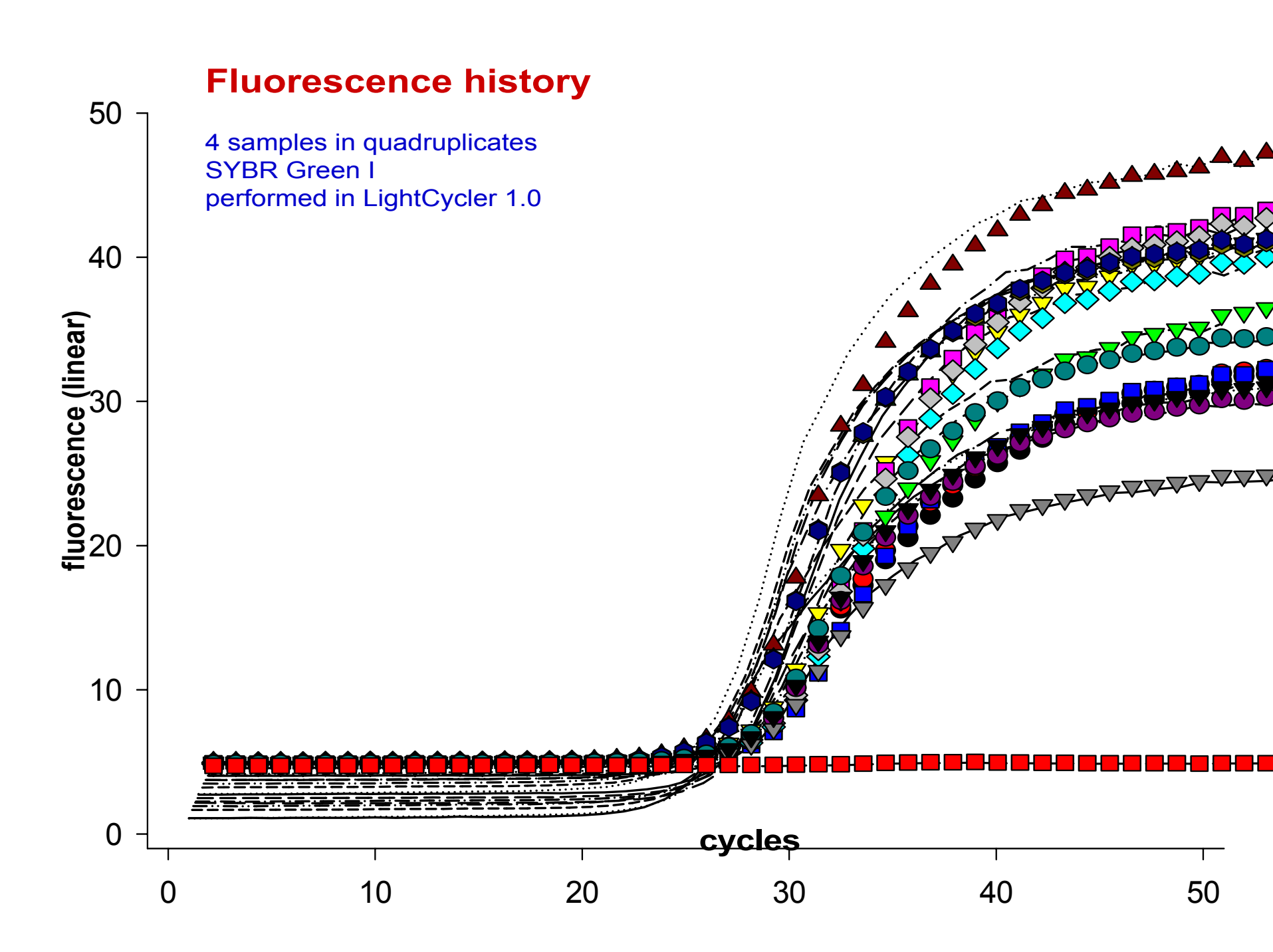
qPCR 2005 5 – 9th September 2005
Symposium & Exhibition & Workshop
2nd international qPCR Event, Technische Universität München,
Freising-Weihenstephan, Germany

