Strategies in qRT-PCR:

Considerations from sample collection to data analysis

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Quantification of specific mRNAs and microRNAs

(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, …)

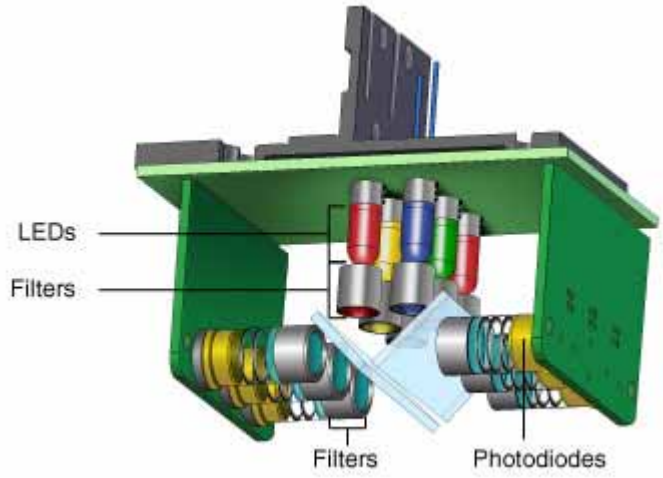
microRNA quantification assays:

- real-time qRT-PCR
- microRNA array

RNA integrity:

Bioanalyzer 2100, Experion

- Improvement of RNA extraction
- Total-RNA and microRNA integrity measurement
- Algorithm development



Software application development:

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

Genotype => Phenotype => Function

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

↓ Transcription

↓ Splicing

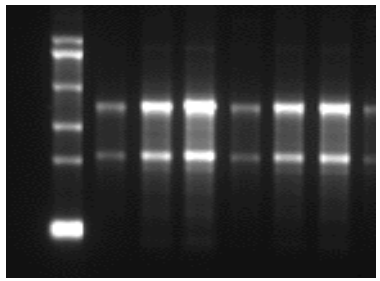
↑ *microRNA*

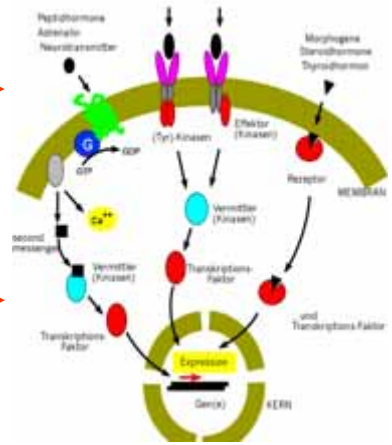
↓ Translation
post-translatoric modifications

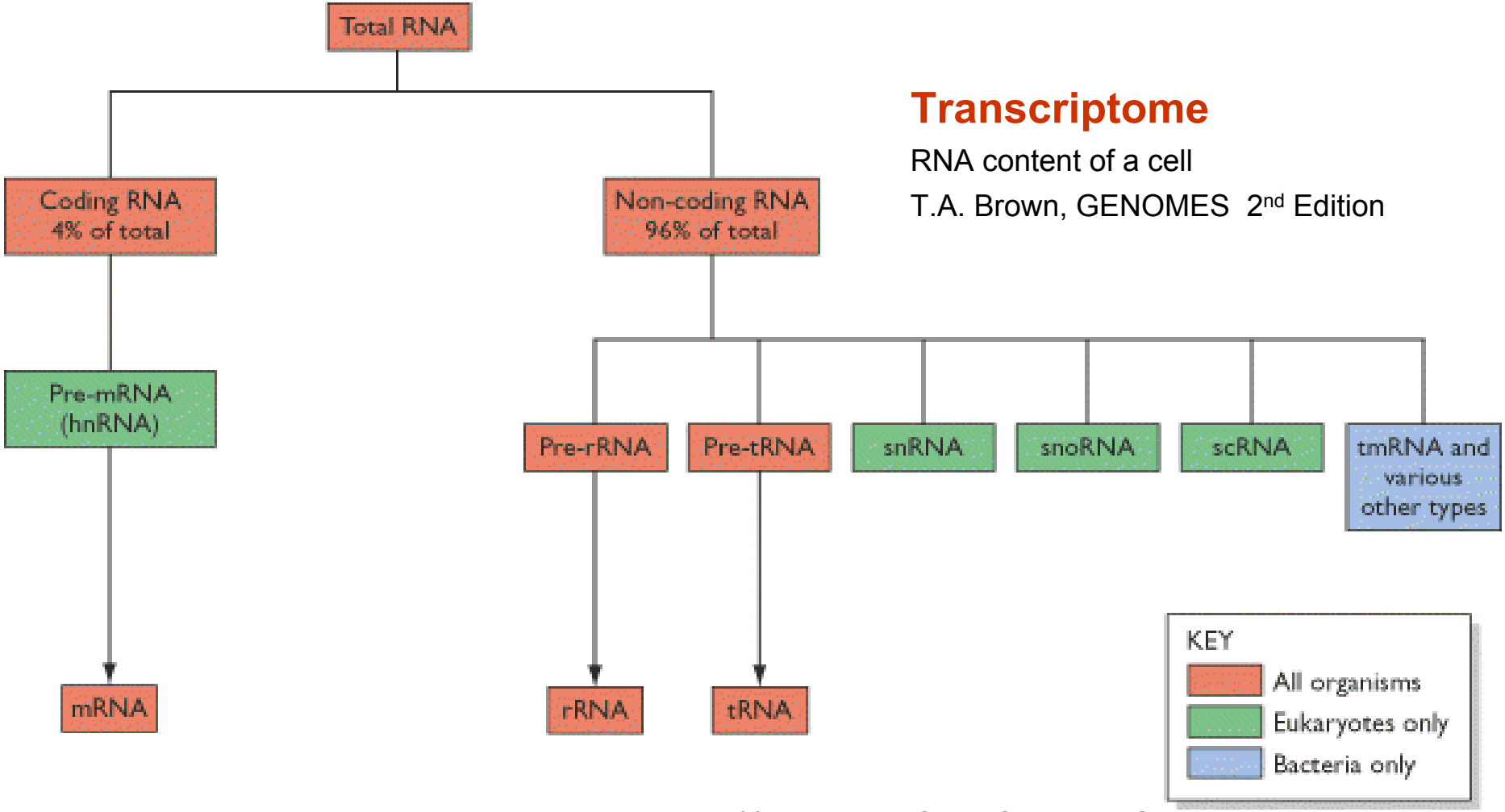
Genome

Transcriptome
& microTranscriptome
& Splicome

Proteome **Metabolome**







Transcriptome

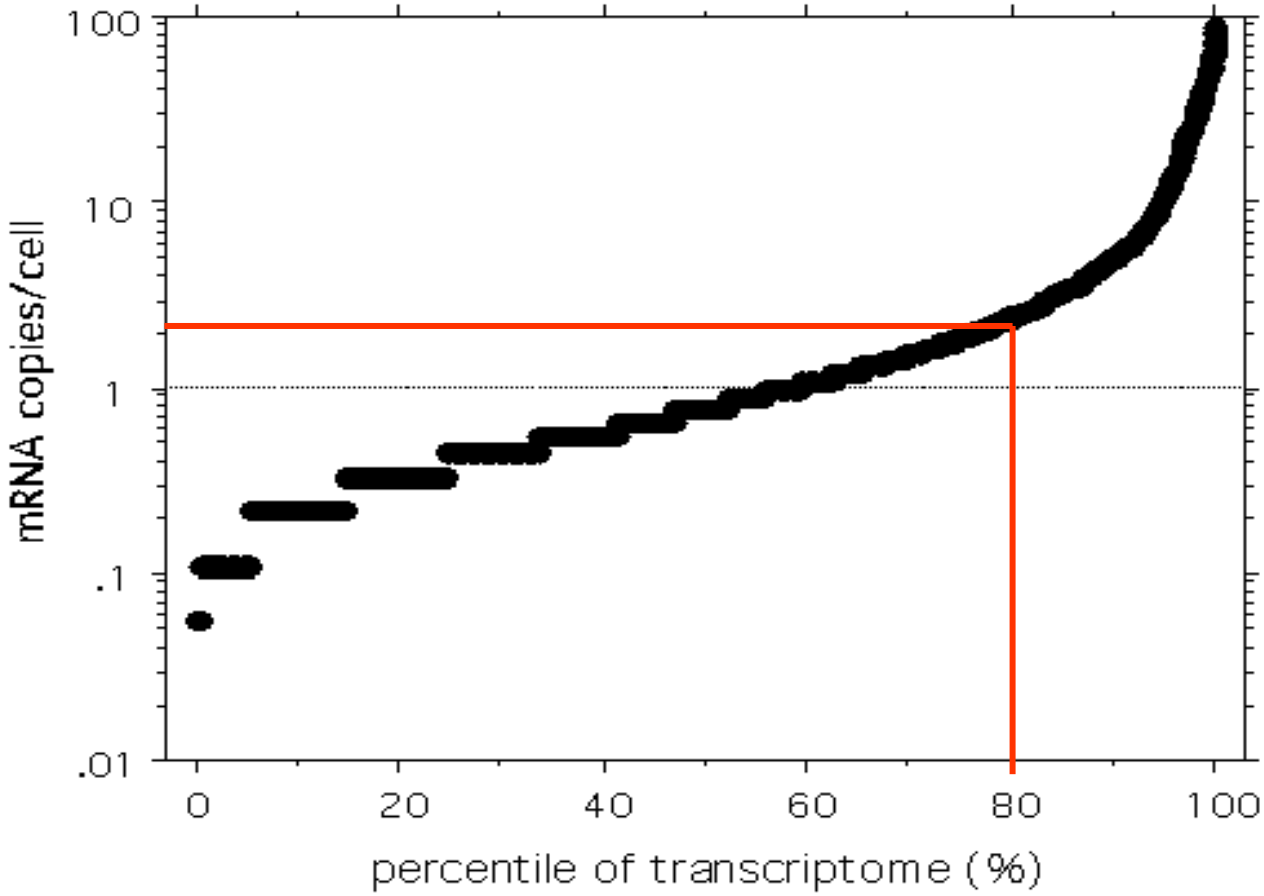
RNA content of a cell

T.A. Brown, GENOMES 2nd Edition

- ribosomal RNA rRNA 80-85% (5S, 18S und 28S)
- transfer RNA tRNA 10-15%
- **microRNA** **1-10%**
- **messenger RNA** **mRNA 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** - 20 copies/cell
- RNA quantity & RNA quality

Transcriptomics in Yeast

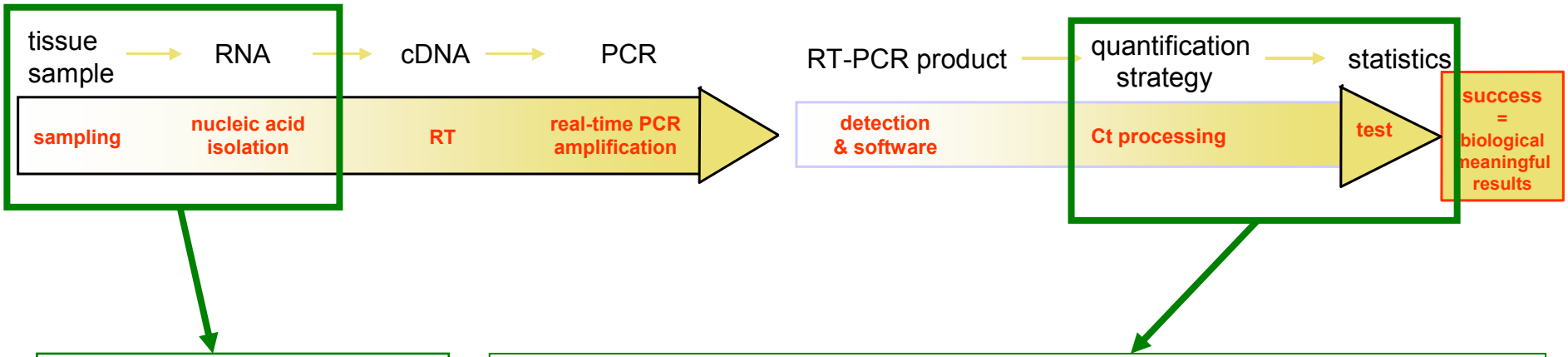
5460 transcript were investigated
estimated 15000 poly-A RNAs per cell
average level: 2.8 copies/cell
median level: 0.9 copies/cell
80% of the yeast transcriptome is expressed at 0.1-2 copies/cell



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

Pre-analytical RNA processing & post-analytical data analysis



Extraction method:

- total RNA
  - mRNA
  - microRNA
- liquid-liquid
- columns
- Automatic via robot
- **RNA integrity:**
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Quantification strategy:

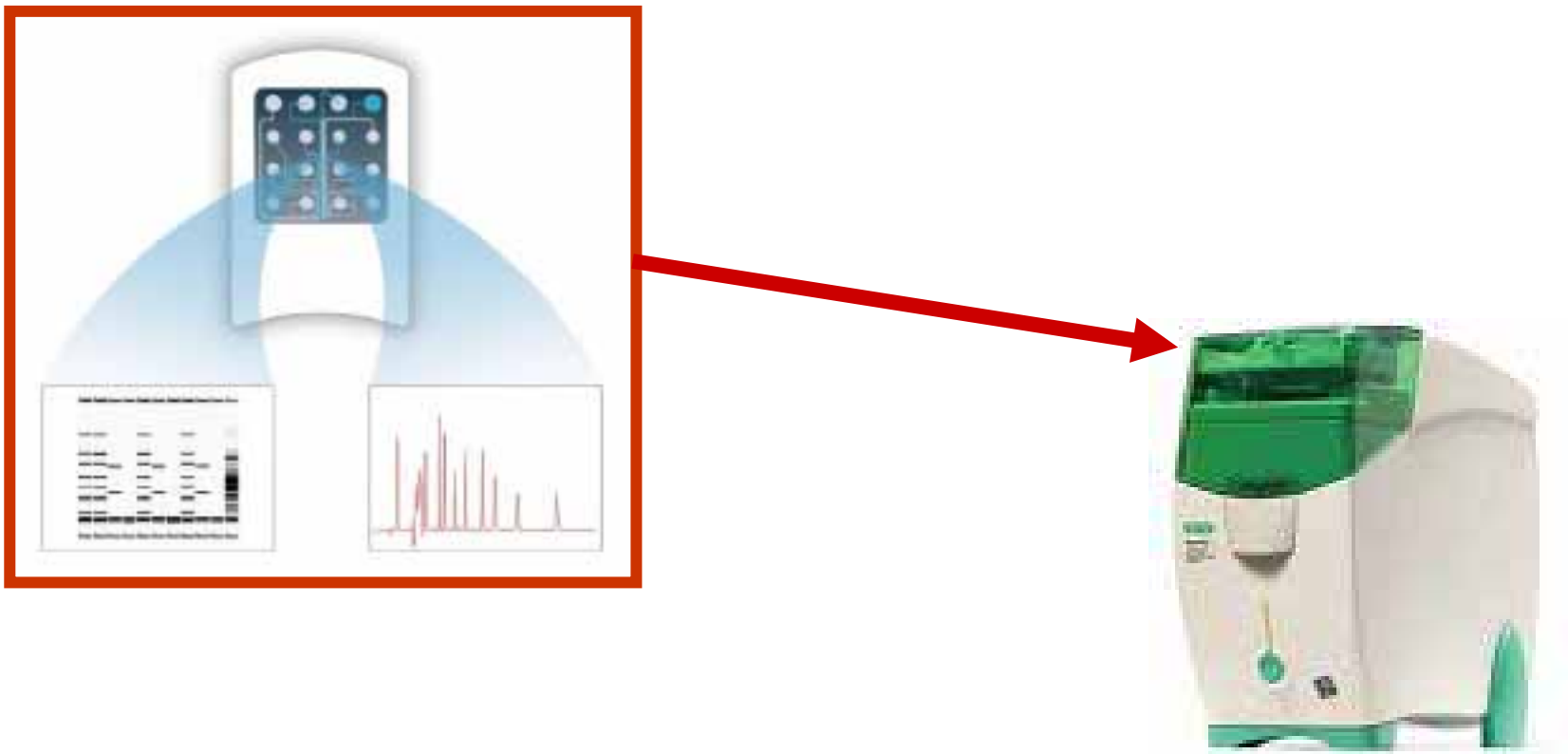
- **"absolute" quantification**
  - type of calibration curve?
  - normalization with RG
- **relative quantification**
  - total RNA, cells, tissue mass
  - normalization with RG
  - normalization via an RG Index (> 3 RGs)
  - geNorm, REST, BestKeeper, qBASE, Normfinder, etc.

BioStatistics & BioInformatics:

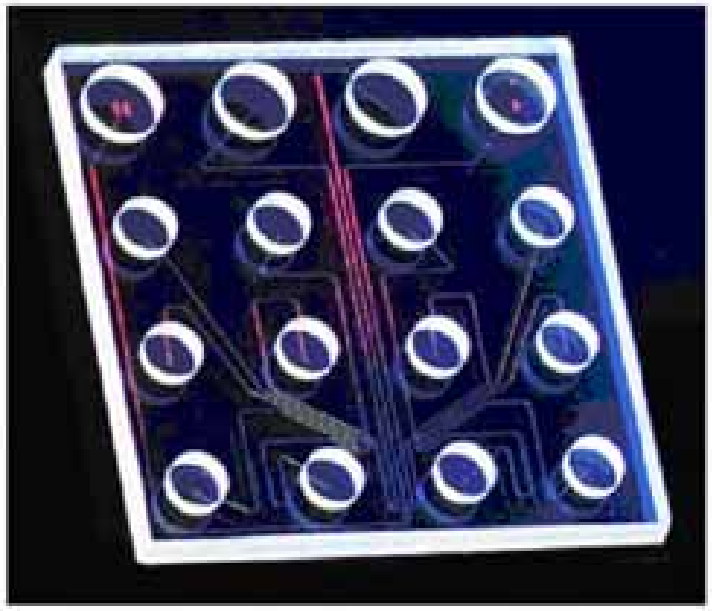
- **CP vs. quantified molecules**
- Normality of data (???)
- t-Test (?)
- ANOVA (on the ranks ?)
- SAS, SPSS, Excel, Sigma Stat
- Permutation test
- Randomization test (REST)
- Bootstrapping (REST-2008)
- Cluster analysis
- Multiple regression analysis
- Multi-dimensional modeling

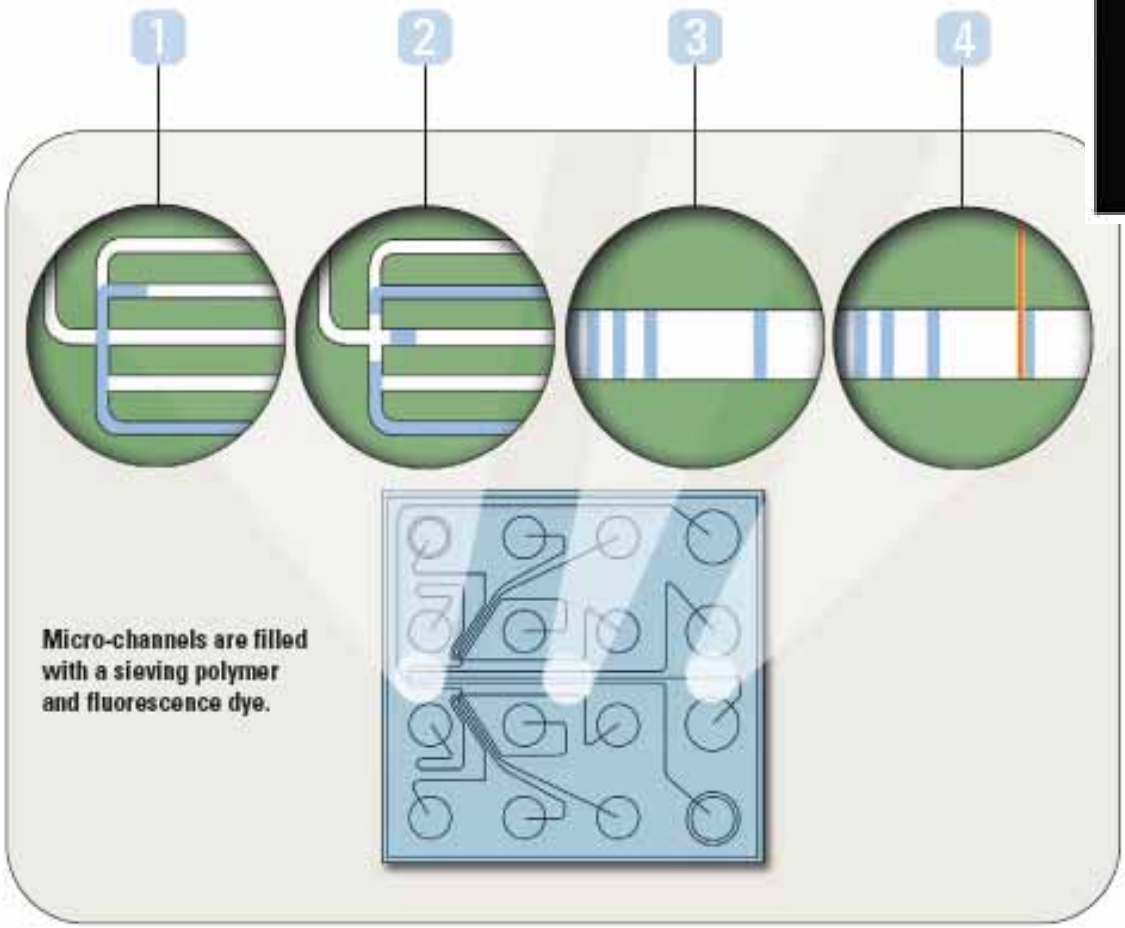
Experion & Bioanalyzer 2100

- Lab-on-chip technology
- Electrophoretic separation of total-RNA on mikrofabricated chips
- RNA samples are detected via laser induced fluorescence detection



Experion & Bioanalyzer 2100
RNA chip



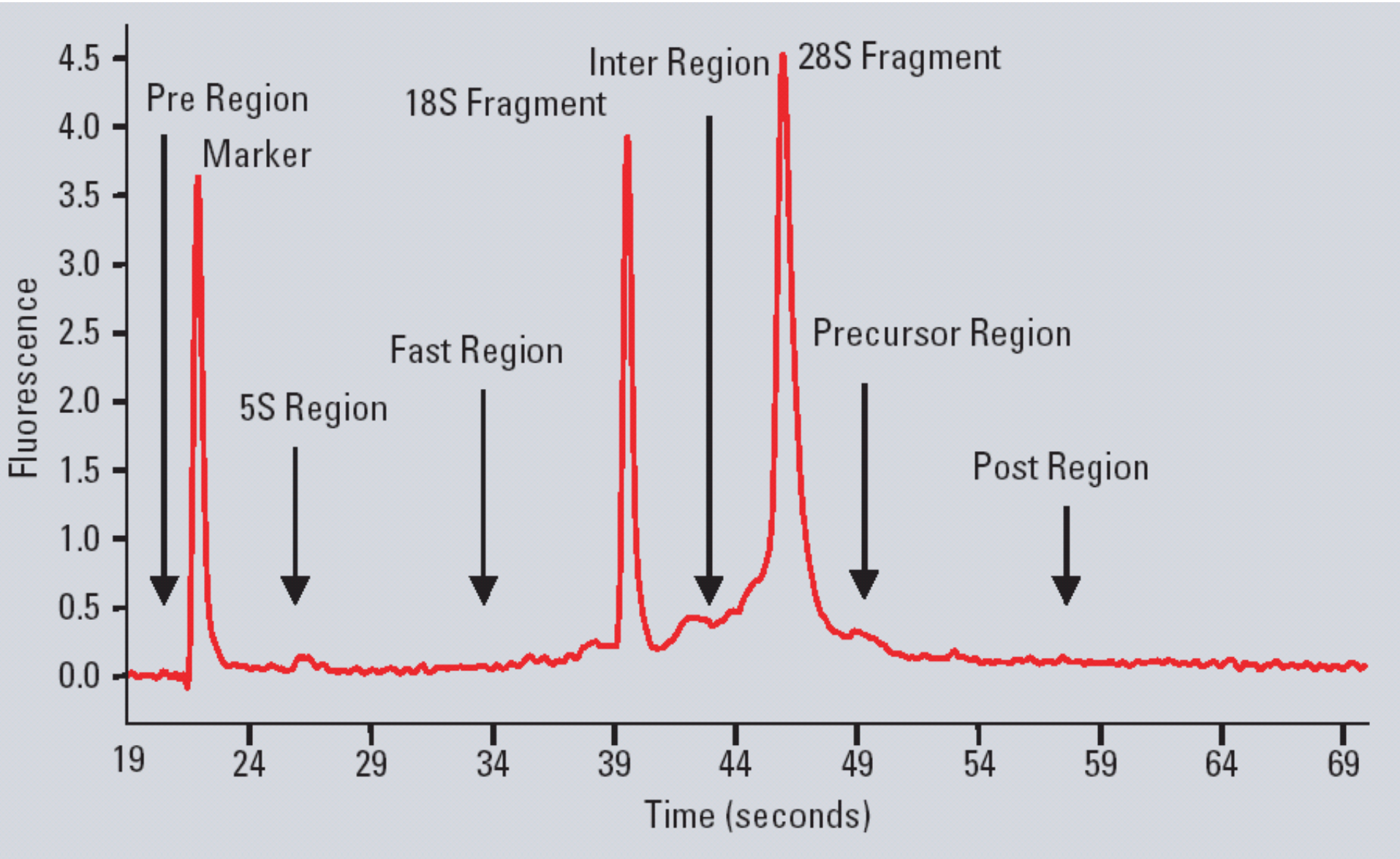


2 The sample is injected into the separation channel.

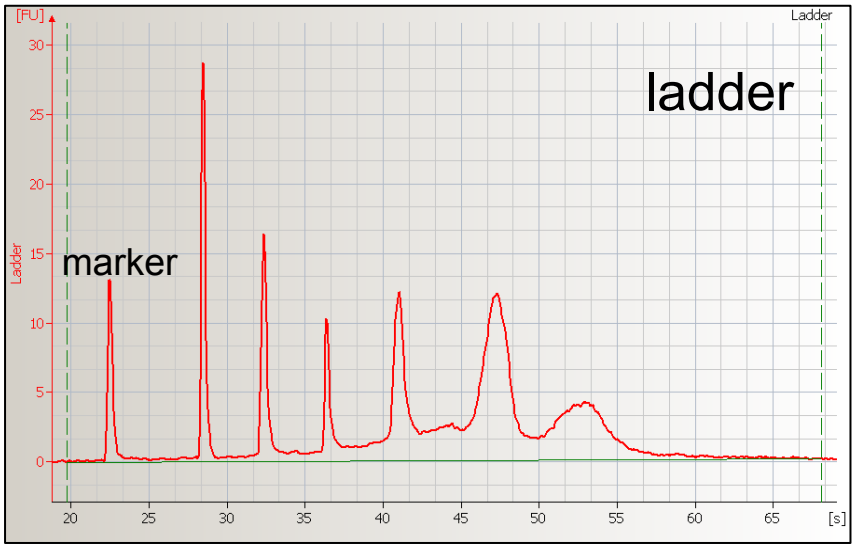
3 Sample components are electrophoretically separated.

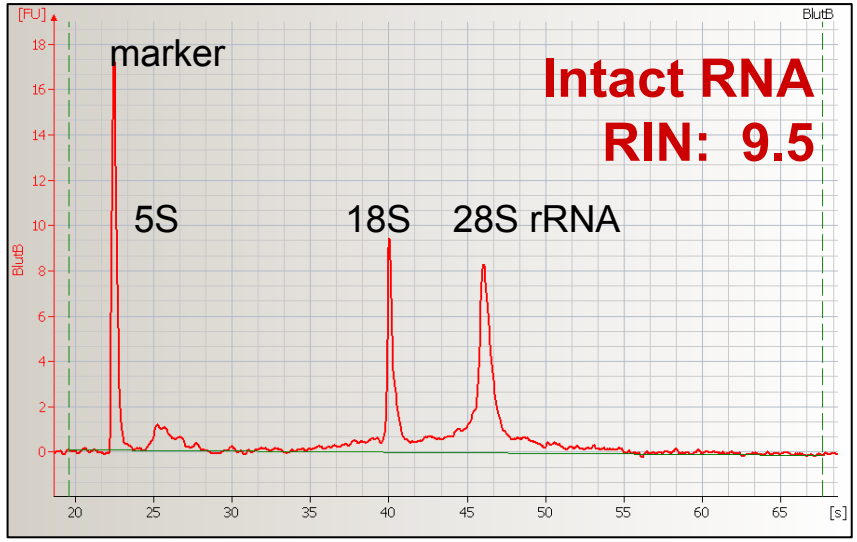
4 Components are detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks).

