Recent advances and new perspectives in real-time RT-PCR Quantification

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Michael W. Pfaffl
Physiology – Weihenstephan
TATAA Biocenter Germany
Technical University of Munich
Weihenstephaner Berg 3
85350 Freising-Weihenstephan
Germany

Michael.Pfaffl@wzw.TUM.de
Michael.Pfaffl@TATAA.com

www.gene-quantification.info
TATAA.gene-quantification.info

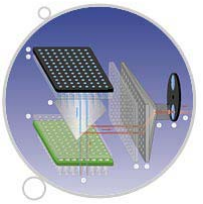
Physiology – Weihenstephan | City of Freising | Research station
Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL)
Center of Life and Food Sciences, TUM

Quantification of specific mRNAs

(cattle, sheep, pig, rat, horse, monkey, buffalo, humans, …. etc.)

Molecular Physiology – Immunology - Endocrinology:

- Immuno-modulation and immuno-stimulation of the gastro-intestinal tract of farm animals (cattle, pig & sheep)
- Growth Physiology (cattle & pig)
- Lactation Physiology
- Immunology in Mammary Gland (cattle & sheep)



mRNA quantification assays:

competitive RT-PCR, real-time qRT-PCR

- Hormone and Hormone Receptors
- Cytokines, growth factors and their receptors
- Cytokines, factors and receptors of the Immune System
- Enzymes
- Housekeeping Genes (UBQ, β-actin, GAPDH, Histon, 18S, etc.)

RNA integrity:

Bioanalyzer 2100, Experion

- Improvement of RNA extraction
- RNA integrity measurement

Software application development:

- Relative Expression Software Tool (REST)
- BestKeeper
- Efficiency calculation (algorithms development)
- Kineret

Genotype -> Phenotype -> Function

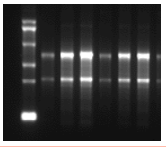
DNA => pre-mRNA => mRNA => Protein => Function

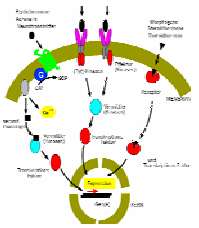
↓ Transcription

↓ Splicing

↓ Translation
post-translatoric modifications

Genome | **Transcriptome & *Splicome*** | **Proteome** | **Metabolome**



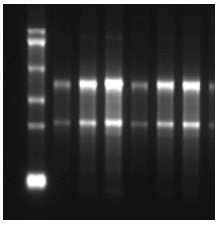


Transcriptome

RNA sub-classes in a mammalian total RNA

ribosomal RNA	rRNA	80-85%	(5S, 18S und 28S)
transfer RNA	tRNA	10-15%	
messenger RNA	**mRNA**	**2% (1-5%)**	(∅ length 1930 bases)

high abundant	> 100 genes	> 1,000	copies/cell
intermediate abundant	~ 500 - 1,000 genes	~ 100-500	copies/cell
low abundant	**> 27,000 genes**	**< 1-5**	**copies/cell**



28S rRNA 3898-6333 bases

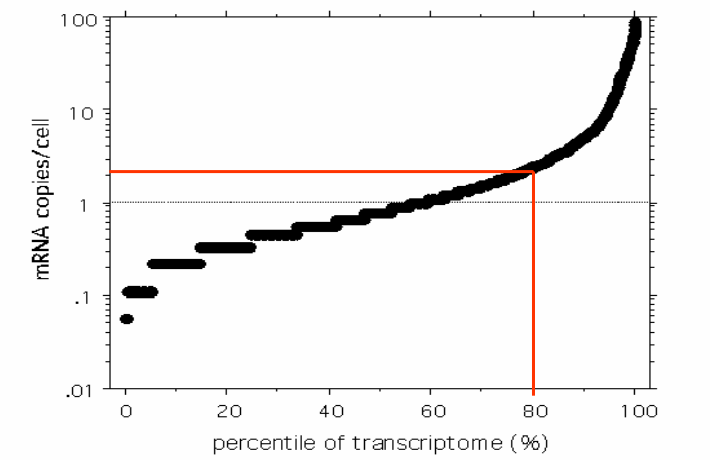
18S rRNA 1898–1976 bases

5S rRNA 120 bases

www.Qiagen.com

Transcriptomics in Yeast

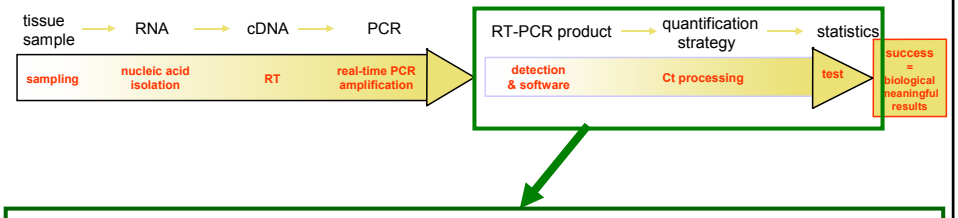
5460 transcript were investigated
estimated 15000 poly-A RNAs per cell
average level: 2.8 copies/cell
median level: 0.9 copies/cell
80% of the yeast transcriptome is expressed at 0.1-2 copies/cell



Richard A. Young http://web.wi.mit.edu/young/expression/transcriptome.html

Gene	Expression Level (Copies/Cell)	mRNA Half-life (min)	Transcriptional Frequency (mRNAs/hr)	YPD Title Line™ ©2000 Proteome, Inc. Reprinted with permission. [last updated: 11/23/98]
TAS1	1.2	21	2.2	Histone acetyltransferase of the MYST family
TBF1	0.9	12	2.5	Teleomere binding protein that binds to TTAGGG repeats
TCI1	1.2	11	4.3	Protein that interacts with protein phosphatase 2C
TCM10	0.6	38	0.6	Protein of unknown function
TCP1	2.7	16	6.4	Component of Chaperonin-containing T-complex (TCP ring complex, TRiC), homologous to mouse TCP1/CCT1
TDH1	3.6	10	12.7	Glyceraldehyde-3-phosphate dehydrogenase 1, converts D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate
HHF1	17.3	17	38	Histone H4
HHF2	23.9	14	65.3	Histone H4
HHO1	1.6	10	5.8	Histone H1
HHT1	45.5	16	103.3	Histone H3;
HHT2	37.6	12	111.7	Histone H3
UBP1	3.5	14	9	Ubiquitin-specific protease (ubiquitin C-terminal hydrolase), cleaves at the C-terminus of ubiquitin
UBP11	0.2	30	0.3	Ubiquitin-specific protease
UBP12	0.6	16	1.3	Ubiquitin-specific protease
UBP13	0.6	14	1.6	Ubiquitin C-terminal hydrolase
UBP14	0.7	16	1.7	Ubiquitin-specific protease
UBP2	1.3	20	2.4	Ubiquitin-specific protease (ubiquitin C-terminal hydrolase), cleaves at the C-terminus of ubiquitin
UBP3	0.9	27	1.3	Ubiquitin-specific protease
UBP5	0.6	26	0.7	Ubiquitin-specific protease (ubiquitin C-terminal hydrolase), homologous to Doa4p and human Tre-2
UBP7	0.6	#N/A-nc	#N/A	Putative ubiquitin-specific protease
UBP9	0.1	#N/A-nc	#N/A	Ubiquitin C-terminal hydrolase, has similarity to Ubp13p
UBR1	0.7	30	0.8	Ubiquitin-protein ligase (N-recognin or E3 enzyme), involved in selection of substrates for the N-end rule pathway

Steps and variables of a successful mRNA quantification using real-time RT-PCR



Detection method:

- **SYBR Green I**
- **Probes:** Beacons, Scorpions, etc.
- raw data vs. background correction
- Fit point method
- Background fitting (10x SD)
- 1st or 2nd derivative maximum
- other models: logistic / sigmoidal / NLR / CalQPlex / ***E***-method
- Multiple and/or mixed models
- Other curve "manipulations"
- 2-step, 3-step, or 4-step qPCR

Quantification strategy:

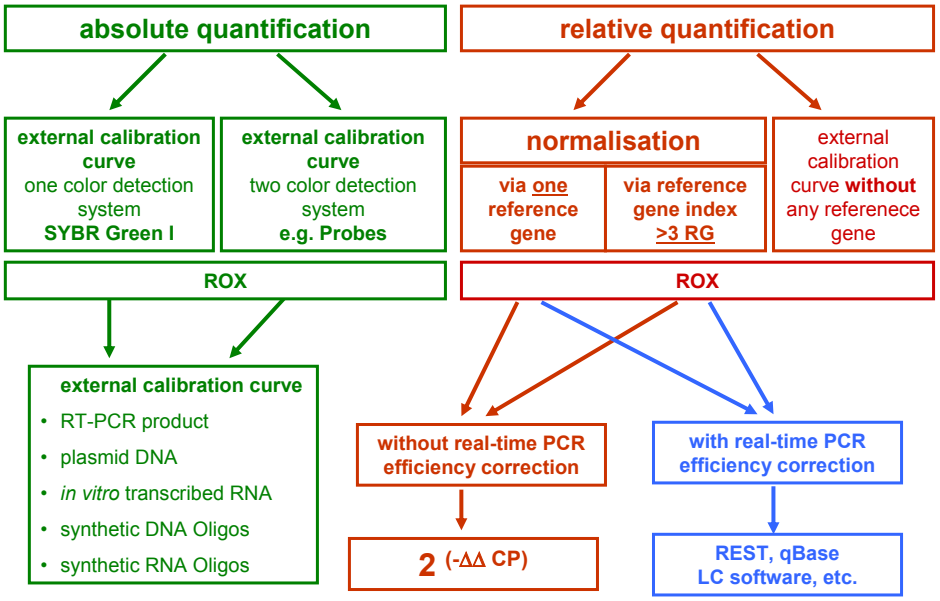
- **"absolute" quantification**
  - type of calibration curve?
  - normalization with RG
- **relative quantification**
  - total RNA, cells, tissue mass
  - normalization with RG
  - normalization via an RG Index (> 3 RGs)
  - geNorm, REST, BestKeeper, qBASE, Normfinder, etc.