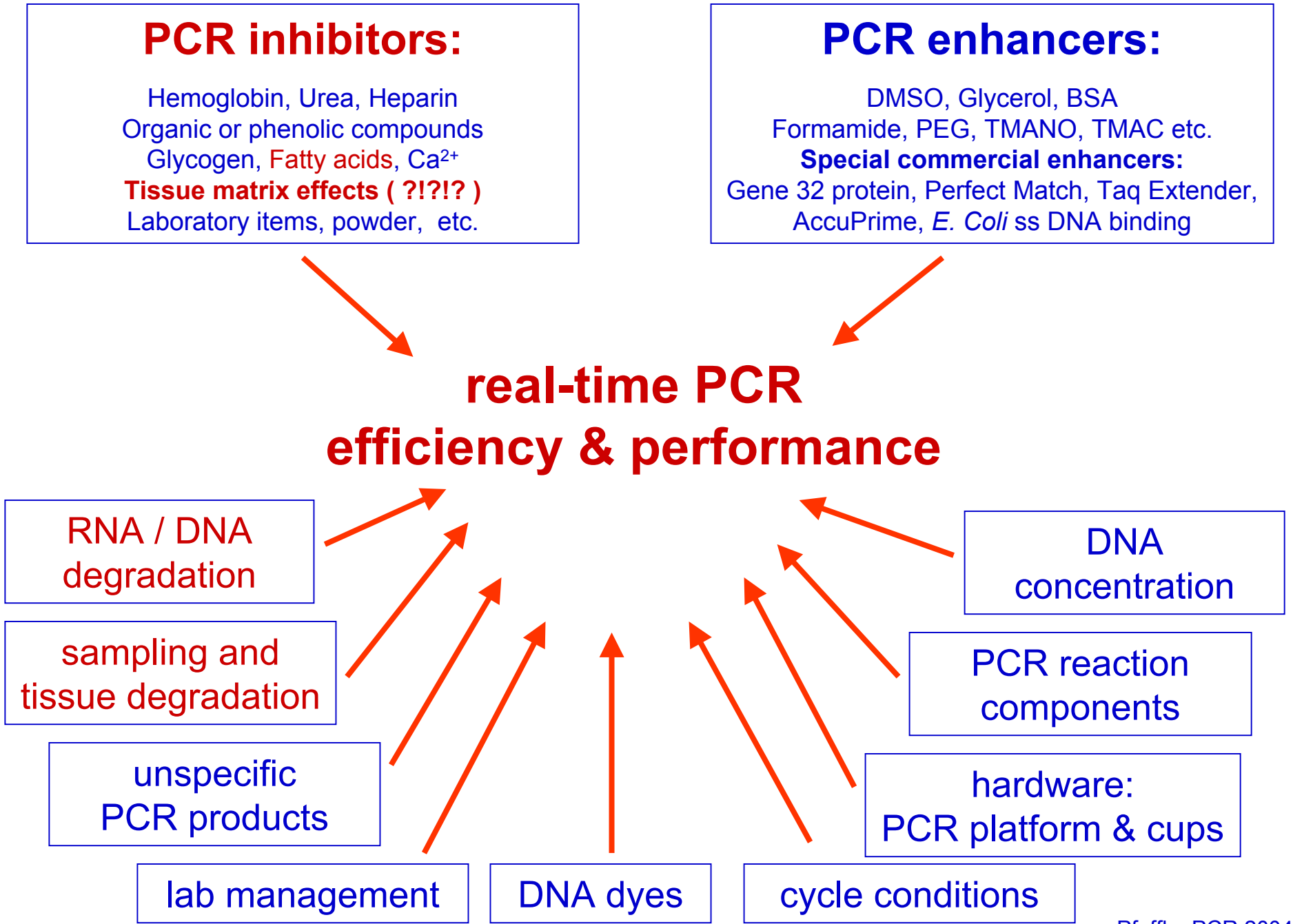
Influence of RNA matrix effect on qRT-PCR results - an overview