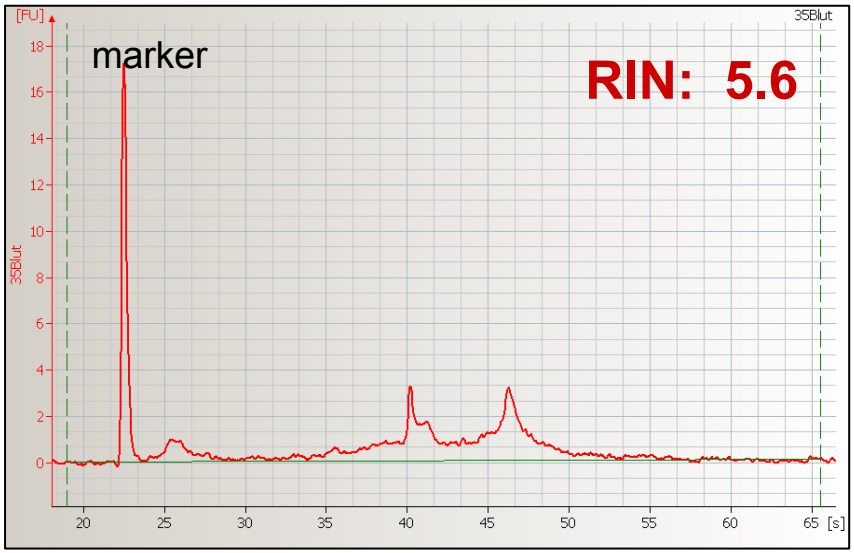
E-Gram & Electopherogram

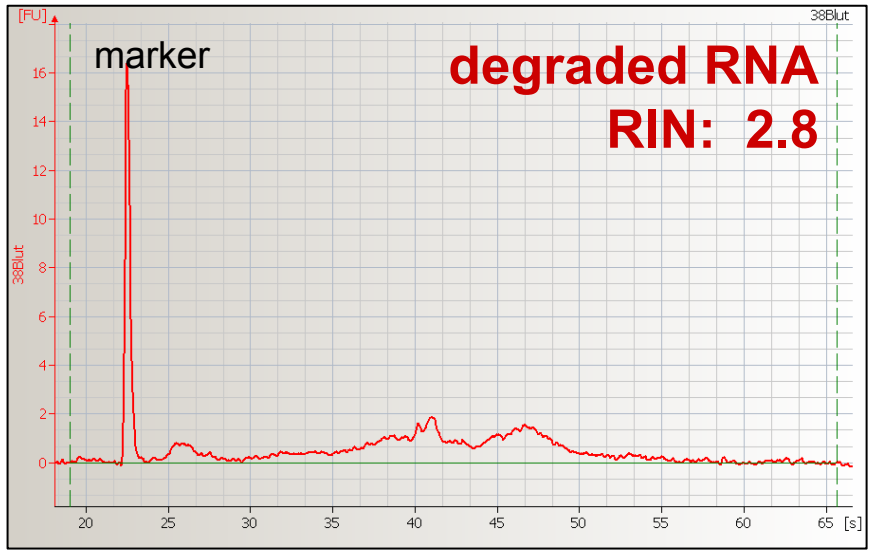


Various total-RNA qualities analysed in the Bioanalyzer 2100



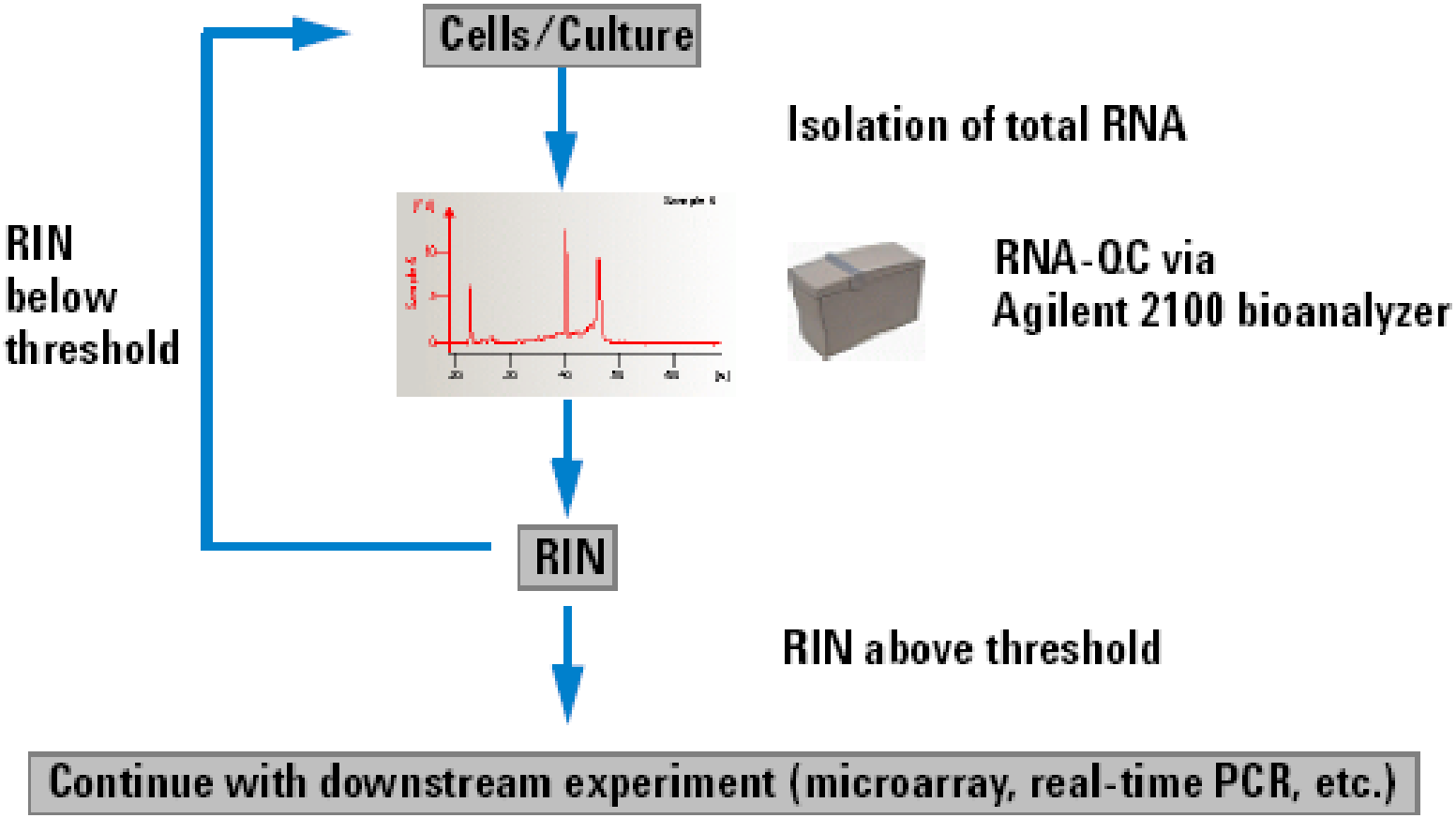






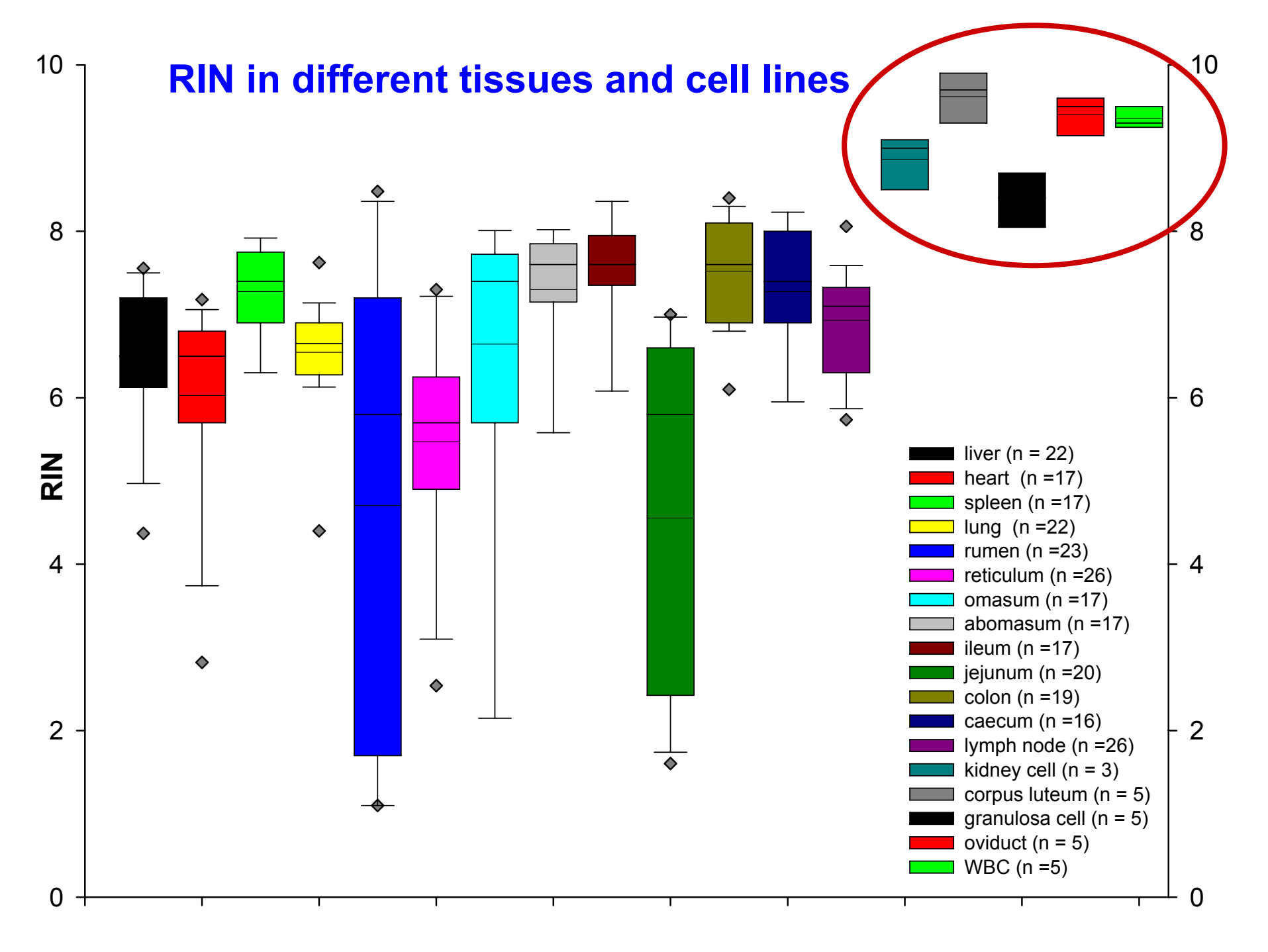
Fleige & Pfaffl, et al., Mol Aspects Med 2006; Fleige, et al., Biotechnolgy Letters 2006

Run standard experiment and use RIN to determine if sample integrity id sufficient:

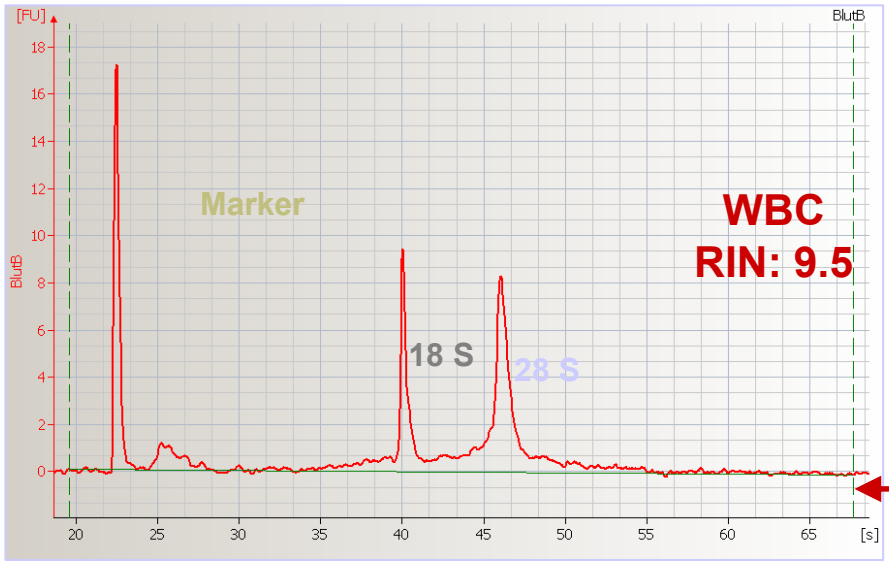


Q: Impact of RNA integrity on the qRT-PCR performance ?

Q: Impact on physiological result ?


RIN in different tissues and cell lines
RIN
10
8
6
4
2
0
liver (n = 22)
heart (n =17)
spleen (n =17)
lung (n =22)
rumen (n =23)
reticulum (n =26)
omasum (n =17)
abomasum (n =17)
ileum (n =17)
jejunum (n =20)
colon (n =19)
caecum (n =16)
lymph node (n =26)
kidney cell (n = 3)
corpus luteum (n = 5)
granulosa cell (n = 5)
oviduct (n = 5)
WBC (n =5)

Degradation scale



total RNA extracted from bovine tissues

artificial degradation

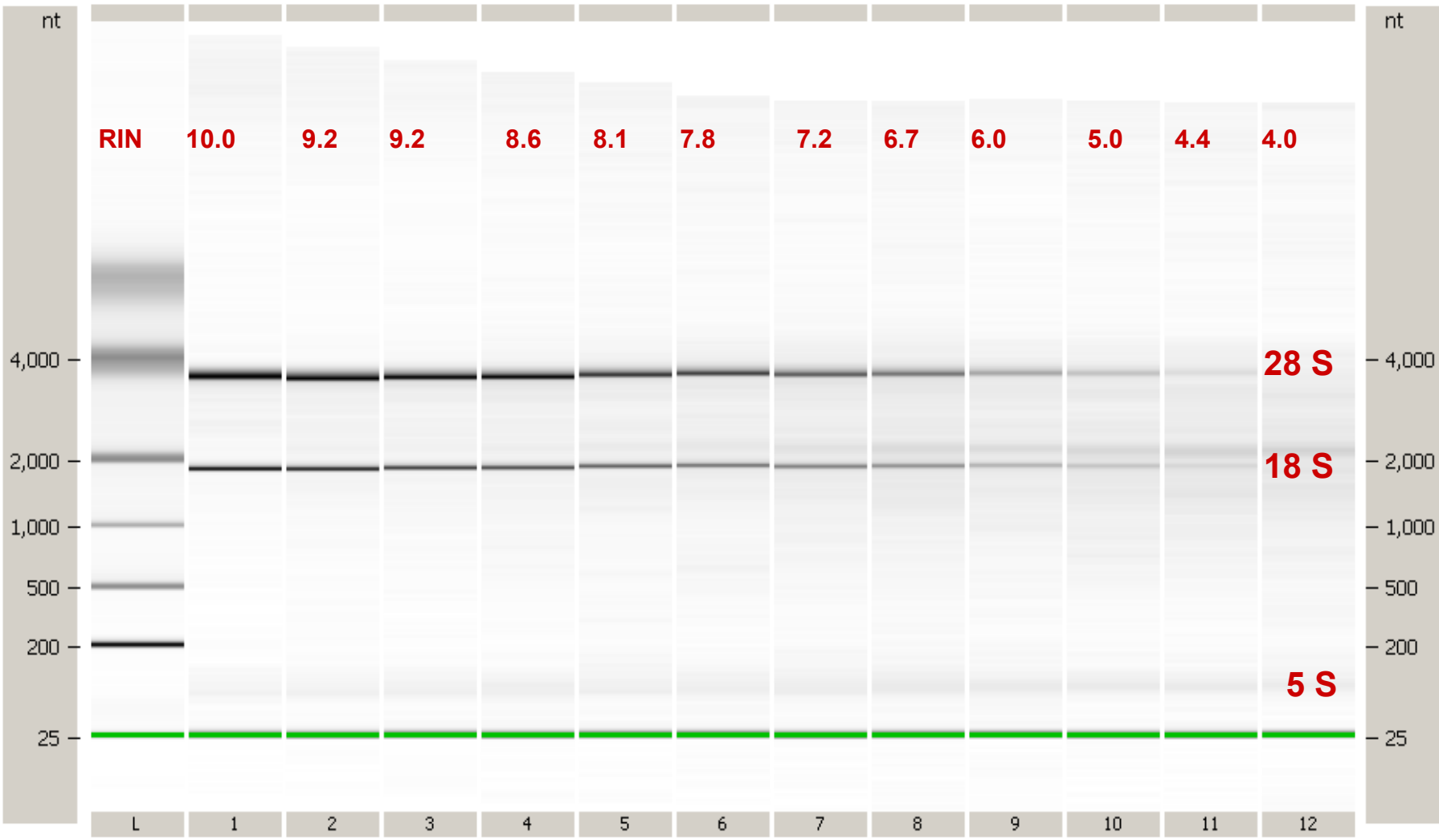
analyzed in Bioanalyzer

analyzed in Bioanalyzer

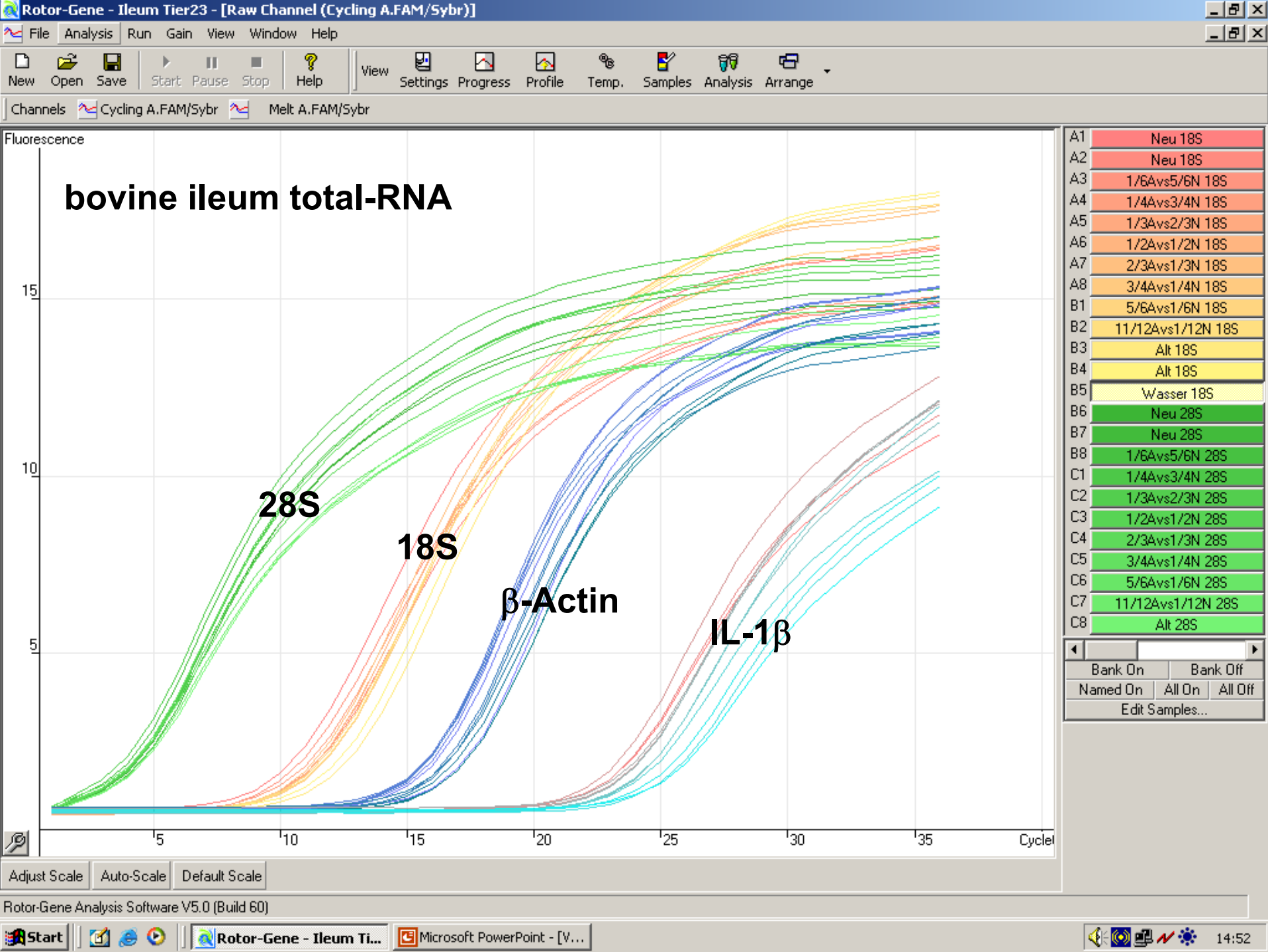
degradation scale of mixtures between good and bad samples

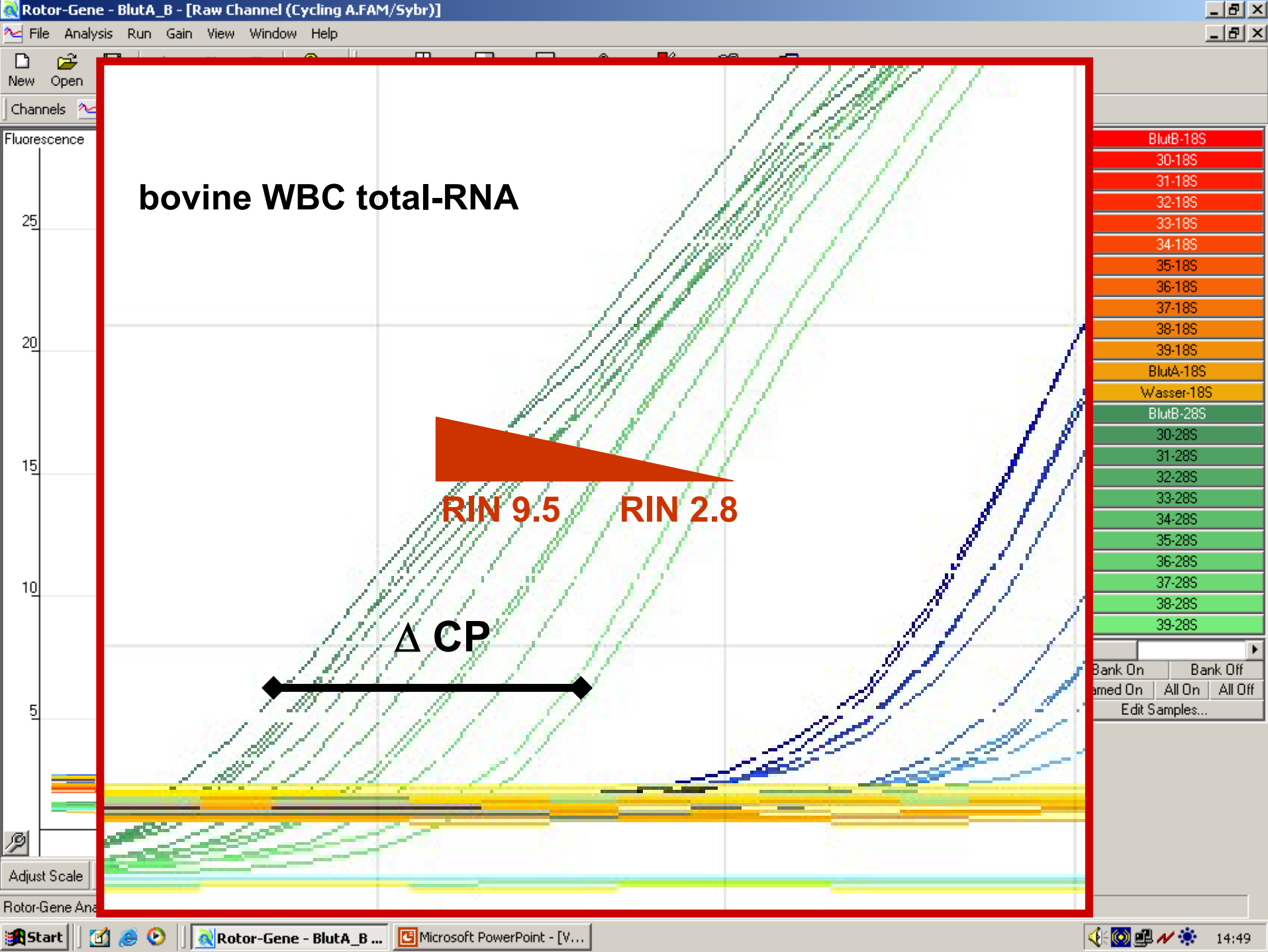
Fleige, et al., Biotechnolgy Letters 2006

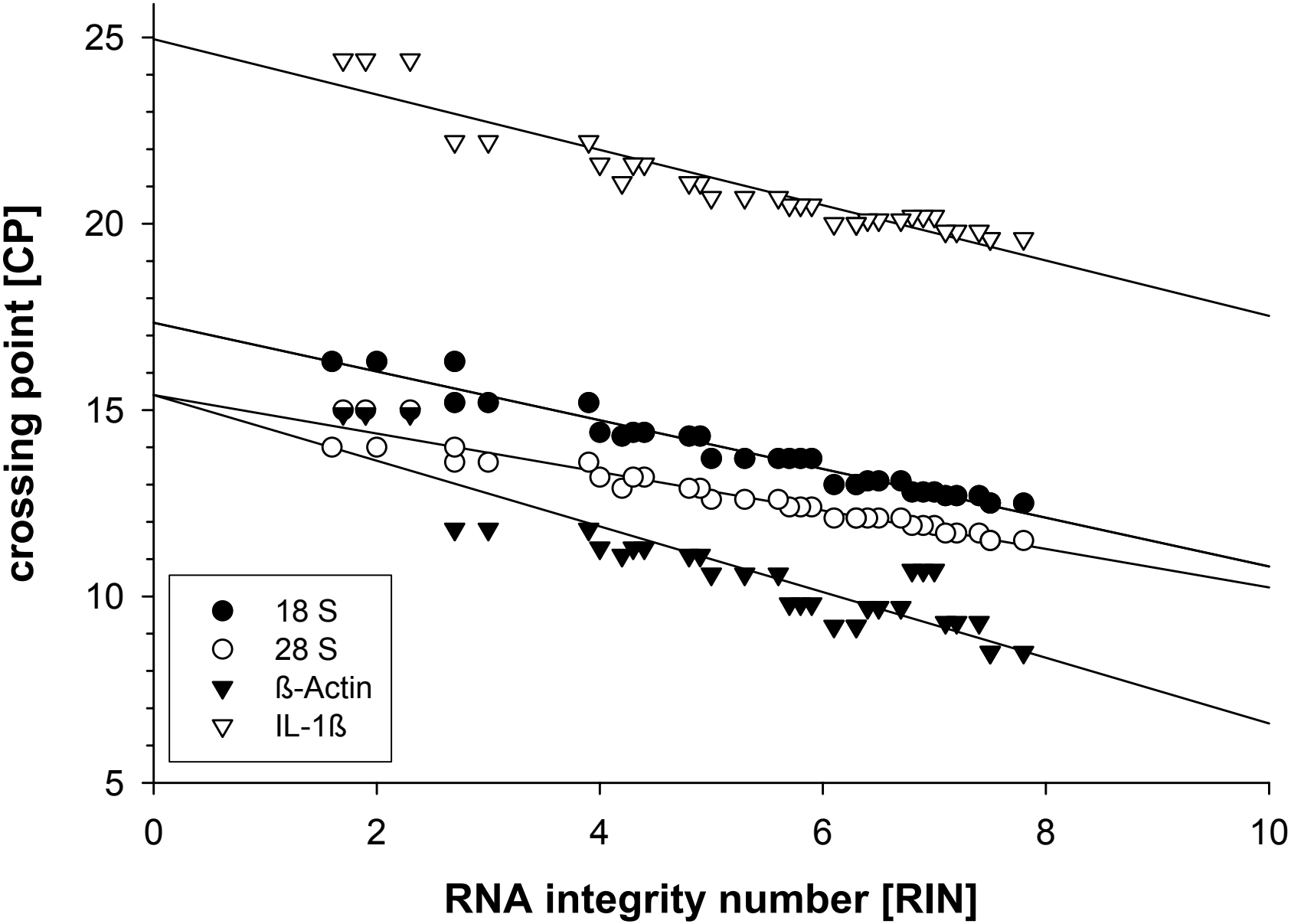
Degradation of extracted total-RNA



The intensity of bands decreases with increasing total-RNA degradation


Rotor-Gene - Ileum Tier23 - [Raw Channel (Cycling A.FAM/Sybr)]
File Analysis Run Gain View Window Help
New Open Save Start Pause Stop Help View Settings Progress Profile Temp. Samples Analysis Arrange
Channels Cycling A.FAM/Sybr Melt A.FAM/Sybr
Fluorescence
bovine ileum total-RNA
28S
18S
β-Actin
IL-1β
15
10
5
5 10 15 20 25 30 35
Cycle
A1 Neu 18S
A2 Neu 18S
A3 1/6Avs5/6N 18S
A4 1/4Avs3/4N 18S
A5 1/3Avs2/3N 18S
A6 1/2Avs1/2N 18S
A7 2/3Avs1/3N 18S
A8 3/4Avs1/4N 18S
B1 5/6Avs1/6N 18S
B2 11/12Avs1/12N 18S
B3 Alt 18S
B4 Alt 18S
B5 Wasser 18S
B6 Neu 28S
B7 Neu 28S
B8 1/6Avs5/6N 28S
C1 1/4Avs3/4N 28S
C2 1/3Avs2/3N 28S
C3 1/2Avs1/2N 28S
C4 2/3Avs1/3N 28S
C5 3/4Avs1/4N 28S
C6 5/6Avs1/6N 28S
C7 11/12Avs1/12N 28S
C8 Alt 28S
Bank On Bank Off
Named On All On All Off
Edit Samples...
Adjust Scale Auto-Scale Default Scale
Rotor-Gene Analysis Software V5.0 (Build 60)
Start Rotor-Gene - Ileum Ti... Microsoft PowerPoint - [V...
14:52


