Quantification strategies in real-time RT-PCR

with special focus on RNA quality, relative expression and data normalisation

qPCR Satellite Symposium

Bio City Leipzig, 10 - 11th March 2005

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www.gene-quantification.info

Genotype -> Phenotype -> Function

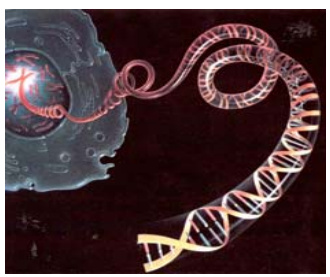
DNA => pre-mRNA => mRNA => Protein => Function

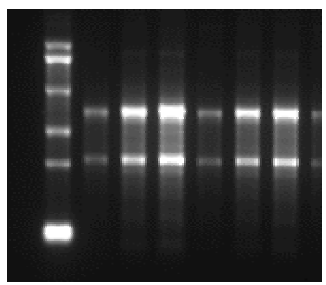
↓ Transcription

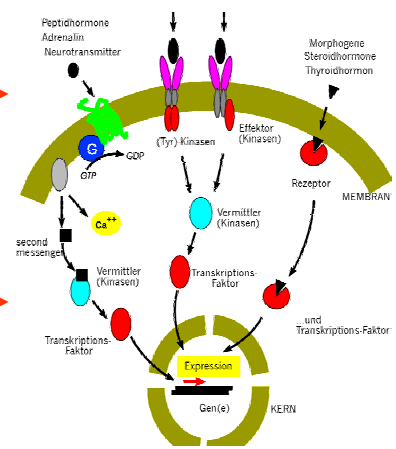
↓ Splicing

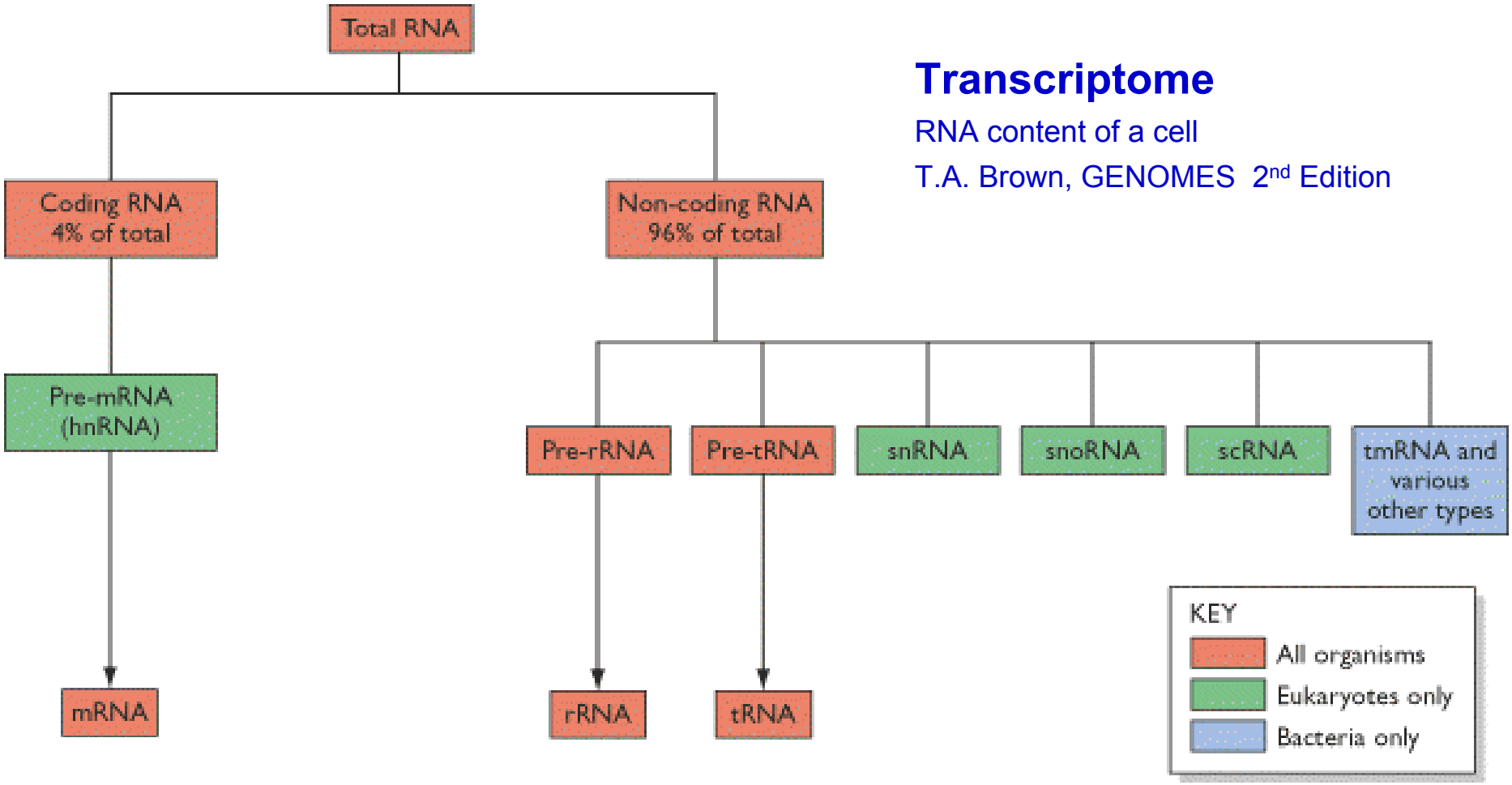
↓ Translation
post-translatoric modifications

Genom | **Transcriptome** *& Splicome* | **Proteome** | **Metabolome**









Transcriptome

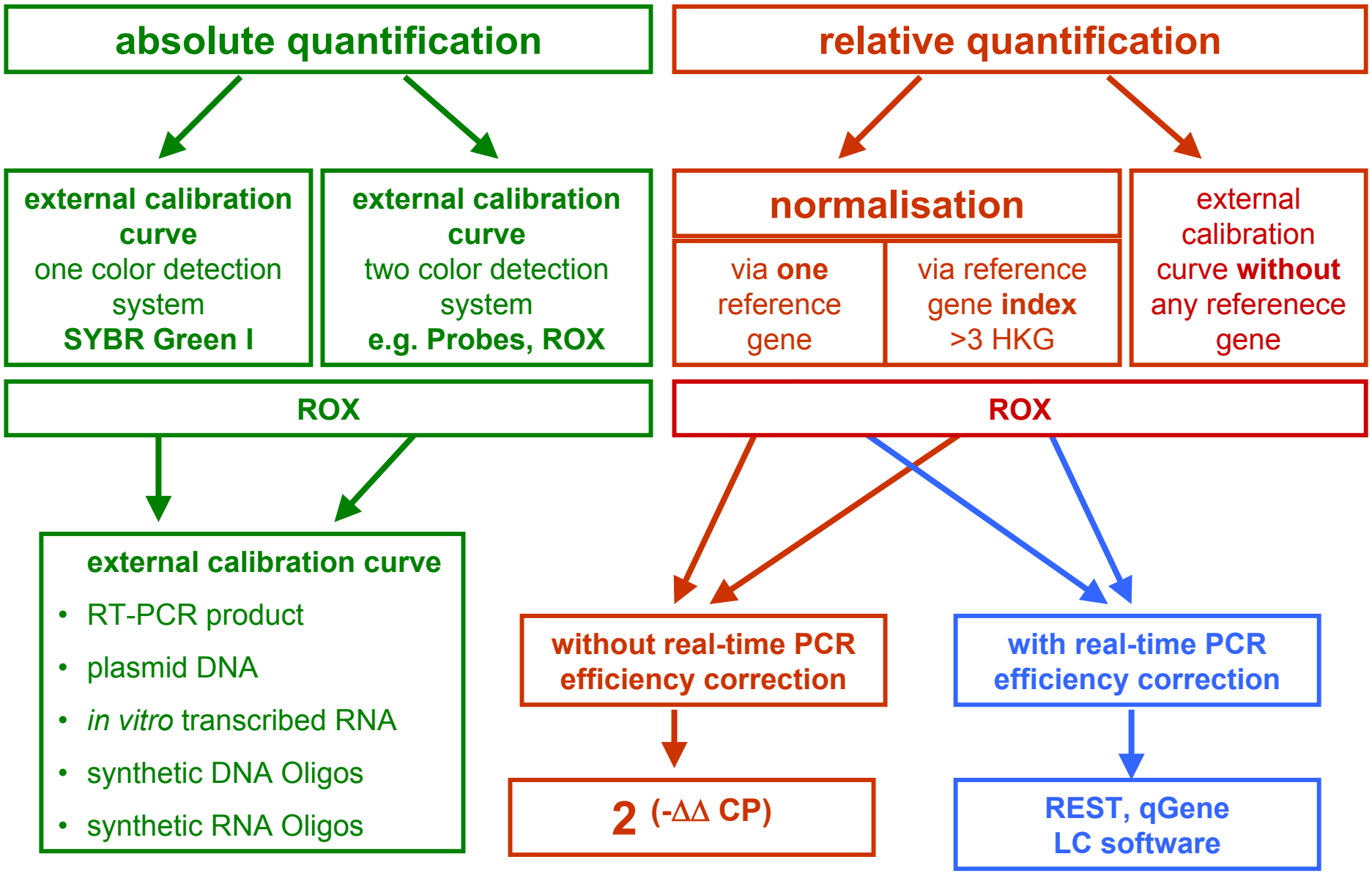
RNA content of a cell

T.A. Brown, GENOMES 2nd Edition

- ribosomal RNA rRNA 80-85% (5S, 18S und 28S)
- transfer RNA tRNA 10-15%
- **messenger RNA** **mRNA 2% (1-5%)** (∅ length 1930 bases)
  - *high abundant* < 100 genes > 10,000 copies/cell
  - *intermediate abundant* ~ 500 - 1,000 genes 200 - 400 copies/cell
  - *low abundant* ~ 27,000 genes **< 10** - 50 copies/cell
- **RNA quantity & RNA quality**

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 (*Pfaffl & Hageleit, Biotechnol.Lett. 2001)*
- **recombinant RNA** (recRNA) calibration curve (*Pfaffl & Hageleit, Biotechnol.Lett. 2001)*
- calibration curve using a synthetic **DNA** oligo-nucleotide *(Bustin, JME 2000)*
- calibration curve using a synthetic **RNA** oligo-nucleotide *(Bustin et al. 2000)*
- calibration curve using a purified **RT-PCR product** *(Einspanier et al. 1999)*
- *„Copy & Paste“* of previously performed **calibration curves** *(LC software)*

Absolute quantification using calibration curves

- ***Calibration curve using a purified RT-PCR product or a synthetic ss/ds oligo-nucleotide***
  two-step RT-PCR
  advantages: quick, highly defined DNA content for the synthetic oligo
  disadvantages: instable, “often problems with re-amplification”, „short“ templates

- ***Calibration curve using a recombinant DNA (recDNA), e.g. plasmid DNA***
  two-step RT-PCR
  advantages: very stabile, no problems with re-amplification, „mimic of mRNA“
  disadvantages: cloning, linearization and purification of recDNA