Michael W. Pfaffl & Simone Fleige

qPCR 2005, Freising-Weihenstephan, 5 – 7th September 2005

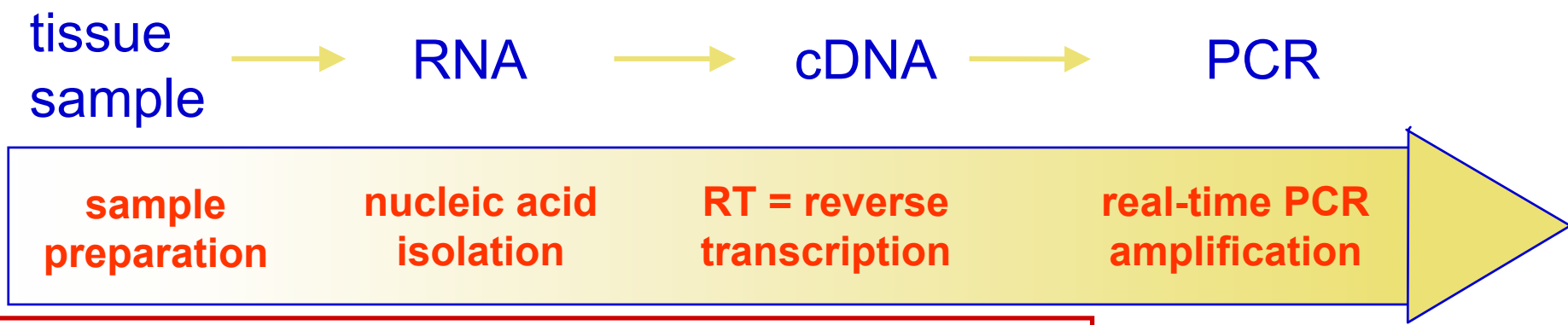
Michael W. Pfaffl
Physiology - Weihenstephan
Center of Life and Food Sciences
Technical University of Munich Weihenstephaner Berg 3
85350 Freising-Weihenstephan; Germany
michael.pfaffl@wzw.tum.de
www.gene-quantification.info


Fluorescence history
4 samples in quadruplicates
SYBR Green I
performed in LightCycler 1.0
fluorescence (linear)
cycles
0
10
20
30
40
50



Pfaffl, qPCR 2004

Steps and variables of a successful mRNA quantification using real-time RT-PCR (1)



Pre-Analytical steps:

- Sampling method:
  - Biopsy
  - Fixed material
  - Fresh blood
  - Tissue storage
  - Liquid Nitrogen
  - RNA Later
  - 1st extraction buffer

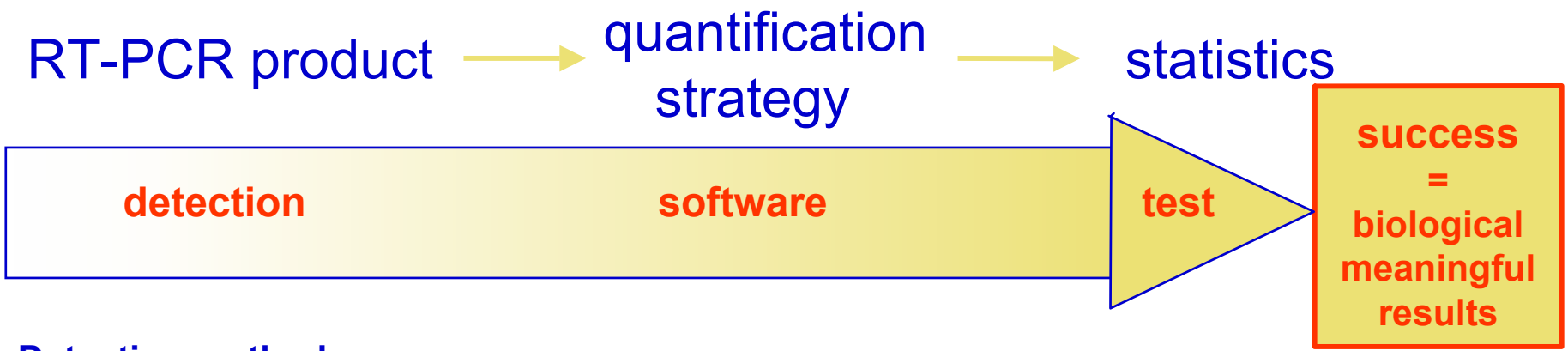
=> native RNA

- Extraction method:
  - total RNA vs. mRNA
  - liquid-liquid
  - columns
  - by Robot
  - **RNA integrity:**
    - Bioanalyzer, Experion
    - Nano Drop
    - various dyes
  - RNA storage –80°C