BioStatistics & BioInformatics:

- **CP vs. "quantified molecules"**
- Normality of data (?)
- t-Test (?)
- ANOVA (on the ranks ?)
- SAS, SPSS, Excel, Sigma Stat
- Permutation test
- Randomization test (REST)
- Bootstrapping (REST-2007)
- Cluster analysis
- Multiple regression analysis
- Multi-dimensional modeling

Quantification Strategies in real time qRT-PCR

M.W. Pfaffl, BioSpektrum 2004 (Sonderausgabe PCR)



Quantification strategies in real-time RT-PCR

Absolute quantification using calibration curves

- **recombinant DNA** (recDNA) calibration curve (Bustin, 2000; *Pfaffl & Hageleit, 2001)*
- **recombinant RNA** (recRNA) calibration curve *(Pfaffl & Hageleit, 2001)*
- calibration curve using a synthetic **DNA** oligo-nucleotide *(Bustin, 2000; Bustin 2005)*
- calibration curve using a synthetic **RNA** oligo-nucleotide *(Bustin et al. 2000, 2004, etc……)*
- calibration curve using a purified **RT-PCR product** *(Einspanier et al. 1999, etc……)*
- *„Copy & Paste"* of previously performed **calibration curves** *(LC software; Roche Diagnostics)*

Absolute quantification using calibration curves

- ***Calibration curve using a purified RT-PCR product or a synthetic ss / ds oligo-nucleotide***
  application: two-step setup => **RT-qPCR**
  advantages: quick set up, highly defined DNA content
  disadvantages: instable, "problems with re-amplification", „short templates"

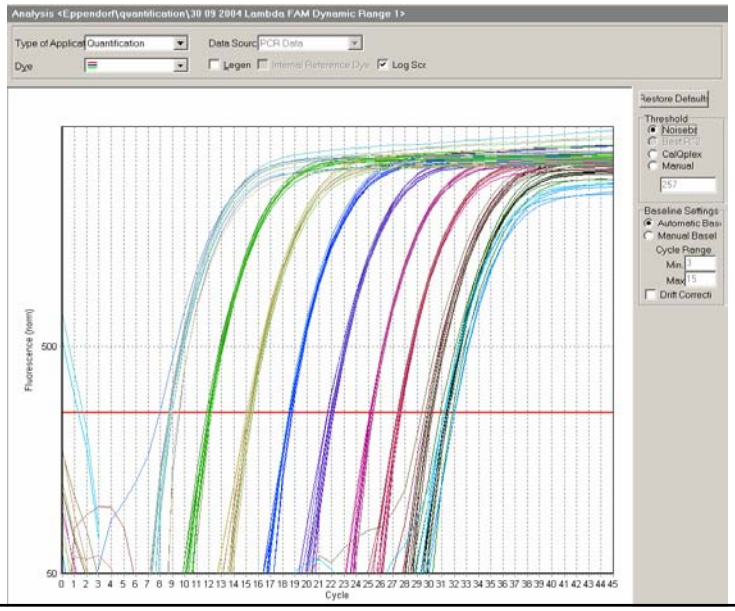
- ***Calibration curve using a cloned recombinant DNA (recDNA)***
  application: two-step setup => **RT-qPCR**
  advantages: very stabile, no problems with re-amplification, „mimic mRNA better"
  disadvantages: cloning, linearization and purification of recDNA

- ***Calibration curve using a in vitro transcribed recombinant RNA (recRNA)***
  application: one-step setup => **qRT-PCR**
  => recRNA and native mRNA undergoing RT and PCR in parallel
  advantages: mimics the natural mRNA situation best (mRNA ~ recRNA)
  disadvantages: very instable recRNA, complicate cloning, linearization, purification of recRNA, storage and stability problems, **reproducibility (???)**
  => Production and storage of stable recRNA is necessary !!!

- ***„Copy & Paste" of previously performed calibration curves***
  advantages: very easy and very high reproducibility (at least for calibration curve !!!)
  disadvantages: do NOT cover any variation in real RT-PCR experiment: e.g. slope of std. curve, PCR efficiency, batch to batch variations, etc. => ***truth ???***

Standard curve FAM label with Lambda Phage

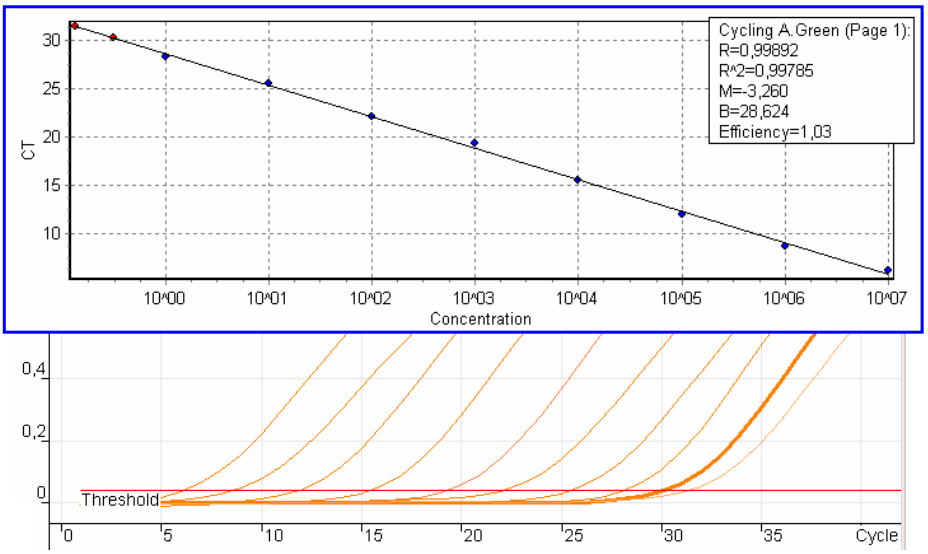
10^9 to 10^1 start molecules (Eppendorf 2004)



SYBR GreenER (Invitrogen) standard curve with Estrogen Receptor alpha (ERα)

10^7 to one start molecule (s.s. plasmid DNA)

The *"fat line"* represents one ERα start molecule



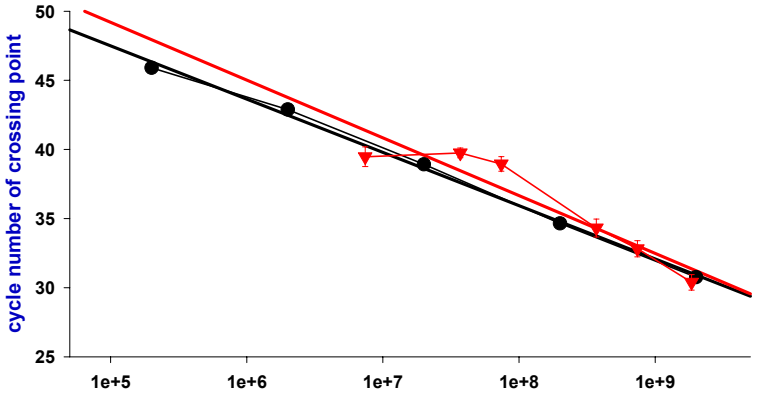
Absolute quantification of IGF-1 receptor

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)



IGF-1 receptor recombinant RNA and native mRNA start molecules

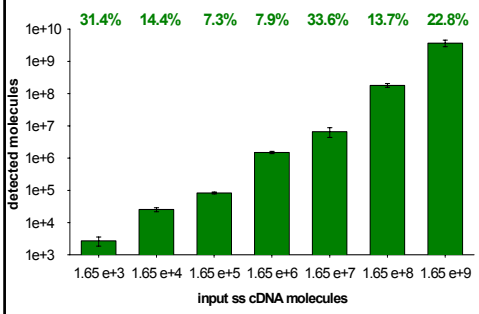
Pfaffl et al., 1998

ERα intra-assay & inter-assay variation

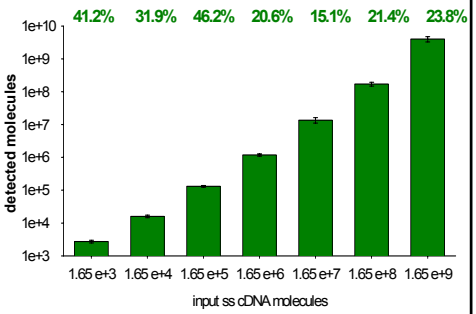
intra-assay variation: within one LightCycler 1.0 run

inter-assay variation: between different LightCycler 1.0 runs

ER-alpha intra-assay variation CV = 18.7% (n = 3)



ER-alpha inter-assay variation CV = 28.6% (n = 7)



using a recombinant plasmid DNA calibration curve (mean ± std.dev.; on molecule basis)

Pfaffl, unpublished 1998

Precision in the estimates

$$SE_{\lg \hat{c}_i}(test) = \frac{SE_{y.x}}{k}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{\left(\overline{CT}_i-\overline{CT}\right)^2}{k^2\sum_{i=1}^{n}\left(\lg c_i-\overline{\lg c}\right)^2}}$$