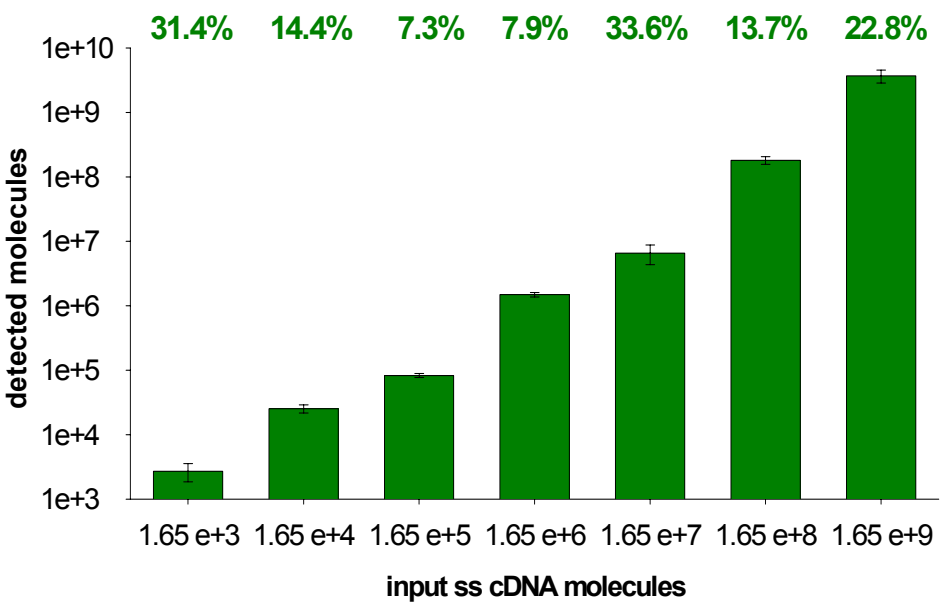
- ***Calibration curve using a recombinant RNA (recRNA)***
  one-step RT-PCR => recRNA and native mRNA undergoing RT and PCR in parallel
  advantages: mimics the natural mRNA situation best (recRNA = native mRNA)
  disadvantages: very instable recRNA, complicate cloning, linearization, purification of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

- ***„Copy & Paste“ of previously performed calibration curves (e.g. LightCycler Software)***
  advantages: very easy and very high reproducibility (at least for the calibration curve)
  disadvantages: do not covers variations in real RT-PCR experiment: RNA quality, slope, qPCR efficiency ??? batch to batch variations, etc. ***=> truth ???***

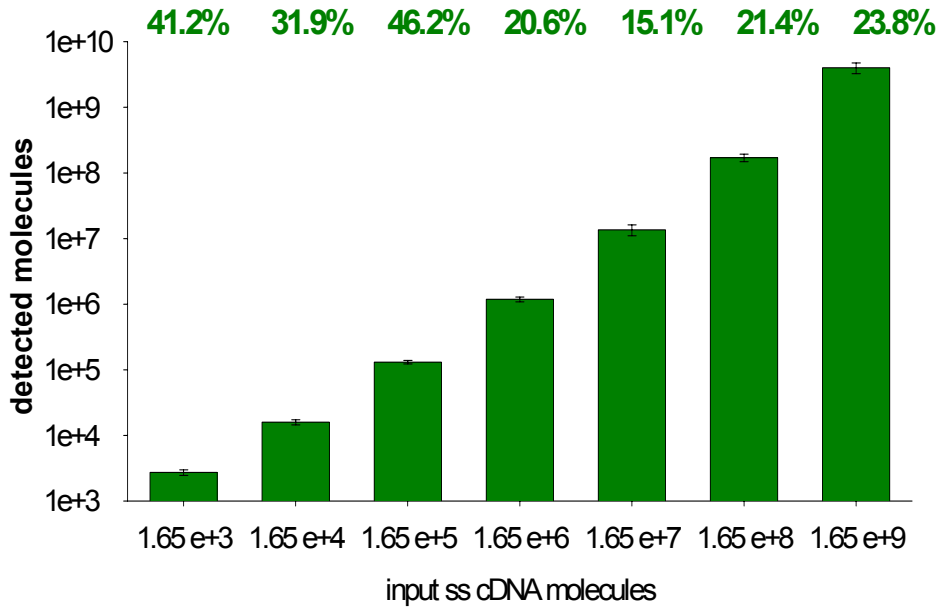
ERα intra-assay & inter-assay variation

variation on the basis of detected molecules
using a recombinant plasmid DNA calibration curve
intra-assay variation: within one run
inter-assay variation: between different runs

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



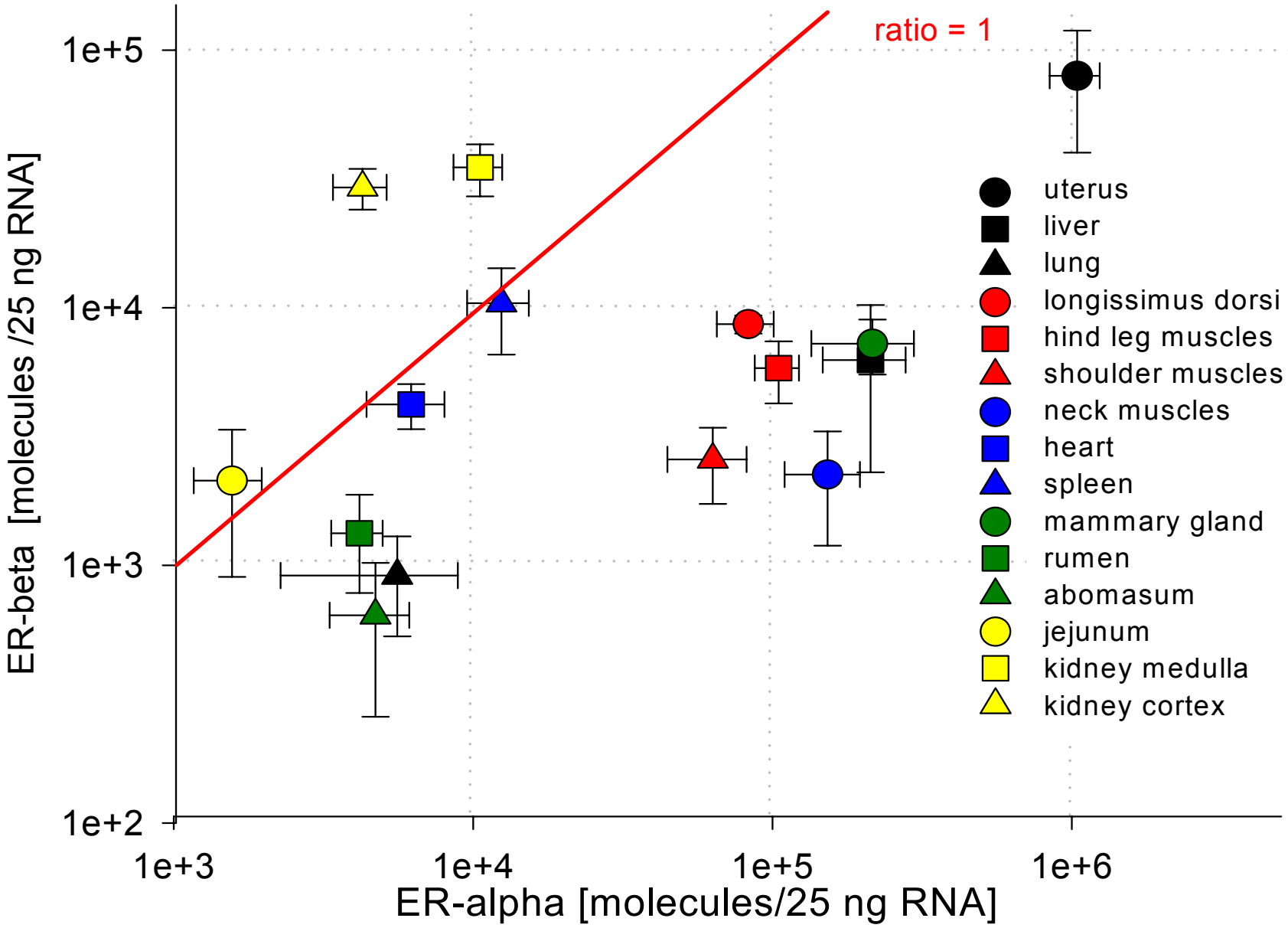
ER-alpha inter-assay variation CV = 28.6% (n = 7)



Validation: absolute quantification of steroid receptors

	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)	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	31.2% (n = 3)	18.7% (n = 4)	17.6% (n = 4)	5.7% (n = 4)
inter-assay variation	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

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**
- **relative to the mean expression of a target gene**
- **relative to an universal calibration curve**
- **relative to the expression of one constant expressed HKG**

  GAPDH, tubulins, various actins, albumins, cyclophilin, micro-globulins, histone subunits, ribosomal units (18S or 28S rRNA), ……..etc.

- **relative to a HKG Index containing more HKGs (> 3)**

  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)

- **etc. ???**

Normalisation strategies

According to known amounts of extracted RNA

(RIN quality check, molecules/ng RNA; ag transcript/ng RNA)

According to mass or volume of extracted tissue

(molecules/mg tissue; ag transcript/mg tissue; transcript/cells)

According to one known and NOT regulated HKG

GAPDH, tubulins, actins, albumins, cyclophilin, micro-globulins, histone subunits, ribosomal units (18S or 28S rRNA),.....

According to a HKG Index containing more HKGs (> 3)

geNorm (Vandesompele et al. 2002, Genome Biology)

Accurate normalization of real-time quantitative RT-PCR data by geometric veraging of multiple internal control genes.

BestKeeper (Pfaffl et al. 2004; Biotechnology Letters 2004)

Determination of most stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper Excel based tool using pair-wise correlations.

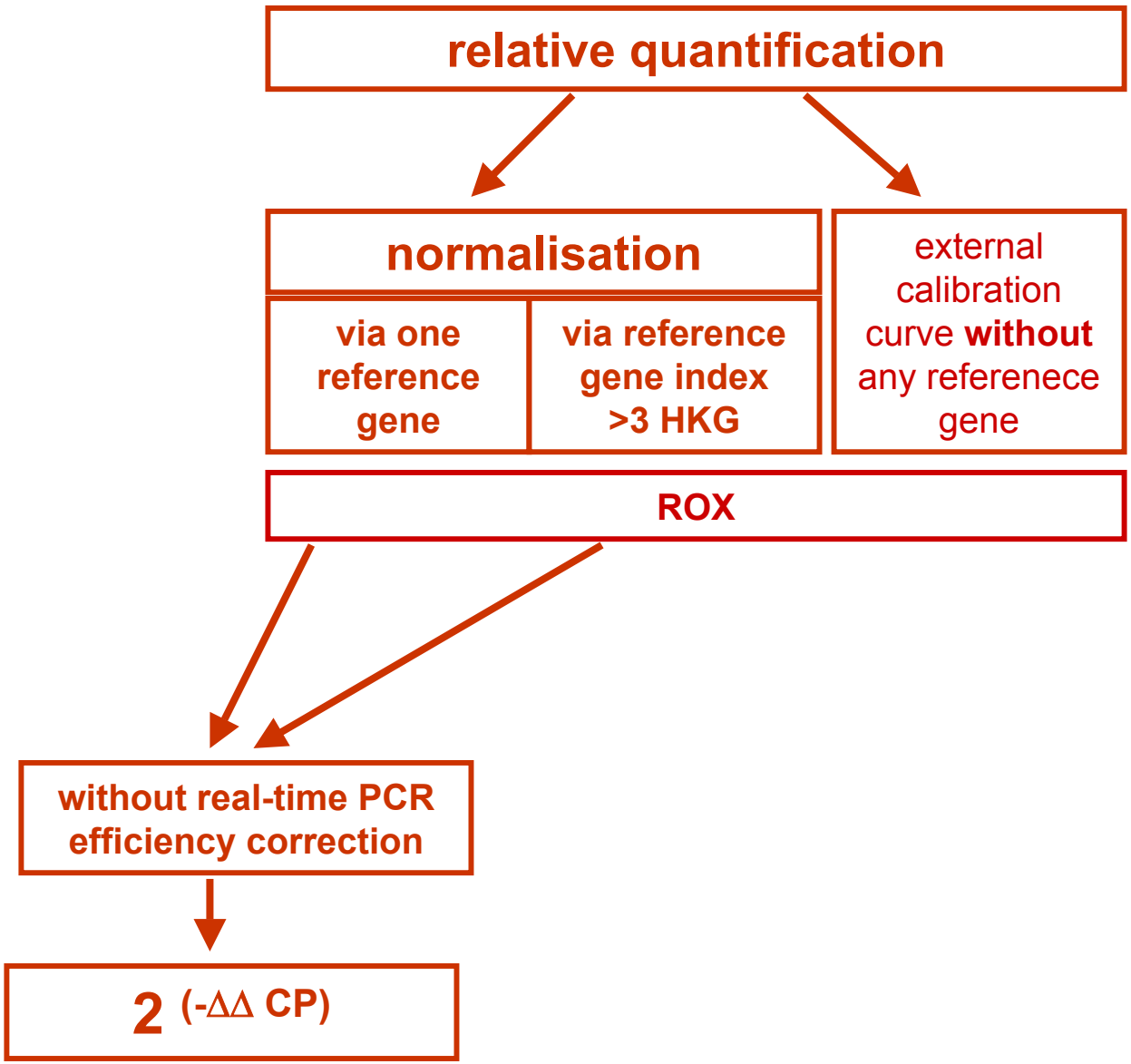
Normfinder (Andersen et al., 2004 Genome Biology)

Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization.

