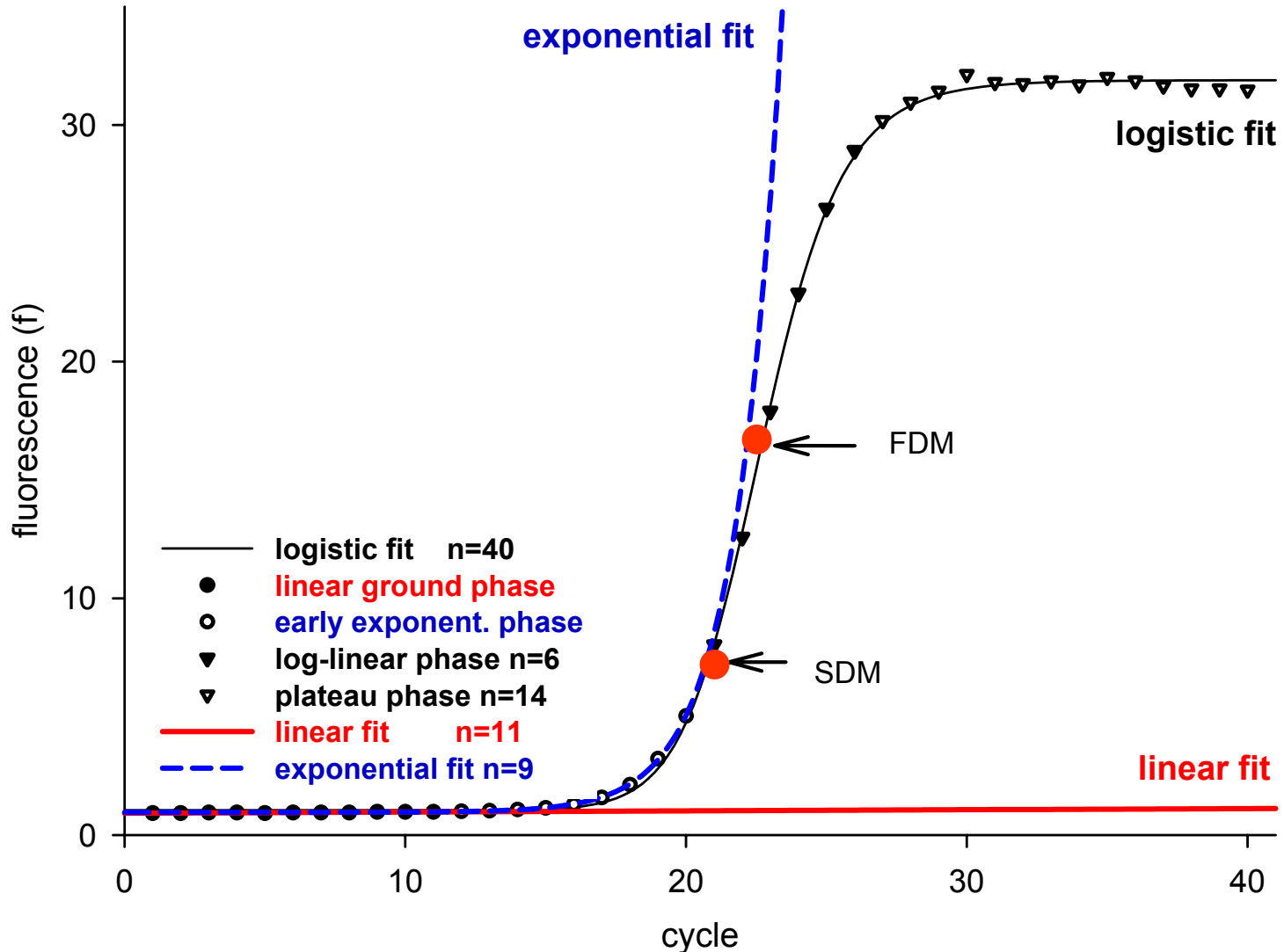


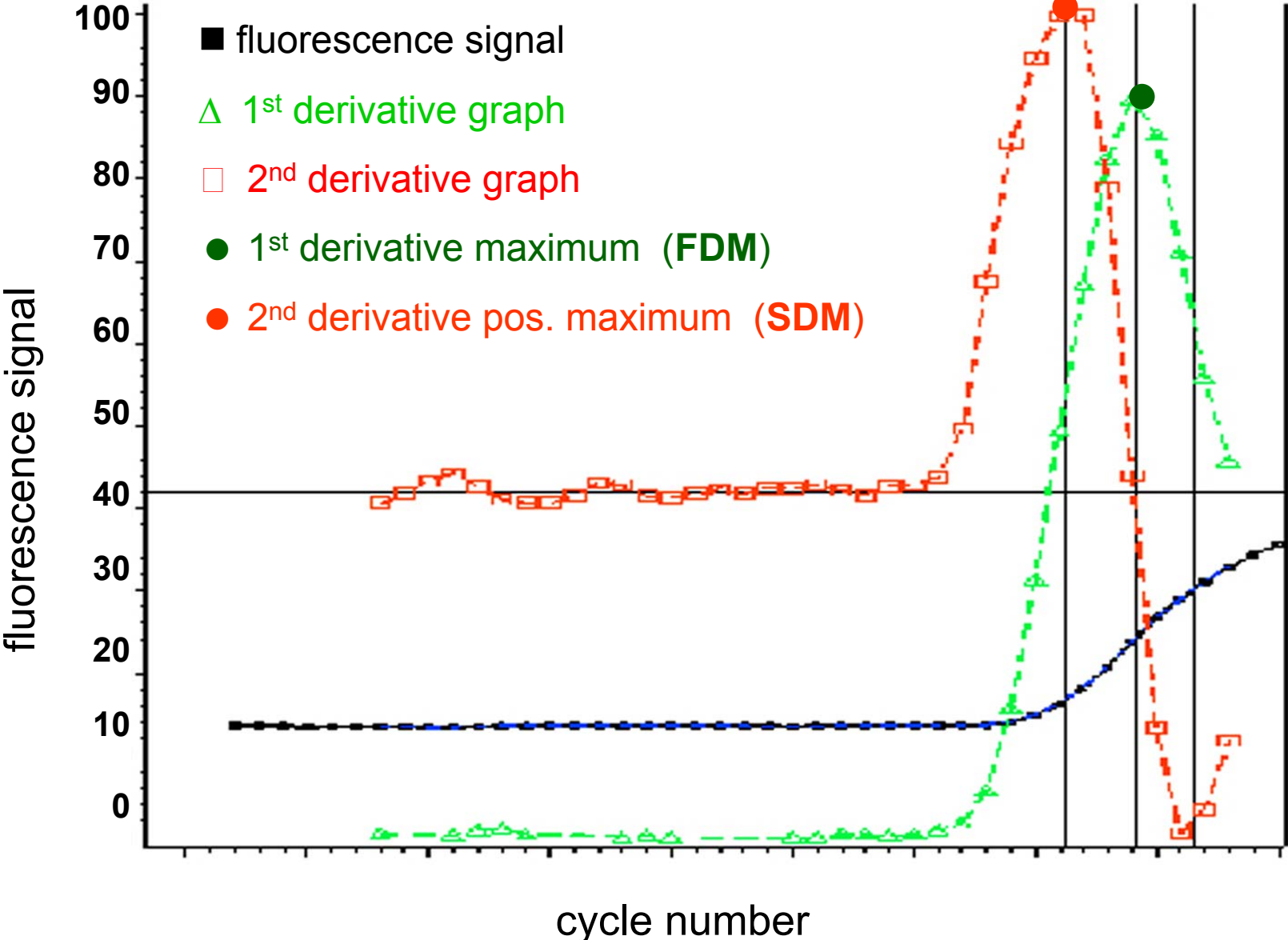
Figure 6. Dilution series of purified PCR product was used as a training set (crosses) for outlier detection in test samples (circles) containing equal starting numbers of template molecules as the encircled concentration in the dilution series, but with elevated concentrations of dNTP as inhibitor. 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.