Rotor-Gene - BlutA_B - [Raw Channel (Cycling A.FAM/Sybr)]
File Analysis Run Gain View Window Help
New Open
Channels
Fluorescence
25
20
15
10
5
BlutB-18S
30-18S
31-18S
32-18S
33-18S
34-18S
35-18S
36-18S
37-18S
38-18S
39-18S
BlutA-18S
Wasser-18S
BlutB-28S
30-28S
31-28S
32-28S
33-28S
34-28S
35-28S
36-28S
37-28S
38-28S
39-28S
Bank On
Bank Off
All On
All Off
Edit Samples...
Adjust Scale
Start
Rotor-Gene - BlutA_B ...
Microsoft PowerPoint - [V...
14:49
bovine WBC total-RNA
RIN 9.5
RIN 2.8
Δ CP


crossing point [CP]
25
20
15
10
5
0
2
4
6
8
10
RNA integrity number [RIN]
18 S
28 S
ß-Actin
IL-1ß

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)

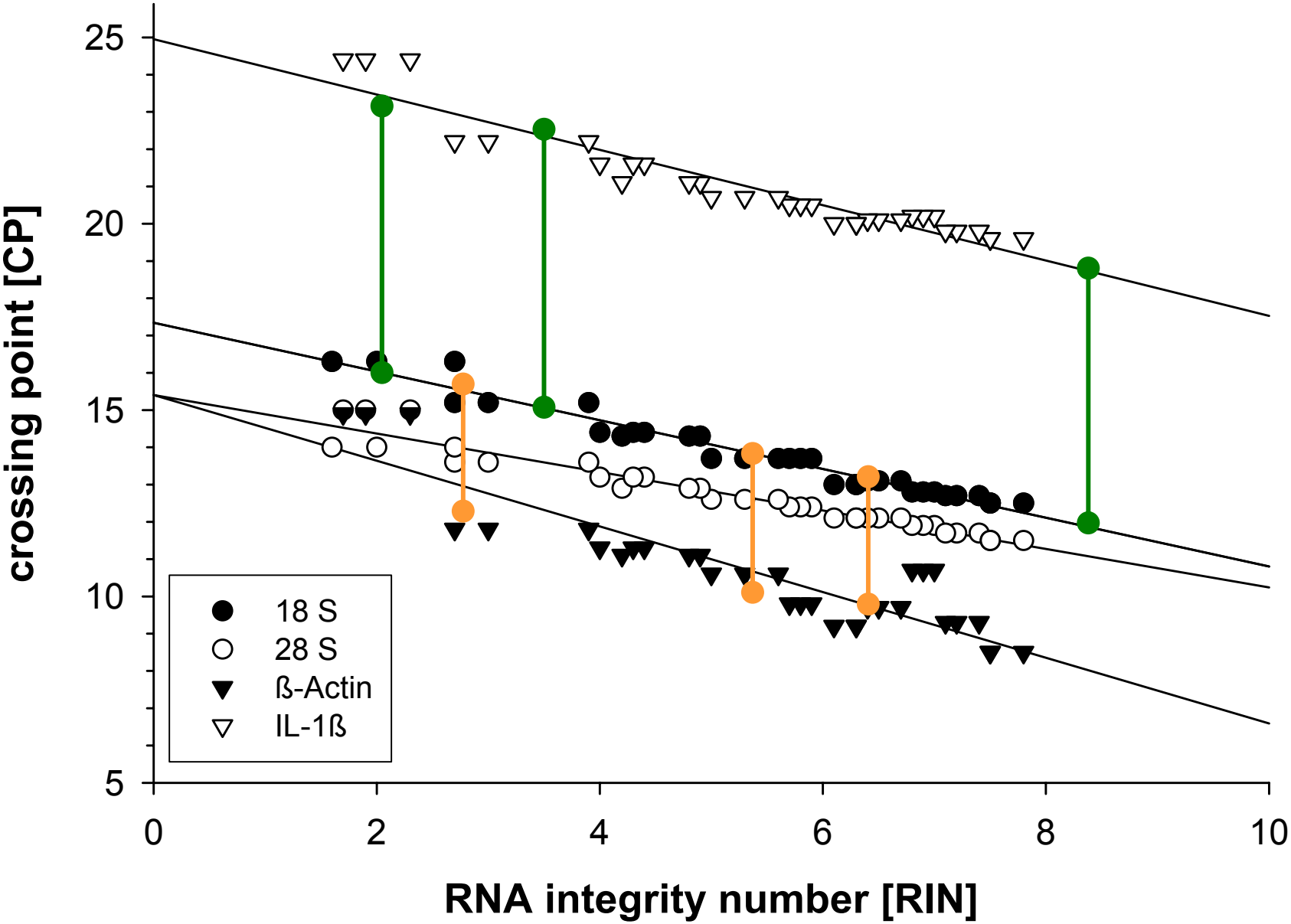
Relative quantification of gene expression

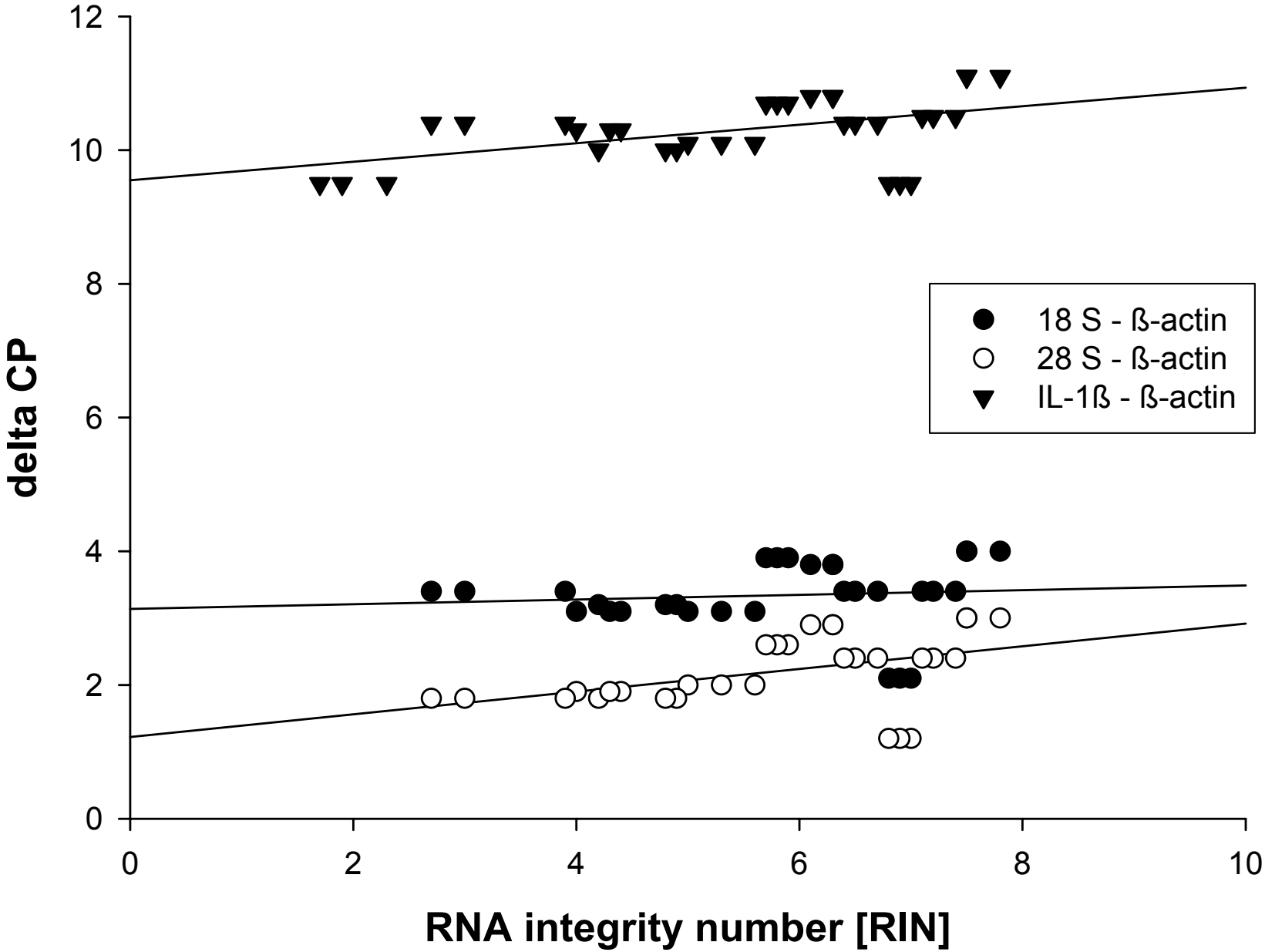
$$\Delta CP = CP_{\text{target gene}} - CP_{\text{refence gene}}$$

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

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

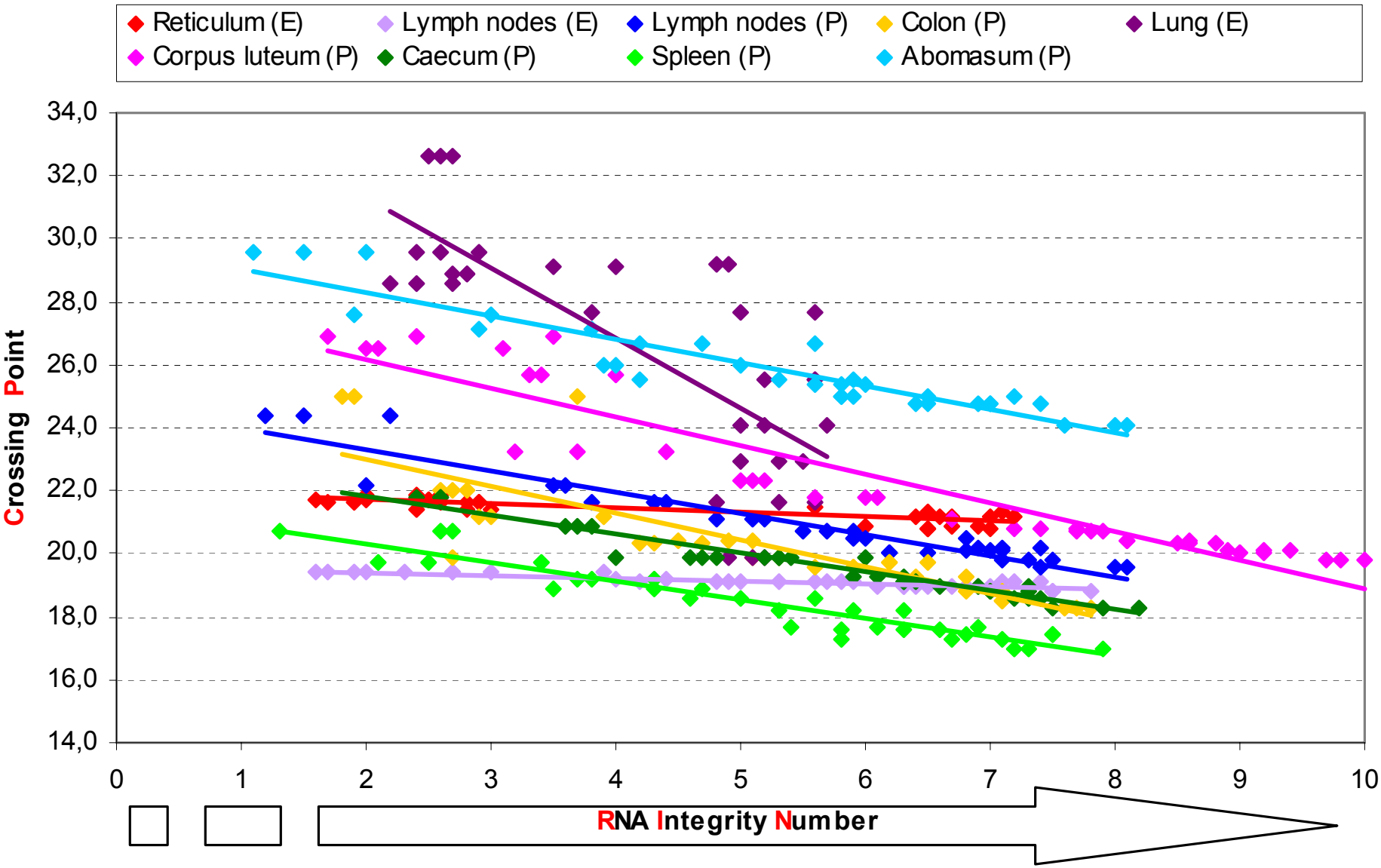
Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 $^{[-\text{ delta deltaC(T)}]}$ method.
Methods, 2001 ***25(4):*** *402-408.*

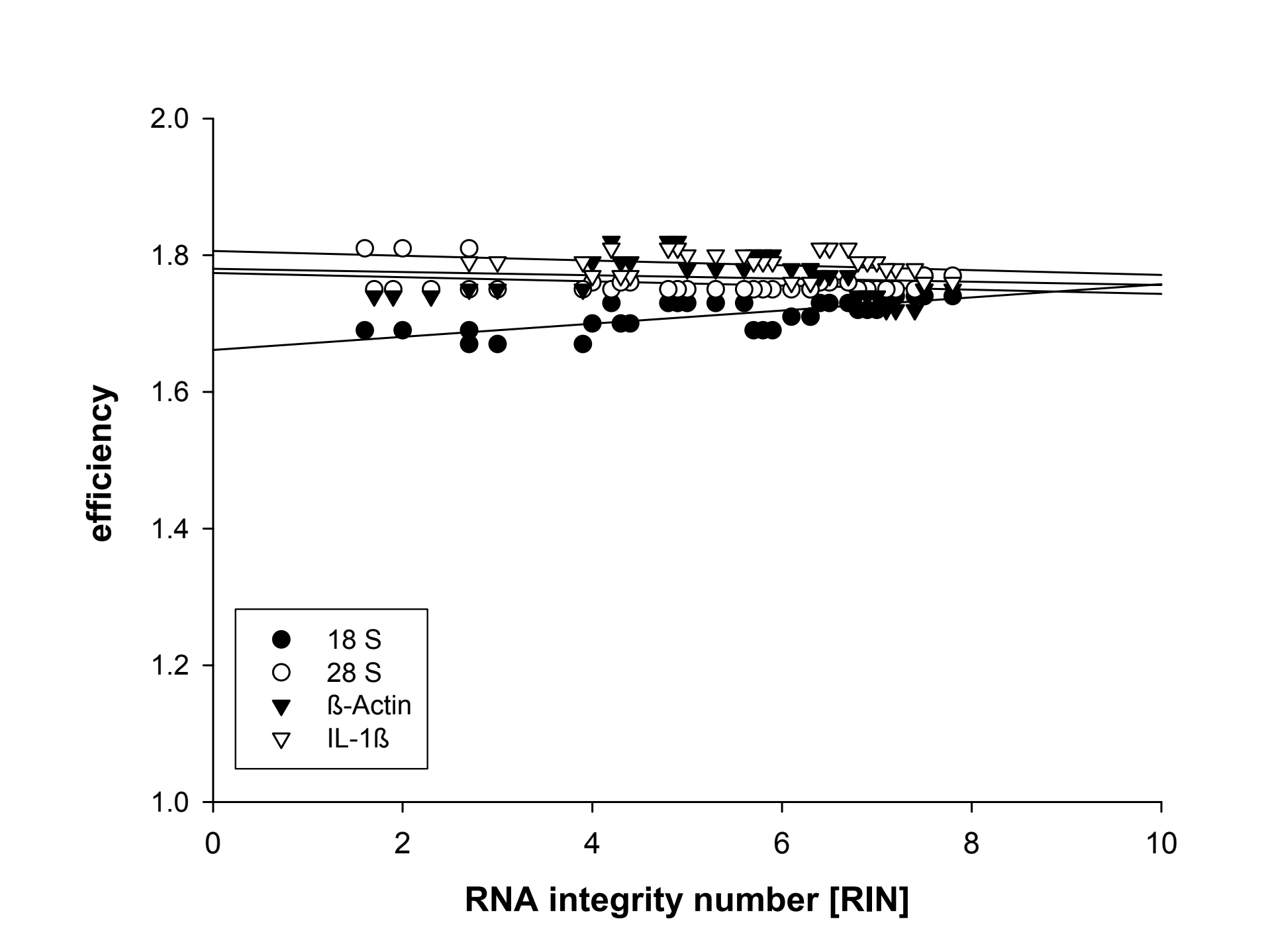

crossing point [CP]
25
20
15
10
5
0
2
4
6
8
10
RNA integrity number [RIN]
18 S
28 S
ß-Actin
IL-1ß


12
10
8
6
4
2
0
delta CP
0
2
4
6
8
10
RNA integrity number [RIN]
18 S - ß-actin
28 S - ß-actin
IL-1ß - ß-actin

Impact of total-RNA integrity on qRT-PCR **CP (Ct)**

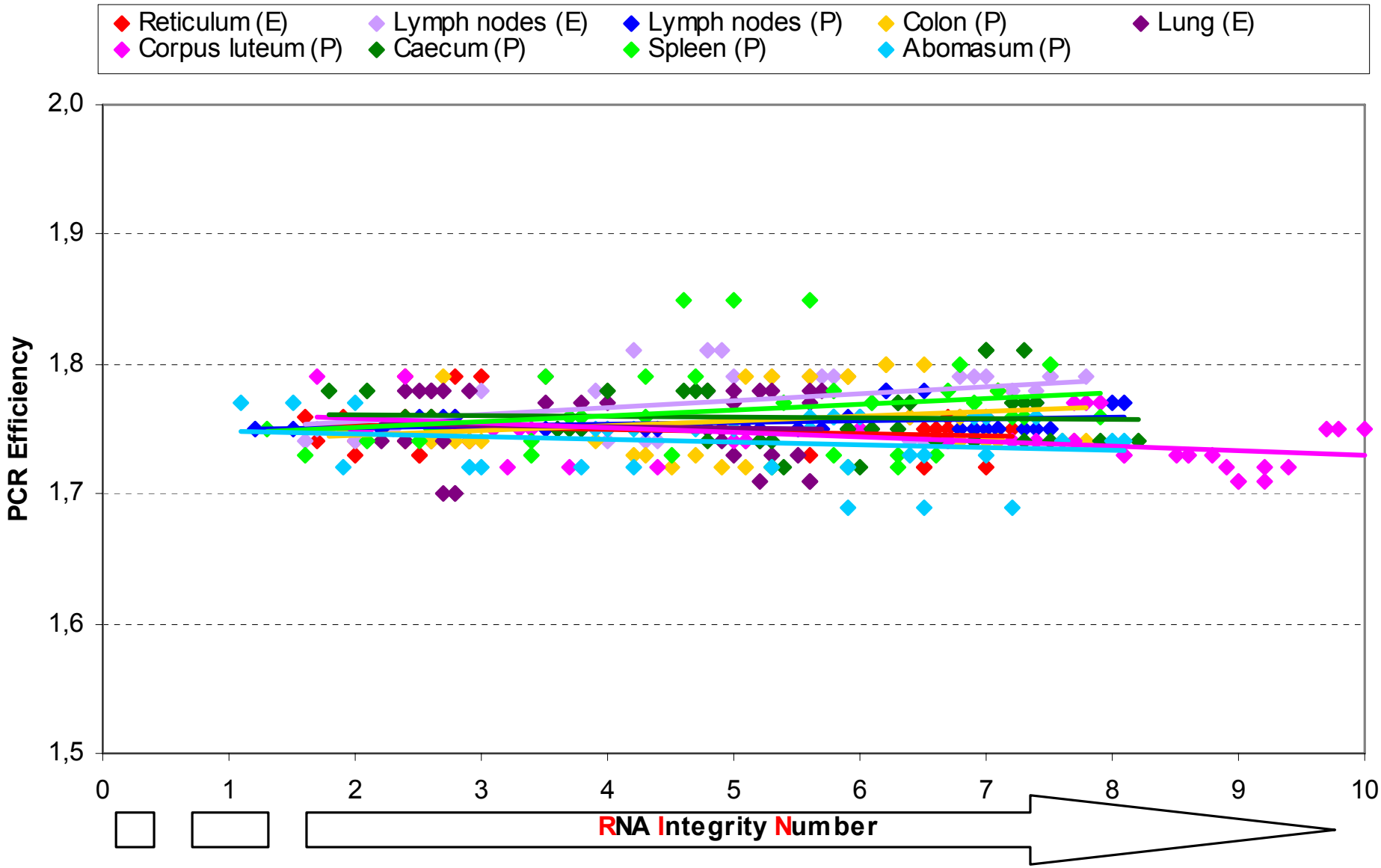
IL-1: ***Crossing Point***

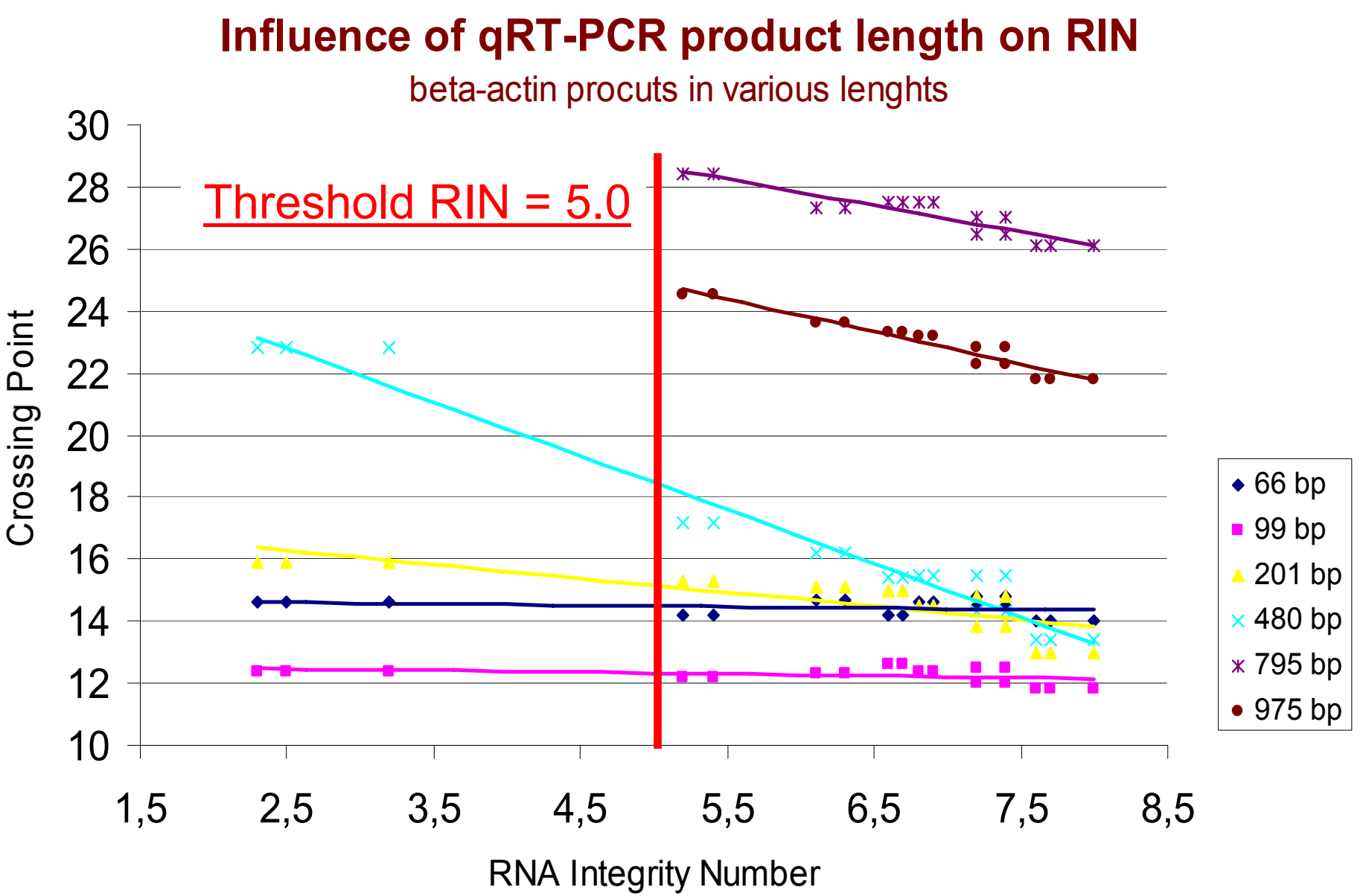



2.0
1.8
1.6
1.4
1.2
1.0
efficiency
0
2
4
6
8
10
RNA integrity number [RIN]
18 S
28 S
ß-Actin
IL-1ß

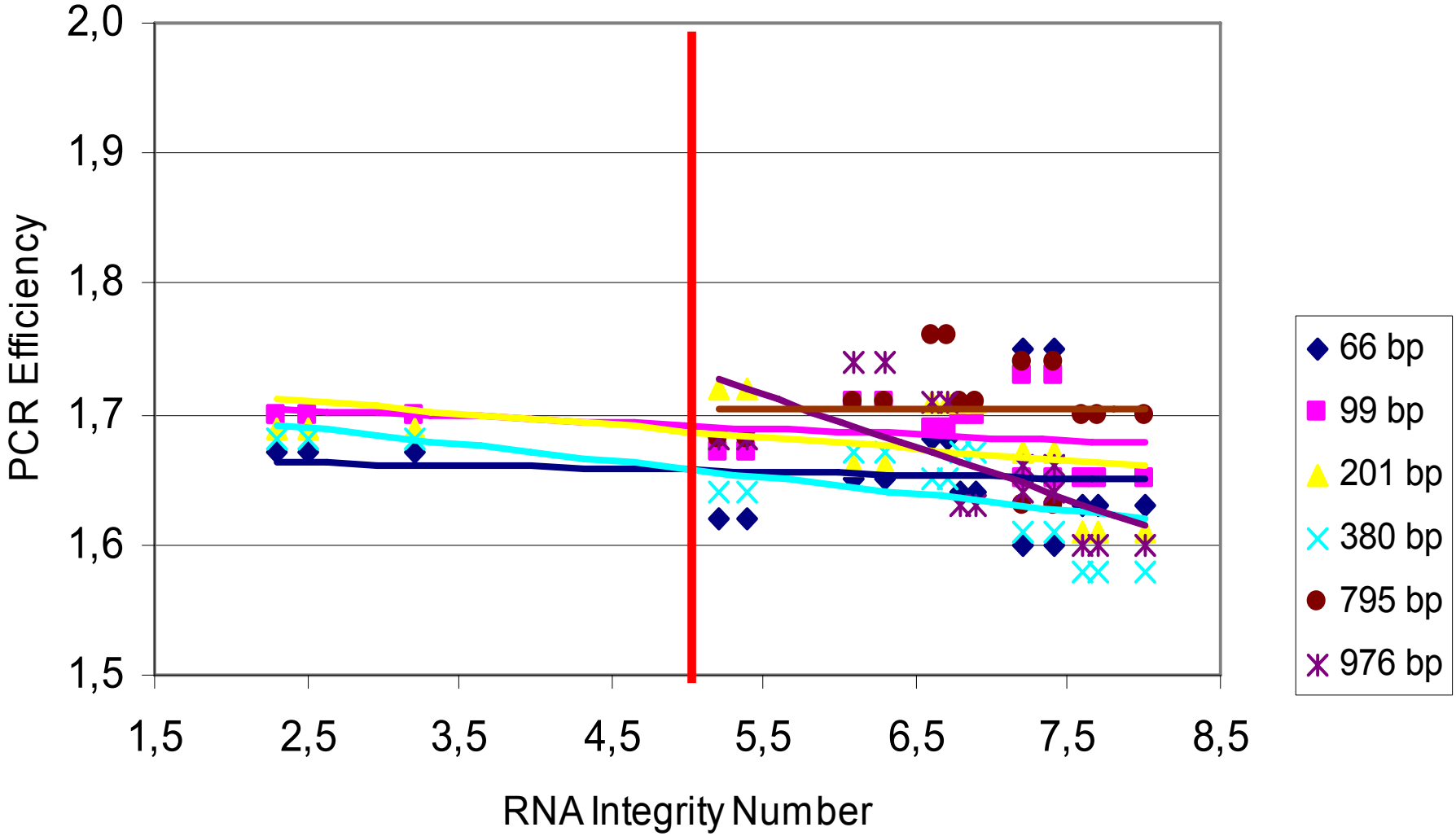
Impact of total-RNA integrity on qRT-PCR **efficiency**