Statistical modeling for selecting housekeeper genes

(Szabo et al. 2004, Genome Biology)

Relative Quantification in real time qRT-PCR



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

presented by PE Applied Biosystems (Perkin Elmer, Forster City, CA, USA)

ABI Prism 7700 Sequence detection System User Bulletin #2 (2001)

Relative quantification of gene expression.
http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf

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

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

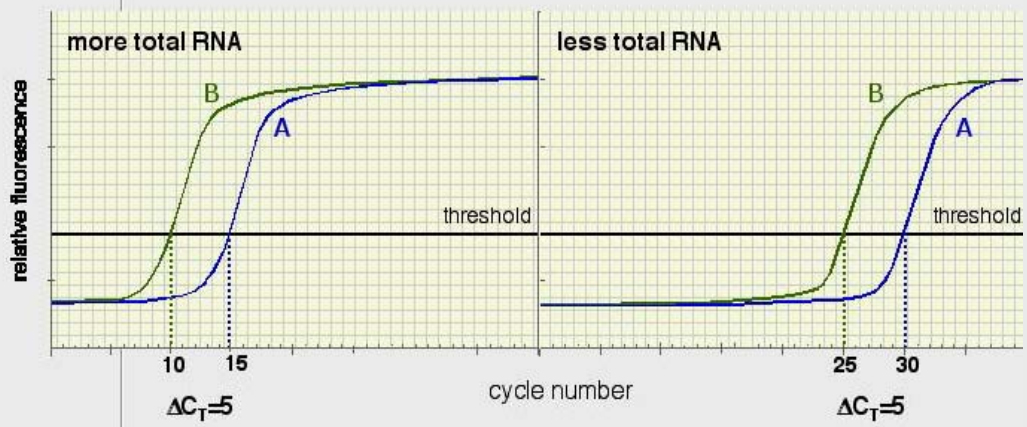
Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 $^{(-\ delta\ deltaC(T))}$ method.
Methods. 2001 ***25(4):*** *402-408.*

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

presented by ABI (Applied Biosystems Inc.)

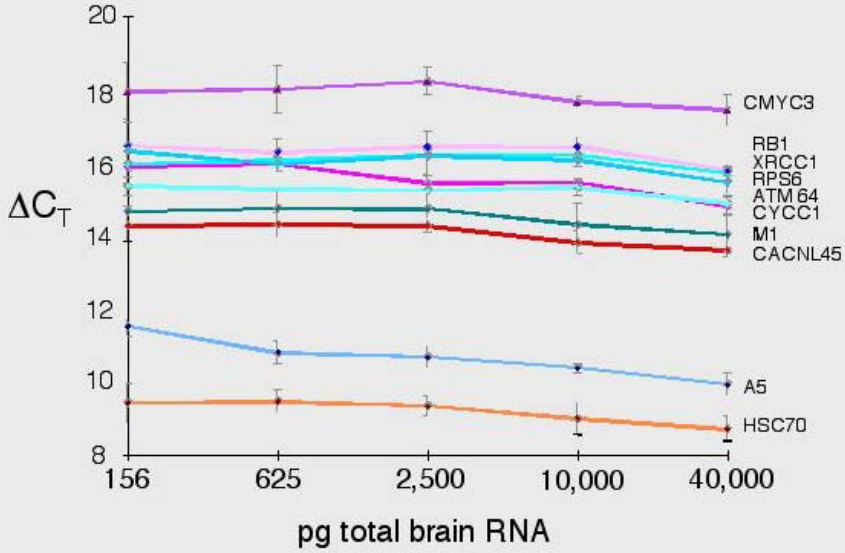
assumptions: E = 2 & ΔCP is constant over a wide range

ΔC_T is a constant value



When the PCR efficiency of both systems is the same the ΔC_T remains constant

ΔC_Ts for 10 systems



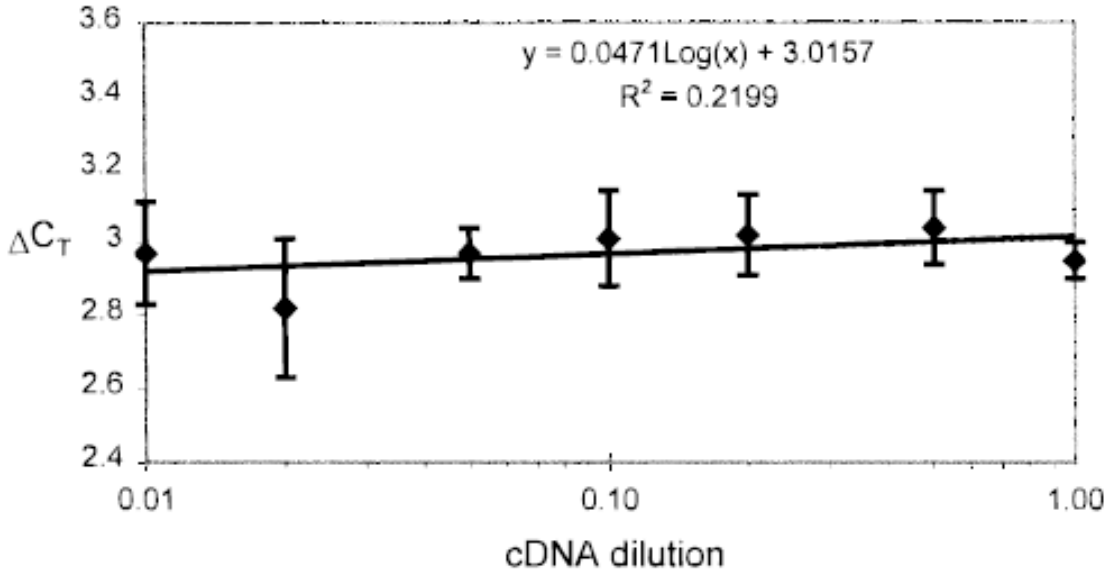


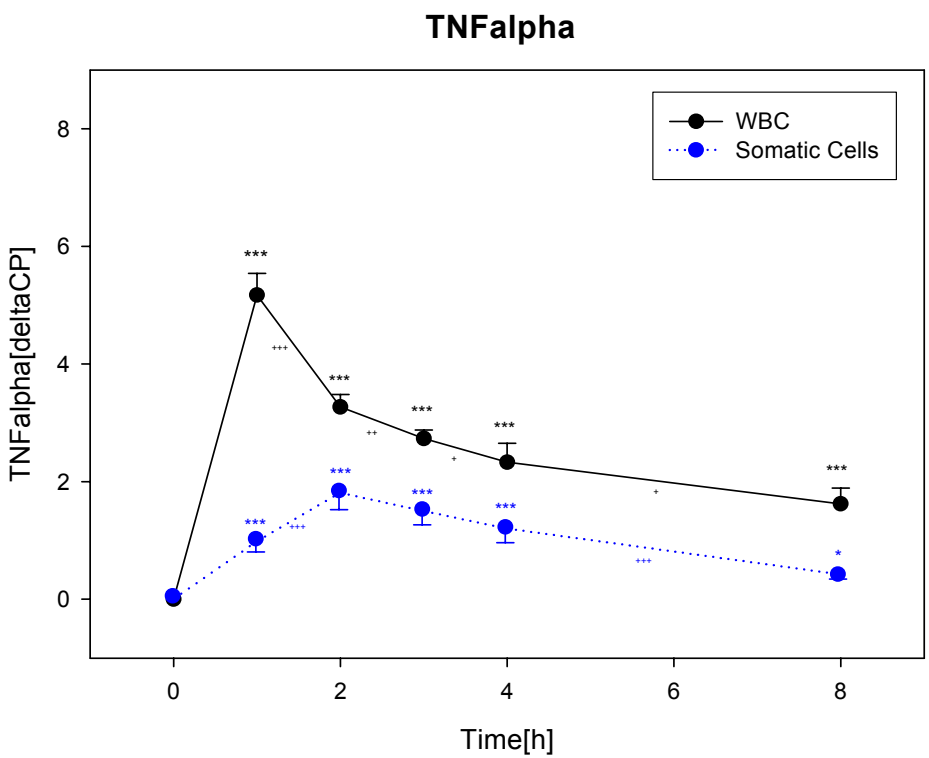
FIG. 1. Validation of the $2^{-\Delta\Delta CT}$ method: Amplification of cDNA synthesized from different amounts of RNA. The efficiency of amplification of the target gene (c-*myc*) and internal control (GAPDH) was examined using real-time PCR and TaqMan detection. Using reverse transcriptase, cDNA was synthesized from 1 μg total RNA isolated from human Raji cells. Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers. The most concentrated sample contained cDNA derived from 1 ng of total RNA. The ΔC_T ($C_{T,c-myc} - C_{T,GAPDH}$) was calculated for each cDNA dilution. The data were fit using least-squares linear regression analysis ($N = 3$).

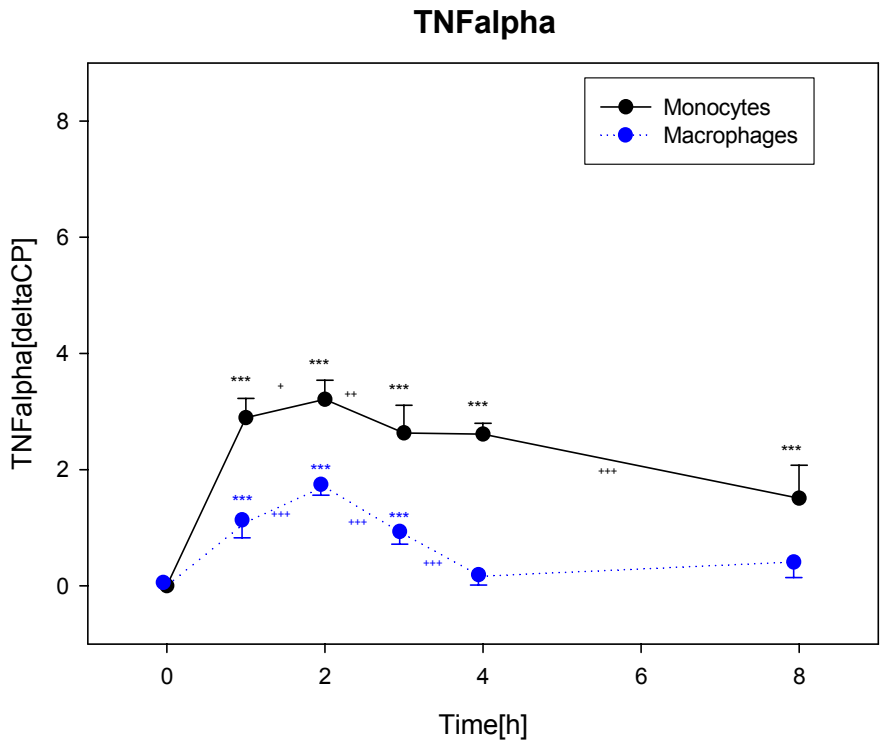
Fig. 1. Relative quantificatio

expression relative to the time point zero & normalised by a HKG:

TNFα mRNA expression in cultured leukocytes after LPS stimulation:
white blood cells [WBC] vs. somatic milk cells [SMC]
isolated blood monocytes vs. isolated milk macrophages

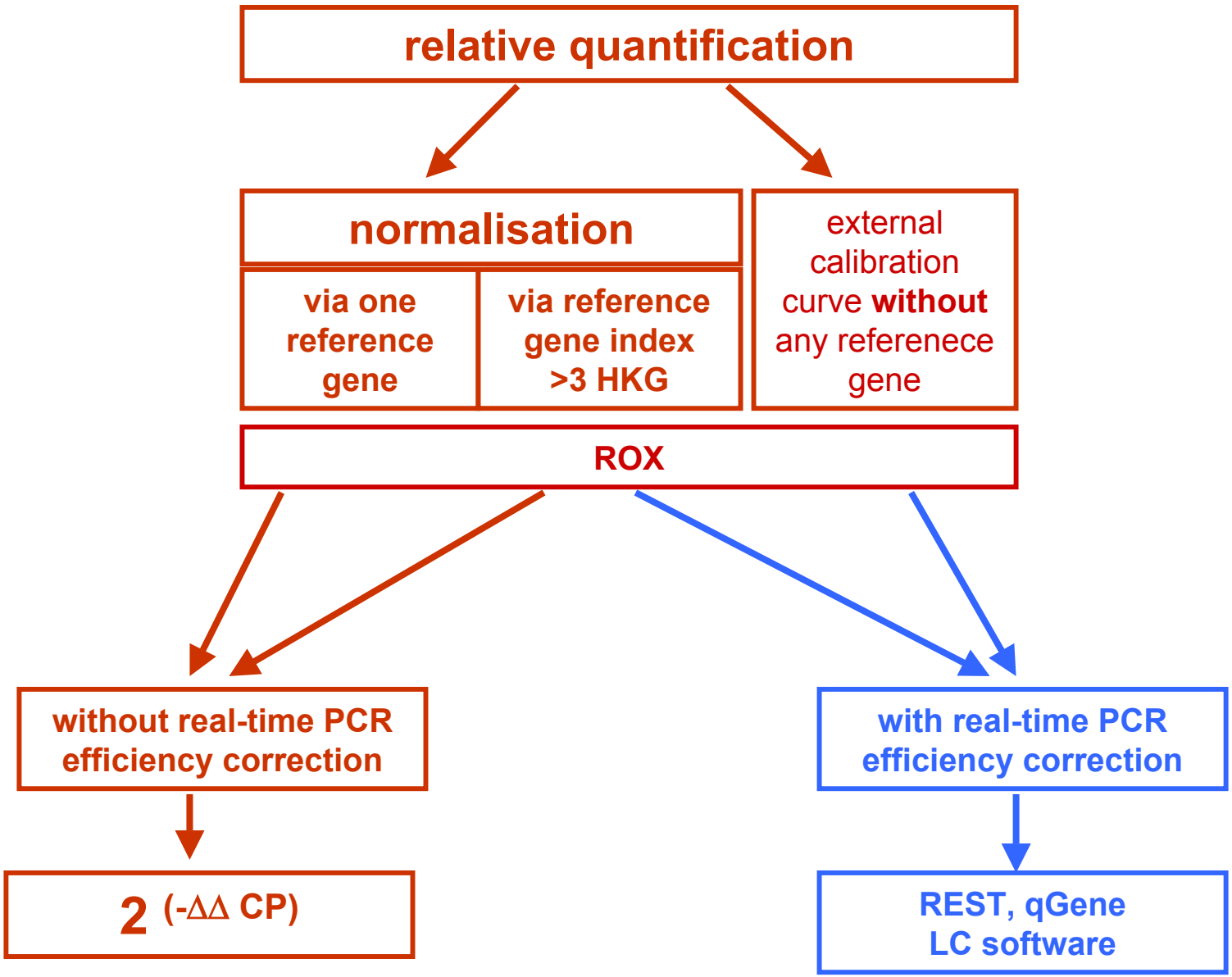
PCR Efficiency = 2 ***n = 6*** ***mean ± sem***





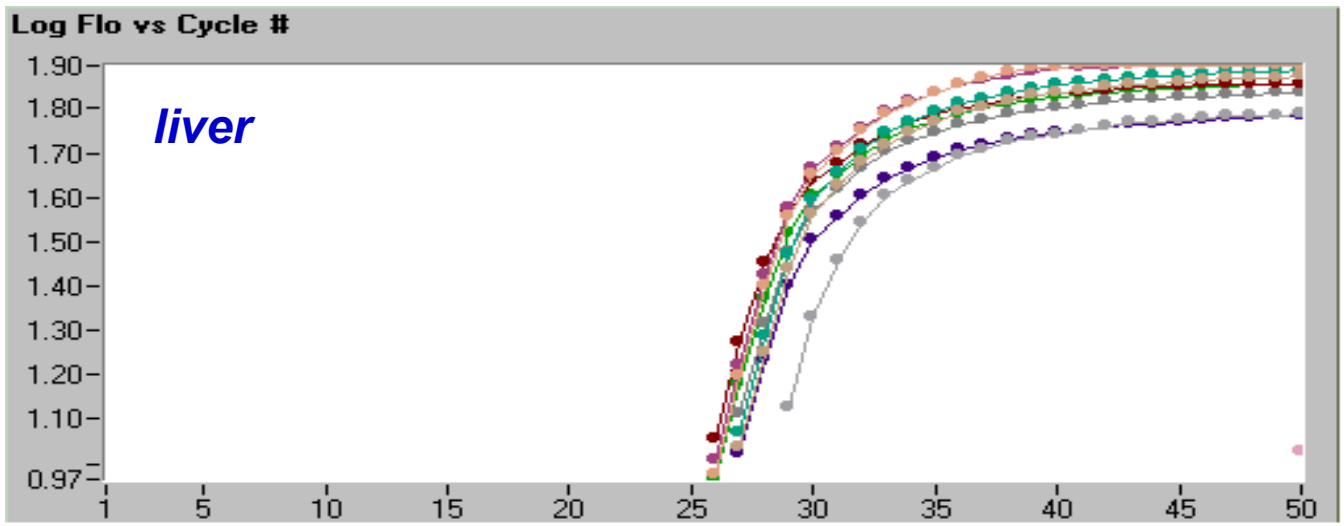
Prgomet et al., 2004

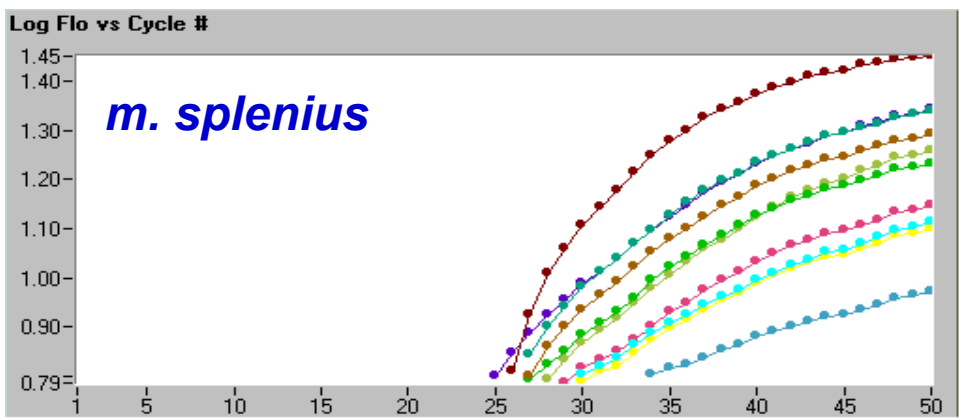
Relative Quantification in real time qRT-PCR

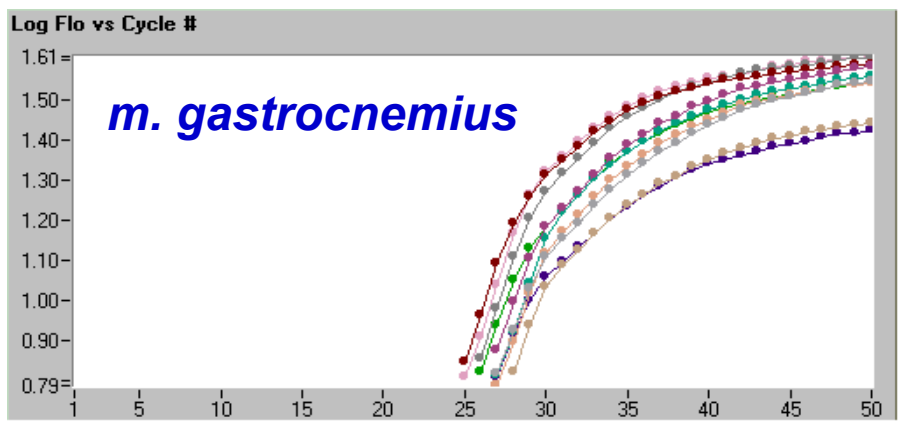


Tissue *"background"* interfere with real-time PCR efficiency and amplification fidelity

IGF-1 mRNA amplification in three cattle tissues







Efficiency variation in real-time RT-PCR

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

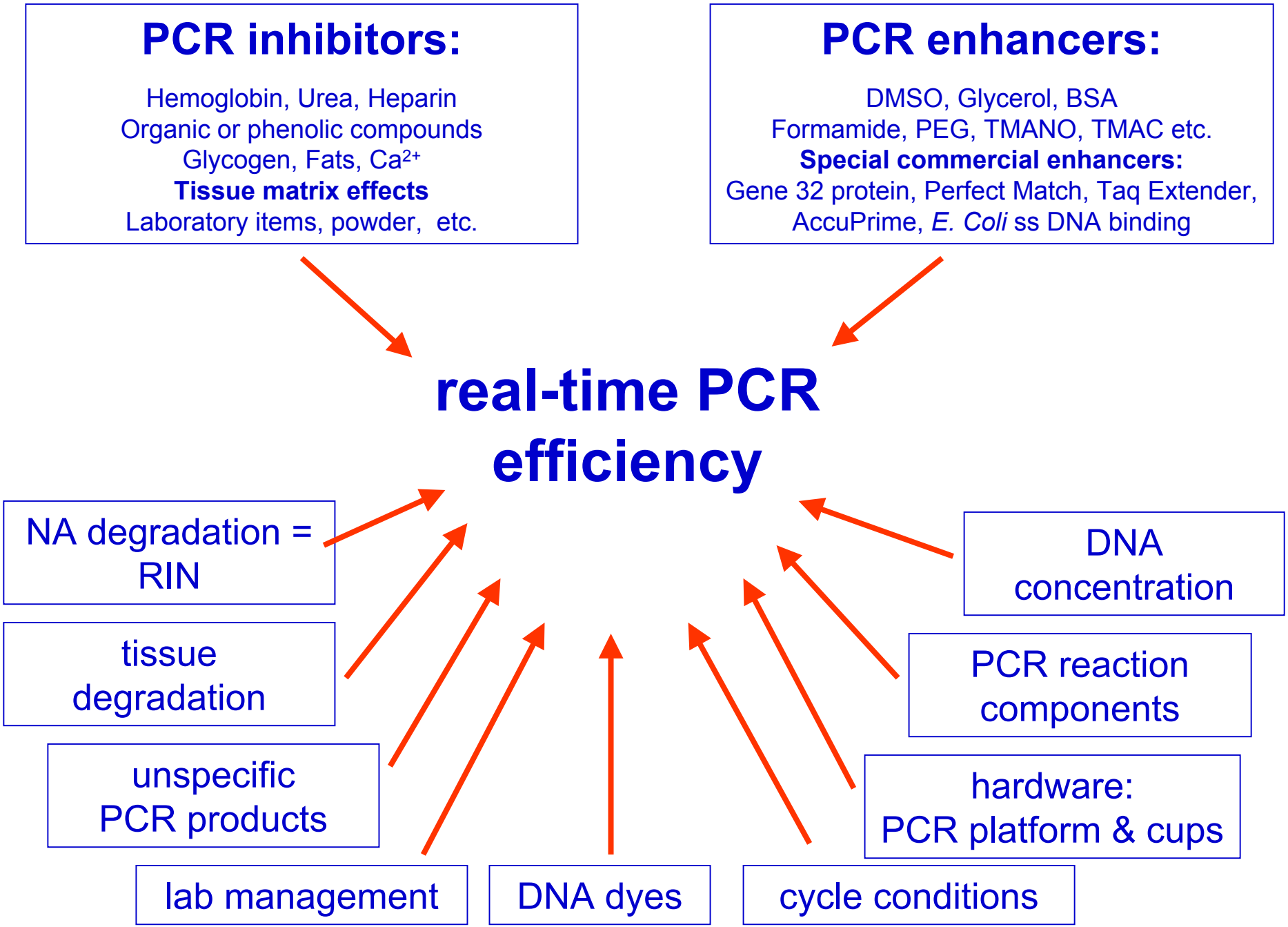
Table 1: Errors of quantification relative to a theoretical PCR efficiency of 2.00