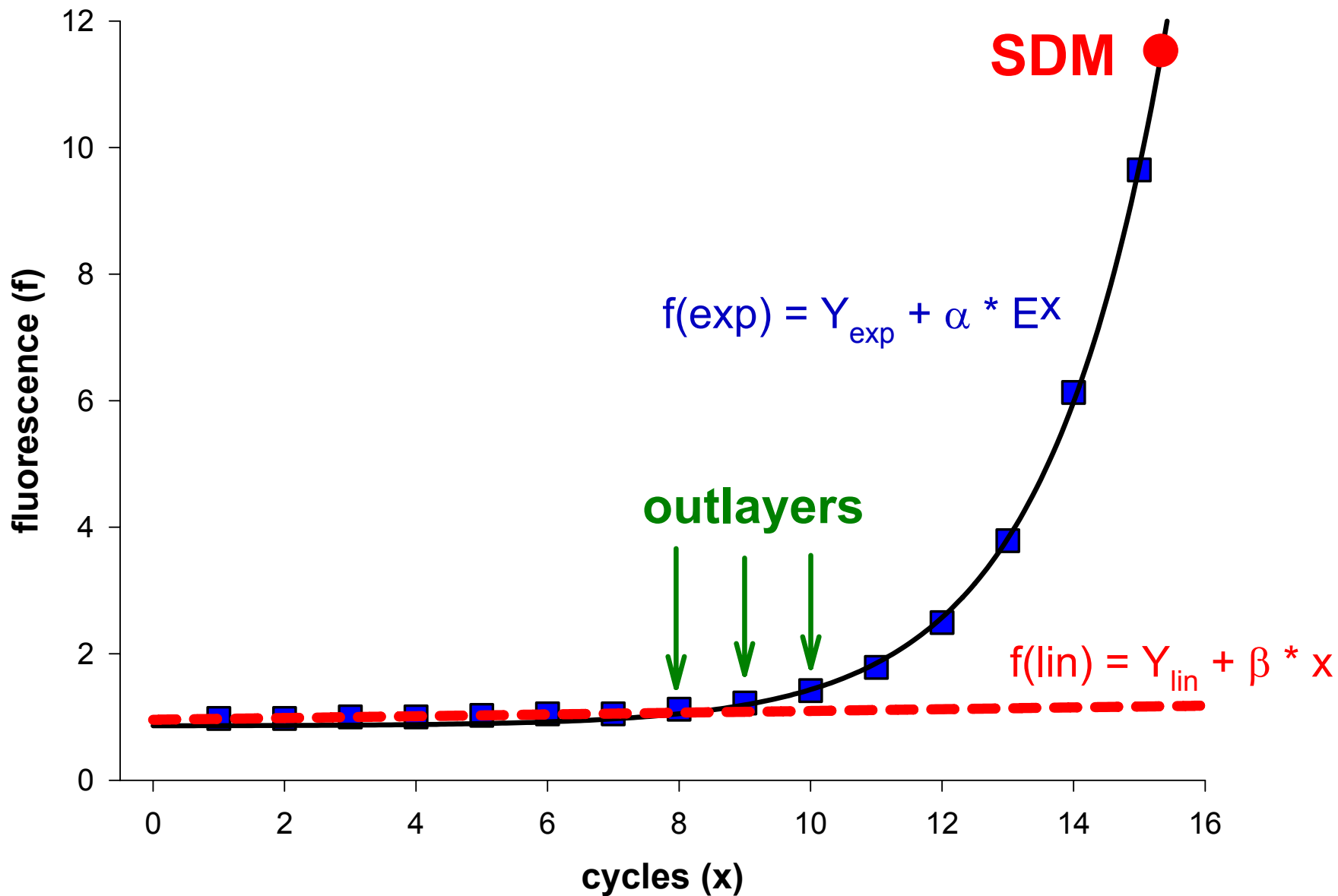
Standardized determination of real-time PCR efficiency from a single reaction setup