28S: ***Amplification***

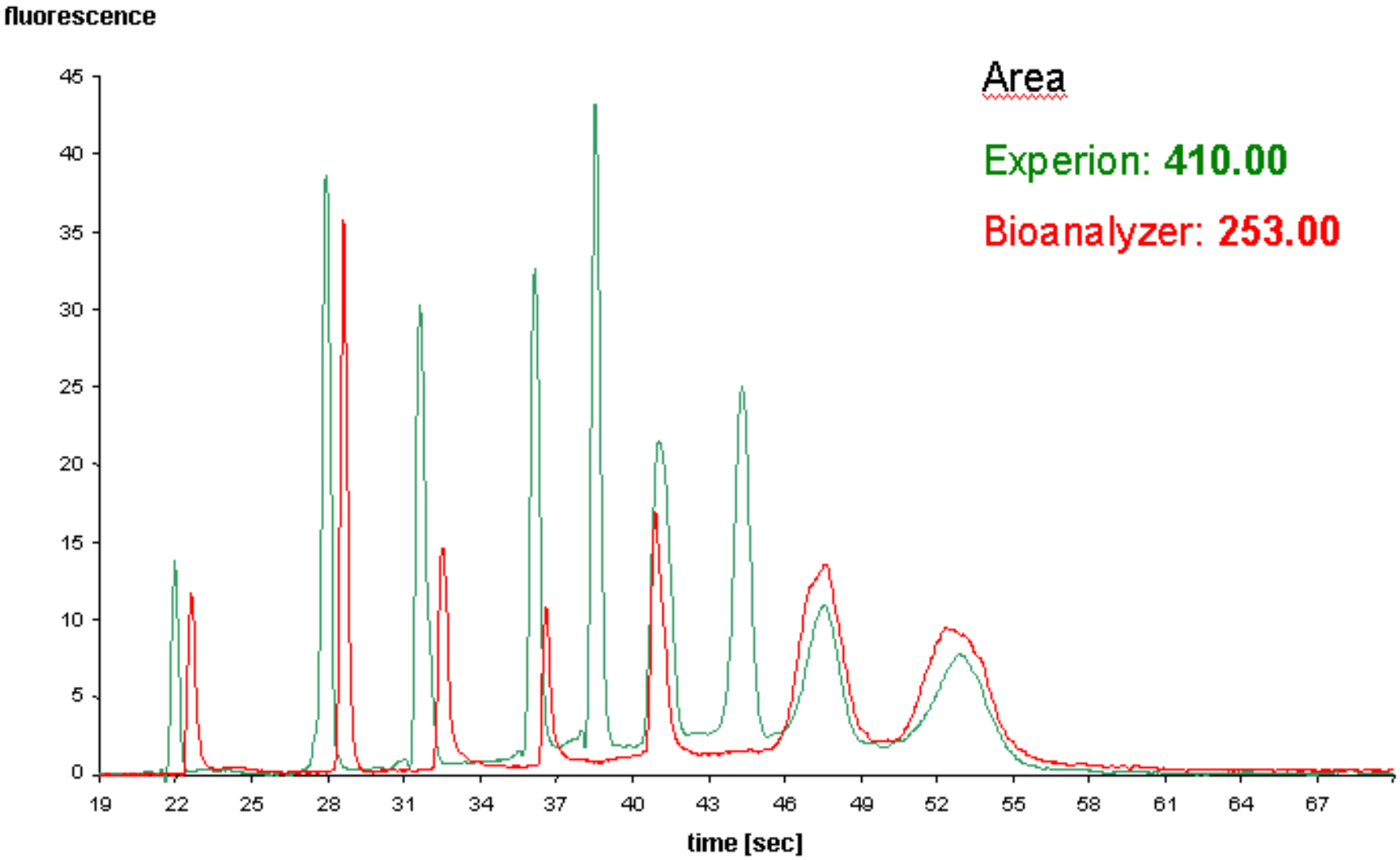



Influence of qRT-PCR product length on RIN
beta-actin procuts in various lenghts
Threshold RIN = 5.0
Crossing Point
30
28
26
24
22
20
18
16
14
12
10
1,5
2,5
3,5
4,5
5,5
6,5
7,5
8,5
RNA Integrity Number
66 bp
99 bp
201 bp
480 bp
795 bp
975 bp

PCR efficiency in dependence of RIN

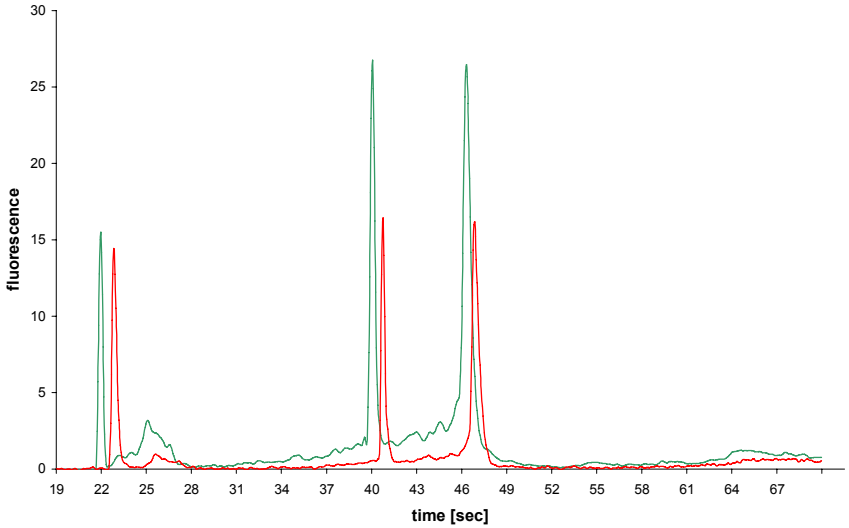


Comparison of E-Grams: Experion & Bioanalyzer 2100



Run performance

Experion & Bioanalyzer 2100

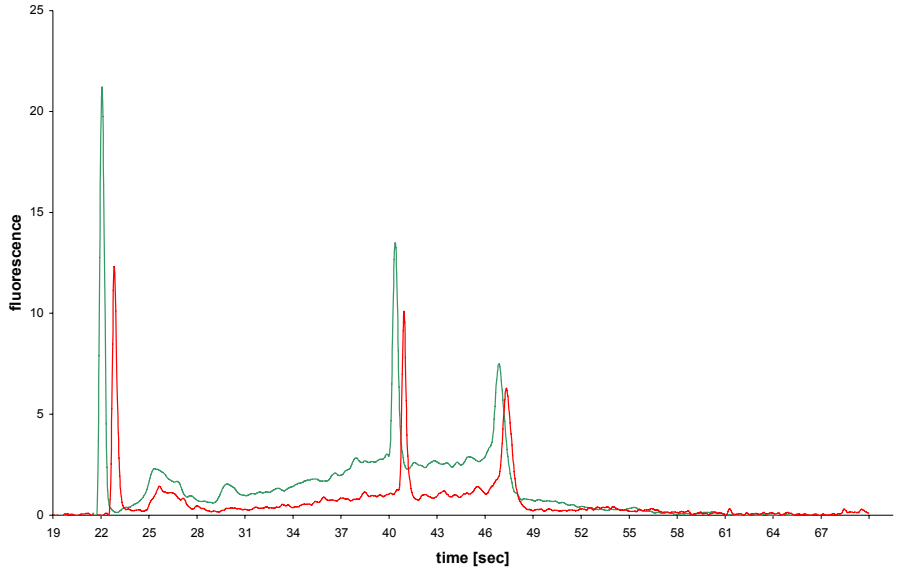


Experion: 165.34 [71.47 ng/µl]
Ratio [28S/18S]: **0.93**

Ladder Area: **370.14**

Bioanalyzer: 63.3 [27.0 ng/µl]
Ratio [28S/18S]: **1.30**
RIN: 7.4

Ladder Area: **354.1**



Experion: 130.31 [45.07 ng/µl]
Ratio [28S/18S]: **1.36**

Ladder Area: **----**

Bioanalyzer: 44.8 [25.0 ng/µl]
Ratio [28S/18S]: **1.80**
RIN: 5.2

Ladder Area: **----**

Variability in total-RNA quantification

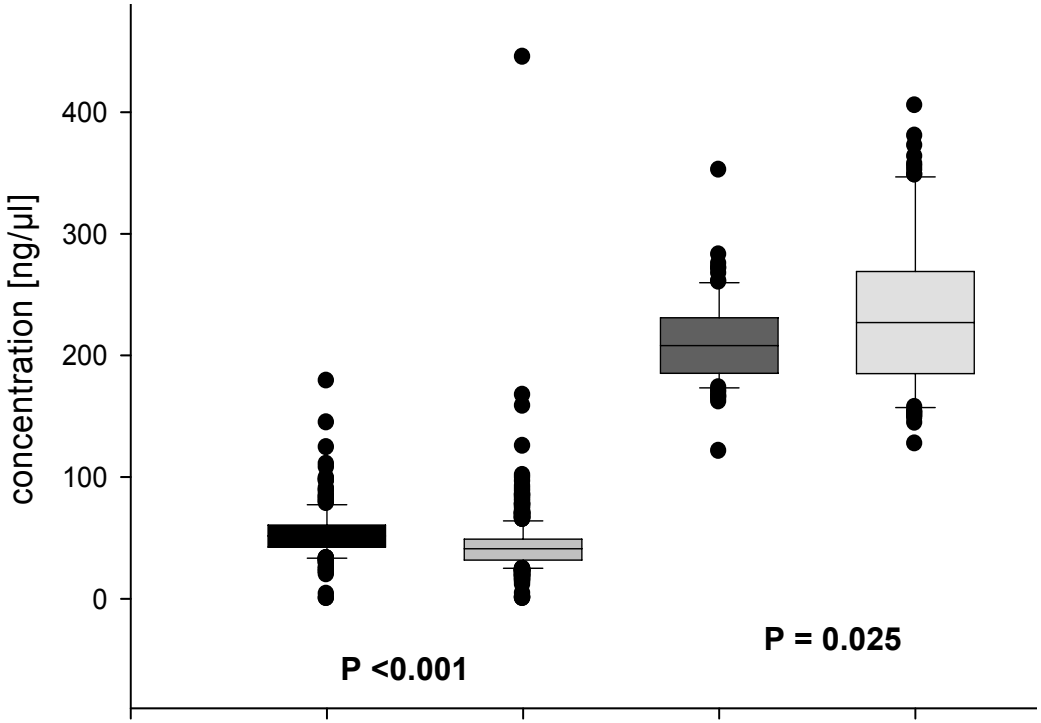
Experion & Bioanalyzer 2100

A: Experion (50 ng/µl)

B: Bioanalyzer (50 ng/µl)

C: Experion (200 ng/µl)

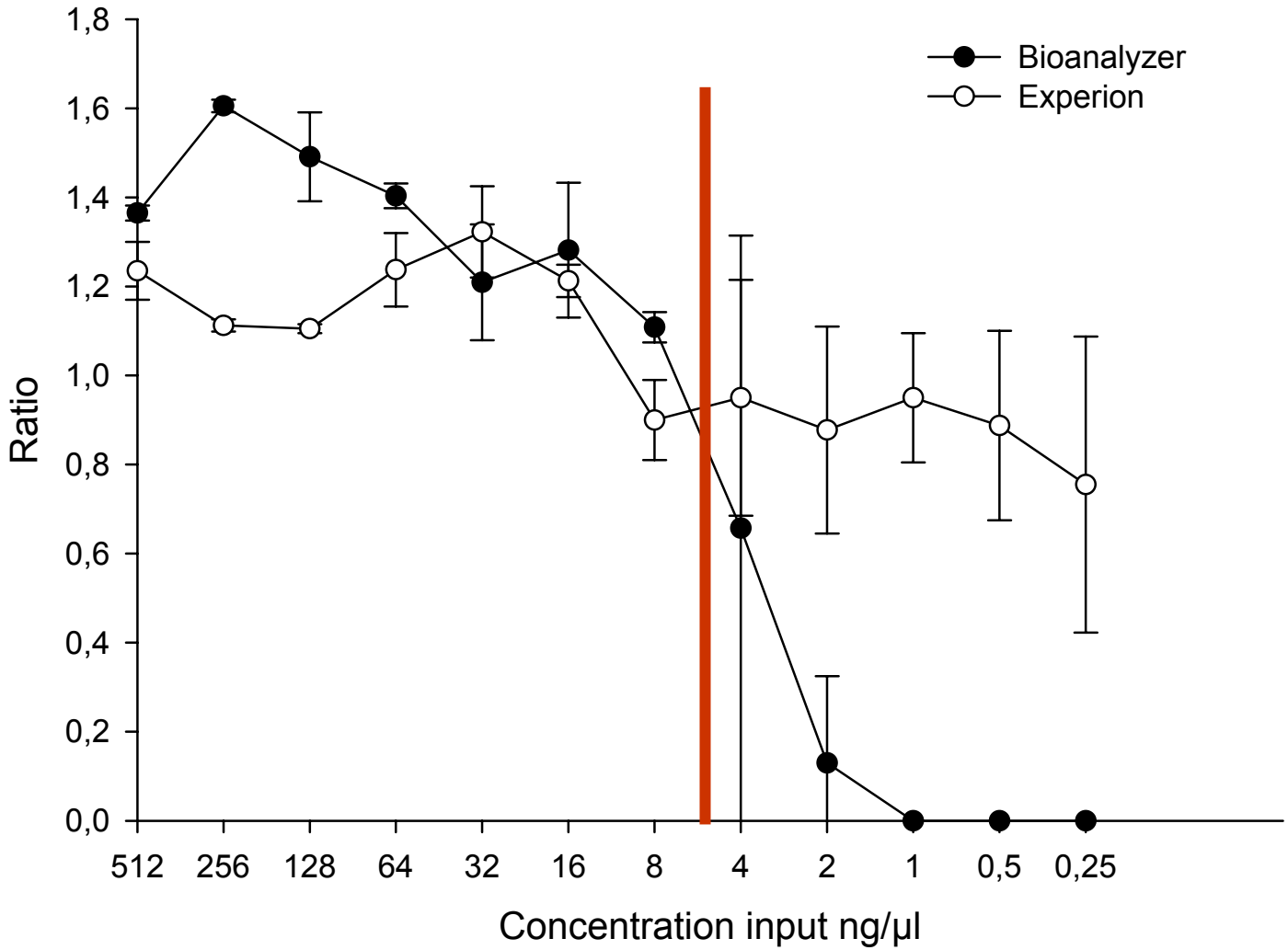
D: Bioanalyzer (200 ng/µl)



	A	B	C	D
mean [ng]	54.2	43.4	211.1	235.8
CV [%]	39.1	57.1	14.7	27.4
	n = 207		n = 171	

Pfaffl et al., submitted 2008

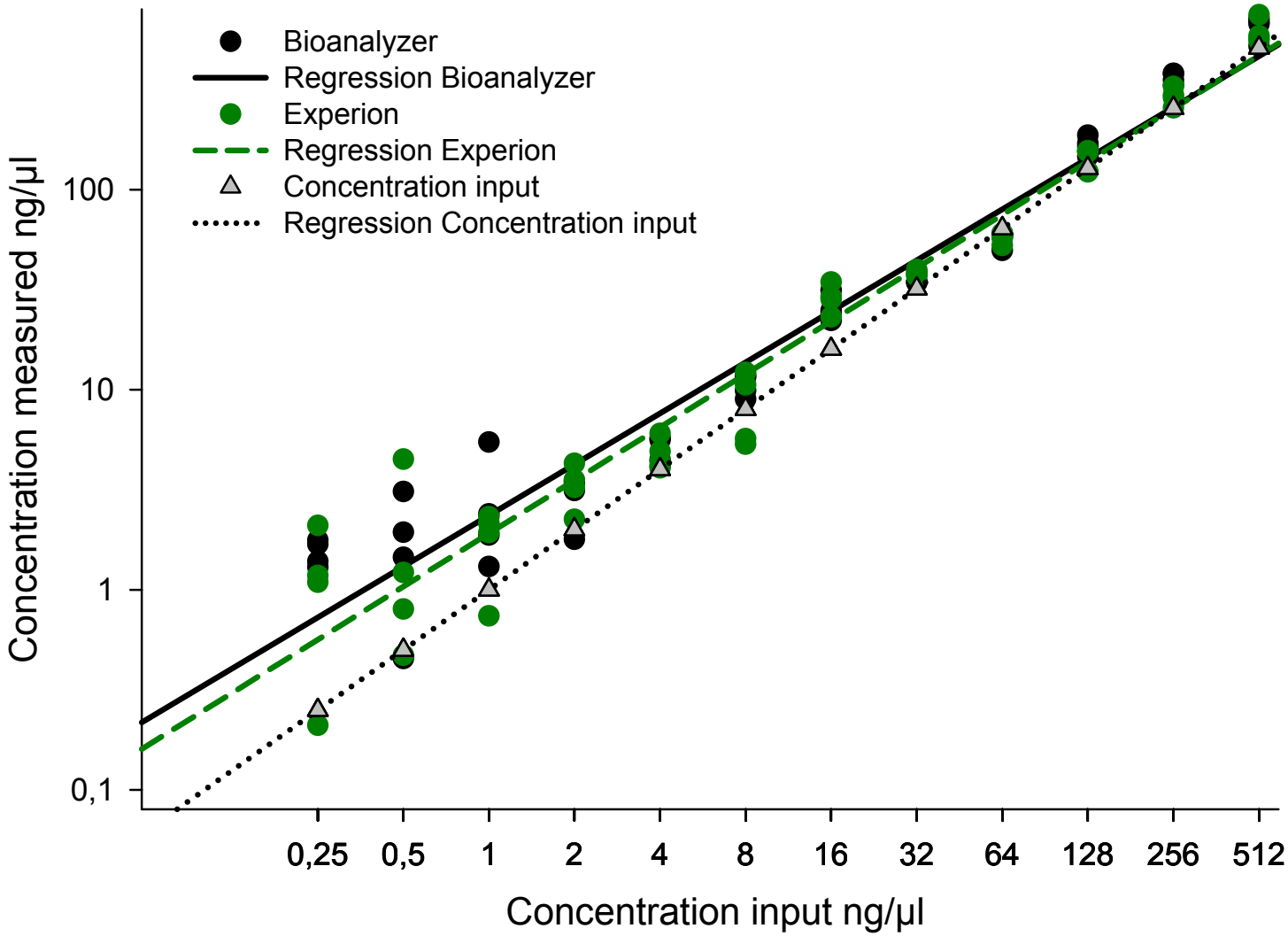
Linearity of 28S/18S rRNA ratio



Pfaffl et al., submitted 2008

Linearity of quantification

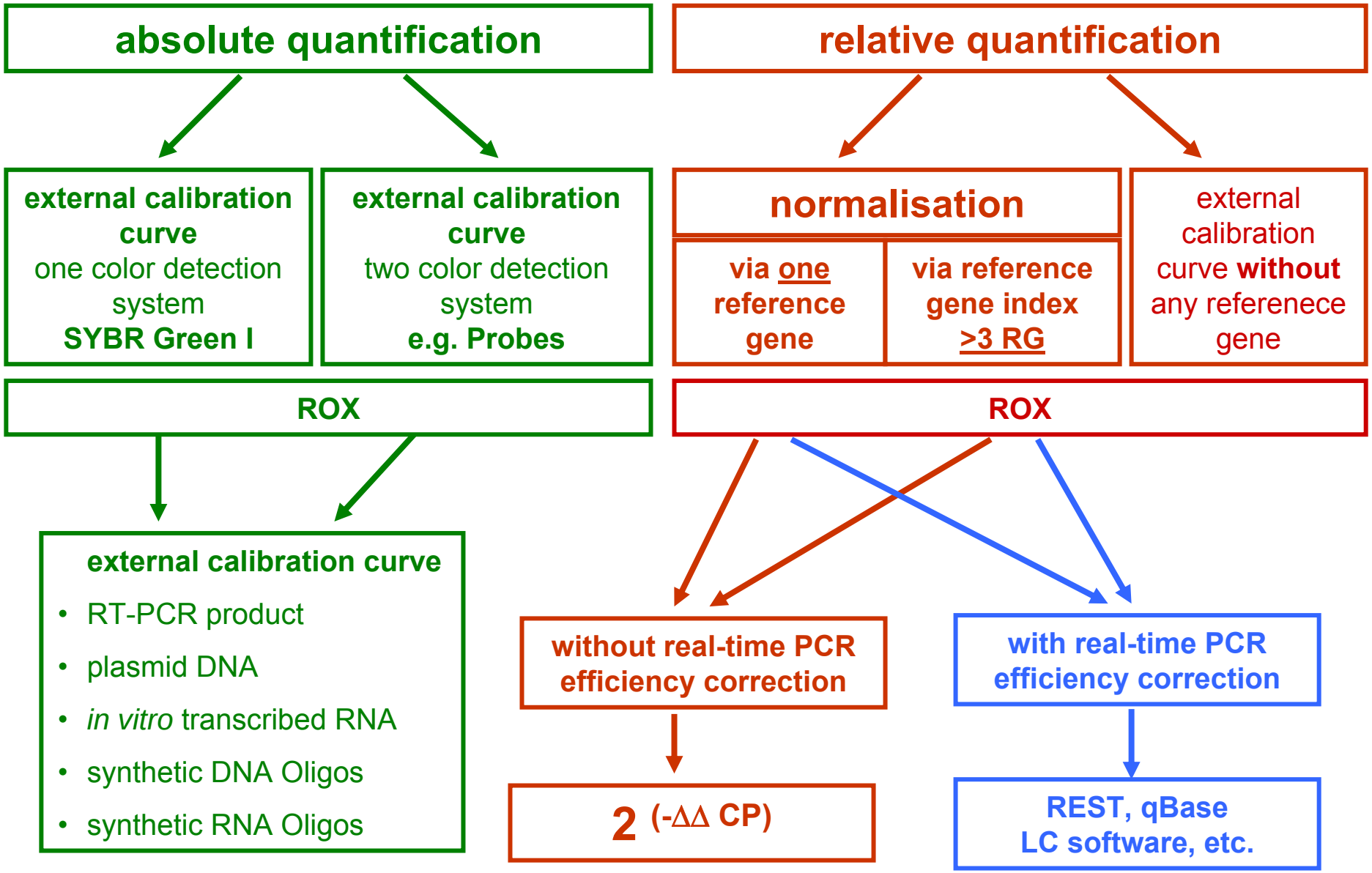
RNA input vs. RNA concentration measured



Pfaffl et al., submitted 2008

Quantification Strategies in real time qRT-PCR

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



“Absolute quantification” using calibration curves

- calibration curve using a **purified RT-PCR product** *(Einspanier et al. 1999, etc……)*
- **recombinant DNA** (recDNA) calibration curve (Bustin, 2000; *Pfaffl & Hageleit, 2001)*
- calibration curve using a synthetic **DNA** oligo-nucleotide *(Bustin, 2000; Bustin 2005)*
- **recombinant RNA** (recRNA) calibration curve (*Pfaffl & Hageleit, 2001)*
- calibration curve using a synthetic **RNA** oligo-nucleotide *(Bustin et al. 2000, 2004, etc……)*
- calibration curve using a ***pool of biological samples***

⇒ Valid calibration curve needs to have comparable ***biological matrix background*** like the biological sample!

⇒ Valid calibration curve needs ***same RNA integrity*** like biological sample!

⇒ Amplification efficiency and over all reaction performance of calibration curve needs to be ***identical*** to the biological sample!

⇒ ***„Copy & Paste“*** of previously performed curves is NOT the right approach!