Distance from center

Number of test replicates

Number of standards

Confidence interval for estimated concentrations

$$\log \hat{c}_i \pm t_{95\%,2tails,n-2} \times SE_{\log \hat{c}_i}$$

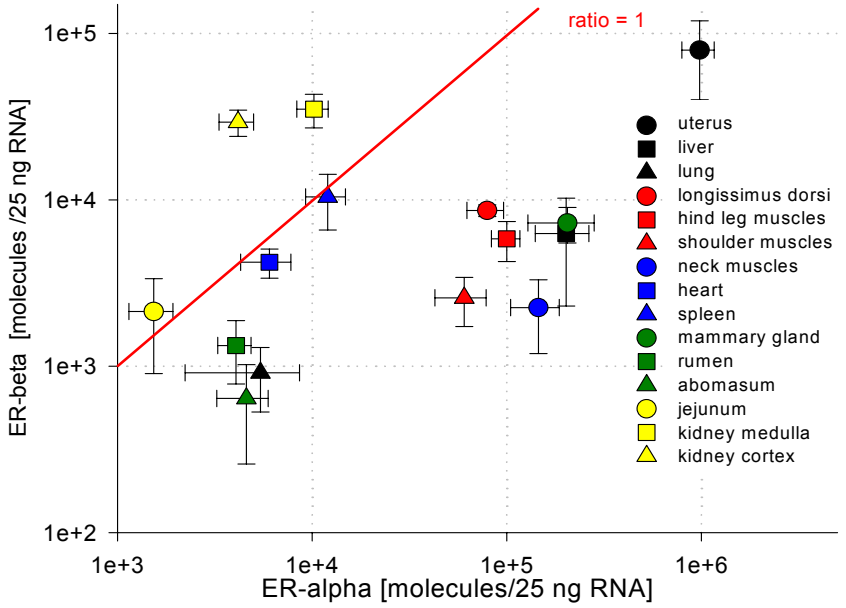
Validation of an „absolute quantification“ of steroid receptors

suitable for multiple species

	AR	ERα	ERβ	PR
product length	172 bp	234 bp	262 bp	227 bp
detection limit	12 molecules	2 molecules	10 molecules	14 molecules
quantification limit	120 molecules	165 molecules	106 molecules	760 molecules
quantification range test linearity Pearson correlation coefficient	120 - $1.20*10^{10}$ molecules (r = 0.998)	165 - $1.65*10^{9}$ molecules (r = 0.995)	106 - $1.06*10^{10}$ molecules (r = 0.996)	760 – $7.60*10^{9}$ molecules (r = 0.998)
PCR efficiency	90.7%	81.2%	81.3%	93.9%
intra-assay variation [CV] molecule basis	31.2% (n = 3)	18.7% (n = 4)	17.6% (n = 4)	5.7% (n = 4)
inter-assay variation [CV] molecule basis	24.3% (n = 7)	28.6% (n = 4)	29.7% (n = 4)	25.7% (n = 4)
Species specific T_{melt} (°C)				
Homo sapiens	85.4	86.0	[87.9]	83.5
Rattus norvegicus	84.4	85.0	89.0	[82.9]
Callithrix jacchus (primate)	85.0	--	[89.9]	83.9
Bos taurus	85.5	85.3	90.1	83.8
Ovis aries	--	85.4	90.5	83.1
Sus scrofa	84.5	86.0	90.2	83.5

Pfaffl et al., APMIS 2001

Estrogen receptors (ERα & ERβ) expression pattern in cattle tissues



Pfaffl et al., APMIS 2001

Relative Quantification

The mRNA expression is relative to WHAT ???

- **relative to a non treated control**
- **relative to a time point zero**
- **relative to another gene-of-interest (GOI)**
- **relative to the mean expression of all GOIs**
- **relative to an universal calibration curve**
- **relative to the expression of one constant expressed reference-gene**
  GAPDH, tubulins, various actins, albumins, cyclophilin, micro-globulins, histone subunits, 18S, 28S…
- **relative to an index containing more reference-genes (>3 RGs)**
  geNorm (Vandesompele et al.; Genome Biology, 2002)
  BestKeeper (Pfaffl et al.; Biotechnology Letters 2004)
  Normfinder (Andersen et al.; Cancer Research 2004)
  Statistical modeling (Szabo et al.; Genome Biology 2004)
  REST versions: REST–384, REST-MCS, REST-RG, ……. (Pfaffl 2007; review in press)
  qBASE (Hellemans & Vandesompele 2006, in preparation)
- **…… ???**

Commonly used normalisation strategies

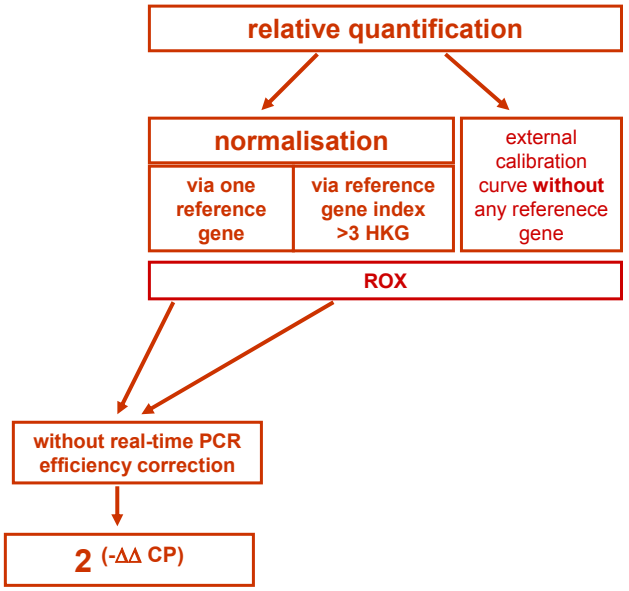
First GOI expression is normalised.....

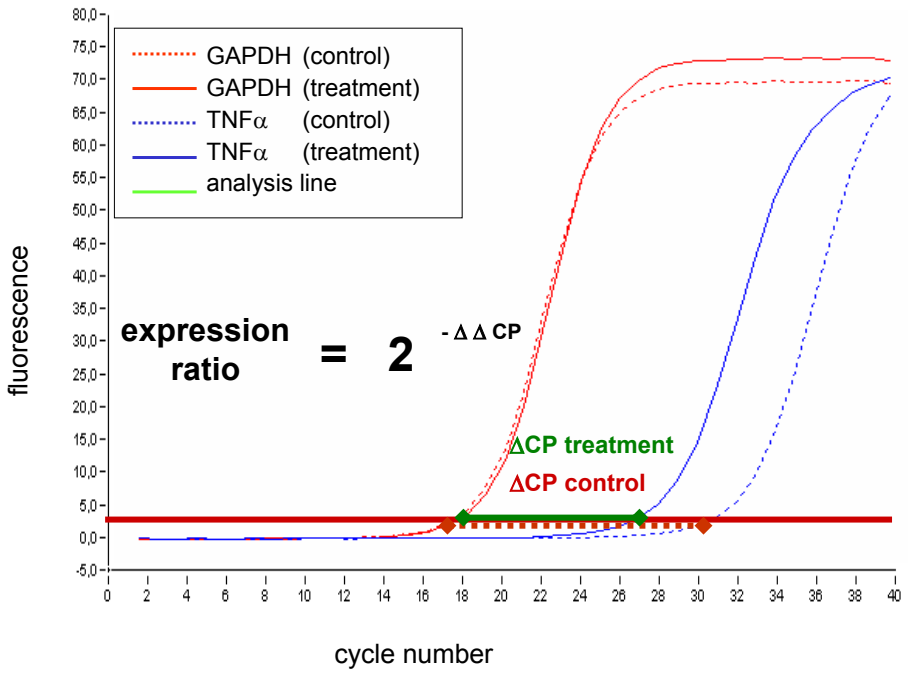
- **according to known amounts of extracted RNA**
  (molecules/ng RNA; ag transcript/ng RNA; RIN quality check ?)
- **according to mass / volume / cells of extracted tissue**
  (molecules/mg tissue; mass of transcript/mg tissue; copies per counted/selected cells, transcripts per single-cell)
- **according to one reference-gene (=> ΔCP)**
  GAPDH, actins, albumins, cyclophilin, micro-globulins, histone subunits, rRNA, …….
- **according to an index containing more reference-genes (> 3) (=> ΔCP)**
  geNorm, BestKeeper, Normfinder, qBASE, REST 384, REST 2005, ……

Second relative parameters, e.g. comparing the normalized GOI (ΔCP) expression level to a further parameter (=> ΔΔCP):

- **a non treated control => ΔΔCP**
- **the time point zero => ΔΔCP**
- **a healthy individual => ΔΔCP**
- **…… ???**

Relative Quantification in real time qRT-PCR





Normalisation according to an internal reference gene

***"delta-delta Ct method"* for comparing relative expression results between treatments in real-time PCR**

ABI Prism Sequence detection System User Bulletin #2 (2001)

Relative quantification of gene expression

$$\Delta CP = CP_{\text{target gene}} - CP_{\text{refence gene}}$$

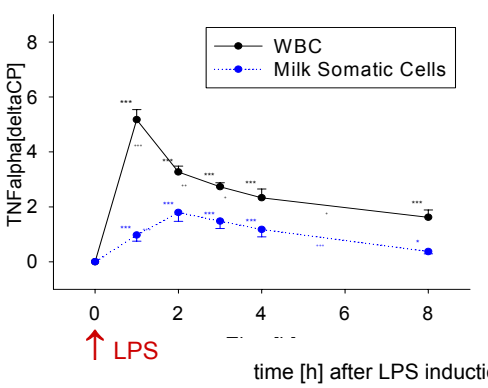
$$\text{expression ratio} = 2^{-[\Delta CP\ \text{treatment} - \Delta CP\ \text{control}]}$$

$$\text{expression ratio} = 2^{-\Delta\Delta CP}$$

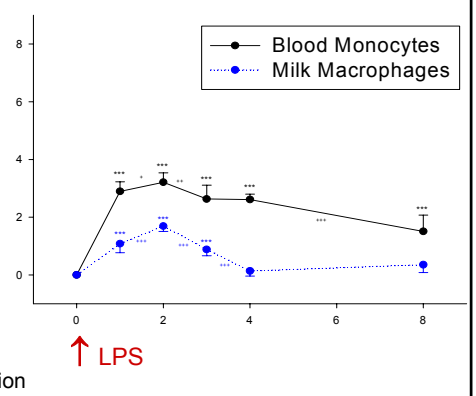
Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 [- delta deltaC(T)] method.
Methods, 2001 ***25(4):*** *402-408.*