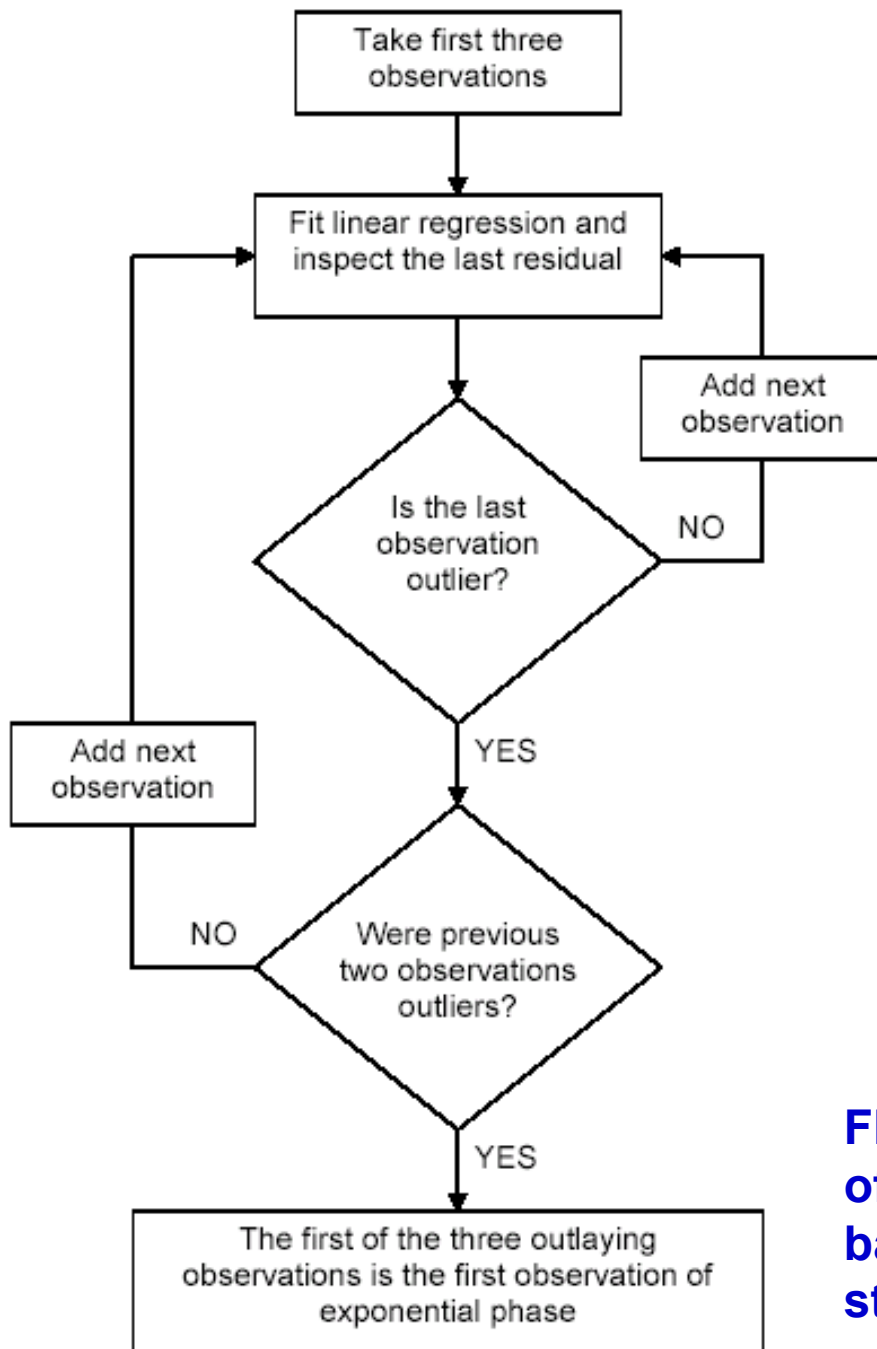
Tichopad et al., 2003 Nucleic Acids Research **31(20)**: e122



Principal of "Second Derivative Maximum" method







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

Conc.	n	CP _{fp}	CP _{sdm}	E1 _{fit point}			E1 _{SDM}			E2 _{FDM}				E2 _{SDM}				E _{new}			
				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		79.7	1.92		41.5	1.37	0.46		159.8	1.47	0.71		195.9	1.84	0.62		30.8
				9.91E+10			2.89E+11					5.93E+08				1.99E+08				1.05E+11	

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 curve¹¹ where crossing points are obtained as Fit–points.

E1_{sdm} – Amplification efficiency computed from calibration curve where crossing points are computed as SDM.