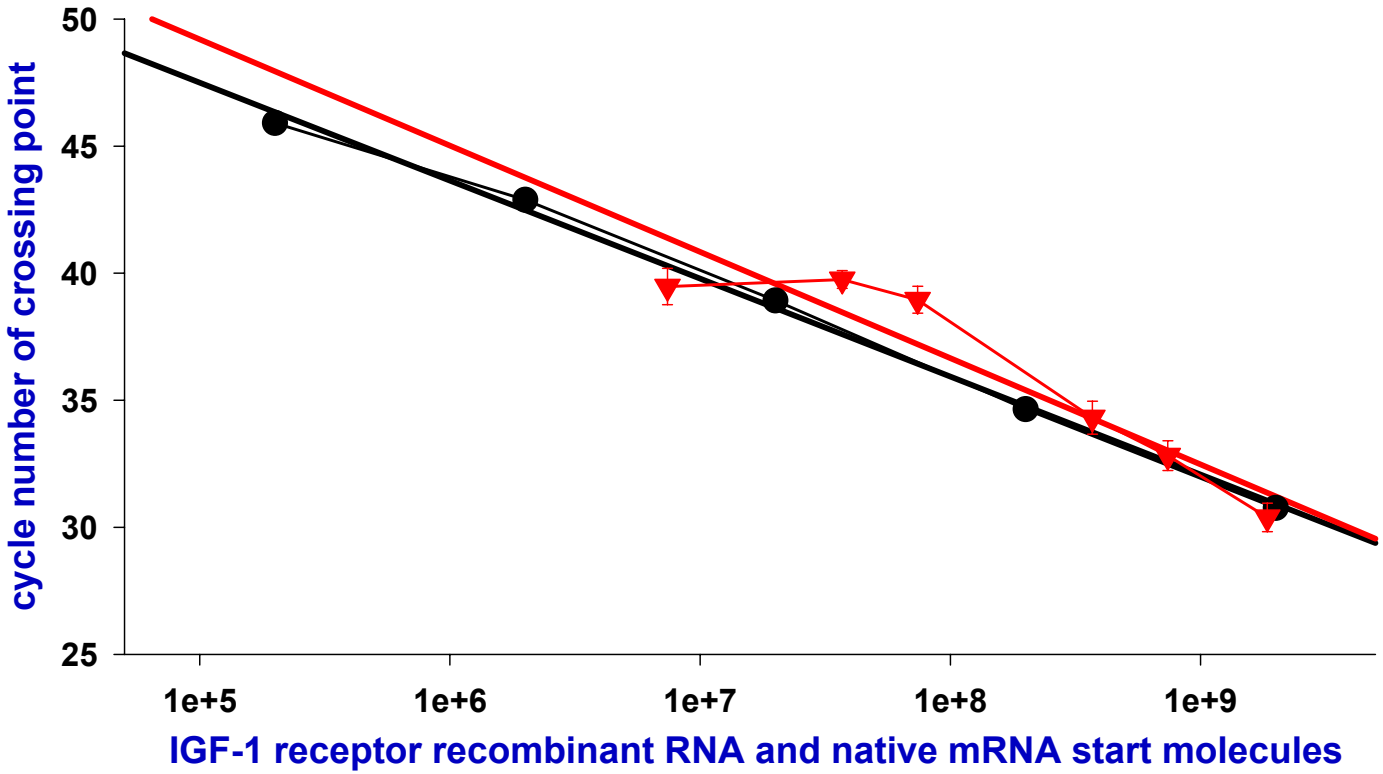
Absolute quantification of IGF-1 receptor

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)



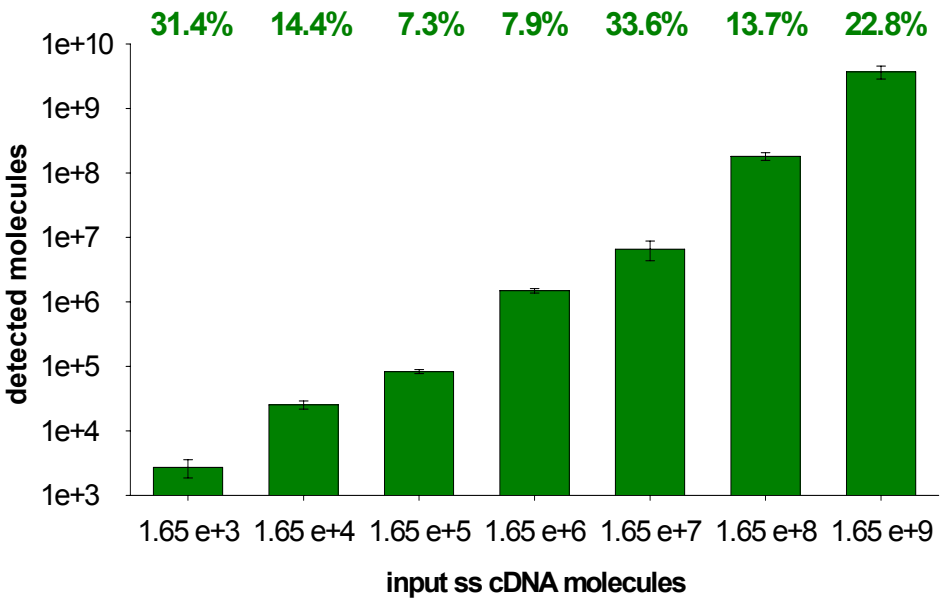
Pfaffl et al., 1998

ERα intra-assay & inter-assay variation

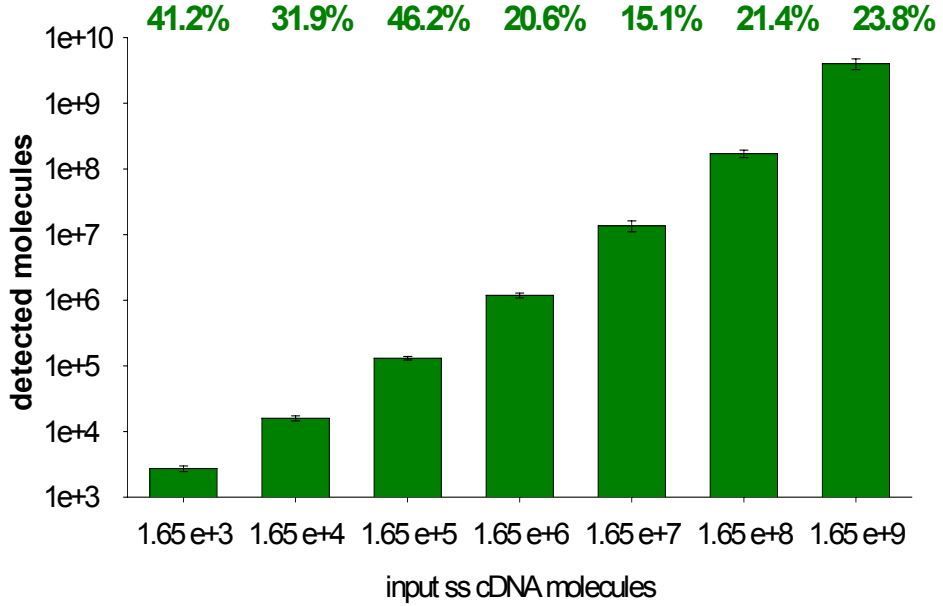
intra-assay variation: within one LightCycler 1.0 run

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)

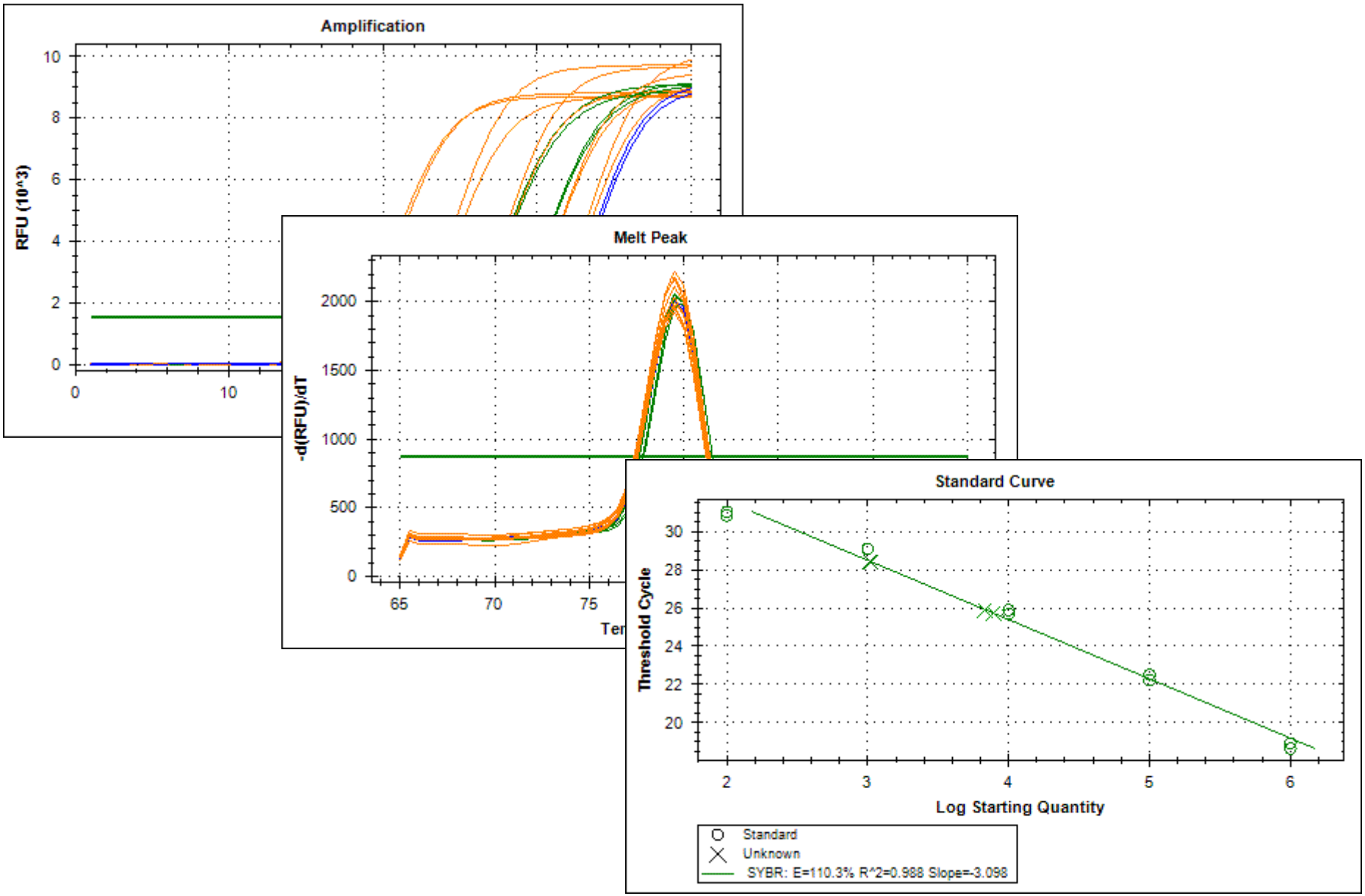


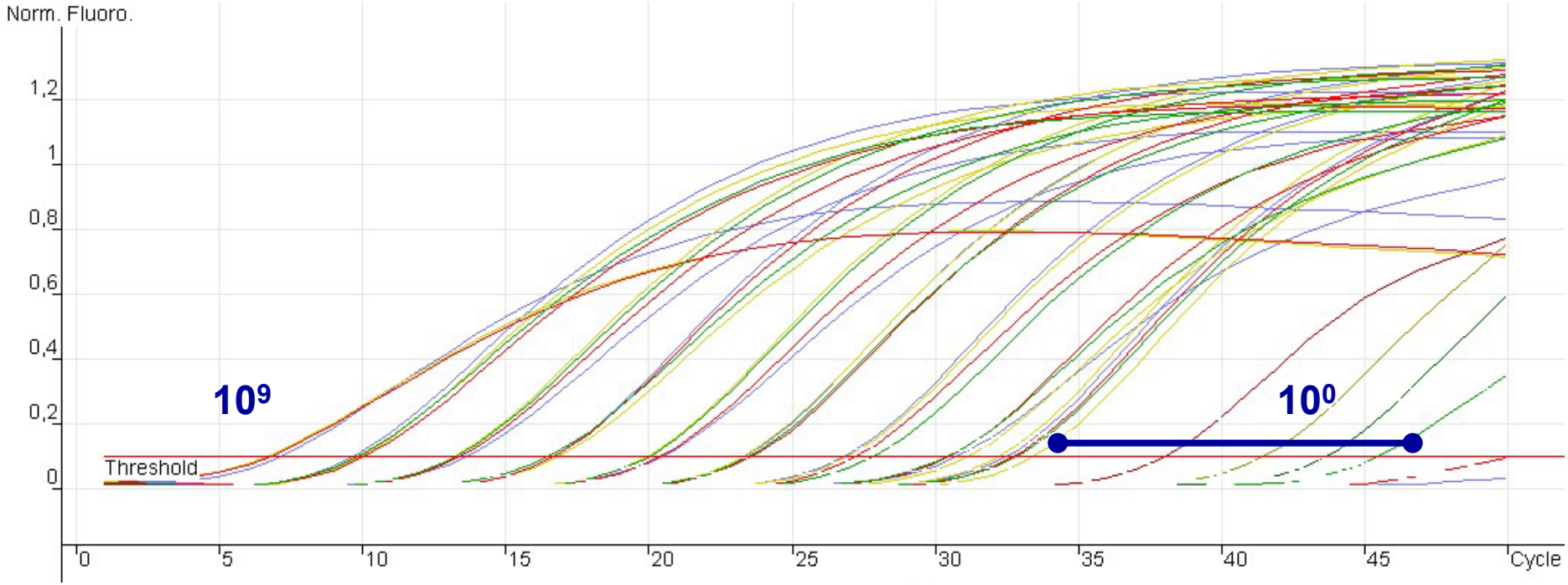
using a recombinant plasmid DNA calibration curve (mean ± std.dev.; on molecule basis)

Pfaffl, unpublished 1998

SYBR Green I standard curve of RT-PCR product

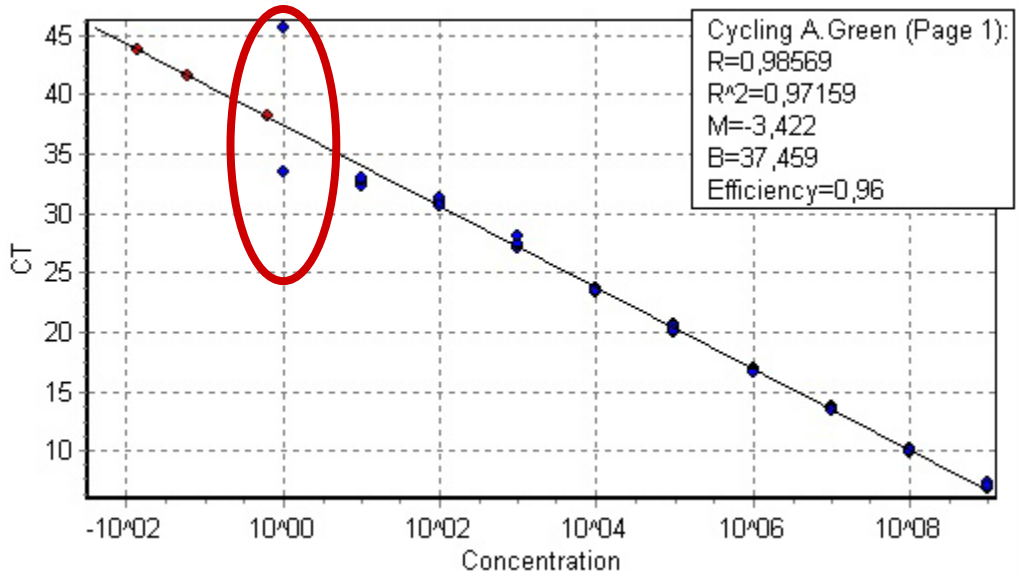
10^6 to 10^2 start molecules in Bio-Rad CFX96





ERα standard curve

10^9 – 10^0 DNA molecules



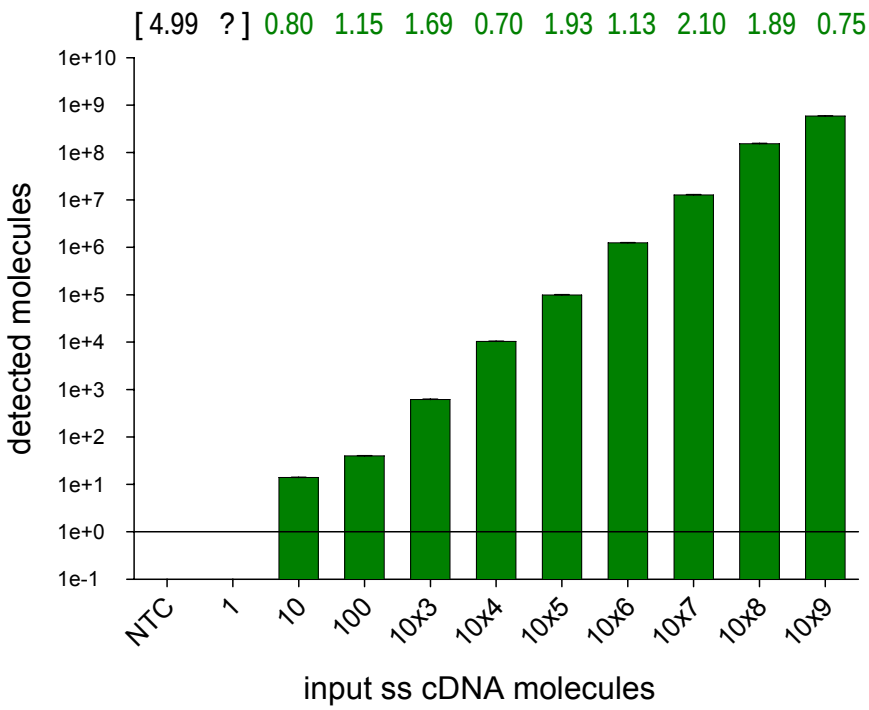
ERα intra-assay & inter-assay variation (2007)

intra-assay variation: within one RG-6000 run (n = 4)

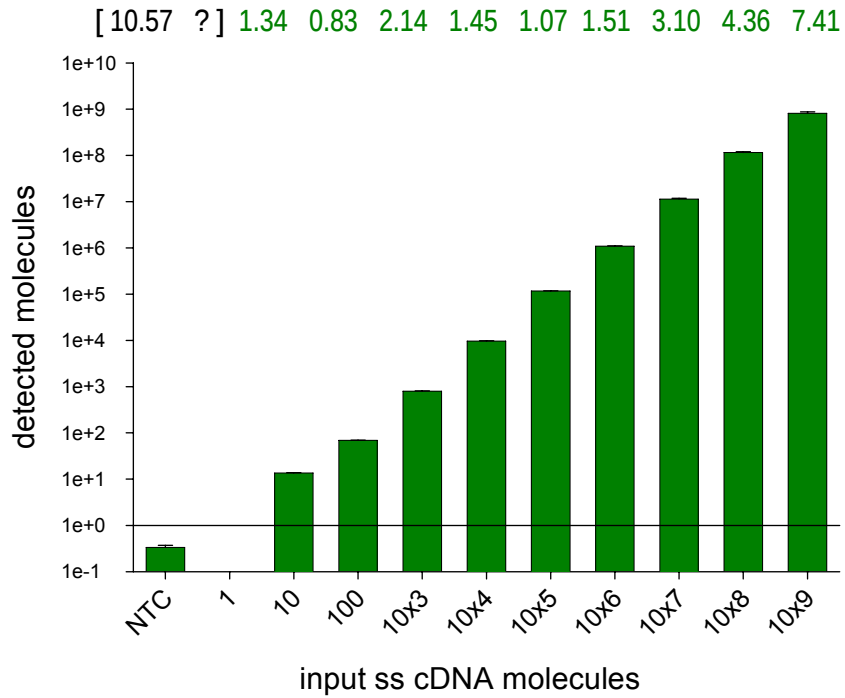
inter-assay variation: between different RG-6000 runs (n = 10)

Invitrogen two-step SYBR GreenER Kit

ERα intra-assay variability (n = 4) over all CV = 1.35%



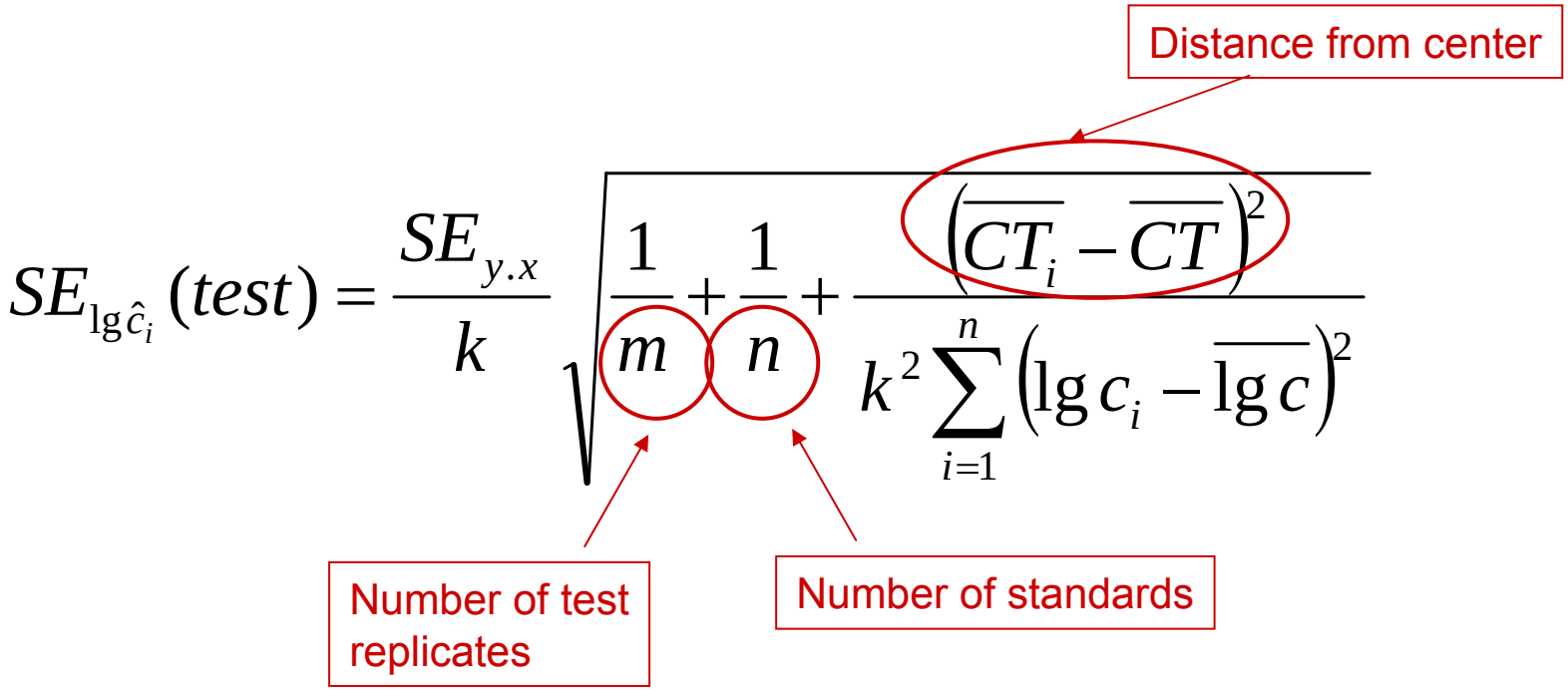
ERα inter-assay variability (n = 10) over all CV = 2.58%



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

Pfaffl, unpublished 2007

Precision in the estimates



Confidence interval for estimated concentrations

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

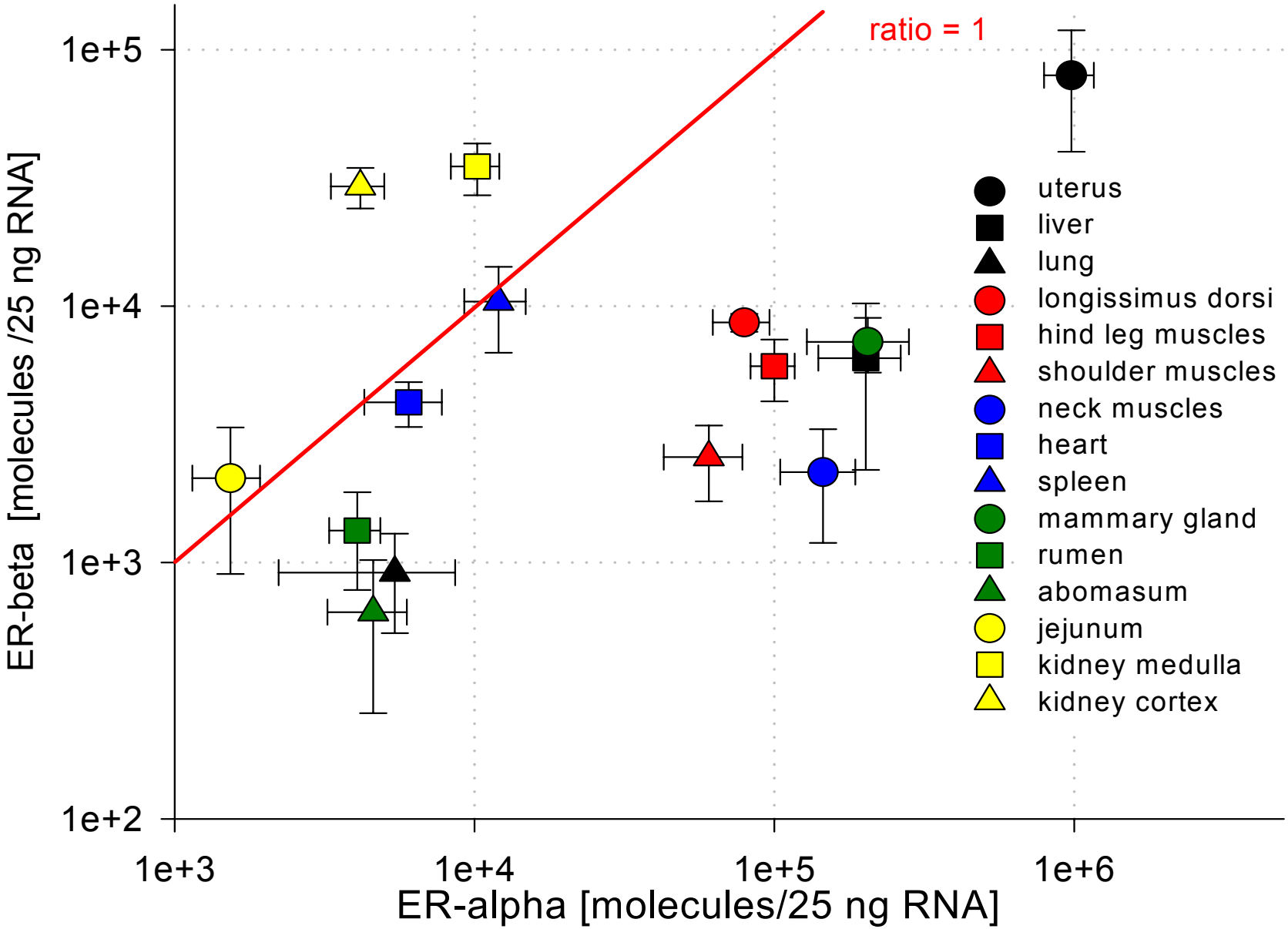
Validation of an „absolute quantification“ of steroid receptors

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



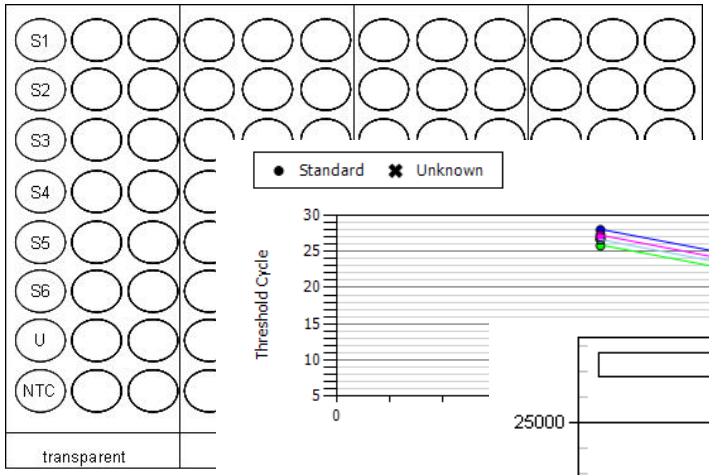
Pfaffl et al., APMIS 2001

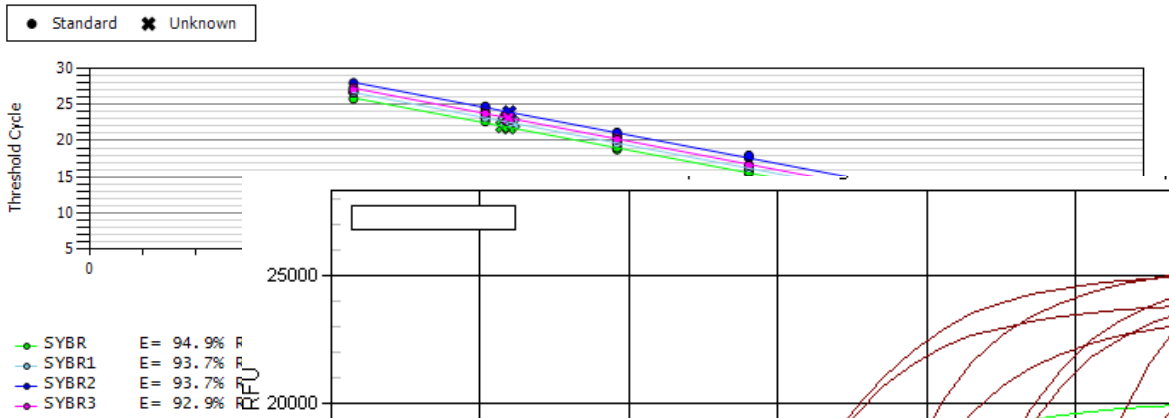
Comparison of bio-equipment:

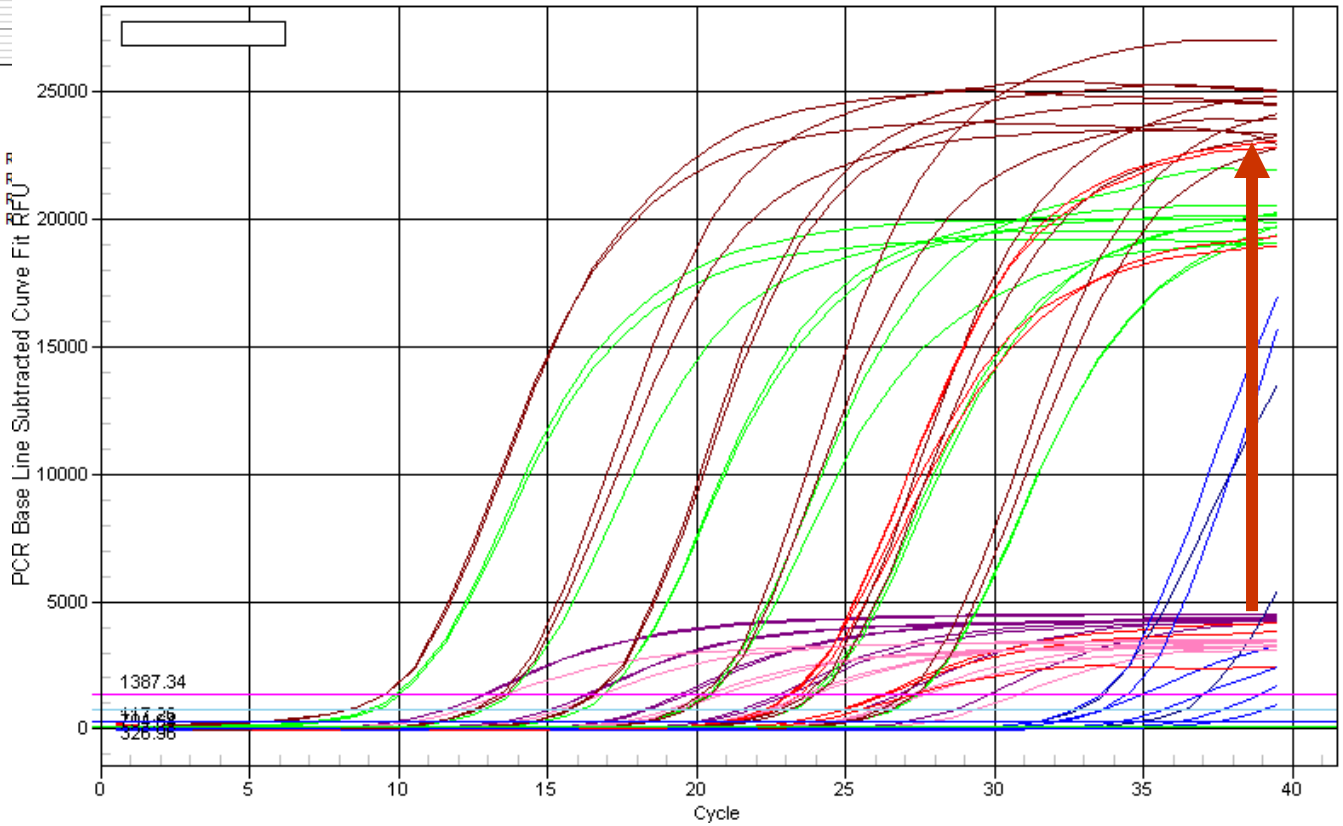
iQ5 vs. Realplex

white plates (EPW) vs. transparent plates (EPD)

heat sealing (hs) vs. adhesive sealing (as)







Comparison of bio-equipment:

iQ5 vs. Realplex

white plates (EPW) vs. transparent plates (EPD)

heat sealing (hs) vs. adhesive sealing (as)

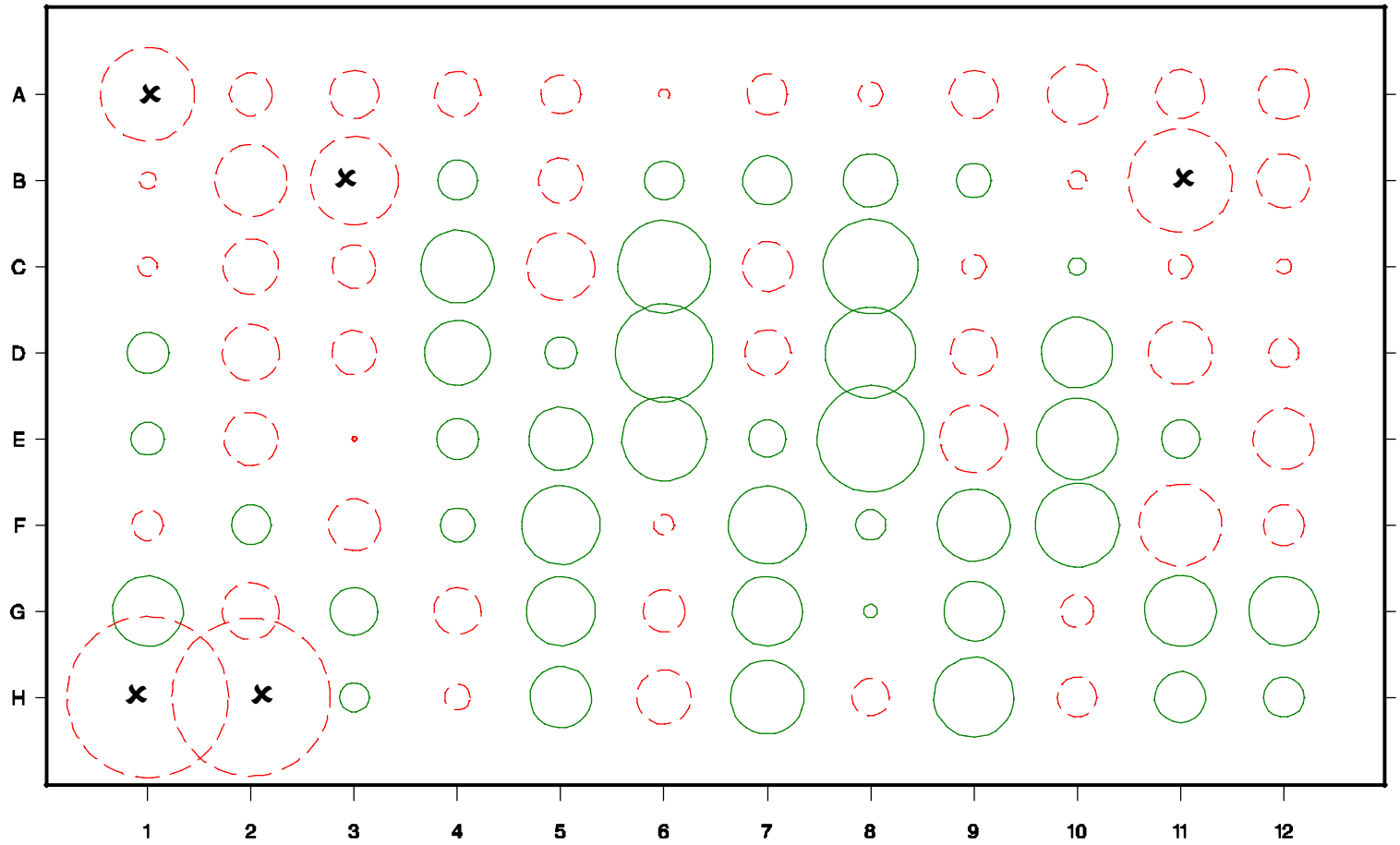
iQ5												
	intra-run data			intra-run data			intra-run data			inter-run data		
Ct	Ct	S.D.	C.V. (%)	Ct	S.D.	C.V. (%)	Ct	S.D.	C.V. (%)	Ct	S.D.	C.V. (%)
AE	AE	S.D.	C.V. (%)	AE	S.D.	C.V. (%)	AE	S.D.	C.V. (%)	AE	S.D.	C.V. (%)
	hsEPD											
Ct	26.35	0.20	0.75	24.60	0.15	0.60	24.56	0.16	0.65	**25.17**	**0.17**	**0.67**
AE	75.50	9.88	13.09	86.60	6.46	7.45	73.90	5.74	7.77	**78.67**	**7.36**	**9.36**
	hsEPW											
Ct	26.38	0.16	0.62	24.50	0.18	0.74	24.45	0.32	1.31	**25.11**	**0.22**	**0.89**
AE	1213.60	82.02	6.76	1375.20	80.55	5.86	1411.60	139.61	9.89	**1333.47**	**100.73**	**7.55**
	asEPD											
Ct	25.23	0.19	0.76	25.25	0.79	3.12	25.12	0.69	2.74	**25.20**	**0.56**	**2.21**
AE	73.20	10.09	13.79	72.10	14.40	19.97	72.50	10.54	14.54	**72.60**	**11.68**	**16.08**
	asEPW											
Ct	24.71	0.14	0.55	25.17	0.19	0.74	25.15	0.17	0.67	**25.01**	**0.16**	**0.65**
AE	1261.70	79.62	6.31	1285.20	84.74	6.59	1246.20	98.89	7.94	**1264.37**	**87.75**	**6.94**
									EPW	**25.06**	**0.19**	**0.77**
									EPD	**25.18**	**0.36**	**1.44**
									hs	**25.14**	**0.19**	**0.78**
									as	**25.10**	**0.36**	**1.43**
	realplex											
	hsEPD											
Ct	25.11	0.25	1.01	24.56	0.44	1.77	25.15	0.41	1.65	**24.94**	**0.37**	**1.48**
AE	122.30	25.00	20.44	120.30	22.33	18.56	133.10	27.01	20.29	**125.23**	**24.78**	**19.79**
	hsEPW											
Ct	24.10	0.48	1.98	23.95	0.38	1.57	22.49	0.17	0.76	**23.51**	**0.34**	**1.44**
AE	1911.30	354.10	18.53	2013.10	345.81	17.18	2410.42	162.89	6.76	**2111.61**	**287.60**	**13.62**
	asEPD											
Ct	23.36	0.52	2.23	23.71	0.70	2.96	23.83	0.33	1.40	**23.63**	**0.52**	**2.20**
AE	181.55	28.68	15.80	188.30	19.90	10.57	177.80	24.79	13.94	**182.55**	**24.46**	**13.40**
	asEPW											
Ct	22.55	0.49	2.17	22.60	0.34	1.50	22.99	0.23	1.00	**22.71**	**0.35**	**1.56**
AE	3003.3	211.679	7.05	2916.20	301.80	10.35	2897.60	218.88	7.55	**2939.03**	**244.12**	**8.31**
									EPW	**23.11**	**0.35**	**1.50**
									EPD	**24.29**	**0.44**	**1.84**
									hs	**24.23**	**0.35**	**1.46**
									as	**23.17**	**0.44**	**1.88**

Comparison of bio-equipment:

iQ5 vs. Realplex
white plates (EPW) vs. transparent plates (EPD)
heat sealing (hs) vs. adhesive sealing (as)

Kineret software detection of significant outliers

Tichopad et al. 2008



Reiter & Pfaffl, Biotechnology & Biotechnology Equipment 2008

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 2008; review in press)
  qBASE (Hellemans & Vandesompele; Genome Biology 2007)
- **…… ???**

Commonly used normalisation strategies

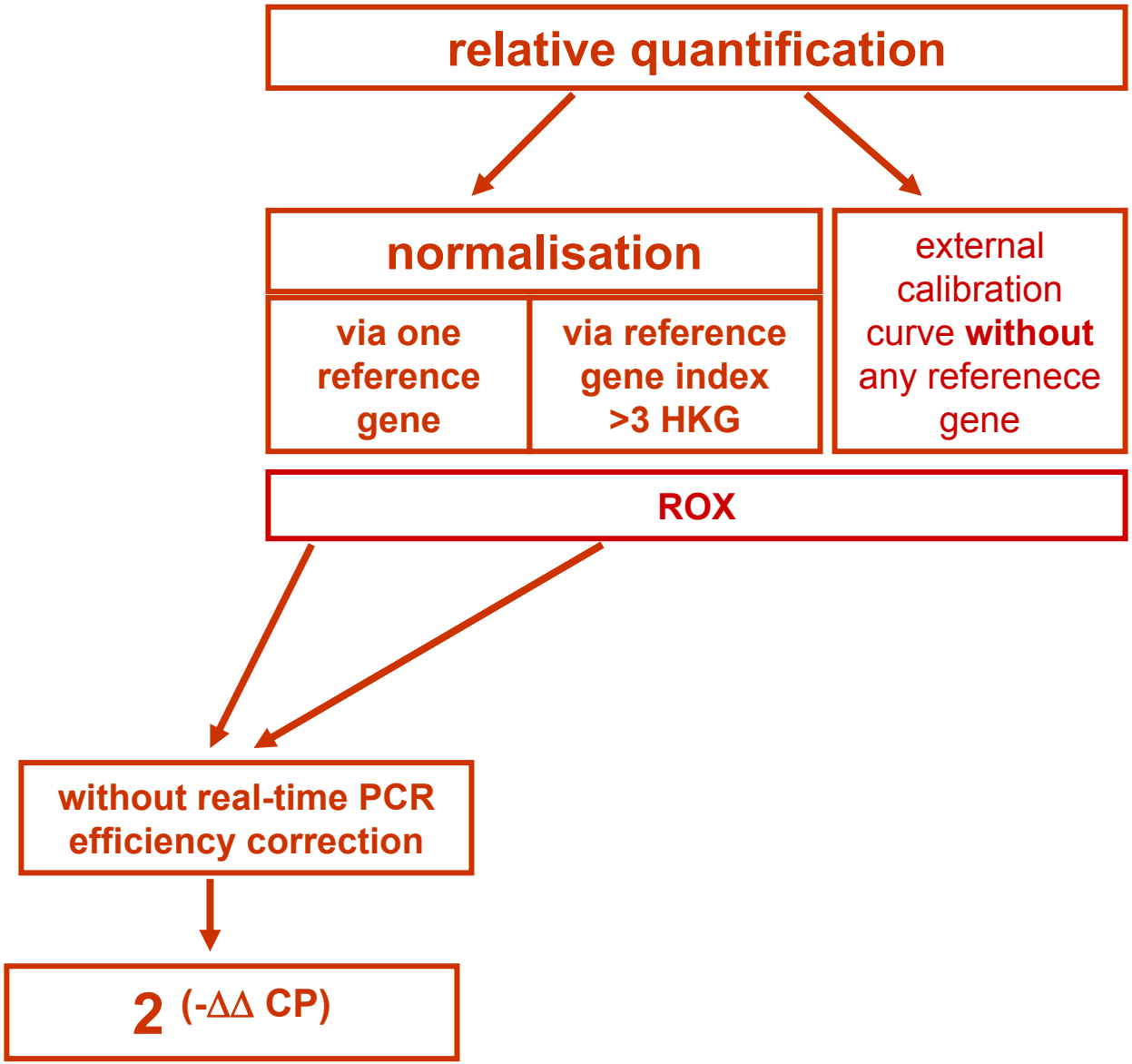
First GOI expression is normalised.....

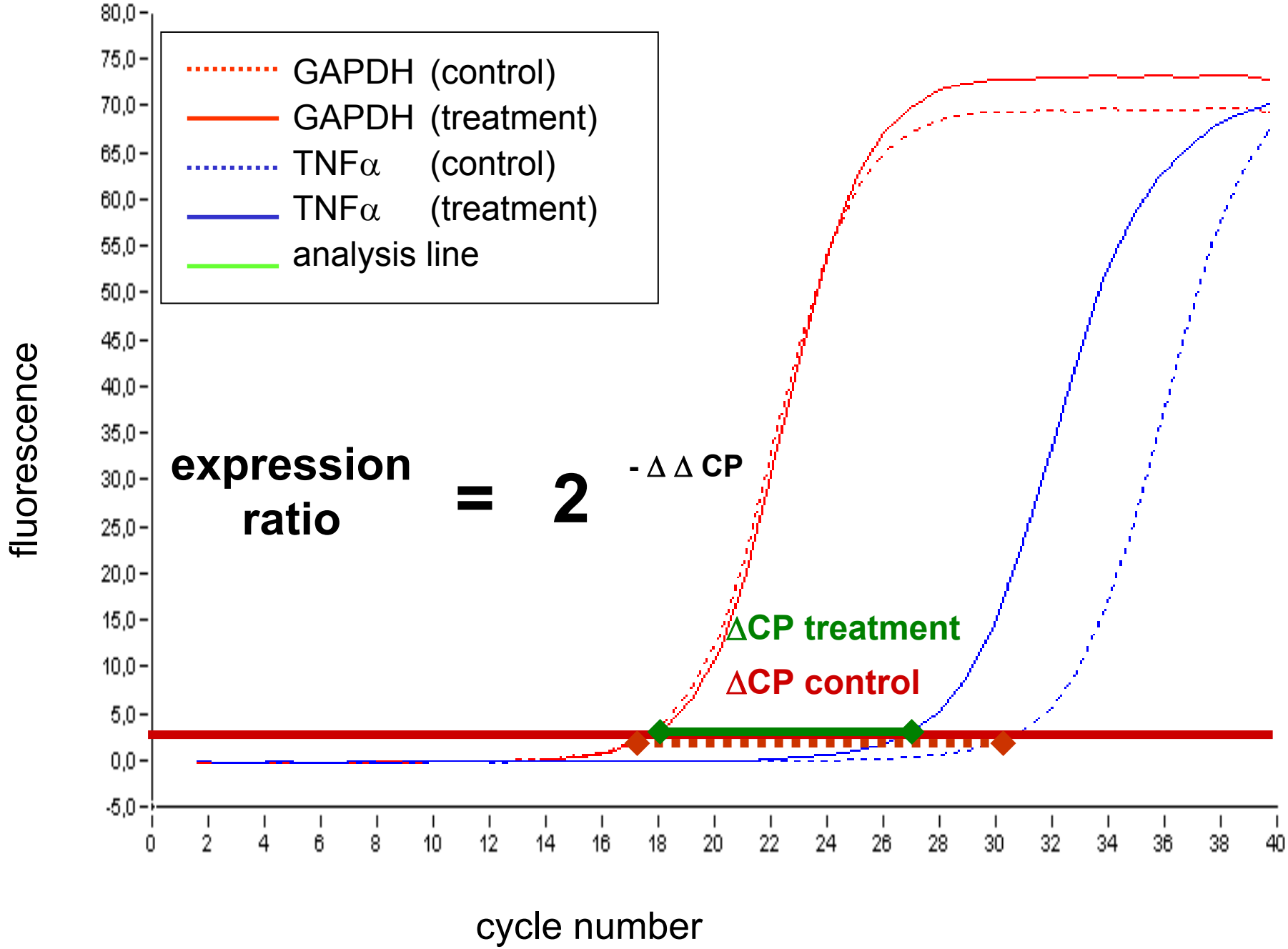
- **according to known amounts of extracted RNA**
  (molecules/ng RNA; ag transcript/ng RNA; RIN quality check ?)
- **according to mass / volume / cells of extracted tissue**
  (molecules/mg tissue; mass of transcript/mg tissue; copies per counted/selected cells, transcripts per single-cell)
- **according 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 versions

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




GAPDH (control)
GAPDH (treatment)
TNFα (control)
TNFα (treatment)
analysis line
fluorescence
cycle number
expression ratio = 2 - Δ Δ CP
ΔCP treatment
ΔCP control
80,0
75,0
70,0
65,0
60,0
55,0
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
-5,0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

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

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

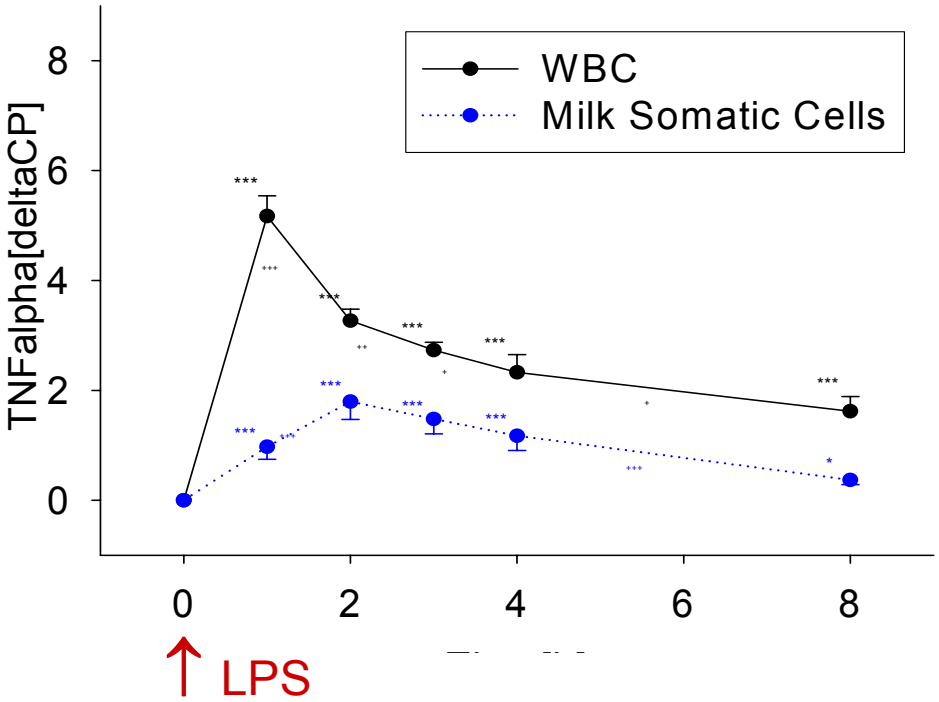
$$\text{expression ratio} = 2^{-[\Delta CP\ \text{treatment} - \Delta CP\ \text{control}]}$$

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

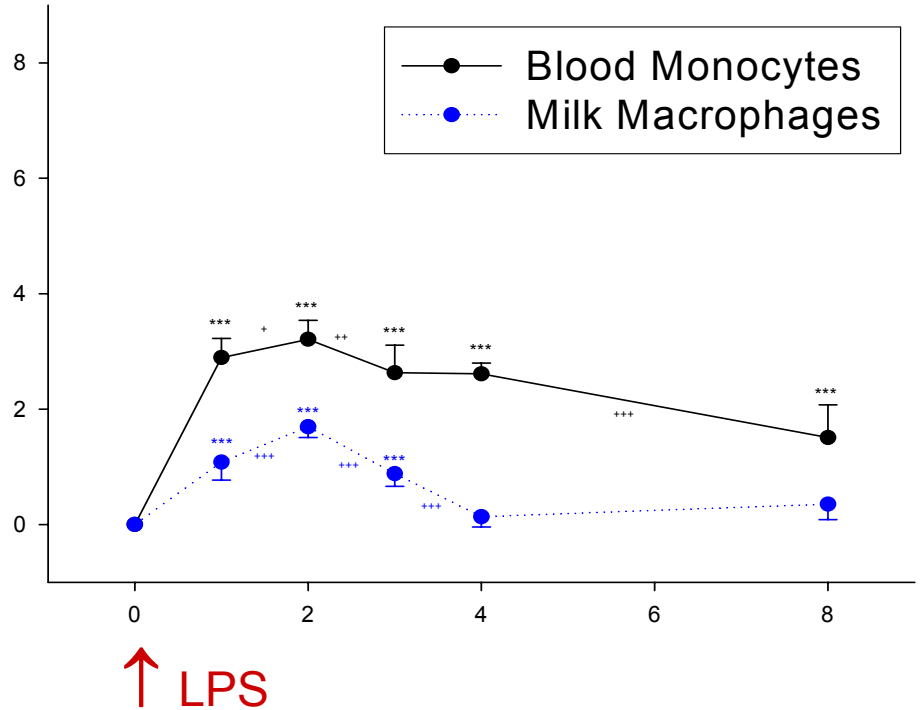
Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 [- delta deltaC(T)] method.
Methods, 2001 ***25(4):*** *402-408.*

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

TNFα response in WBC and milk somatic cells



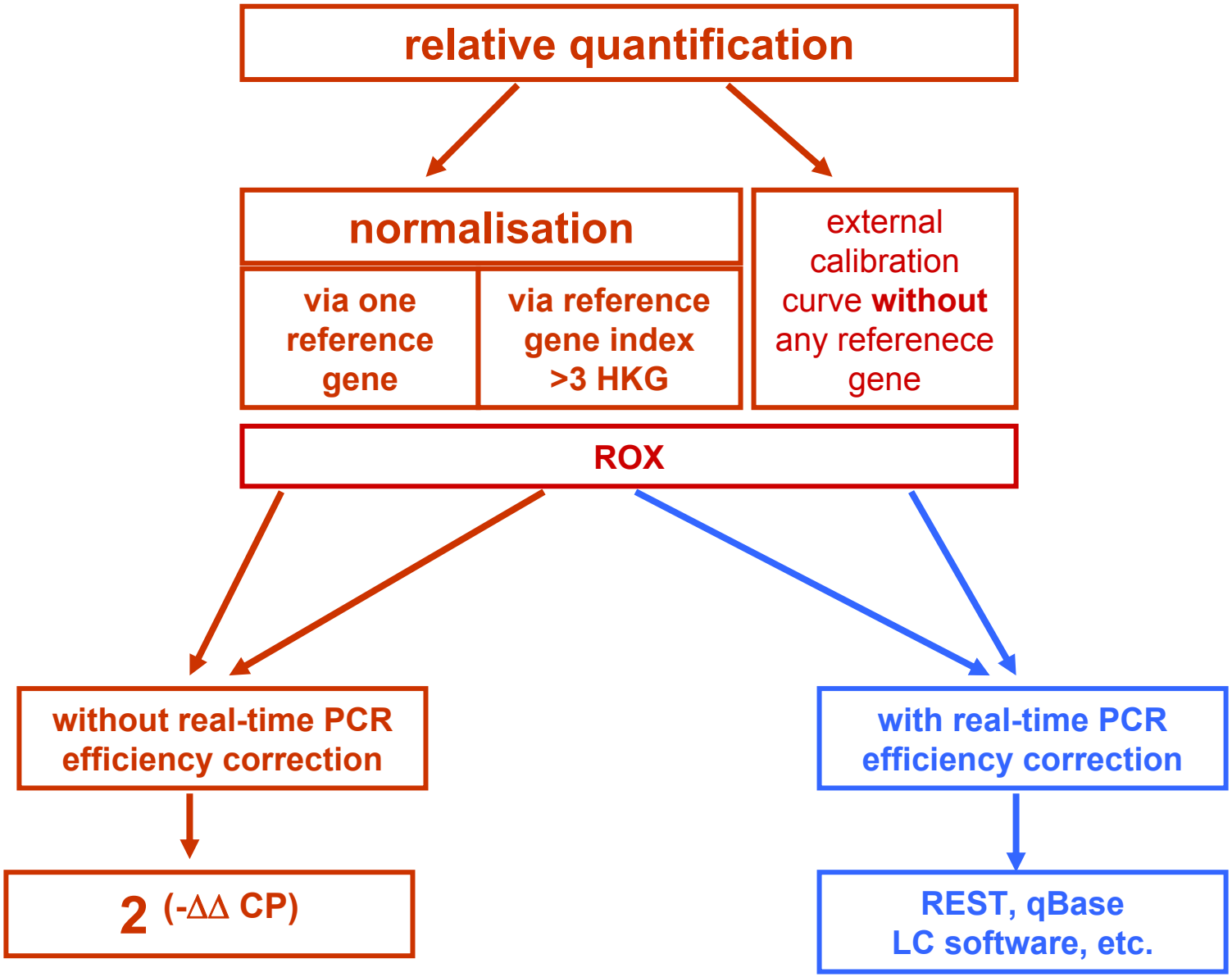
TNFα response in purified monocytes and macrophages



time [h] after LPS induction

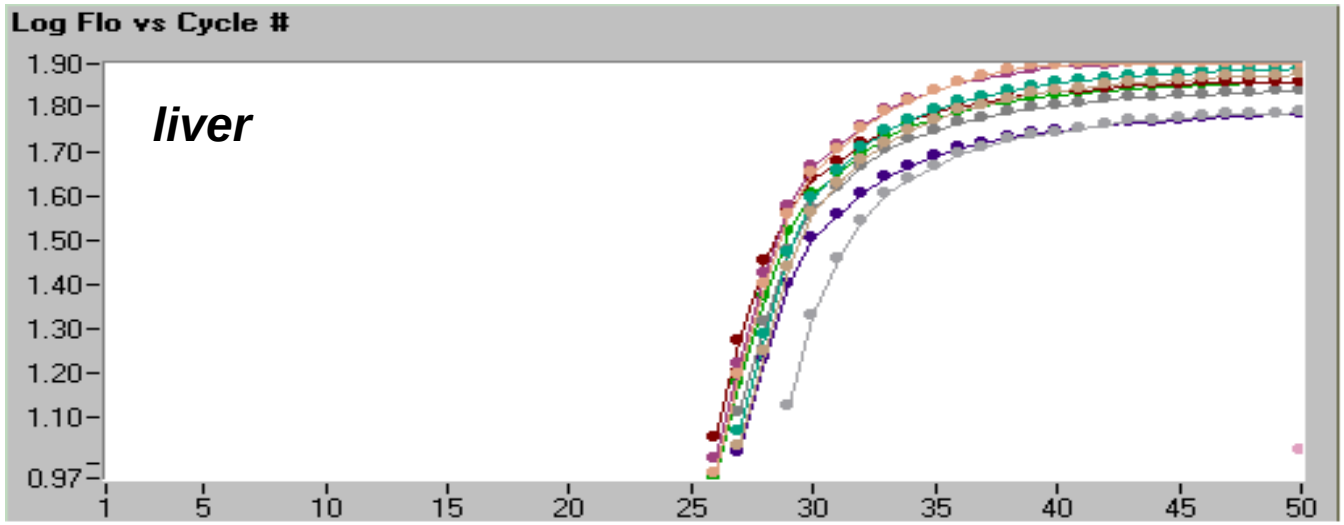
Prgomet et al, 2006

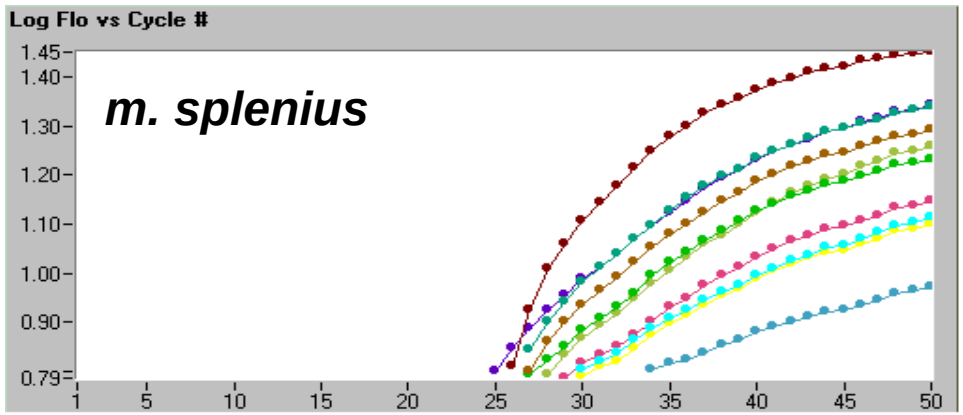
Relative Quantification in real time qRT-PCR