Immunological response of pro-inflammatory marker on LPS stimuli in various bovine cell types

TNFα response in WBC and milk somatic cells

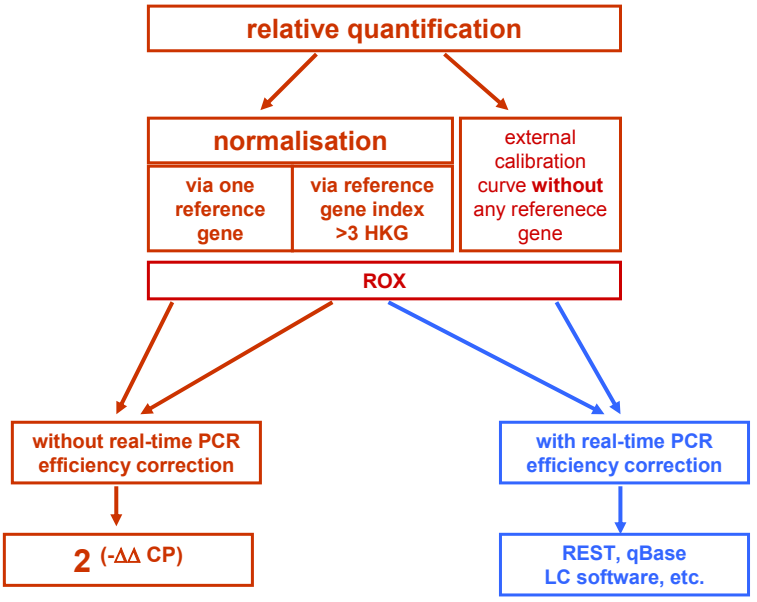


TNFα response in purified monocytes and macrophages



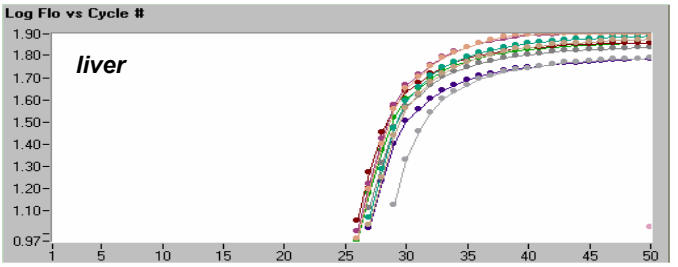
Prgomet et al, 2006

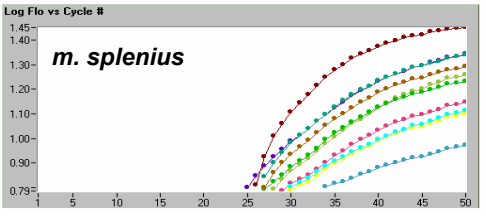
Relative Quantification in real time qRT-PCR

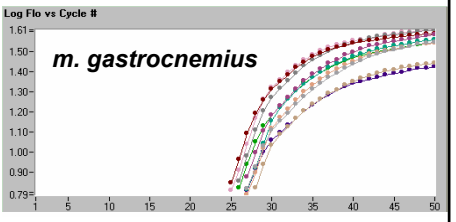


Tissue *"matrix"* interfere with real-time PCR efficiency and amplification fidelity

IGF-1 mRNA amplification in three cattle tissues



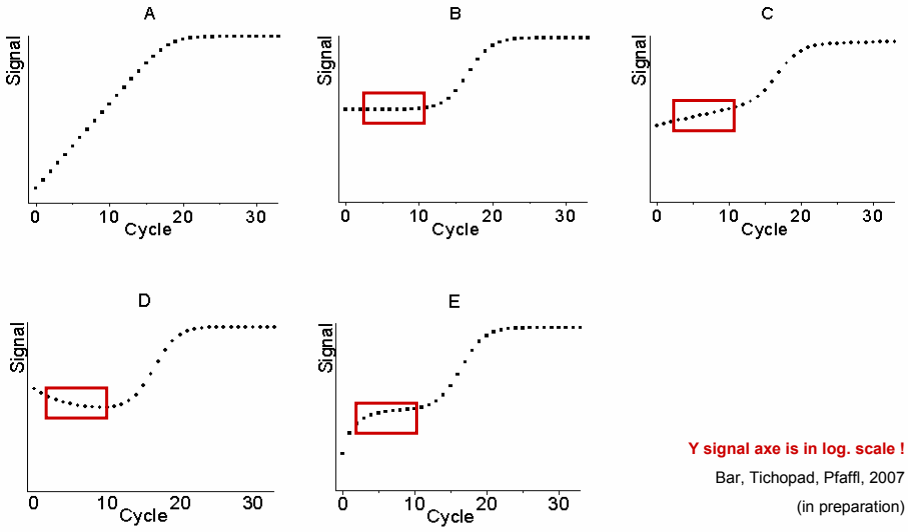




Noise in real-time PCR !

⇒ **Effect of different types of background on an amplification history**

A. No background **B.** Constant background (Roche Biochemicals 1999) **C.** Linear rising background (Tichopad et al., 2000 & 2003) **D.** Decreasing non-linear background (Johnson et al., 2004) **E.** Increasing non-linear background (SoFar, Wilhelm et al., 2003).

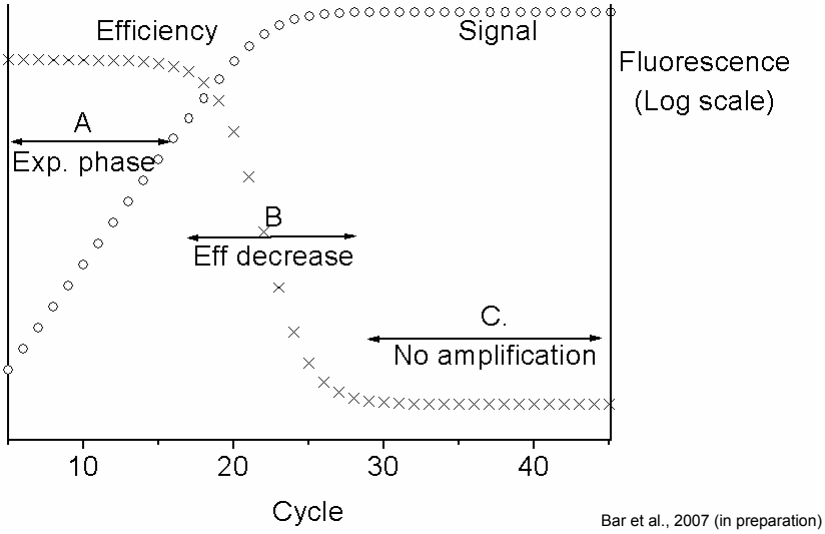


Y signal axe is in log. scale !

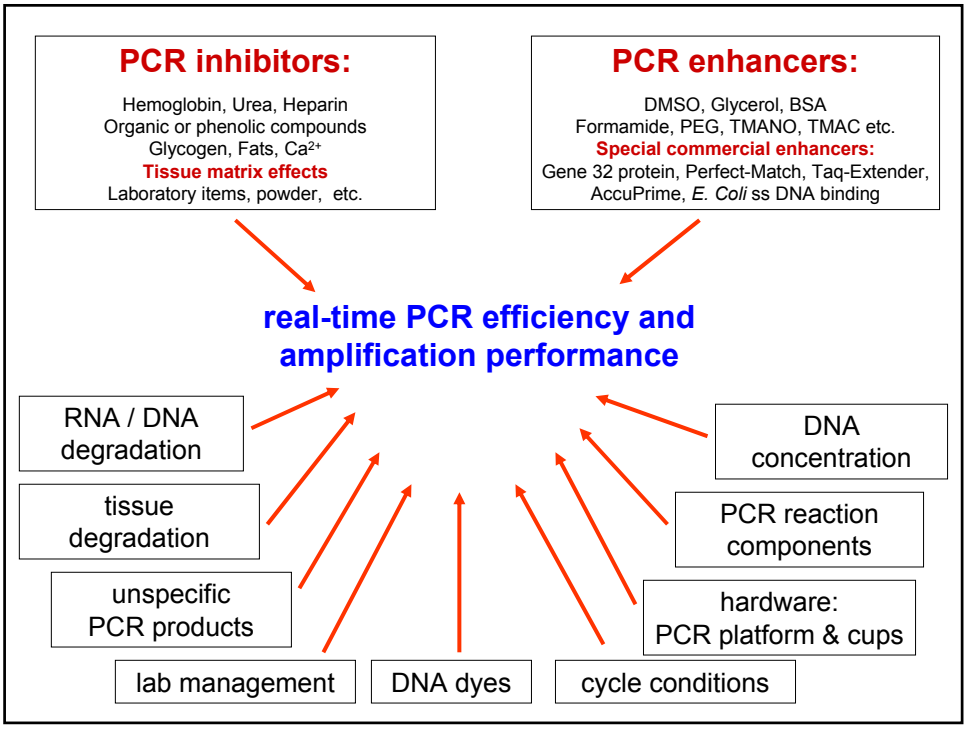
Bar, Tichopad, Pfaffl, 2007

(in preparation)

Theoretical real-time PCR kinetics



Bar et al., 2007 (in preparation)



Relative quantification of a target gene versus an internal control = reference gene *(mostly a housekeeping gene)*

$$\text{relative expression} = 2^{-[\Delta CP\ \text{sample} - \Delta CP\ \text{control}]}$$

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

Pfaffl, Nucleic Acids Research 2001

Determination principles of real-time PCR amplification efficiency

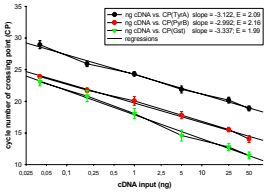
Direct methods:

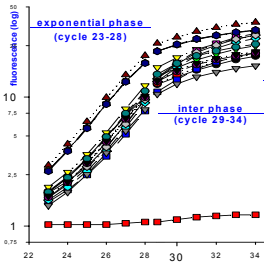
- **Dilution series**
  (Rasmussen 2001, Peirson et al. 2003, etc.)
- **Determination of absolute increase in fluorescence**
  (Rasmusen 2001; Peccoud & Jacob 1998; Pfaffl 2001)