- Efficiency of RT:
  - RT enzyme type
  - RT temperature
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
  - **one-step RT-PCR**
  - **two-step RT-PCR**

- **Efficiency & Specificity of real-time PCR:**
  - Primer design
  - Primer specificity
    - multi-species Primer
  - mRNA abundance
  - cDNA input
  - Polymerase types & Mixtures
  - Robot vs. Technician

Steps and variables of a successful mRNA quantification using real-time RT-PCR (2)

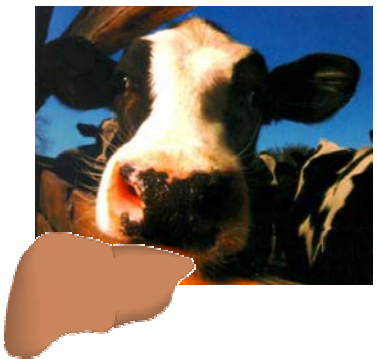


- **Detection method:**
- **SYBR Green I**
- **Probes:** Beacons, Scorpions etc.
- Fit point method
- TaqMan fitting (10x SD)
- 2nd derivative maximum
- other models (Log. or Sig.)
- raw data vs.background correction
- other curve manipulations
- 2-step, 3-step or 4-step PCR

- **Quantification strategy:**
- **"absolute" quantification**
  - which standard curve
  - normalization with HKG
- **relative quantification**
  - Total RNA, mass of tissue
  - normalization with HKG
  - normalization via an index of more (> 3) HKGs
  - geNorm, REST, BestKeeper, qBASE, Normfinder, etc.

- **BioInformatics:**
- **CP vs. molecules**
- **Normality of data (???)**
- t-Test (?)
- ANOVA (on the ranks ?)
- SAS, SPSS, Excel, Sigma Stat
- Permutation test
- Randomization test (REST)
- ……???