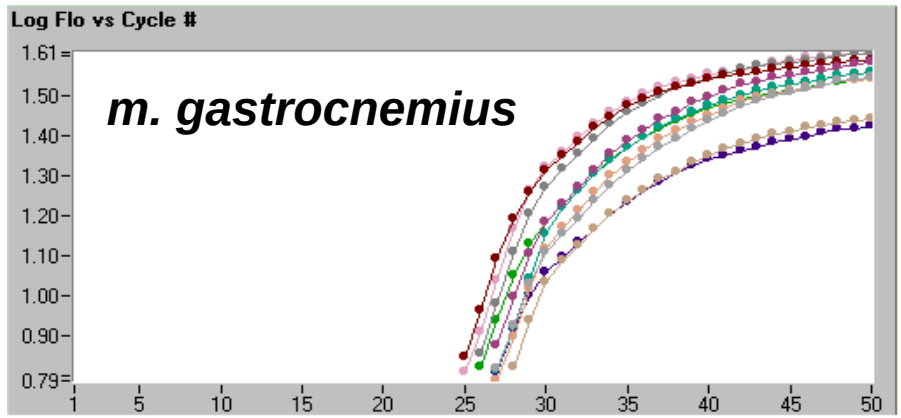


Tissue "*matrix*" interfere with real-time PCR efficiency and amplification fidelity

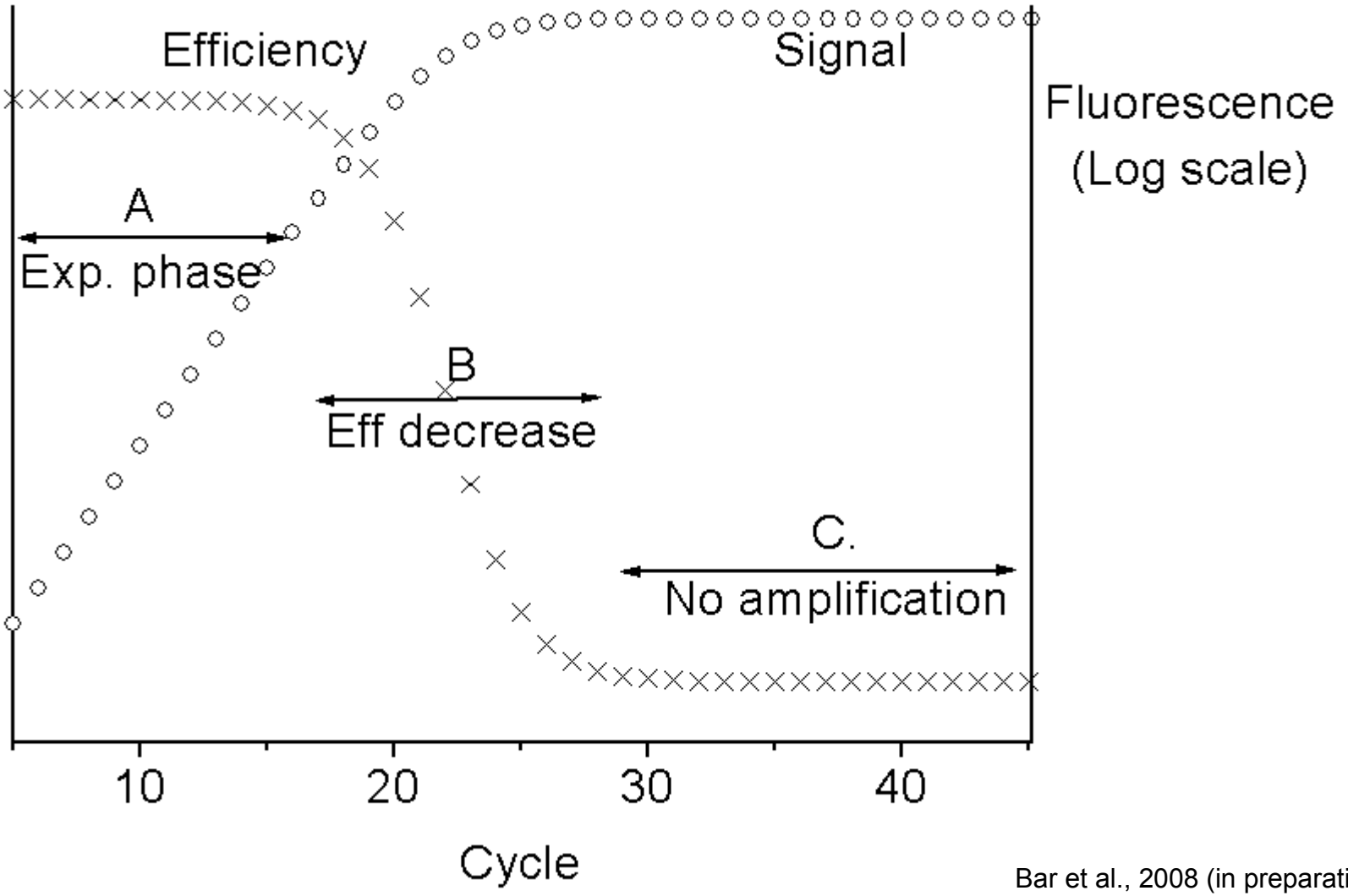
IGF-1 mRNA amplification in three cattle tissues



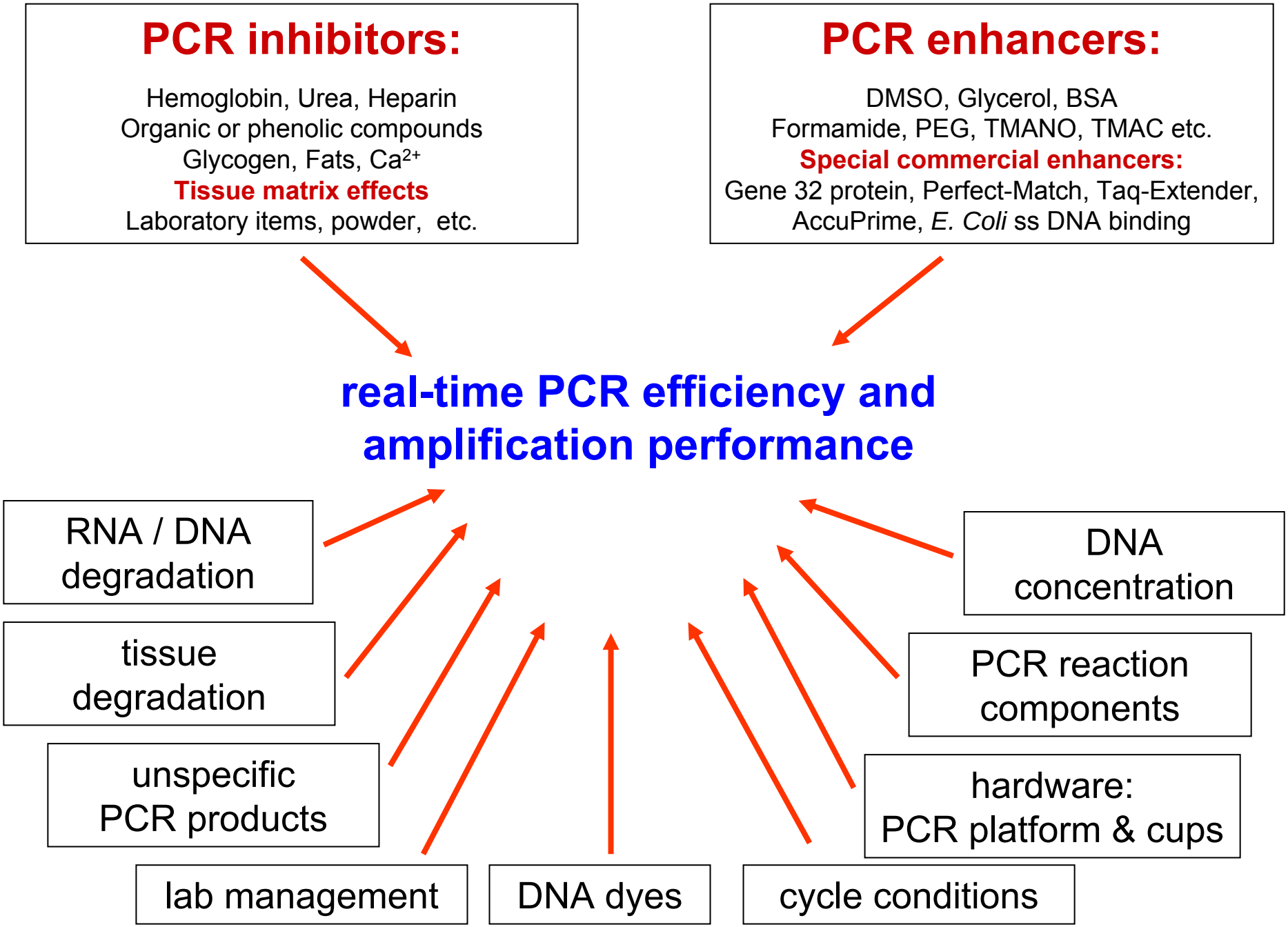




Theoretical real-time PCR kinetics



Bar et al., 2008 (in preparation)


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,
AccuPrime, E. Coli ss DNA binding
real-time PCR efficiency and amplification performance
RNA / DNA degradation
tissue degradation
unspecific PCR products
lab management
DNA dyes
cycle conditions
hardware: PCR platform & cups
PCR reaction components
DNA concentration

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

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

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

Pfaffl, Nucleic Acids Research 2001

Determination principles of real-time PCR amplification efficiency

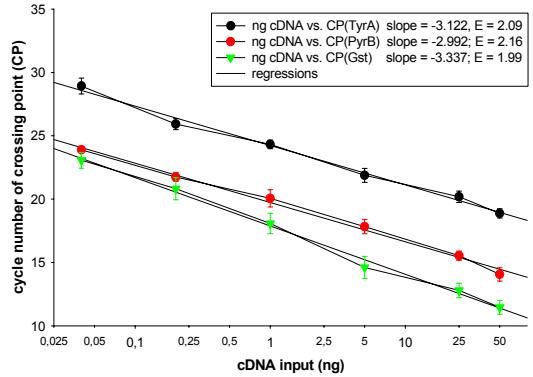
Direct methods:

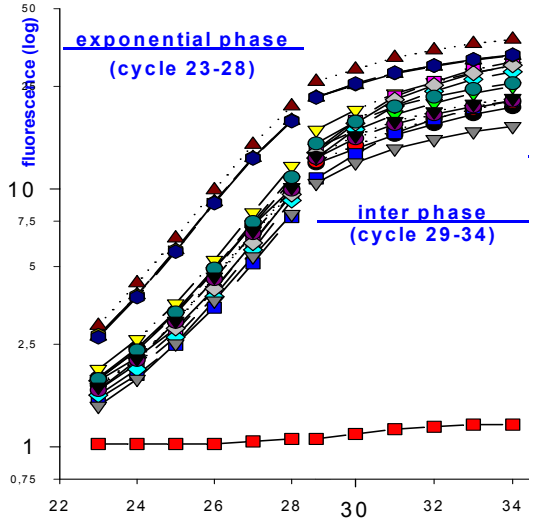
- **Dilution series**
  (Rasmussen 2001, Peirson et al. 2003, etc.)
- **Determination of absolute increase in fluorescence**
  (Rasmusen 2001; Peccoud & Jacob 1998; Pfaffl 2001)

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)

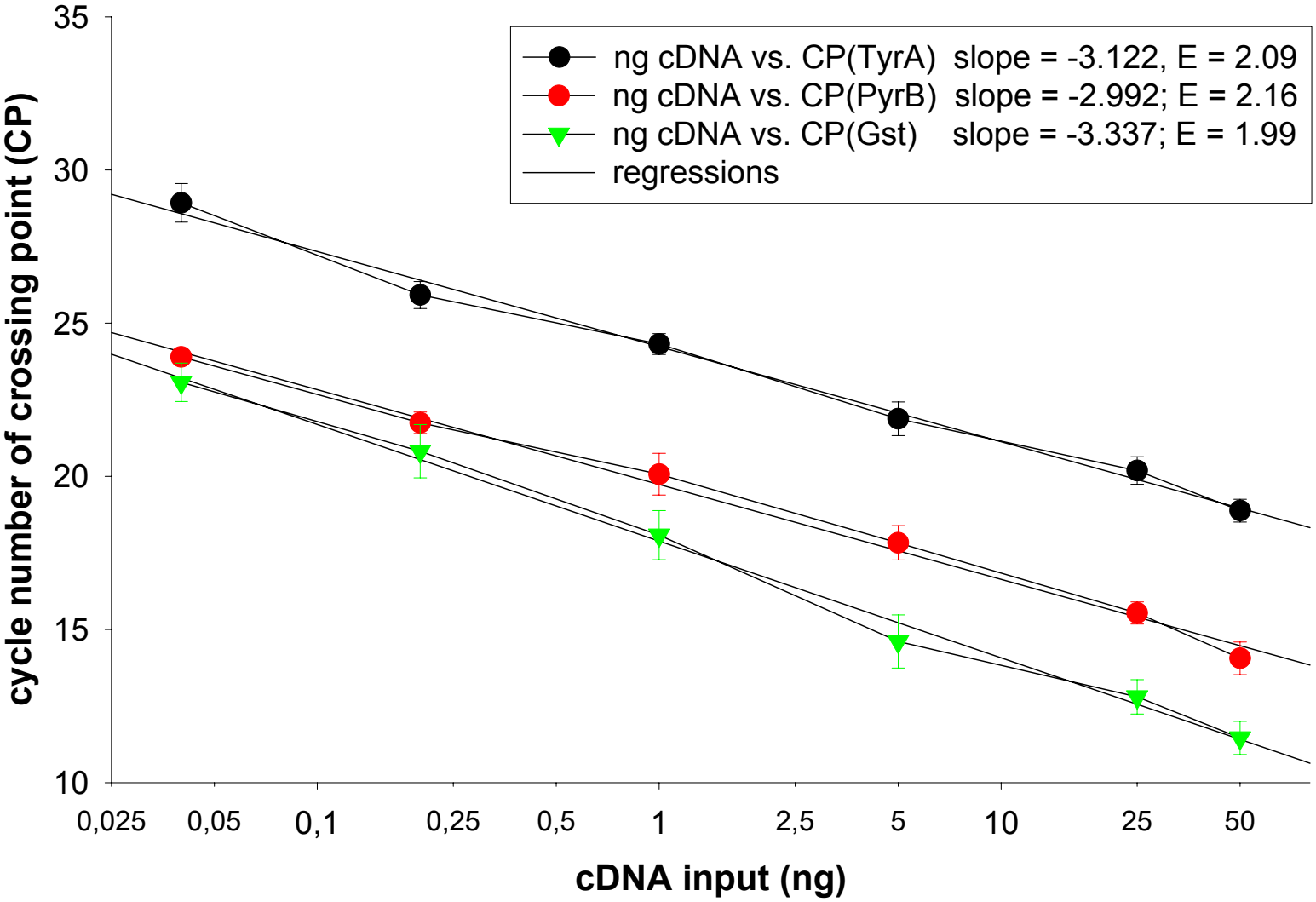
- http://**Efficiency.gene-quantification.info**

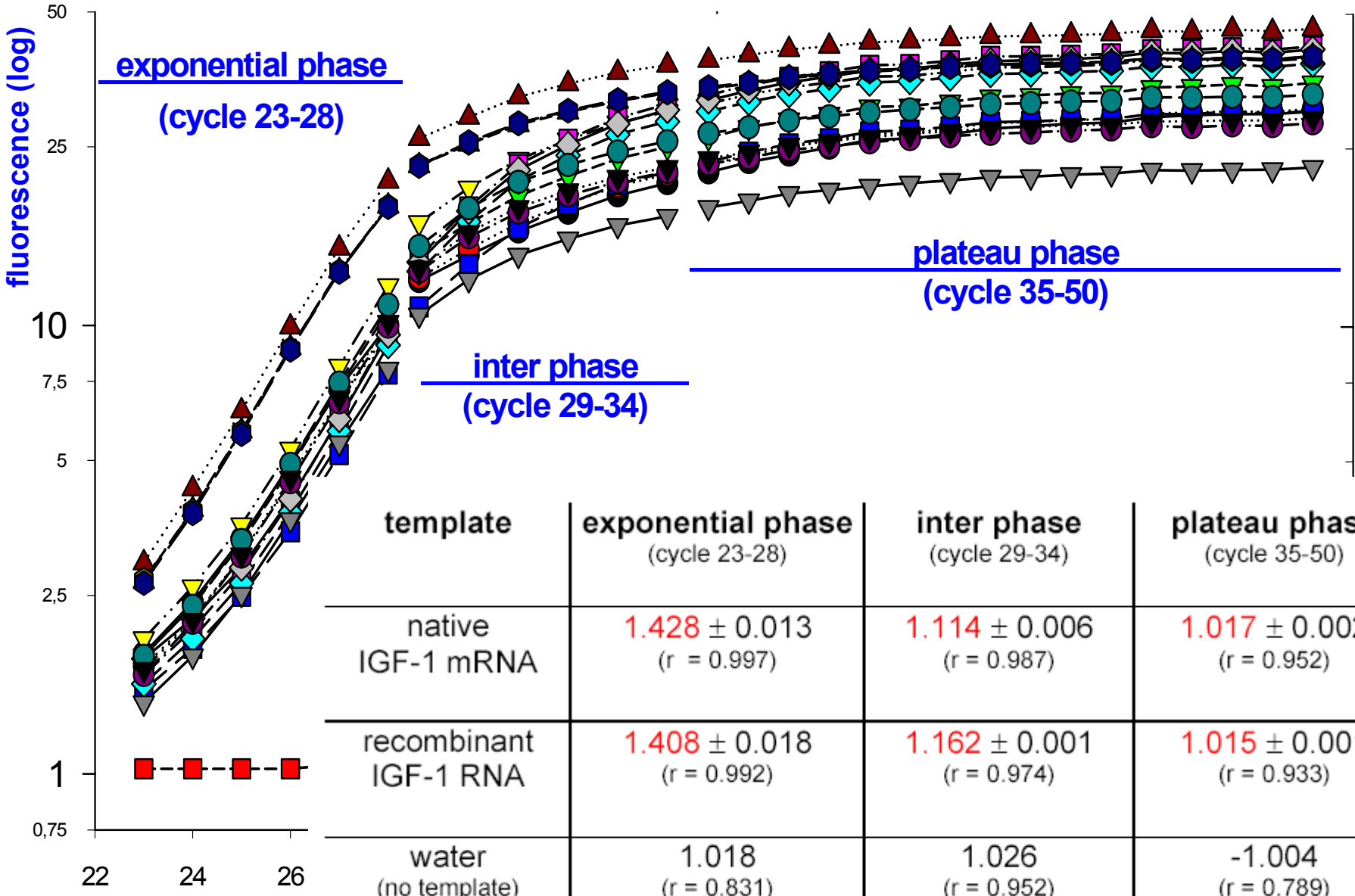




$$f = y_0 + \frac{a}{1+e^{-(\frac{x-x_0}{b})}}$$

Determination principles of real-time PCR efficiency:
Dilution series



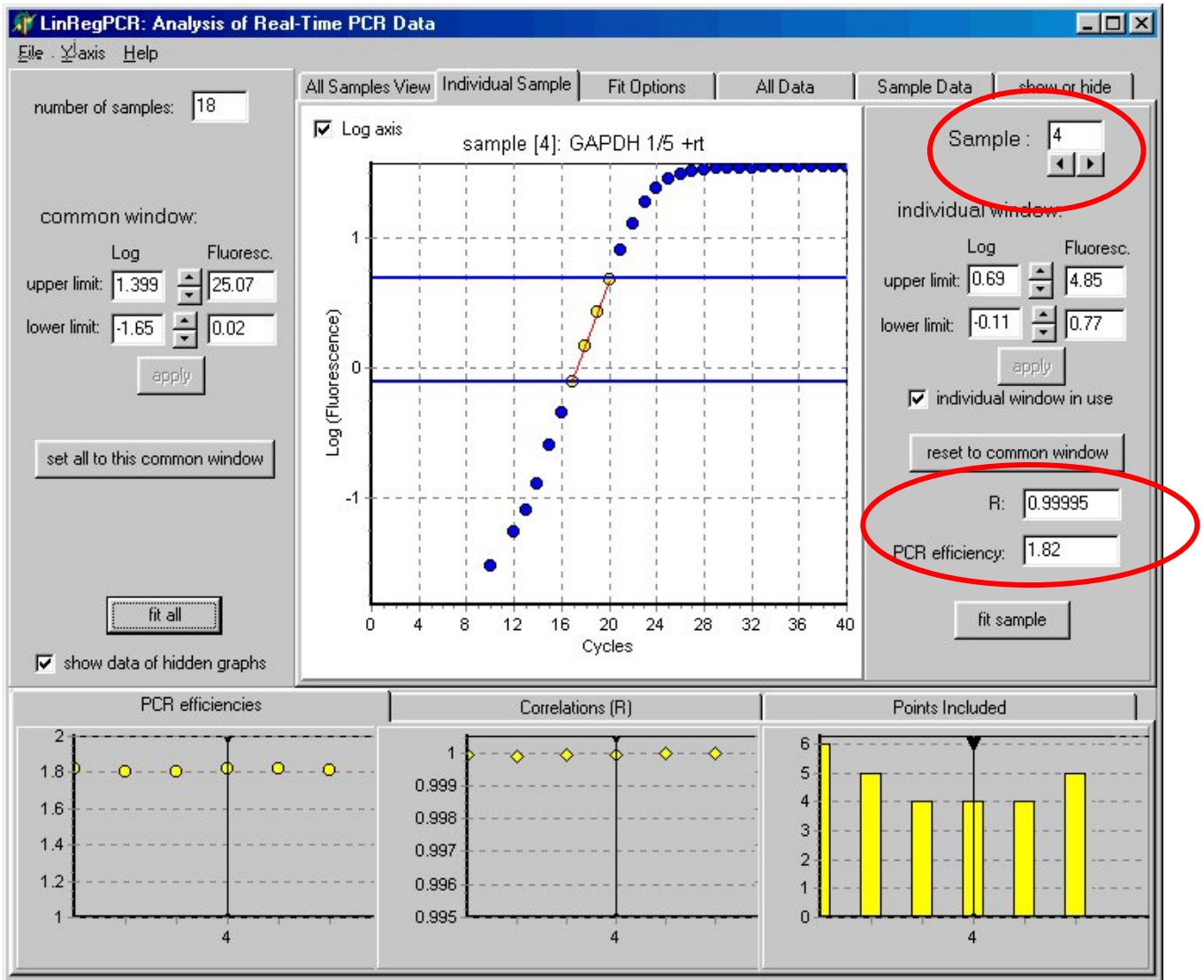


template	exponential phase (cycle 23-28)	inter phase (cycle 29-34)	plateau phase (cycle 35-50)
native IGF-1 mRNA	1.428 ± 0.013 (r = 0.997)	1.114 ± 0.006 (r = 0.987)	1.017 ± 0.002 (r = 0.952)
recombinant IGF-1 RNA	1.408 ± 0.018 (r = 0.992)	1.162 ± 0.001 (r = 0.974)	1.015 ± 0.001 (r = 0.933)
water (no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

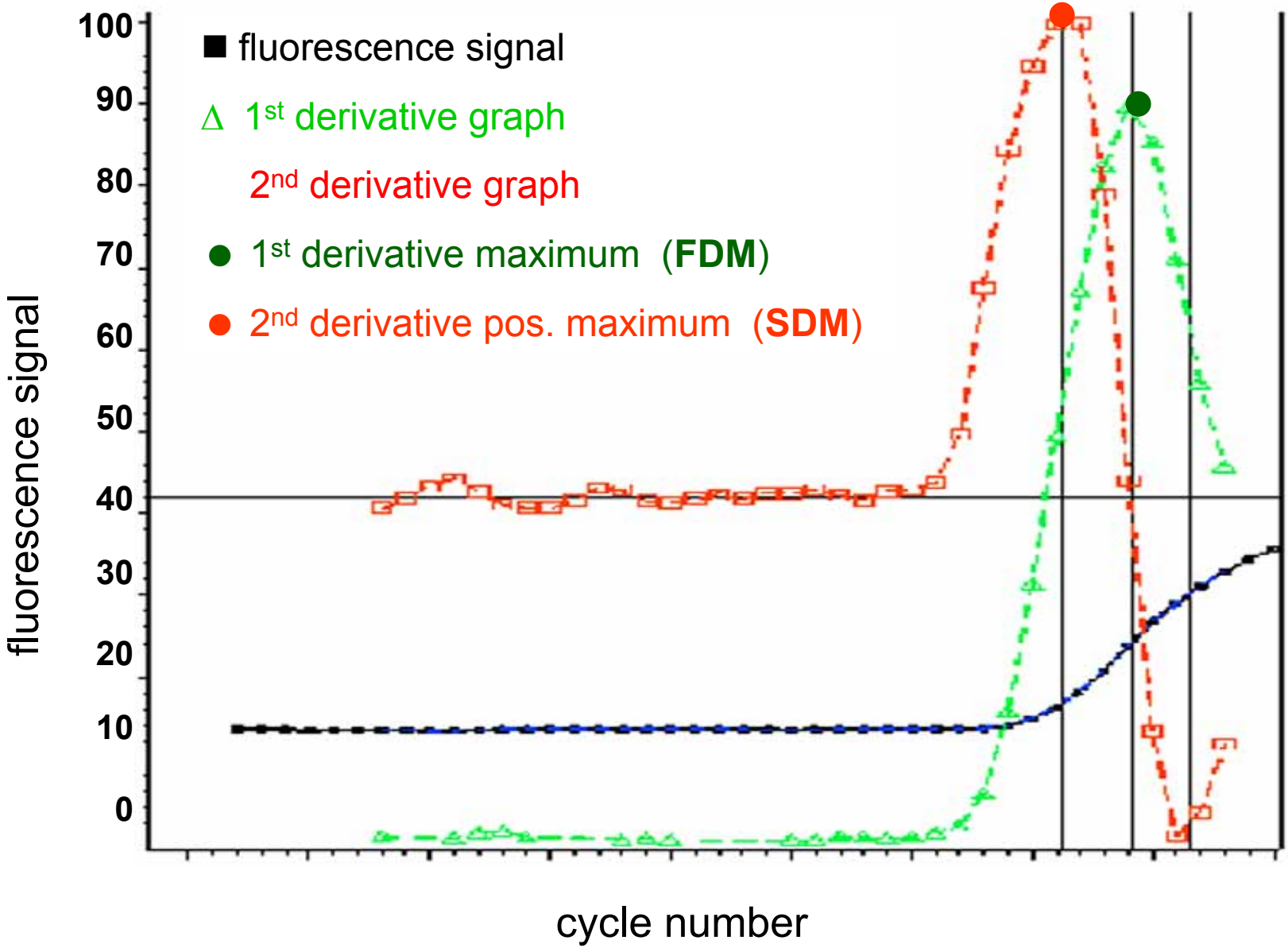
Calculation of real-time PCR efficiency: **LinRegPCR Interface**

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)