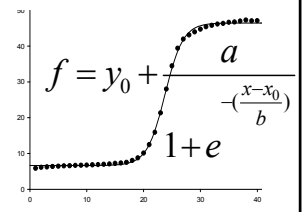
Indirect methods: (fit of mathematical models)

- **Sigmoidal model**
  (Lui & Saint 2002; Rutledge 2003; Tichopad et al. 2002)
- **Logistic model**
  (Wittwer et al. 2000; Tichopad et al. 2003)
- **Exponential model**
  (Tichopad et al. 2003, Bar et al. 2003)
- **Multiple-model fit**
  sigmoidal, linear, and exponential *(Tichopad et al. 2003)*
- **Comparative Quantitation Analysis**
  Rotor-Gene software (Corbett Life Science)
- **[CalQPlex algorithm]**
  realplex software (Eppendorf)
- **E-Method algorithm**
  Light-Cycler software (Roche Applied Science)

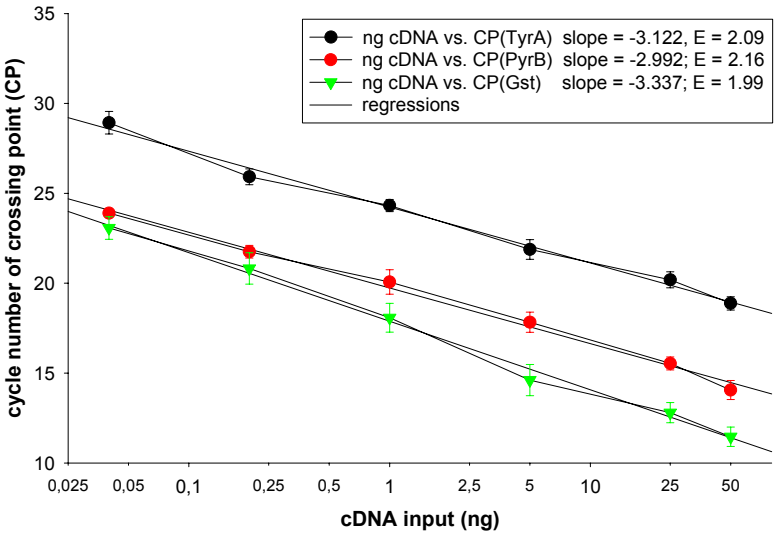
- http://**Efficiency.gene-quantification.info**





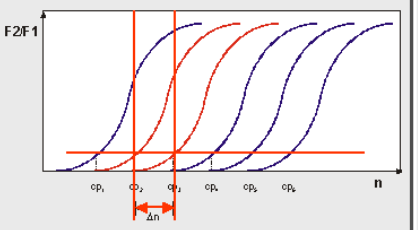


Determination principles of real-time PCR efficiency:
Dilution series



Calculation of real-time PCR efficiency

$E = 10^{-1/slope}$ => $E = 10^{-1/-3.337}$ => $E = 10^{0.299}$ => $E = 1.99$



$N_2 = N_{0,2} \times E^{n_2}$ $N_3 = N_{0,3} \times E^{n_3}$

For $N_2 = N_3$:

$N_{0,2} / N_{0,3} = E^{(n_3 - n_2)} = E^{\Delta n}$

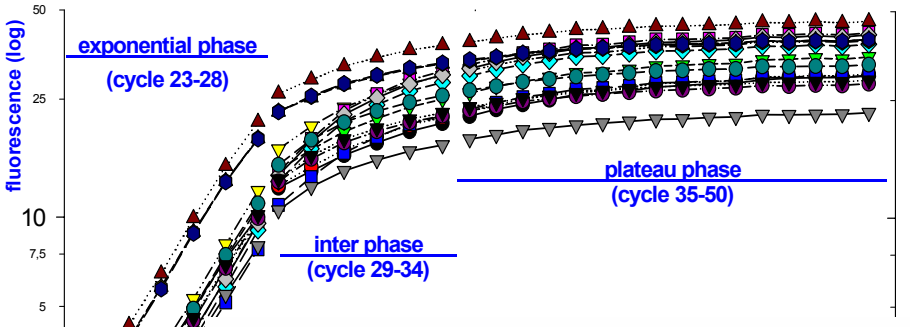
For $N_{0,2} = 10 \times N_{0,3}$ (10-fold dilutions):

$\Delta n = 1 / \log E$ $E = 10^{-1/slope}$

e.g. E = 2.0 Δn = 3.32
E = 1.9 Δn = 3.58
E = 1.8 Δn = 3.91

Roche Diagnostics, LC rel. Quantification software, March 2001

Rasmussen, R (2001) *Quantification on the LightCycler. In: Meuer, S, Wittwer, C, Nakagawara, K, eds. Rapid Cycle Real-time PCR, Methods and Applications Springer Press, Heidelberg; page 21-34*

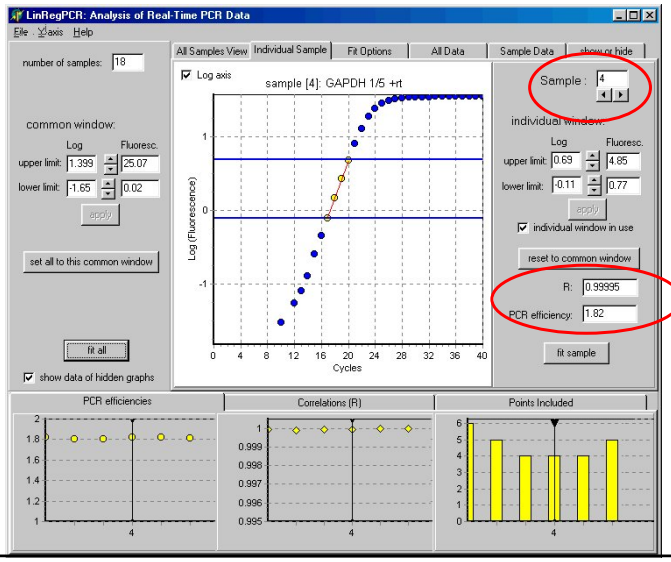


template	exponential phase (cycle 23-28)	inter phase (cycle 29-34)	plateau phase (cycle 35-50)
native IGF-1 mRNA	1.428 ± 0.013 (r = 0.997)	1.114 ± 0.006 (r = 0.987)	1.017 ± 0.002 (r = 0.952)
recombinant IGF-1 RNA	1.408 ± 0.018 (r = 0.992)	1.162 ± 0.001 (r = 0.974)	1.015 ± 0.001 (r = 0.933)
water (no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

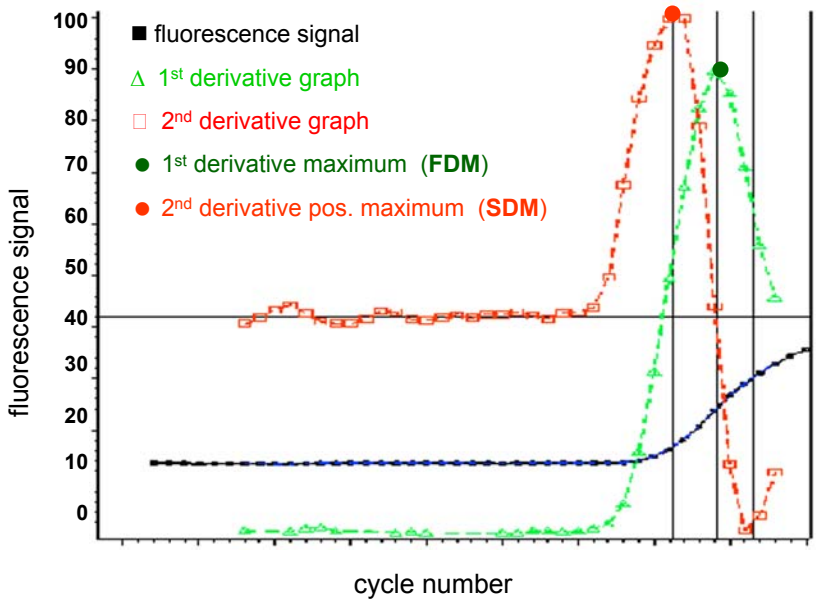
Calculation of real-time PCR efficiency: **LinRegPCR Interface**

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

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



Principal of *"Second Derivative Maximum"* methods (1)



Principal of *"Second Derivative Maximum"* methods (2)

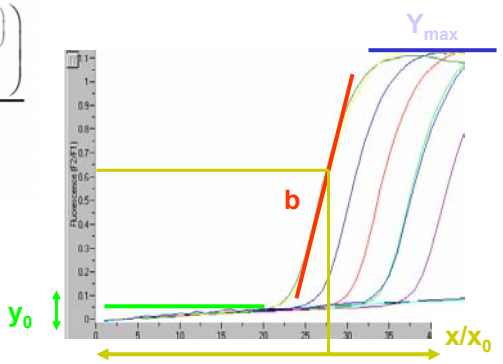
$$f(x) = y_0 + \frac{a}{1 + e^{-\frac{x - x_0}{b}}}$$

$x_0 \sim x_{1/2\,F}$

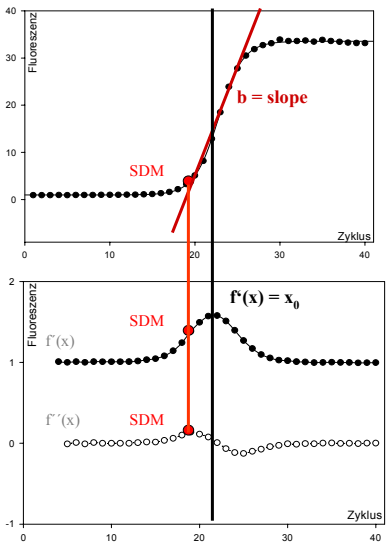
$a = y_{max} - y_0$

$$f'(x) = -a\left(1 + e^{-\frac{x - x_0}{b}}\right)^{-2} \cdot e^{-\frac{x - x_0}{b}} \cdot \left(-\frac{1}{b}\right)$$

$$f''(x) = -\frac{a}{b^2} \cdot \frac{e^{-\frac{x - x_0}{b}}\left(1 - e^{\left(-\frac{x - x_0}{b}\right)}\right)}{\left(1 + e^{-\frac{x - x_0}{b}}\right)^3}$$