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

Example:

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

Roche Diagnostics, LC rel. Quantification software, March 2001


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
NA degradation = RIN
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 a reference gene *(housekeeping gene)*

Single data (n = 1) e.g. array results:

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

Pfaffl, Nucleic Acids Research 2001

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

Roche Diagnostics, LC relative Quantification software, March 2001

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

single data (n = 1) e.g. array results:

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

Pfaffl, Nucleic Acids Research 2001

multiple data (1 < n < 100) e.g. experimental groups via REST-XL©:

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

Pfaffl et al., Nucleic Acids Research 2002

Microsoft Excel - rest-384 beta 14 feb 2005.xls

Datei Bearbeiten Ansicht Einfügen Format Extras Daten Fenster ? Acrobat

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	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
Direct support	http://rest.gene-qu	

name of the g

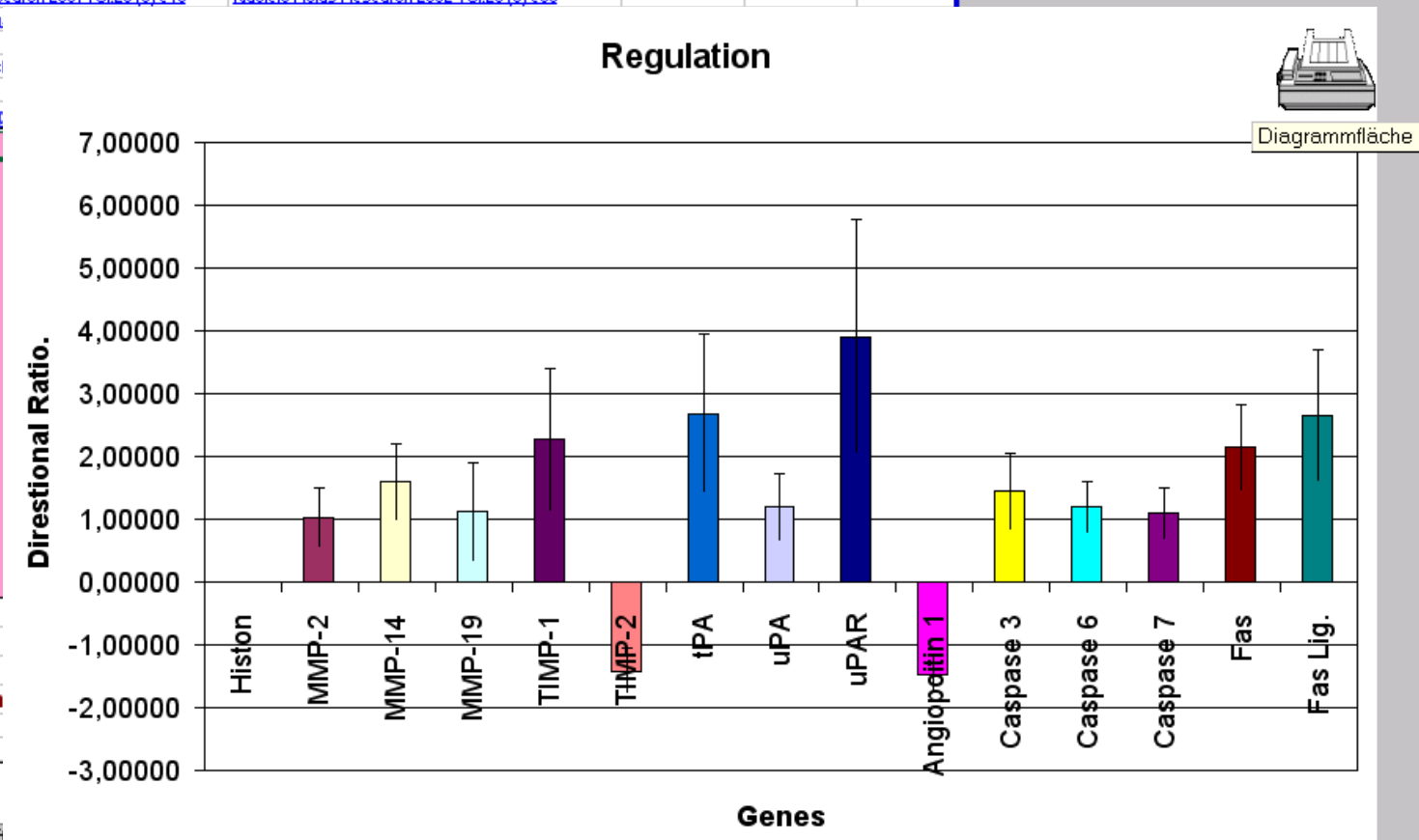
reference gene	
[target gene 1]	
[target gene 2]	
[target gene 3]	
[target gene 4]	
[target gene 5]	
[target gene 6]	
[target gene 7]	
[target gene 8]	
[target gene 9]	
[target gene 10]	
[target gene 11]	
[target gene 12]	
[target gene 13]	
[target gene 14]	
[target gene 15]	

reference gene = red

target gene(s) = blue

general header and settings = da

* Mark gene to use it for calucation of



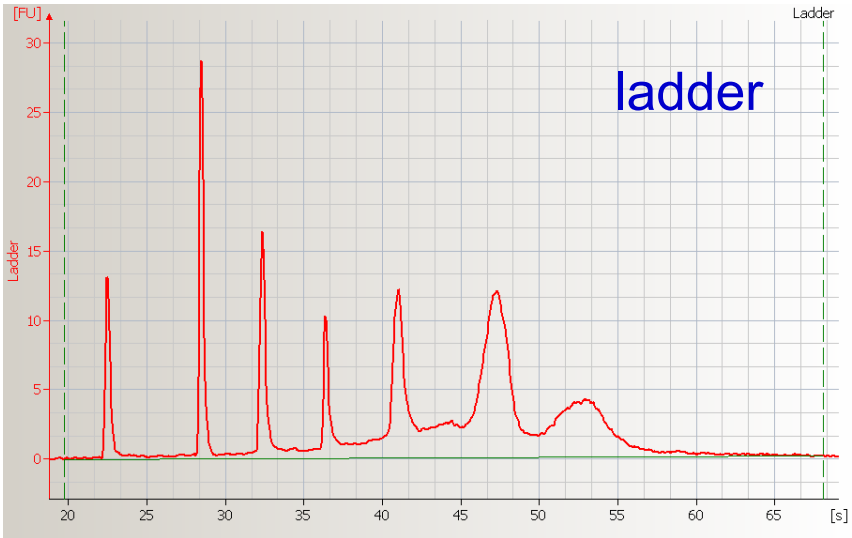
Introduction | PCR effi... | ... | ...