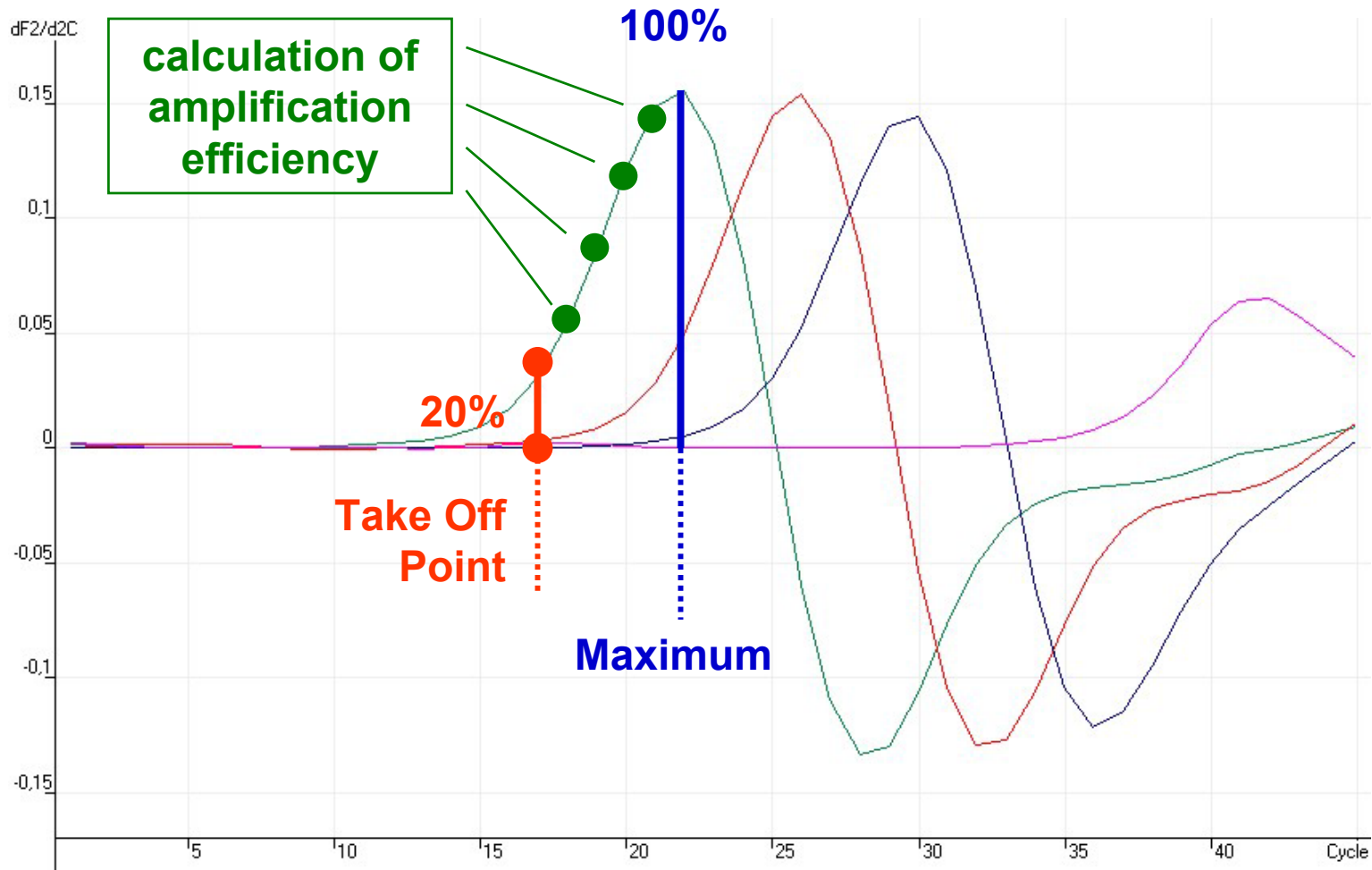
E2_{fdm} – Amplification efficiency computed from absolute fluorescence increment in point of inflexion (first derivative maximum) of amplification trajectory (22).

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.

„Take Off point“ and „Amplification efficiency“ determined by Rotor-Gene 3000 Comparative Quantitation Analysis



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

Bioinformatics

in real-time PCR

[Gene Quantification Home](#) [Physiology Home](#) [Bioinformatics Home](#)

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 !*