Calculation of SDM according to a 4-parametric sigmoidal model



$$f = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

$$f'(x) = \frac{a}{b} \cdot \frac{e^{-\frac{x - x_0}{b}}}{\left(1 + e^{-\frac{x - x_0}{b}}\right)^2}$$

$$f''(x) = -\frac{a}{b^2} \cdot \frac{e^{-\frac{x - x_0}{b}} - e^{-2\frac{x - x_0}{b}}}{\left(1 + e^{-\frac{x - x_0}{b}}\right)^3}$$

$$f'''(x) = \frac{a}{b^3} \cdot \frac{e^{-\frac{x - x_0}{b}} - 4e^{-2\frac{x - x_0}{b}} + e^{-3\frac{x - x_0}{b}}}{\left(1 + e^{-\frac{x - x_0}{b}}\right)^4}$$

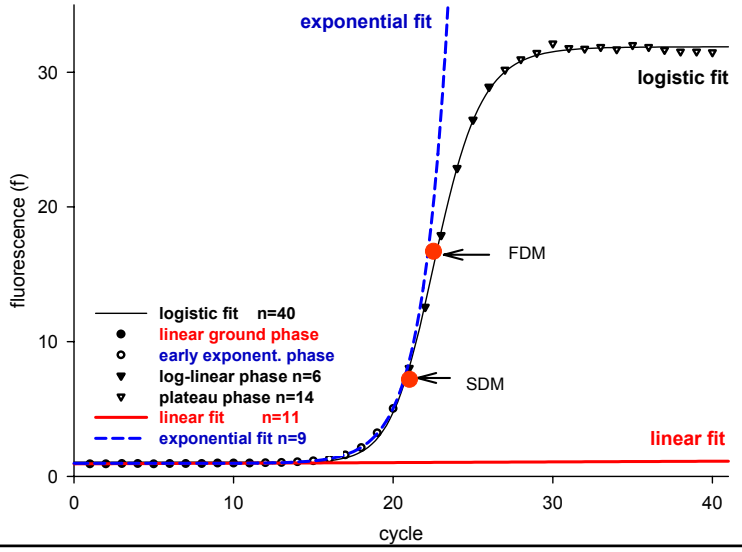
$f'''(x) = 0 \qquad x \approx x_0 \pm 1{,}317 \cdot b$

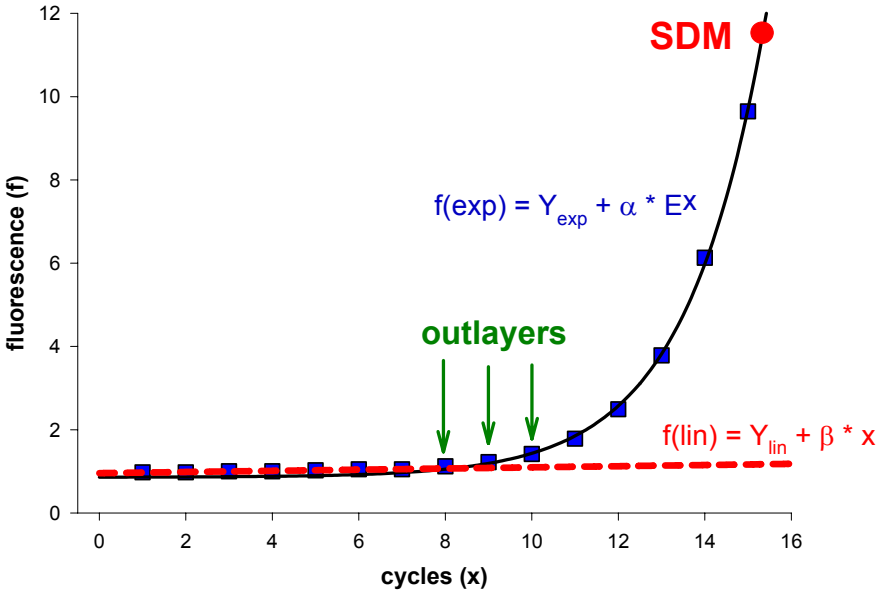
CP (*SDM$_{sm}$*) $\Rightarrow x = x_0 - 1{,}317 \cdot b$

Sigmoidal Model: Tichopad et al. (2002) *Biotechnol Lett* **24**: 2053-2057
Logistic Model: Tichopad et al. (2003) *NAR* **31(20):** e122
Sigmoidal Model: Tichopad et al. (2004) *MCP* **(18):** 45-50

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

multi-model fitting *Tichopad et al., 2003 NAR **31(20):** e122*





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).

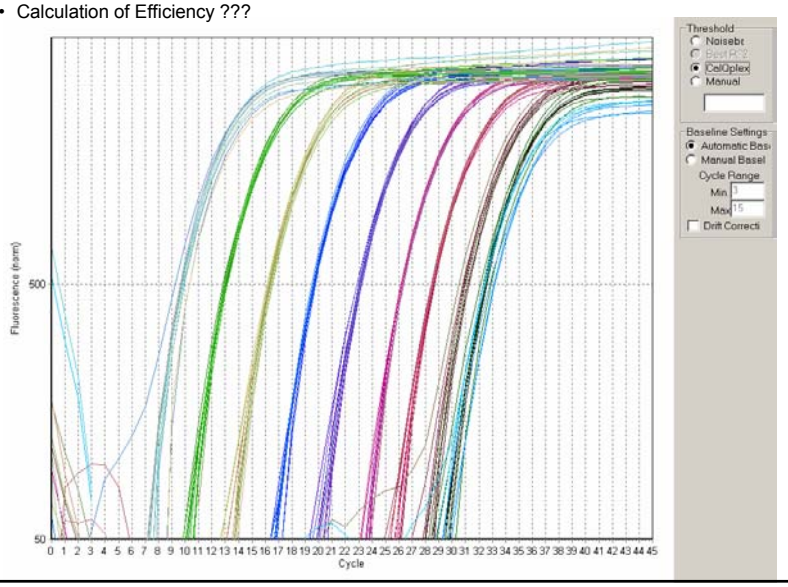
$E1_{fit\ point}$ – Amplification efficiency computed from calibration curve[11] where crossing points are obtained as Fit–points.
$E1_{sdm}$ – Amplification efficiency computed from calibration curve where crossing points are computed as *SDM*.
$E2_{fdm}$ – Amplification efficiency computed from absolute 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.

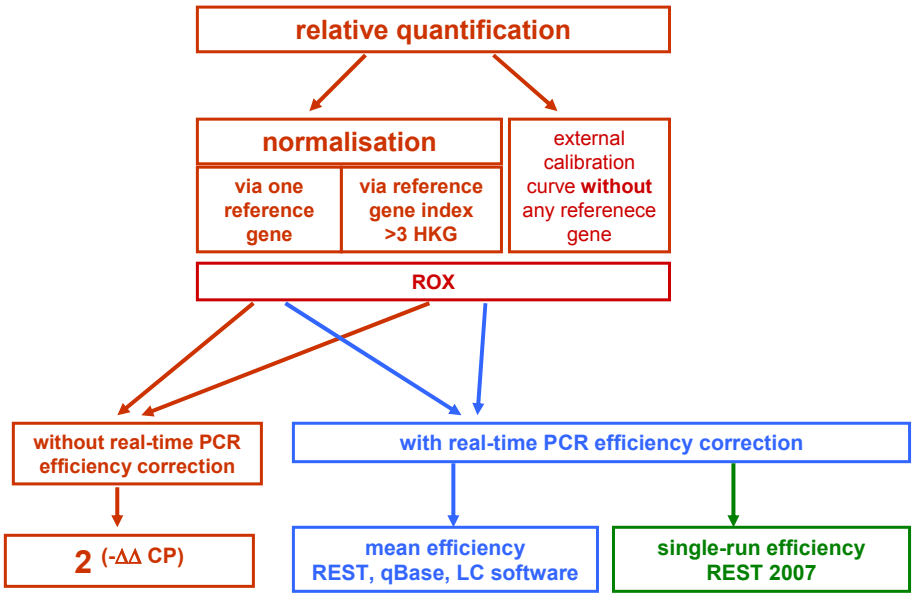
Tichopad et al., 2003 Nucleic Acids Research ***31(20):*** *e122*

CalQPlex algorithm in the new ***ep realplex*** software

- baseline corrected raw data
- sigmoidal fit of each amplification curve
- Quantification point (Ct) is defined as threshold from the exponential => non-exponential amplification
- Calculation of Efficiency ???