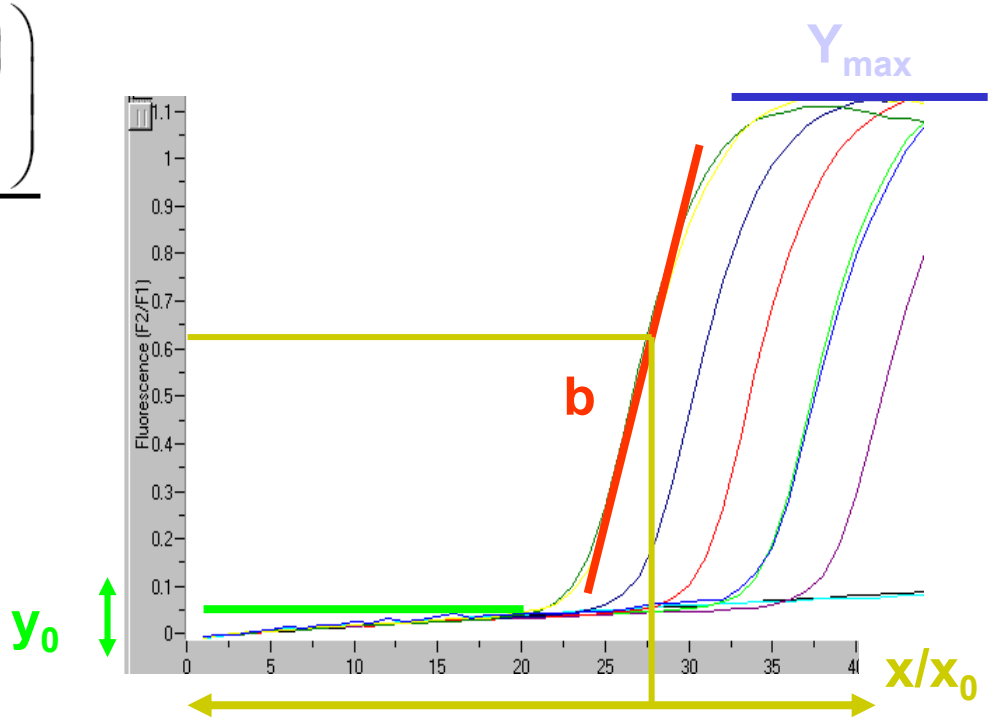
$$f(x) = y_0 + \frac{a}{1 + e^{-\frac{x - x_0}{b}}}$$

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

$a = y_{max} - y_0$

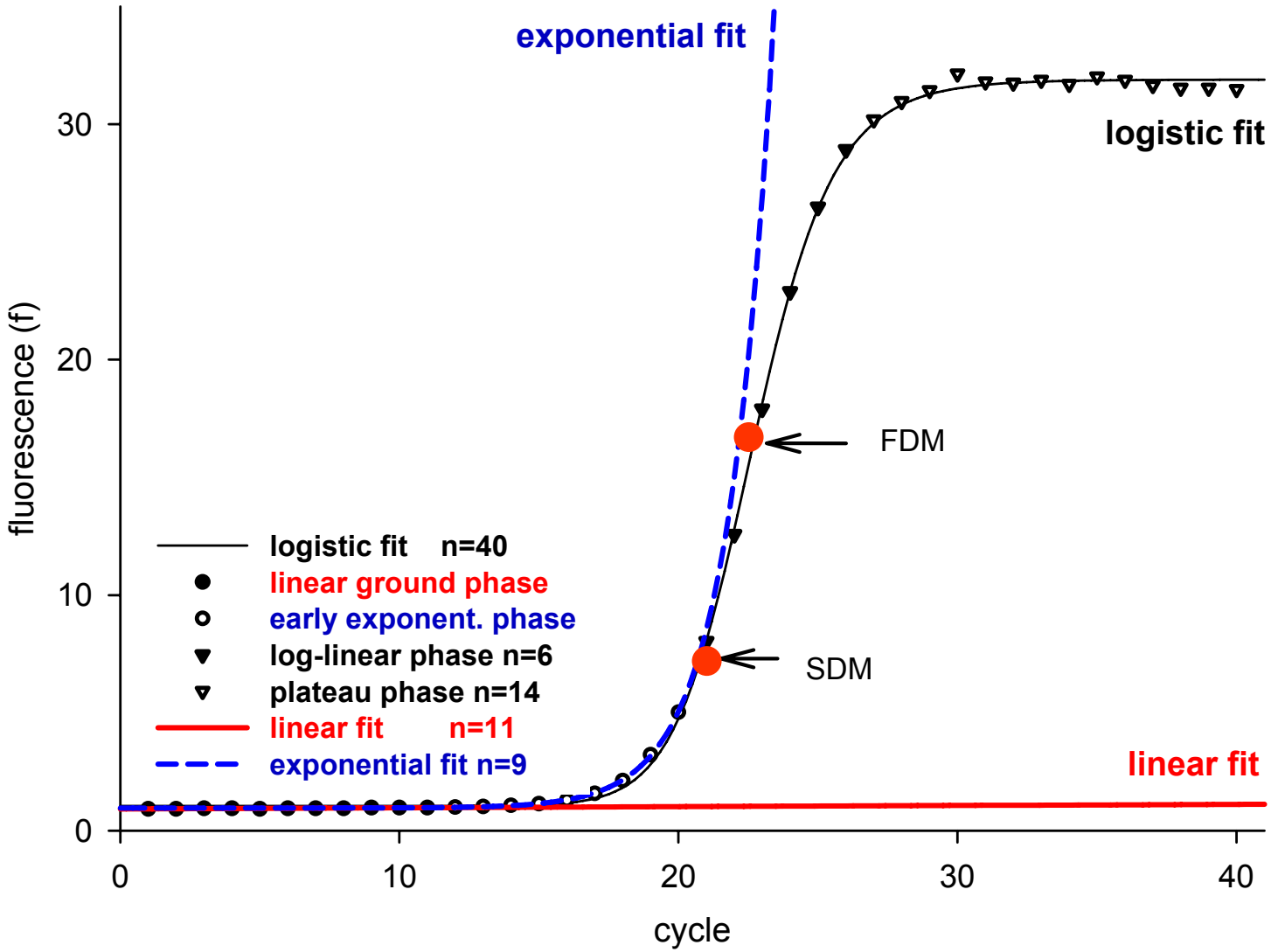
$$f'(x) = -a\left(1 + e^{-\frac{x - x_0}{b}}\right)^{-2} \cdot e^{-\frac{x - x_0}{b}} \cdot \left(-\frac{1}{b}\right)$$

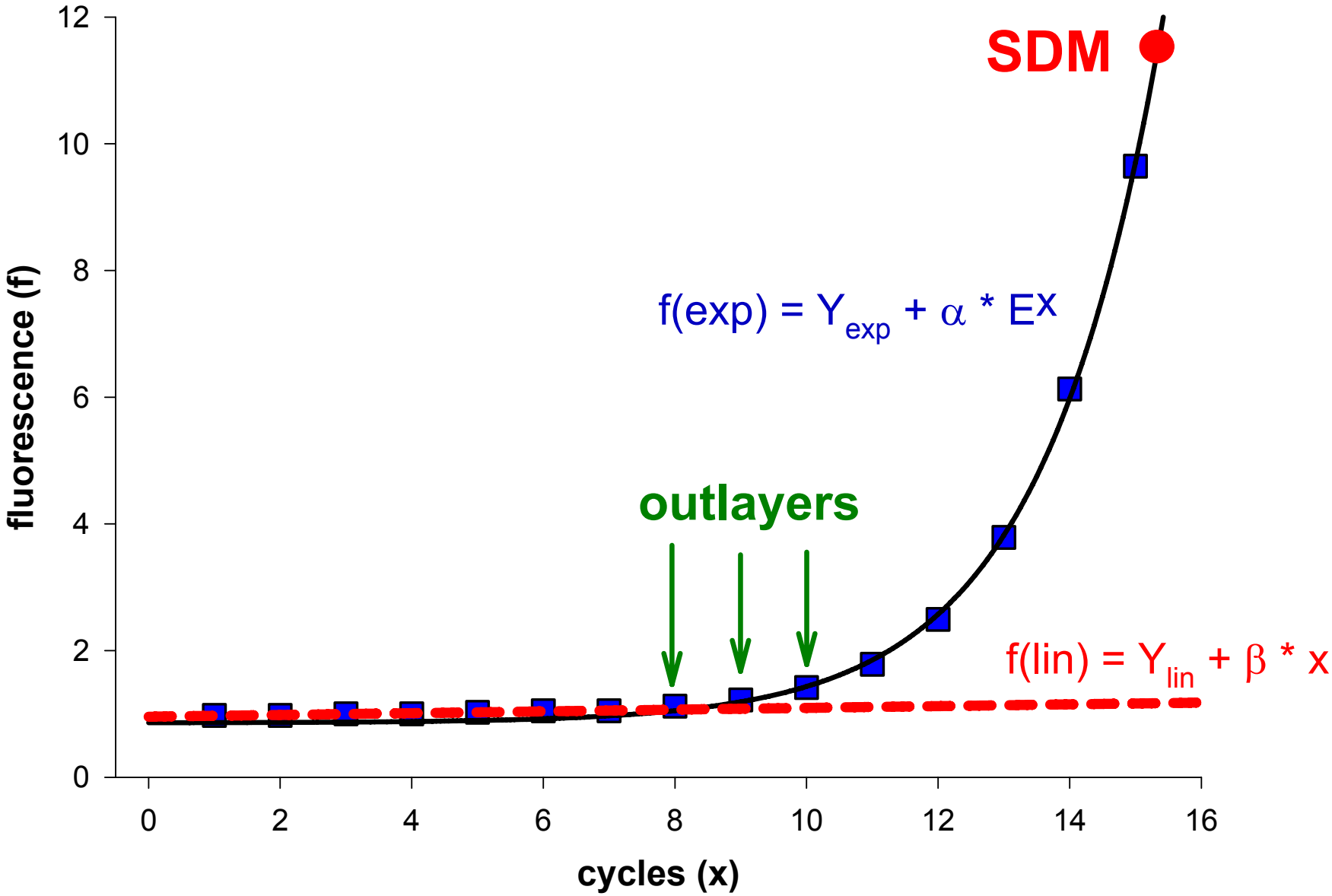
$$f''(x) = -\frac{a}{b^2} \cdot \frac{e^{-\frac{x - x_0}{b}}\left(1 - e^{\left(-\frac{x - x_0}{b}\right)}\right)}{\left(1 + e^{-\frac{x - x_0}{b}}\right)^3}$$



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

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




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

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

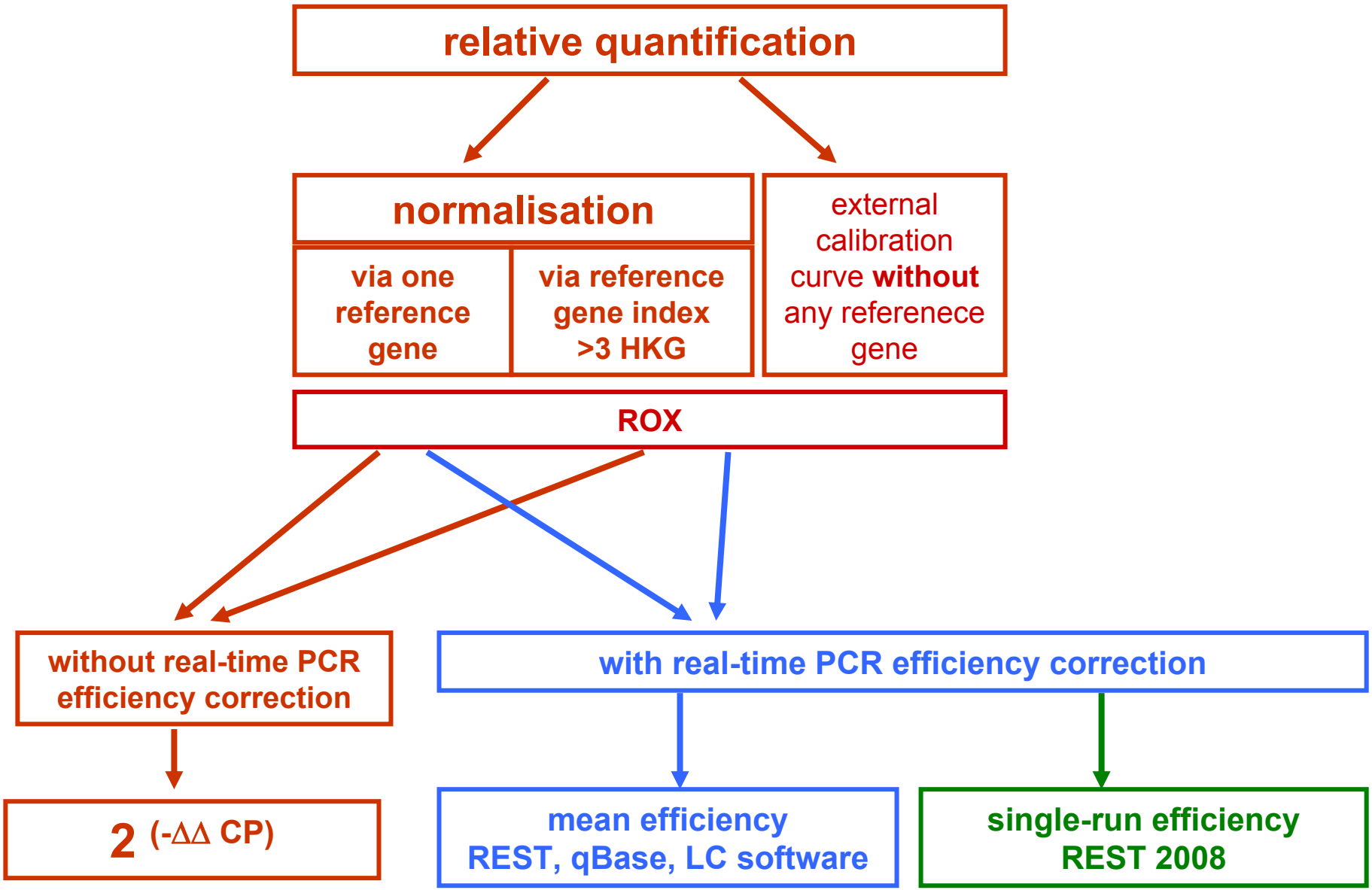
Conc. – input concentration of nucleic acid in sample.
n. - repeats
CP_{fp} – Crossing point based on Fit–point method.
CP_{sdm} – Crossing point based on second derivative maximum – *SDM* computing method by LightCycler software 3.3 (Roche Diagnostics).

$E1_{fit\ point}$ – 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*

Future of relative Quantification in real time qRT-PCR

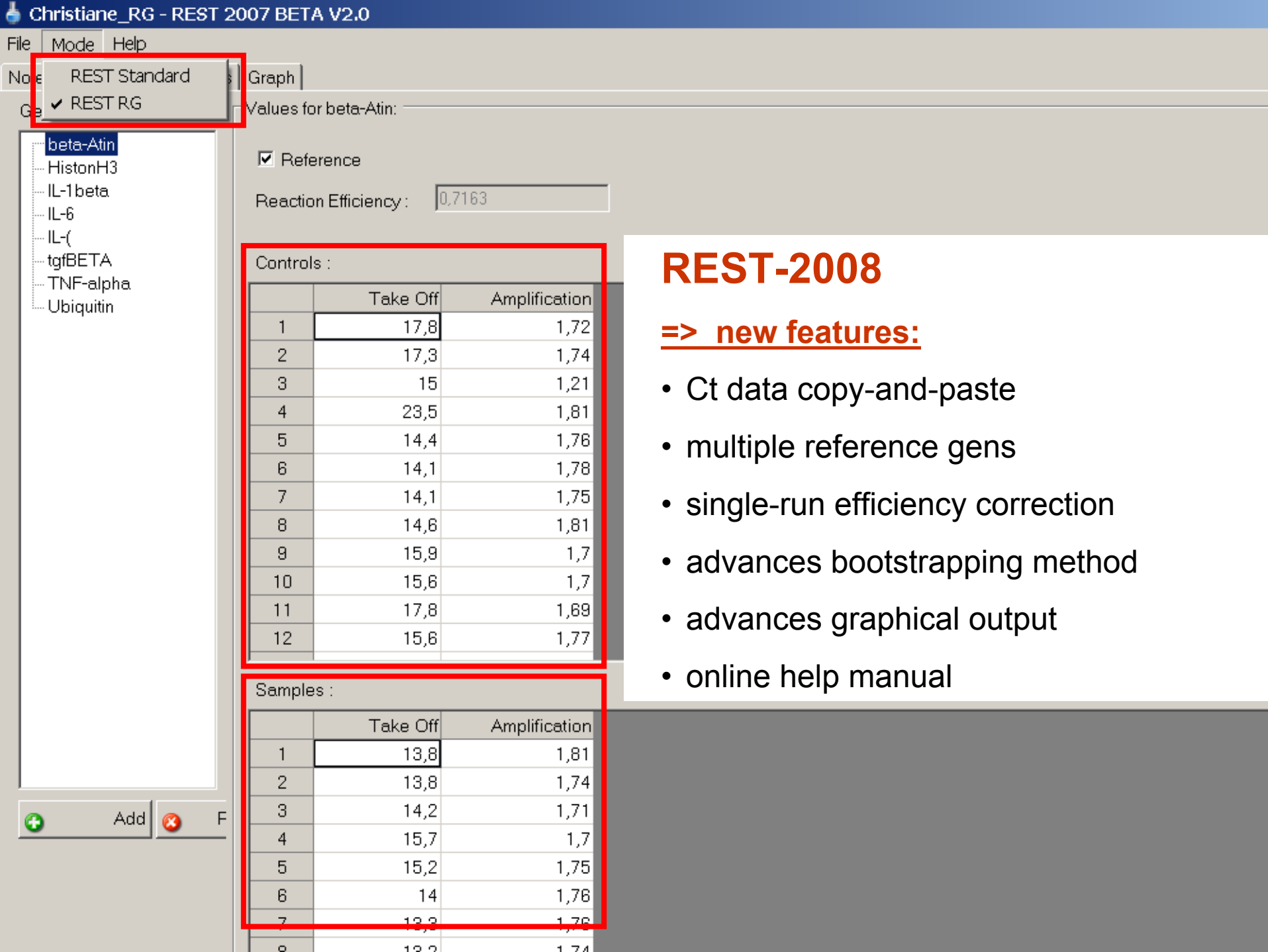


Relative Expression Software Tool (REST)

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-2008	Stand alone application standard Mode + single run efficiency correction	(June 2008)

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.



REST-2008

=> new features:

- Ct data copy-and-paste
- multiple reference gens
- single-run efficiency correction
- advances bootstrapping method
- advances graphical output
- online help manual

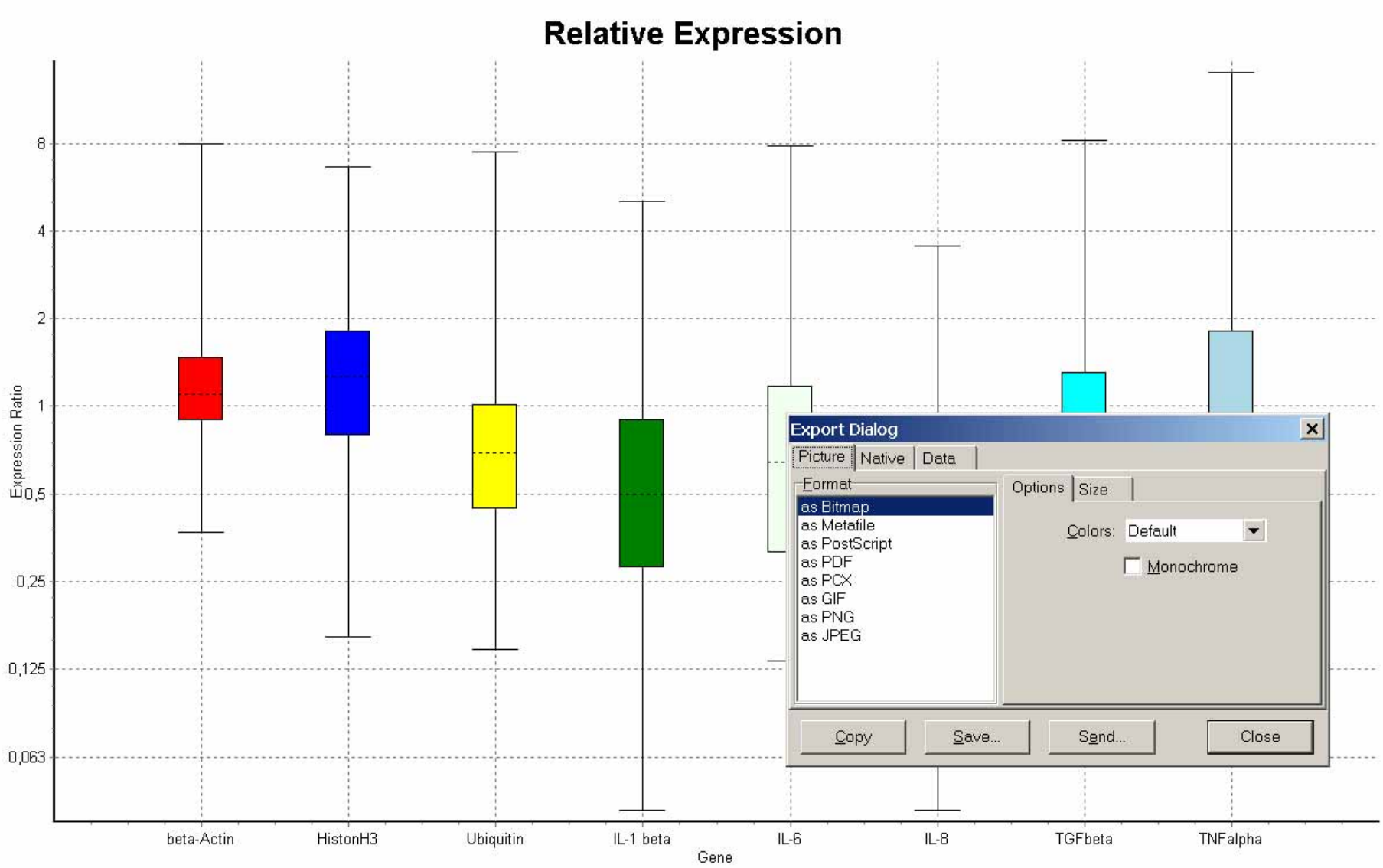
File Mode Help

Notes | Gene Setup | Gene Data | Results | Graph

Relative Expression Results

Parameter	Value
Iterations	2000
Normalisation Fac	1,44

Gene	Type	action Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
beta-Actin	REF	1,0	1,202	0,794 - 1,724	0,536 - 5,657	0,118	
HistonH3	REF	1,0	1,189	0,588 - 2,297	0,315 - 4,287	0,259	
Ubiquitin	REF	1,0	0,700	0,366 - 1,219	0,223 - 3,251	0,025	DOWN
IL-1 beta	TRG	1,0	0,511	0,225 - 1,149	0,104 - 3,257	0,004	DOWN
IL-6	TRG	1,0	0,657	0,268 - 1,571	0,150 - 4,189	0,038	DOWN
IL-8	TRG	1,0	0,413	0,150 - 1,000	0,077 - 2,352	0,000	DOWN
TGFbeta	TRG	1,0	0,771	0,341 - 2,047	0,137 - 5,157	0,228	
TNFalpha	TRG	1,0	1,007	0,406 - 3,001	0,239 - 7,657	0,979	


Relative Expression
Expression Ratio
8
4
2
1
0,5
0,25
0,125
0,063
beta-Actin
HistonH3
Ubiquitin
IL-1 beta
IL-6
IL-8
TGFbeta
TNFalpha
Gene
Export Dialog
Picture
Native
Data
Format
as Bitmap
as Metafile
as PostScript
as PDF
as PCX
as GIF
as PNG
as JPEG
Options
Size
Colors:
Default
Monochrome
Copy
Save...
Send...
Close

The evolution of relative quantification software

- **ΔΔCt method (Livak & Schmittgen, 2001)**
  assumptions:
  - PCR efficiency = 2.00
  - one stable expressed reference gene

$$NRQ = 2^{\Delta\Delta Ct}$$

- **Efficiency correction (Pfaffl, 2001)**
  assumptions:
  - corrected PCR efficiency
  - one stable expressed reference gene

$$NRQ = \frac{E_{goi}^{\Delta Ct,goi}}{E_{ref}^{\Delta Ct,ref}}$$

- **Relative Expression Software Tool - 1st REST version (Pfaffl et al., 2002)**
  assumptions:
  - corrected PCR efficiency
  - multiple stable expressed reference gene (REST 384)
  - statistical testing

- **qBase / qBASE plus (Hellemans et. al, 2007, Vandesompele et al., 2008)**
  assumptions:
  - adjusted PCR efficiency
  - multiple reference genes
  - data management system

$$NRQ = \frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[n]{\prod_{i}^{n} E_{ref_i}^{\Delta Ct,ref_i}}}$$

- **REST 2008 (Pfaffl et al., 2008)**
  assumptions:
  - corrected PCR efficiency
  - multiple stable expressed reference gene
  - statistical testing
  - Single-run efficiency correction (REST 2008)

- **Software download**
  - http://bioinformatics.gene-quantification.info/
  - http://download.gene-quantification.info/

www.Gene-Quantification.info

The Reference in qPCR - Academic & Industrial Information Platform

The Gene Quantification page describes and summarises all technical aspects involved in quantitative gene expression analysis using real-time qPCR & qRT-PCR. It presents a lot of cyclers, kits, dyes, chemistries, methods and services involved. Companies and institutions can present their qPCR technologies, applications and services right here. Directory.Gene-Quantification.info

qPCR.gene-quantification.info	real-time PCR summary / overview of interesting qPCR papers
news.gene-quantification.info	Gene Quantification page NEWS - microRNA (new page!)
strategy.gene-quantification.info	Quantification strategies in qRT-PCR: absolute Quan. - relative Quan.
normalisation.gene-quantification.info	Normalisation strategies & Reference-Genes & multiple RGs
optimisation.gene-quantification.info	Optimisation of reaction setup and qPCR procedure
bioinformatics.gene-quantification.info	DOWNLOAD, REST, DATAN, qBase, algorithm, primers, statistics
events.gene-quantification.info	Meetings, Workshops, Seminars: TATAA qPCR 2005 qPCR 2004
cyclers.gene-quantification.info	real-time PCR hardware
RT.gene-quantification.info	reverse transcription mRNA transcript analysis RNA integrity
dyes.gene-quantification.info	detection dyes and chemistries in real-time PCR
physiology.gene-quantification.info	qPCR in Physiology & Immunology, single-cell qRT-PCR
efficiency.gene-quantification.info	determination of real-time qPCR efficiency; various methods
chips.gene-quantification.info	verification of qRT-PCR via cDNA array / PCR-on-chip / Lab-on-chip

qPCR News

powered by www.Gene-Quantification.info

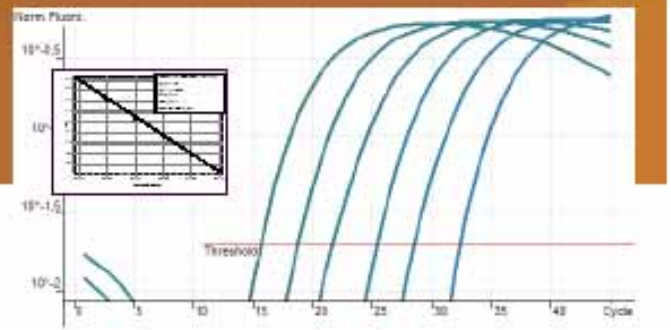
qPCR platform participating companies:

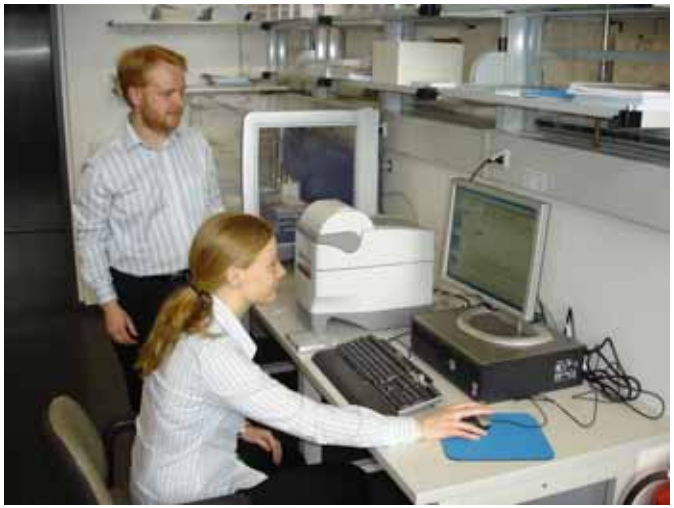
qPCR platform participating companies in alphabetical order

Roche Applied Science.gene-quantification.info

tataabiocenter
germany

qPCR training courses and workshops





http://TATAA-Germany.de

Thank you team !

Thank you for your attention !