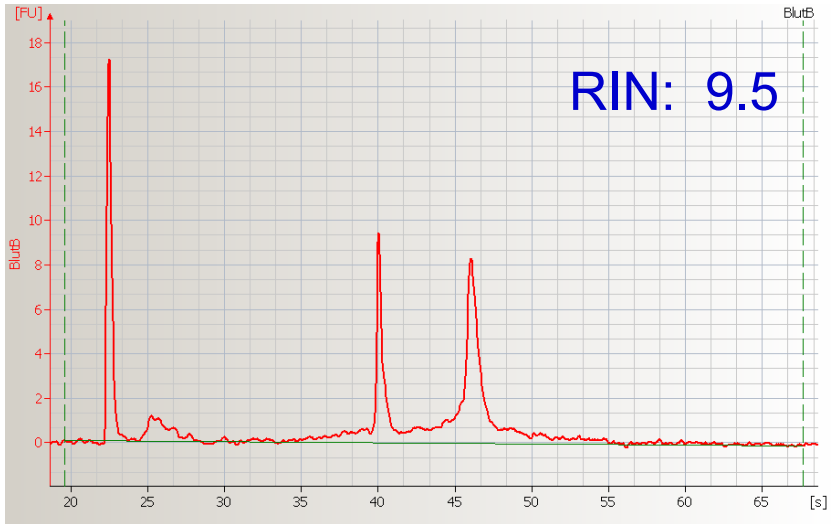
Bereit

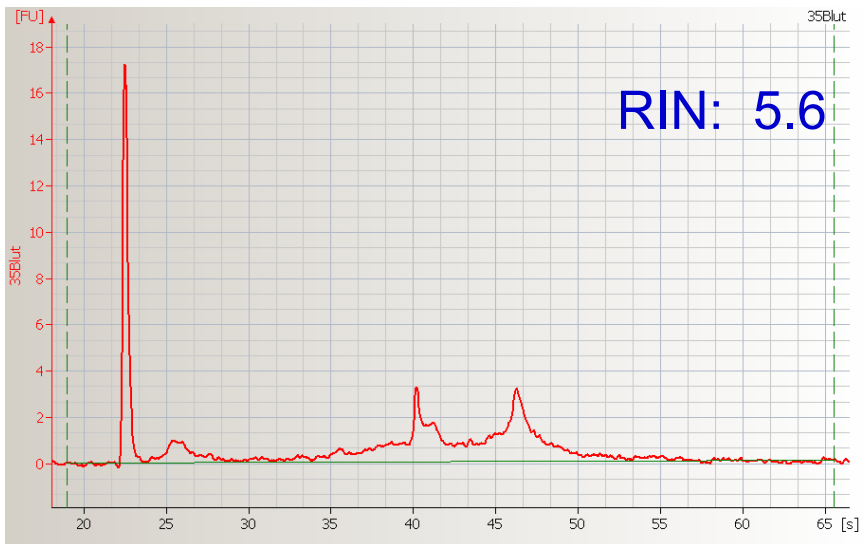
Start 15:33

Influence of total RNA quality, quantity and purity on qRT-PCR results

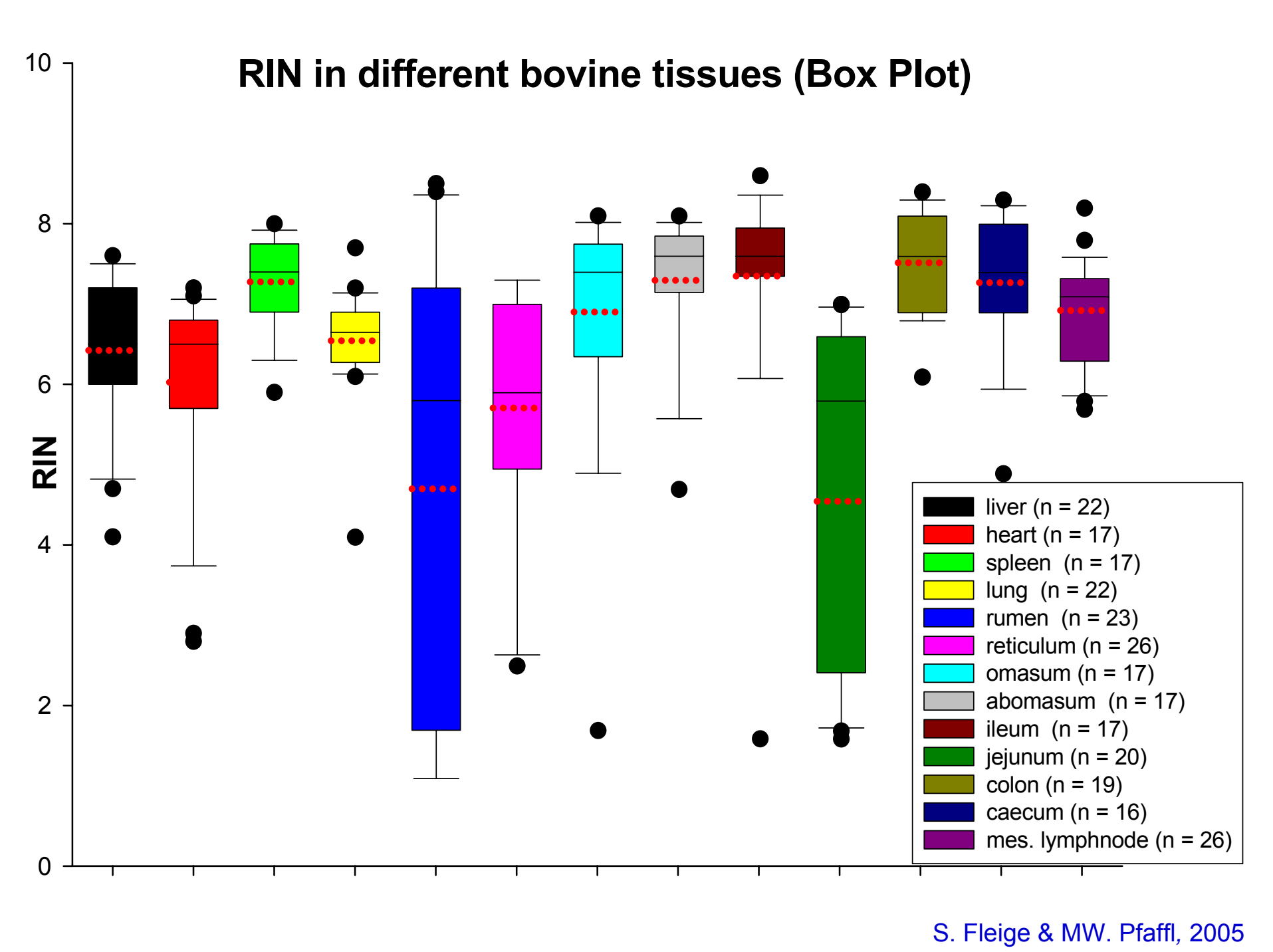
total RNA extracted bovine WBC analyzed in Bioanalyzer 2100