Future of relative Quantification in real time qRT-PCR

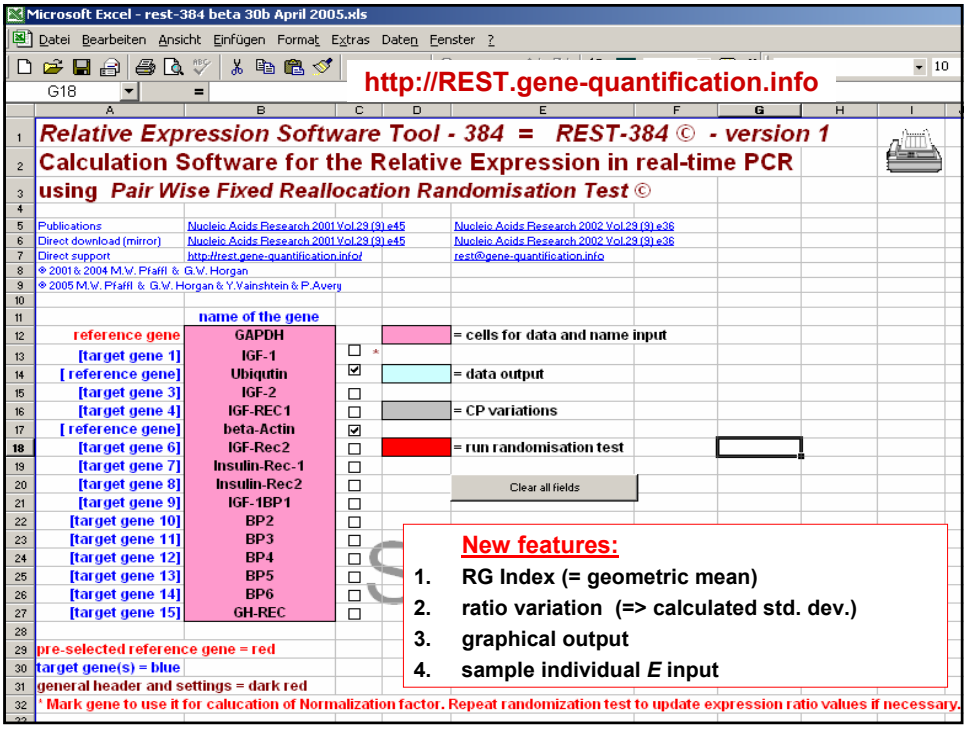


Relative Expression Software Tool (REST)

REST-384	for high throughput applications	(August 2006)
REST-MCS	multiple condition solver	(August 2006)
REST-RG	direct import for sample specific qPCR efficiency and TOP from Rotor-Gene software	(August 2006)
REST-2005	Stand alone application	(March 2005)
REST-2007	Stand alone application standard Mode + single run efficiency correction	(May 2007)

http://REST.gene-quantification.info/

Pfaffl MW, Horgan GW, Dempfle L. (2002) Nucleic Acids Res. 2002 30(9): e36
Relative expression software tool (REST) for group-wise comparison
and statistical analysis of relative expression results in real-time PCR.


Microsoft Excel - rest-384 beta 30b April 2005.xls
http://REST.gene-quantification.info
Relative Expression Software Tool - 384 = REST-384 © - version 1
Calculation Software for the Relative Expression in real-time PCR
using Pair Wise Fixed Reallocation Randomisation Test ©
Publications
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© 2001 & 2004 M.W. Pfaffl & G.W. Horgan
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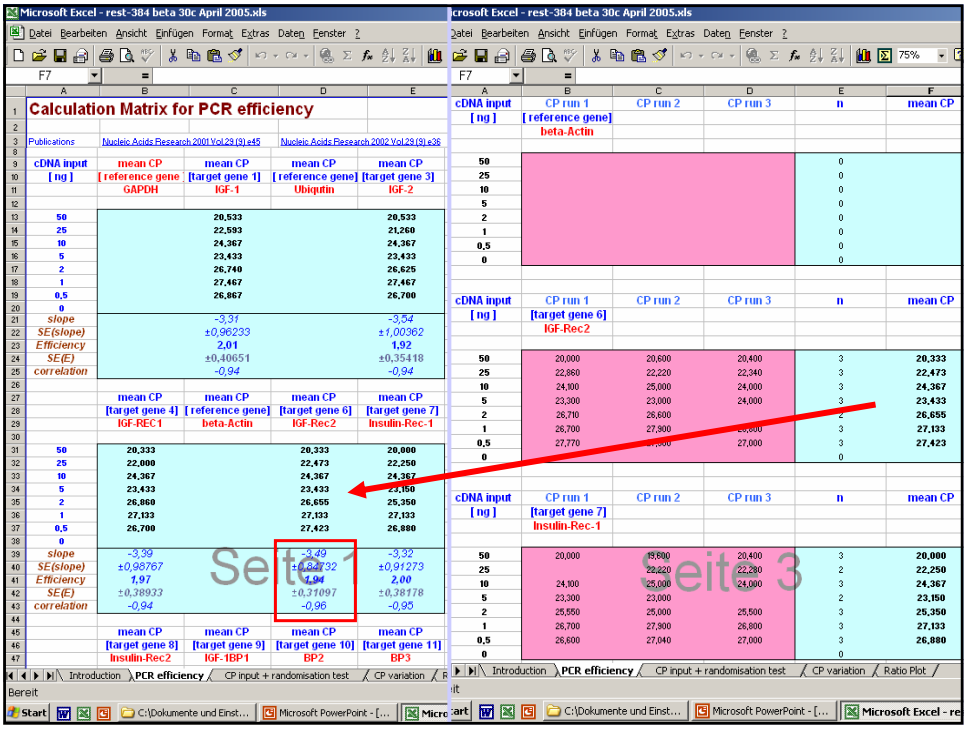
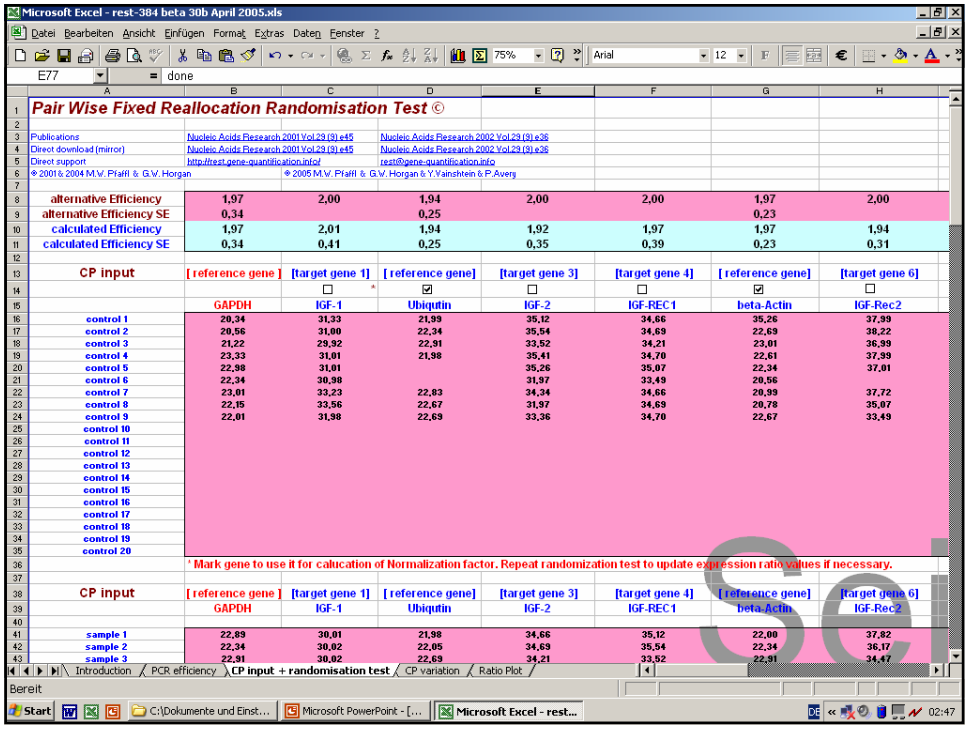
Nucleic Acids Research 2001 Vol.29 (9) e45
Nucleic Acids Research 2002 Vol.29 (9) e36
http://rest.gene-quantification.info/
rest@gene-quantification.info
name of the gene
reference gene
[target gene 1]
[reference gene]
[target gene 3]
[target gene 4]
[reference gene]
[target gene 6]
[target gene 7]
[target gene 8]
[target gene 9]
[target gene 10]
[target gene 11]
[target gene 12]
[target gene 13]
[target gene 14]
[target gene 15]
GAPDH
IGF-1
Ubiqutin
IGF-2
IGF-REC1
beta-Actin
IGF-Rec2
Insulin-Rec-1
Insulin-Rec-2
IGF-1BP1
BP2
BP3
BP4
BP5
BP6
GH-REC
= cells for data and name input
= data output
= CP variations
= run randomisation test
Clear all fields
pre-selected reference gene = red
target gene(s) = blue
general header and settings = dark red
' Mark gene to use it for calucation of Normalization factor. Repeat randomization test to update expression ratio values if necessary.
New features:
1. RG Index (= geometric mean)
2. ratio variation (=> calculated std. dev.)
3. graphical output
4. sample individual E input


Microsoft Excel - rest-384 beta 30c April 2005.xls
Calculation Matrix for PCR efficiency
Seite 2
Seite 3
Introduction
PCR efficiency
CP input + randomisation test
CP variation
Ratio Plot



Microsoft Excel - rest-384 beta 30b April 2005.xls

E77 = done

Pair Wise Fixed Reallocation Randomisation Test ©

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Direct download (mirror)	Nucleic Acids Research 2001 Vol.29 (9) e45	Nucleic Acids Research 2002 Vol.29 (9) e36
Direct support	http://rest.gene-quantification.info/	rest@gene-quantification.info
© 2001 & 2004 M.W. Pfaffl & G.W. Horgan	© 2005 M.W. Pfaffl & G.W. Horgan & Y.Vainshtein & P.Averg	

alternative Efficiency	1,97	2,00	1,94	2,00	2,00	1,97	2,00
alternative Efficiency SE	0,34		0,25			0,23	
calculated Efficiency	1,97	2,01	1,94	1,92	1,97	1,97	1,94
calculated Efficiency SE	0,34	0,41	0,25	0,35	0,39	0,23	0,31