Determination the extraction efficiency



tissue sample:
Liver, Spleen,
Kidney, Caecum
Colon, Reticulum

Spike
with rec. RNA

100 – 100,000
molecules per tube

total RNA extraction

OD260

RT
reverse transcription



standard curve
using rec. DNA
10 – 100,000 molecules

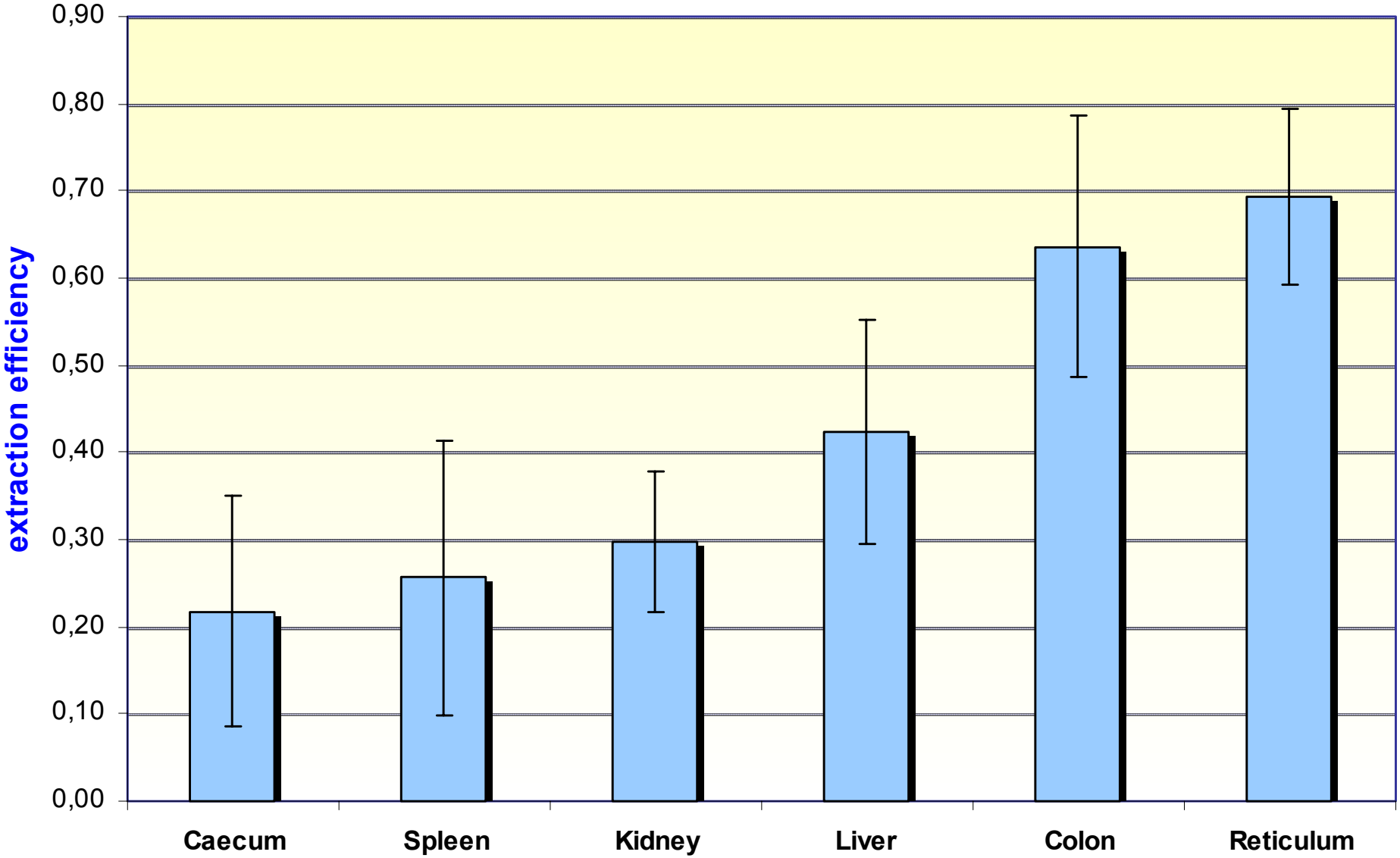


qPCR

CP data analysis

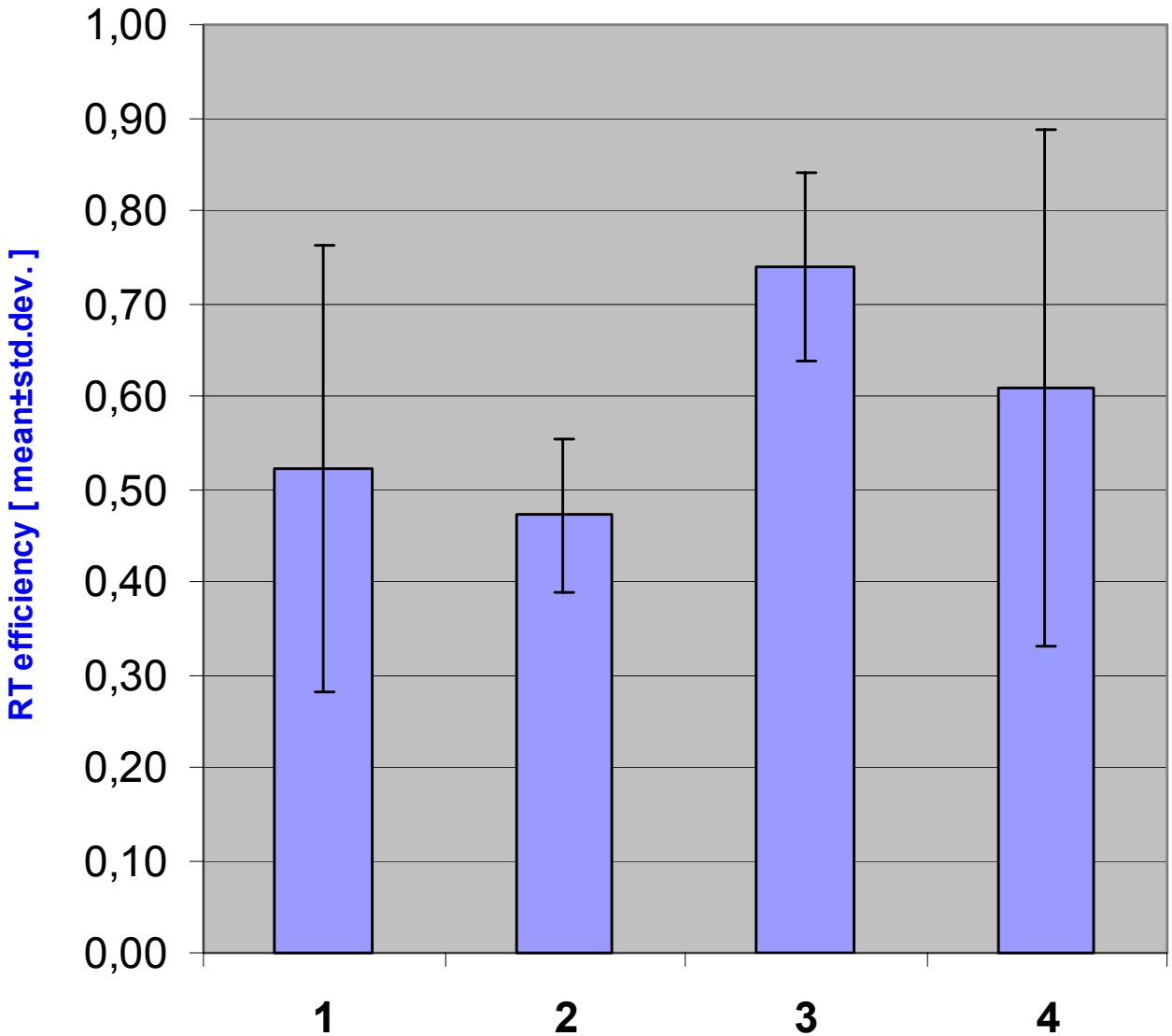
Tissue extraction efficiency [mean±sem]

(3 tissue repeats with 4 different spikes, n =12)