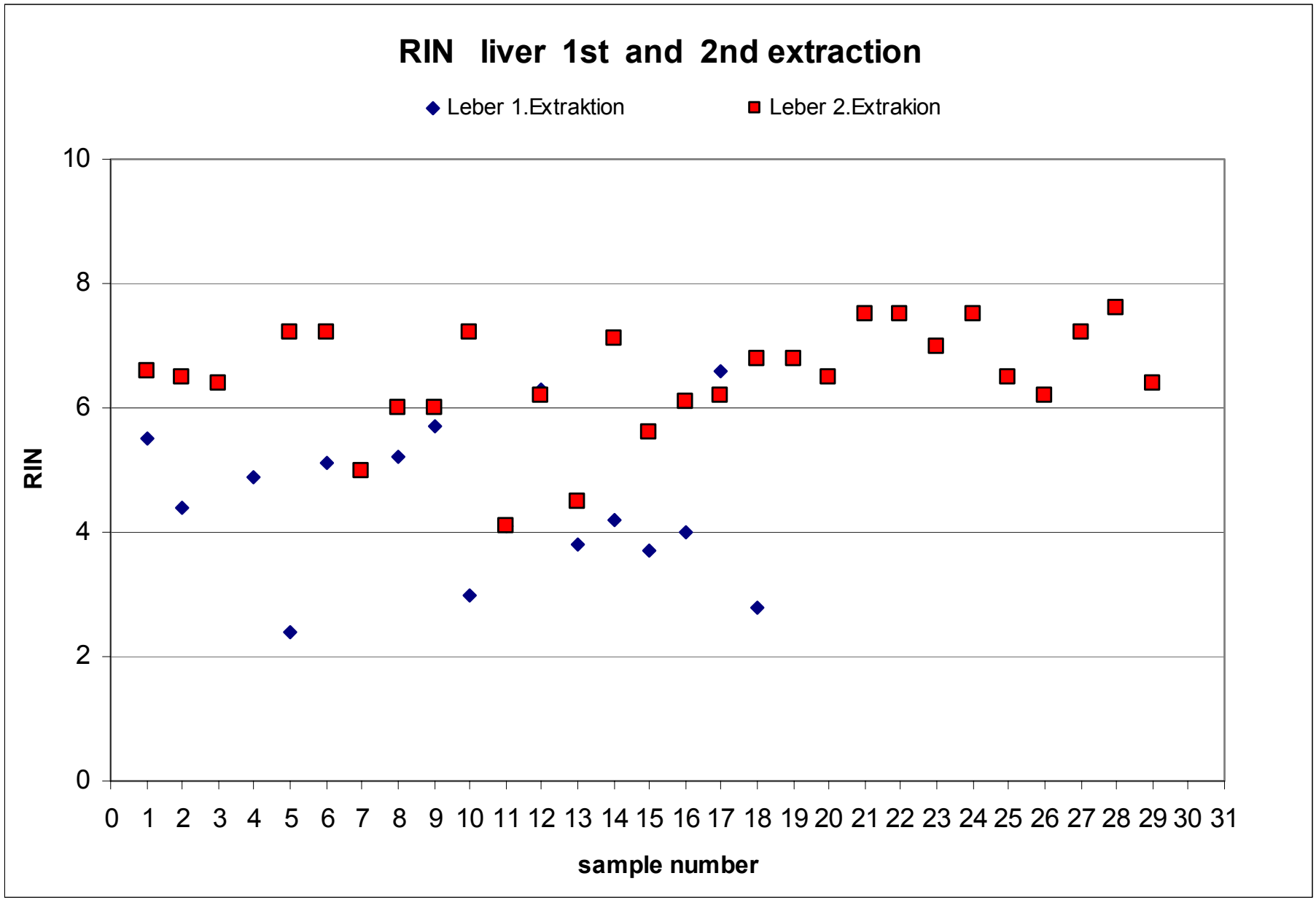




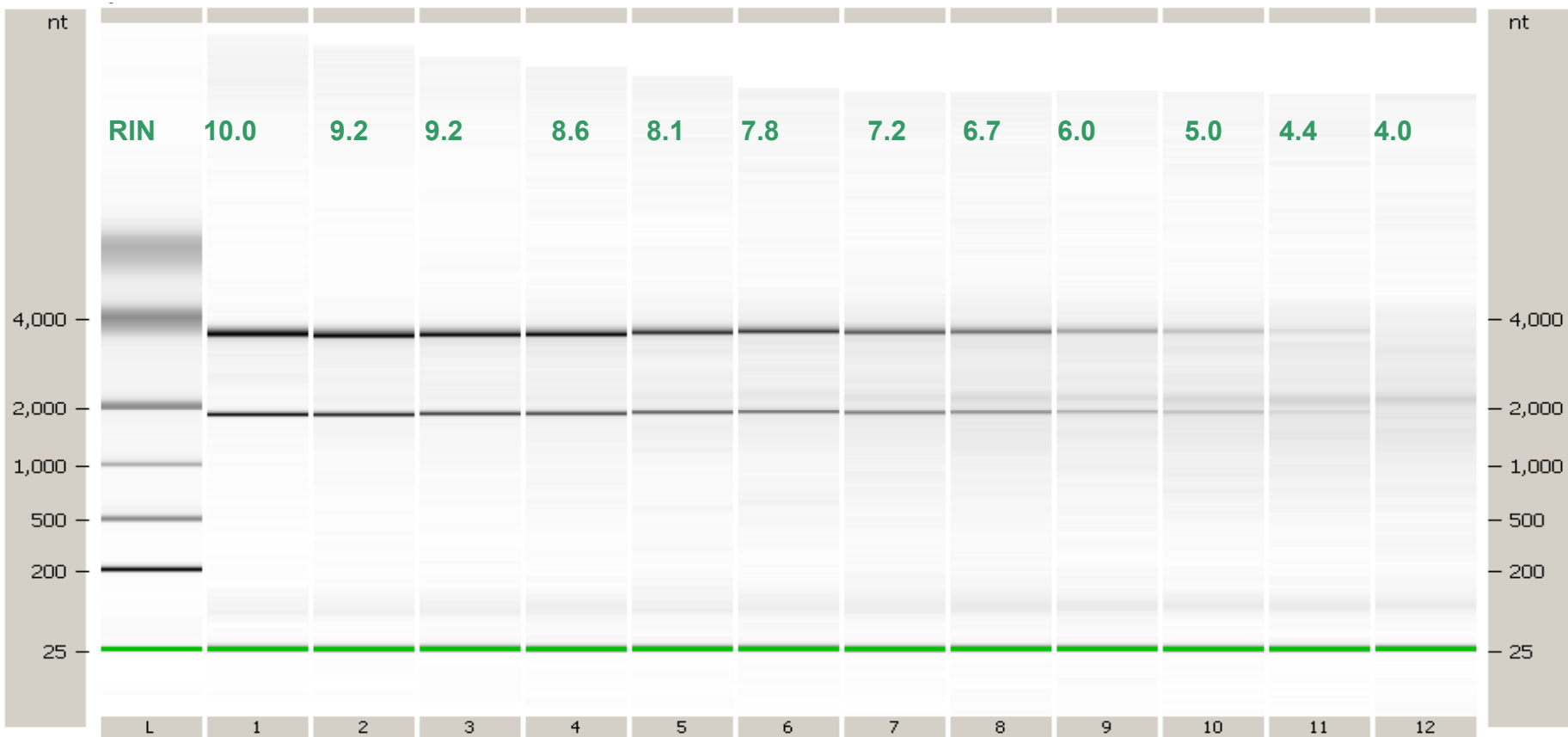
V. Walf, S. Huch, MW. Pfaffl, 2005

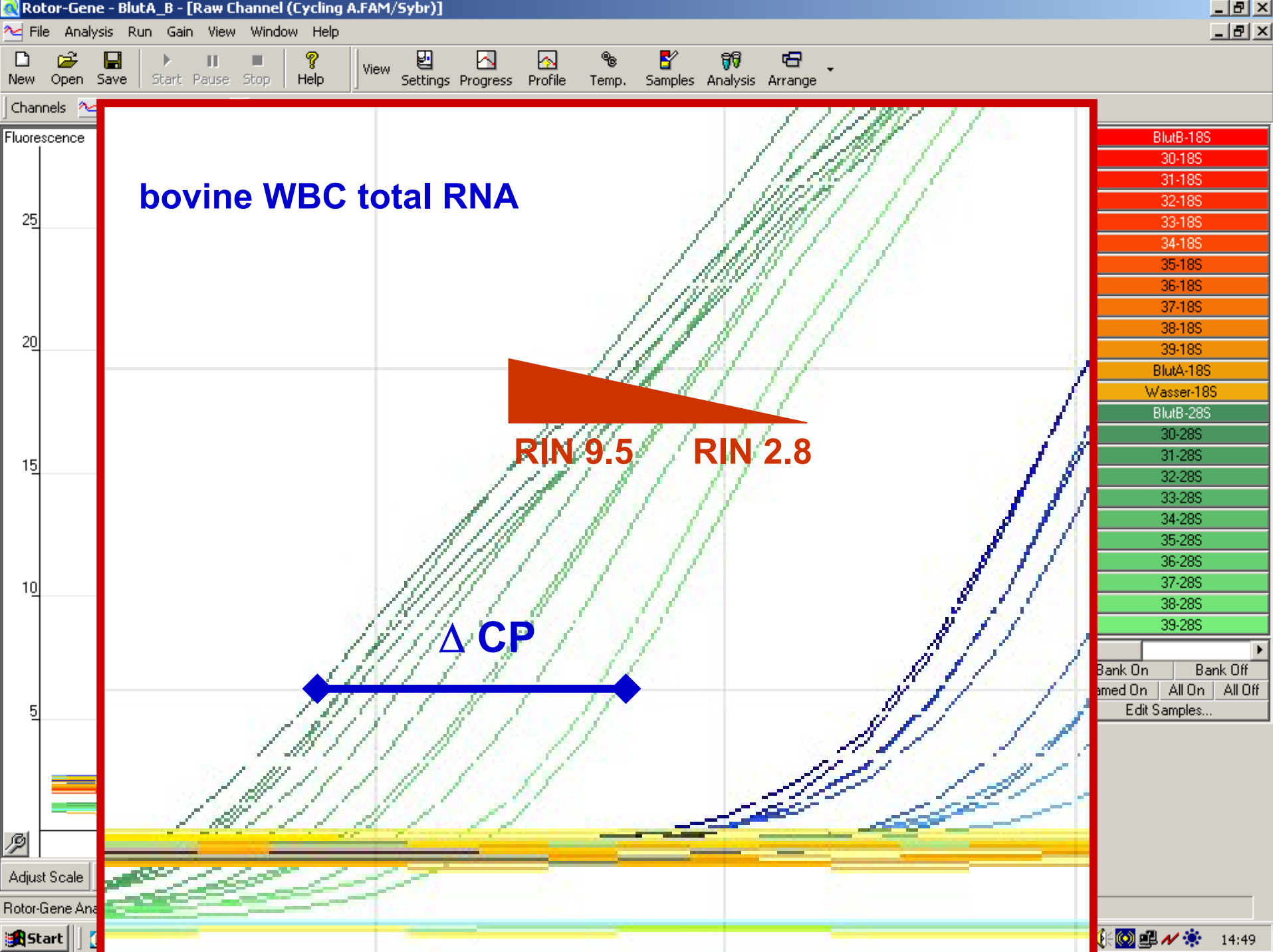


S. Fleige & MW. Pfaffl, 2005


RIN liver 1st and 2nd extraction
Leber 1.Extraktion
Leber 2.Extrakion
RIN
sample number
0
2
4
6
8
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31

Enzymatic or UV degradation of tissue extracted total RNA




Rotor-Gene - BlutA_B - [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
Fluorescence
25
20
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BlutB-18S
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31-18S
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Wasser-18S
BlutB-28S
30-28S
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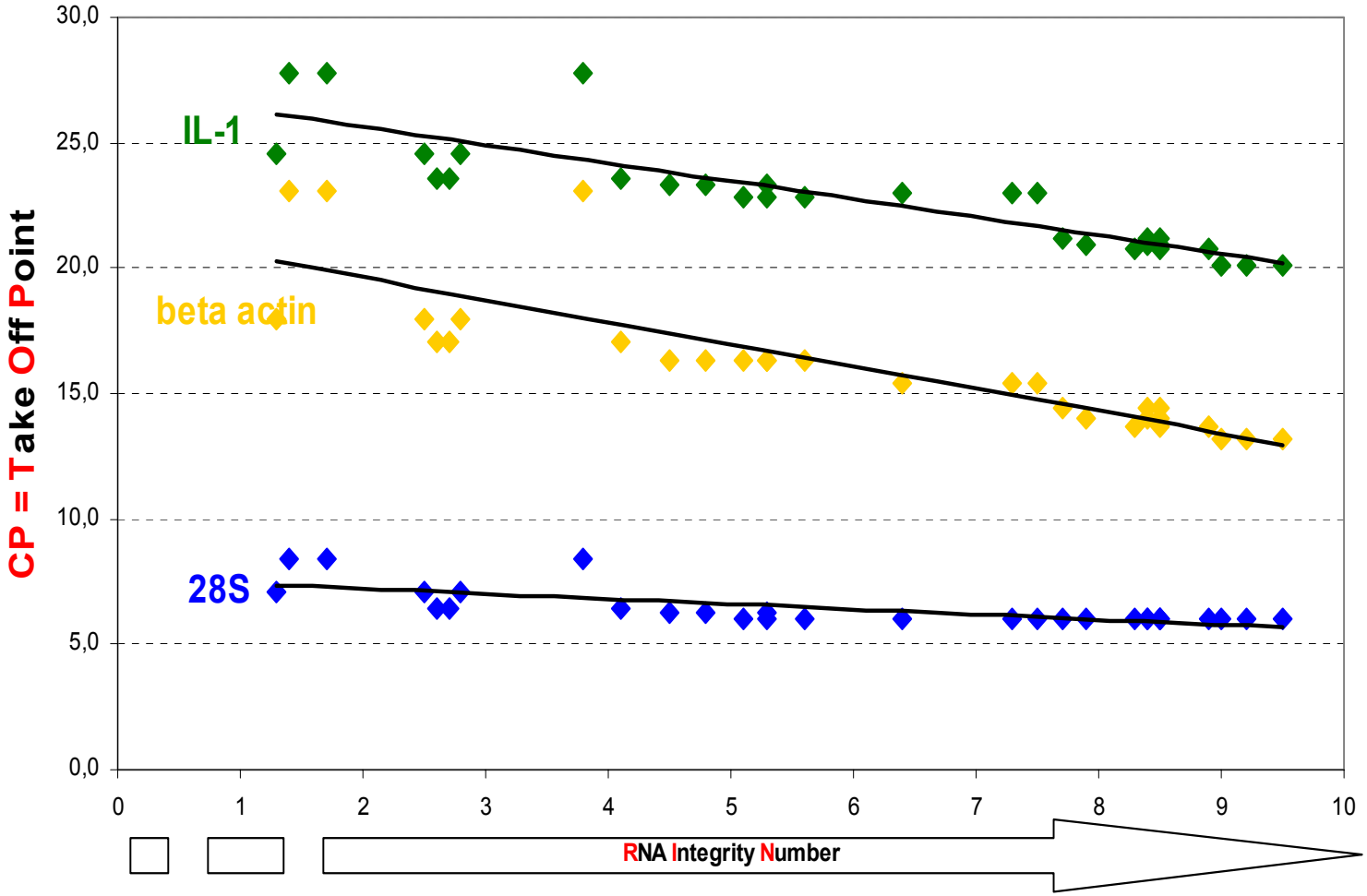
33-28S
34-28S
35-28S
36-28S
37-28S
38-28S
39-28S
Bank On
Bank Off
All On
All Off
Edit Samples...
Adjust Scale
Rotor-Gene Ana
Start
14:49
bovine WBC total RNA
RIN 9.5
RIN 2.8
Δ CP