CP input	[reference gene]	[target gene 1]	[reference gene]	[target gene 3]	[target gene 4]	[reference gene]	[target gene 6]
	☐	☐ *	☑	☐	☐	☑	☐
	GAPDH	IGF-1	Ubiqutin	IGF-2	IGF-REC1	beta-Actin	IGF-Rec2
control 1	20,34	31,33	21,99	35,12	34,66	35,26	37,99
control 2	20,56	31,00	22,34	35,54	34,69	22,69	38,22
control 3	21,22	29,92	22,91	33,52	34,21	23,01	36,99
control 4	23,33	31,01	21,98	35,41	34,70	22,61	37,99
control 5	22,98	31,01		35,26	35,07	22,34	37,01
control 6	22,34	30,98		31,97	33,49	20,56	
control 7	23,01	33,23	22,83	34,34	34,66	20,99	37,72
control 8	22,15	33,56	22,67	31,97	34,69	20,78	35,07
control 9	22,01	31,98	22,69	33,36	34,70	22,67	33,49
control 10							
control 11							
control 12							
control 13							
control 14							
control 15							
control 16							
control 17							
control 18							
control 19							
control 20							

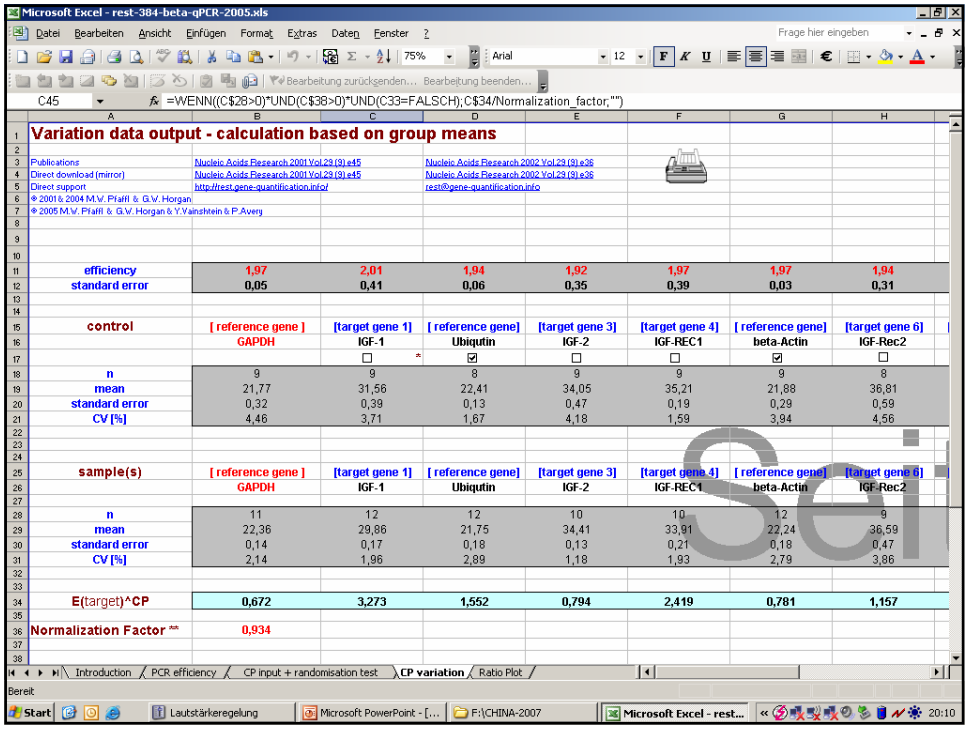
* Mark gene to use it for calucation of Normalization factor. Repeat randomization test to update expression ratio values if necessary.

CP input	[reference gene]	[target gene 1]	[reference gene]	[target gene 3]	[target gene 4]	[reference gene]	[target gene 6]
	GAPDH	IGF-1	Ubiqutin	IGF-2	IGF-REC1	beta-Actin	IGF-Rec2
sample 1	22,89	30,01	21,98	34,66	35,12	22,00	37,82
sample 2	22,34	30,02	22,05	34,69	35,54	22,34	36,17
sample 3	22,91	30,02	22,69	34,21	33,52	22,91	34,47

Introduction | PCR efficiency | CP input + randomisation test | CP variation | Ratio Plot

Bereit

Start | C:\Dokumente und Einst... | Microsoft PowerPoint - [... | Microsoft Excel - rest... | 02:47



Microsoft Excel - rest-384-beta-qPCR-2005.xls

C45 =WENN((C$28>0)*UND(C$38>0)*UND(C33=FALSCH);C$34/Normalization_factor;"")

Variation data output - calculation based on group means

Publications	Nucleic Acids Research 2001 Vol.29 (9) e45	Nucleic Acids Research 2002 Vol.29 (9) e36
Direct download (mirror)	Nucleic Acids Research 2001 Vol.29 (9) e45	Nucleic Acids Research 2002 Vol.29 (9) e36
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© 2005 M.W. Pfaffl & G.W. Horgan & Y.Vainshtein & P.Averg		

efficiency	1,97	2,01	1,94	1,92	1,97	1,97	1,94
standard error	0,05	0,41	0,06	0,35	0,39	0,03	0,31

control	[reference gene]	[target gene 1]	[reference gene]	[target gene 3]	[target gene 4]	[reference gene]	[target gene 6]
	GAPDH	IGF-1	Ubiqutin	IGF-2	IGF-REC1	beta-Actin	IGF-Rec2
	☐	☐ *	☑	☐	☐	☑	☐
n	9	9	8	9	9	9	8
mean	21,77	31,56	22,41	34,05	35,21	21,88	36,81
standard error	0,32	0,39	0,13	0,47	0,19	0,29	0,59
CV [%]	4,46	3,71	1,67	4,18	1,59	3,94	4,56

sample(s)	[reference gene]	[target gene 1]	[reference gene]	[target gene 3]	[target gene 4]	[reference gene]	[target gene 6]
	GAPDH	IGF-1	Ubiqutin	IGF-2	IGF-REC1	beta-Actin	IGF-Rec2
n	11	12	12	10	10	12	9
mean	22,36	29,86	21,75	34,41	33,91	22,24	36,59
standard error	0,14	0,17	0,18	0,13	0,21	0,18	0,47
CV [%]	2,14	1,96	2,89	1,18	1,93	2,79	3,86

E(target)^CP	0,672	3,273	1,552	0,794	2,419	0,781	1,157

Normalization Factor *** 0,934

Introduction | PCR efficiency | CP input + randomisation test | CP variation | Ratio Plot

Bereit

Start | Lautstärkeregelung | Microsoft PowerPoint - [... | F:\CHINA-2007 | Microsoft Excel - rest... | 20:10

REST error calculation using Taylor´s series

by Peter J. Avery, University of Newcastle, Mathematics and Statistics

calculated expression ratio = φ

calculated variation is dependent of 6 different SEs *[2 x SE(Eff.); 4 x SE(mean CP)]*

SE (expression ratio by REST) = around 20-50%

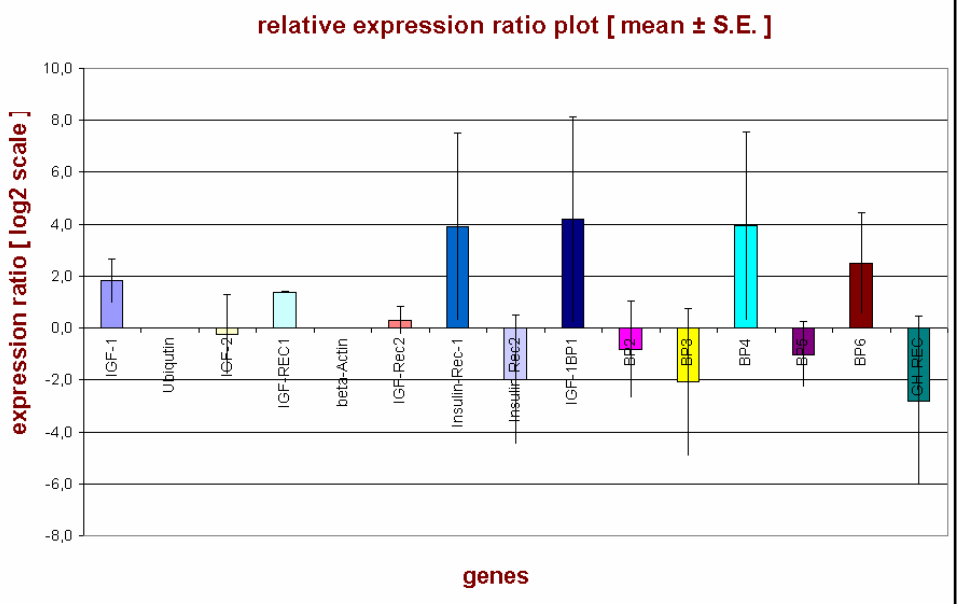
- $CP_{target,test}$ = 32.61; $CP_{target,control}$ = 25.88;
- $CP_{ref,test}$ = 22.35; $CP_{ref,control}$ = 22.53;
- E_{target} =1.670 and E_{ref} = 1.885.
- This gives $1/F$ = 1.12/0.032 = 35.35.
- $SE(E_{target})$ = 0.036 and $SE(E_{ref})$ = 0.102

$$S.E.(\hat{\varphi}) = \varphi\{\frac{(CP_{t\arg,cont} - CP_{t\arg,test})^2}{\hat{E}_{t\arg}^2} SE^2(\hat{E}_{t\arg})$$

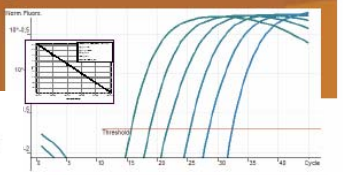
$$+ \frac{(CP_{ref,cont} - CP_{ref,test})^2}{\hat{E}_{ref}^2} SE^2(\hat{E}_{ref})$$

$$+ (\log_e E_{t\arg})^2 (SE^2(CP_{t\arg,cont}) + SE^2(CP_{t\arg,test}))$$

$$+ (\log_e E_{ref})^2 (SE^2(CP_{ref,cont}) + SE^2(CP_{ref,test}))\}^{0.5}$$



qPCR training courses and workshops



tataabiocenter
germany

http://TATAA.gene-quantification.info

Thank you team !

Thank you for your attention !