Influence of total RNA quality on qRT-PCR results



$y = -0{,}7211x + 27{,}077$

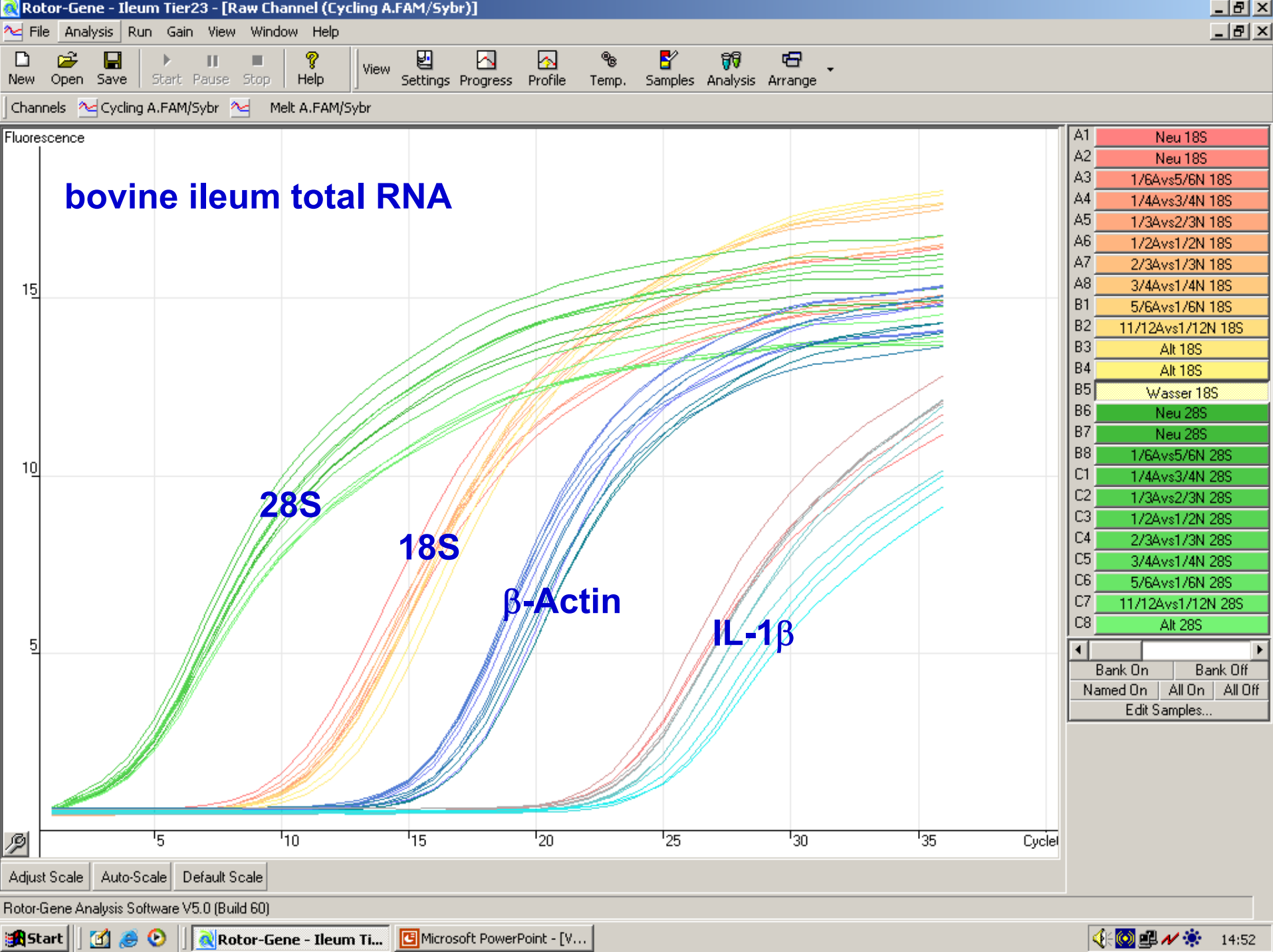
$R^2 = 0{,}7682$

$y = -0{,}887x + 21{,}398$

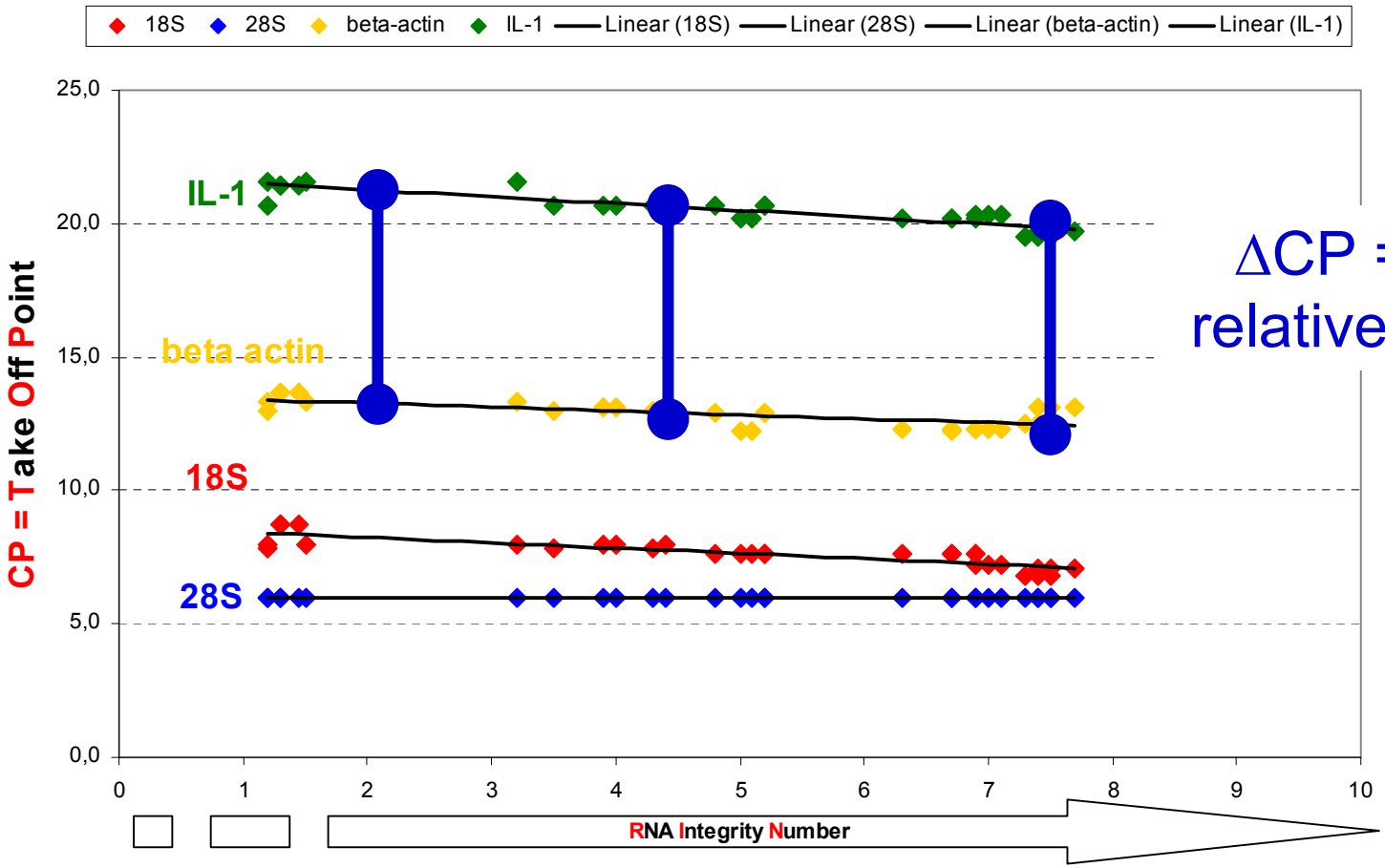
$R^2 = 0{,}7165$

$y = -0{,}206x + 7{,}6396$

$R^2 = 0{,}5363$


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

bovine ileum: CP = TOP



ΔCP = constant in relative quantification

y = -0,2563x + 21,787

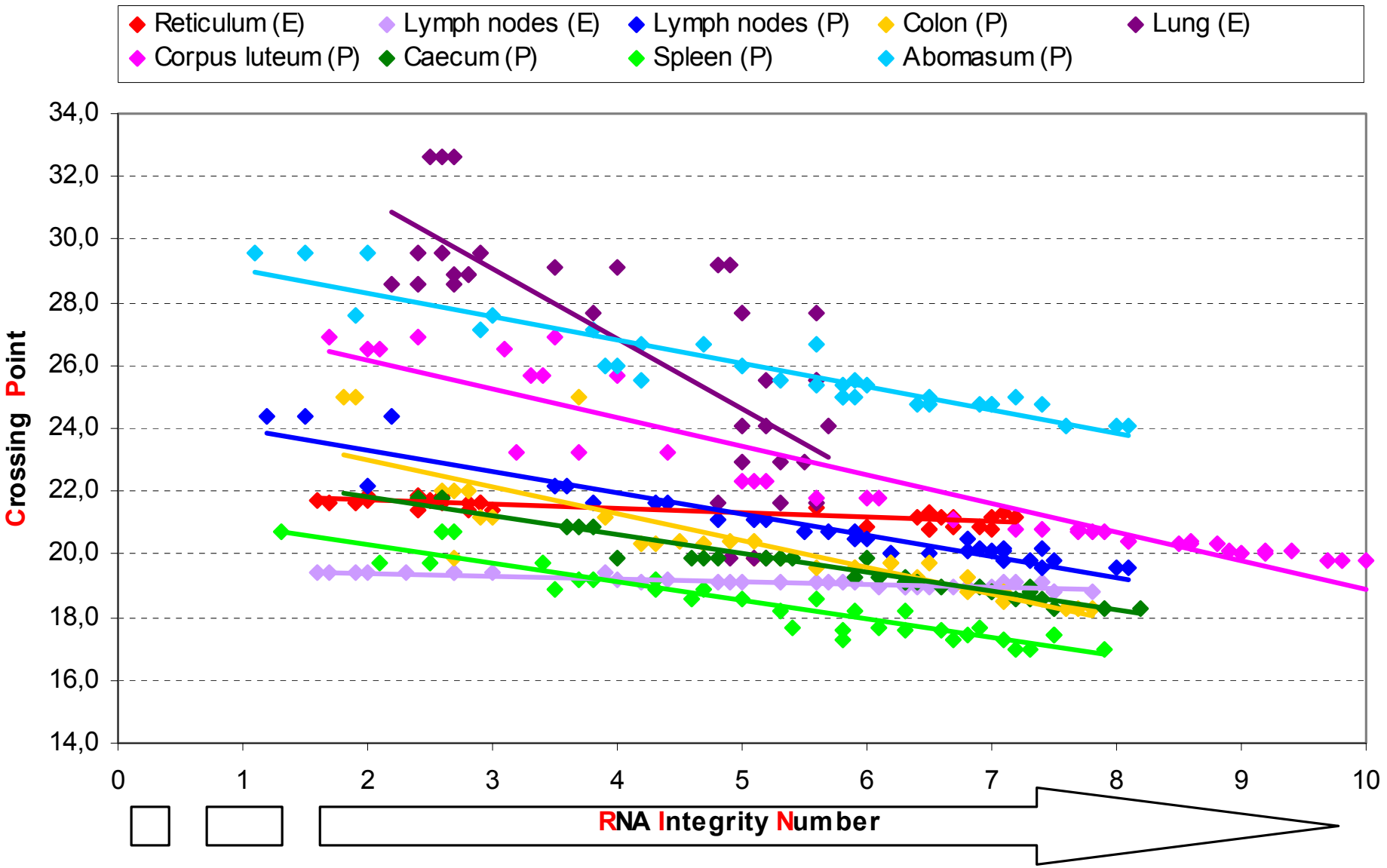
y = -0,1461x + 13,565
R^2 = 0,4903

y = -0,2036x + 8,6557
R^2 = 0,7612

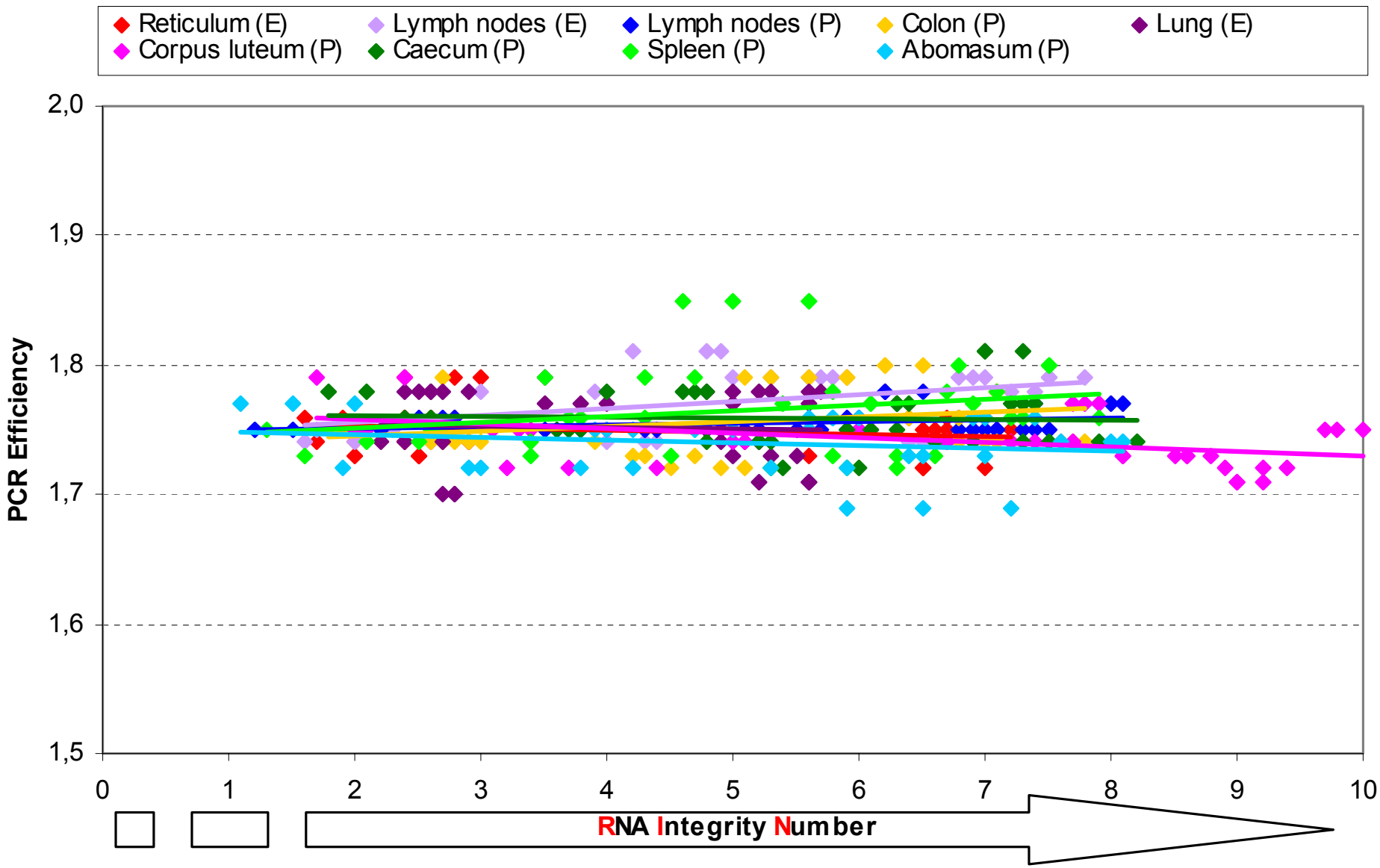
y = -2E-15x + 6,00
R^2 = 1,0

28S	n.s.
Interleukin-1beta	P < 0.001
beta-Actin	P < 0.01
18S	P < 0.001

Influence of total RNA quality on qRT-PCR **CP (Ct)**



Influence of total RNA quality on qRT-PCR **efficiency**



Relative quantification using efficiency corrected calculation

Advantages:

- each sample derived from identical total RNA (one-step quantification) or cDNA source (two-step quantification) = **identical inhibitors and enhancers**
- **tissue specific matrix effects** are given, but not existent in relative quantification
- total **RNA quantity**, **RT efficiency** and **cDNA quantity** are minor relevant
- effect of total **RNA quality** and **influence on qRT-PCR** have to be tested in detail
- optimized assays are **highly reproducibility** (variations only given by researcher, kit, robot and real-time platform)
- each sample will be **normalised with HKG** or better by a **HKG INDEX**
- **no production of a calibration curve:** e.g. cloning, linearization or purification of calibration curve material
- **no data conversion:** molecules, concentrations or molarities
- **=> direct procedure:** measured data were inserted in mathematical model
- **=> relative quantification** by software applications, *e.g. qGene, REST, LightCycler*
- **=> automatic calculation** => bio-informatics session

Disadvantages:

- **No information about the effective molecule concentration** present in the tissues (molecules/mg tissue; molecules/ng RNA; molecules /cell, etc.)

Thank you team !

Thank you for your attention !