RT Efficiency

Qiagen SYBR Green I qRT-PCR Kit, performed in LightCycler

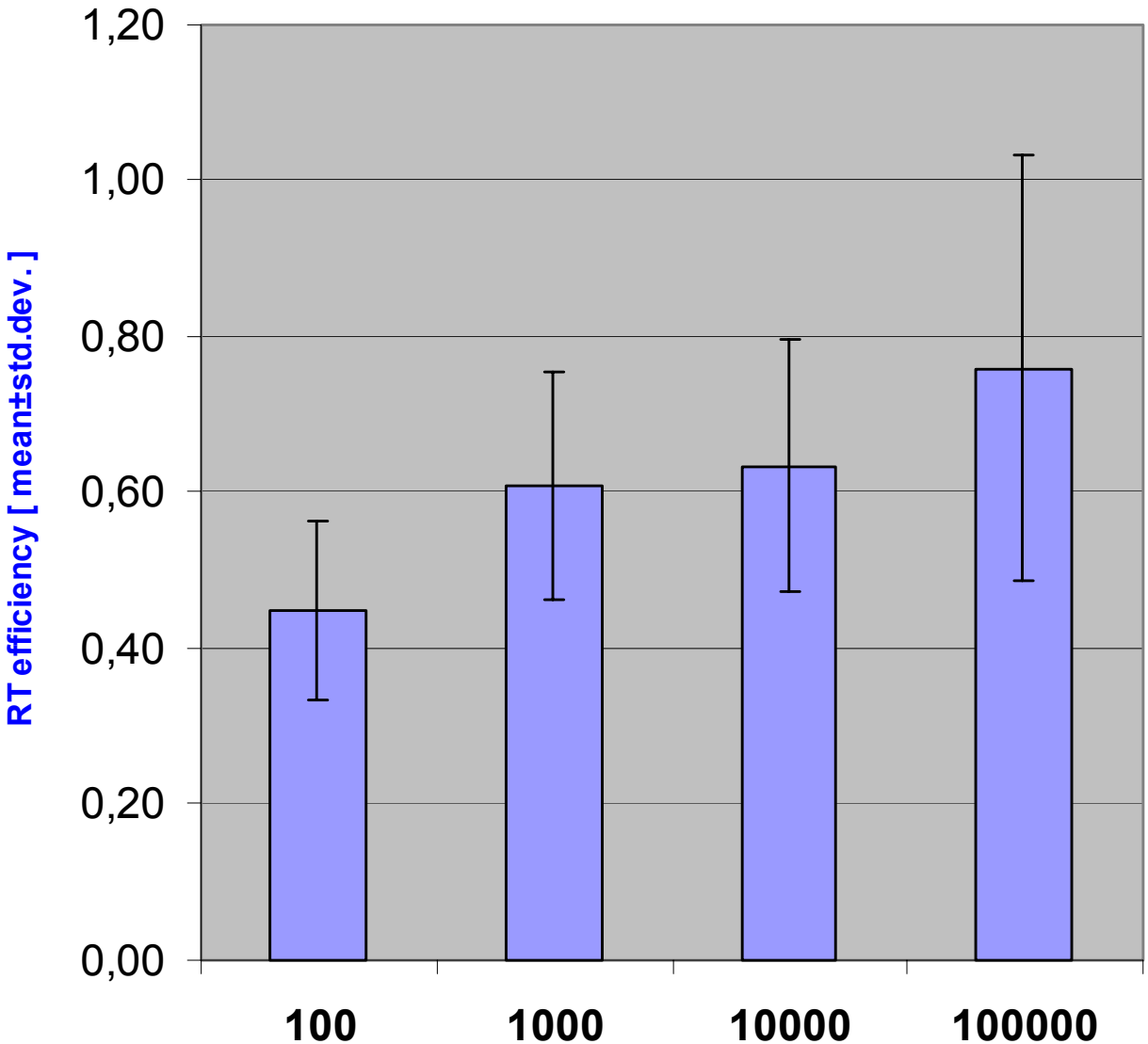


mean daily recovery of recRNA MultiStandard (100 - 10,000 molecules)

overall mean = 58.5±17.5%

RT Efficiency

Qiagen SYBR Green I qRT-PCR Kit, performed in LightCycler



overall mean = 61.3±17.4%

Table

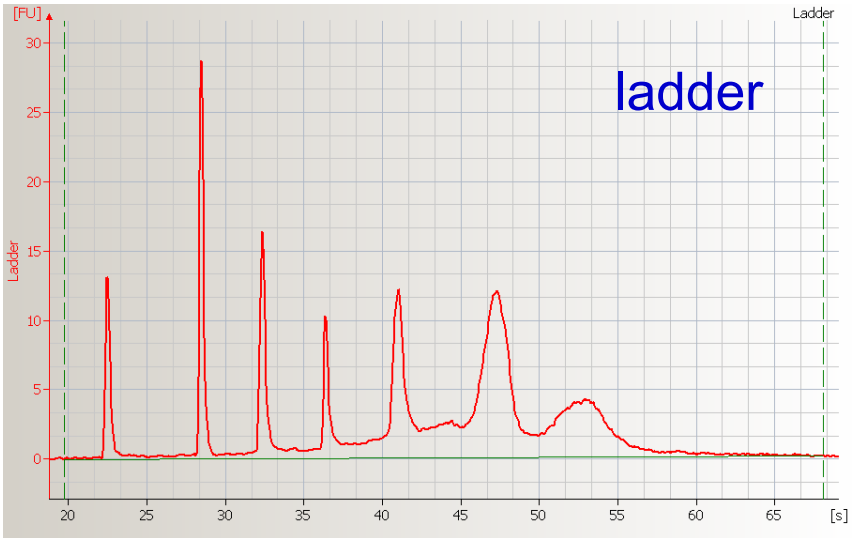
Table 1. Absolute reverse transcription yields for RNA MultiStandard.

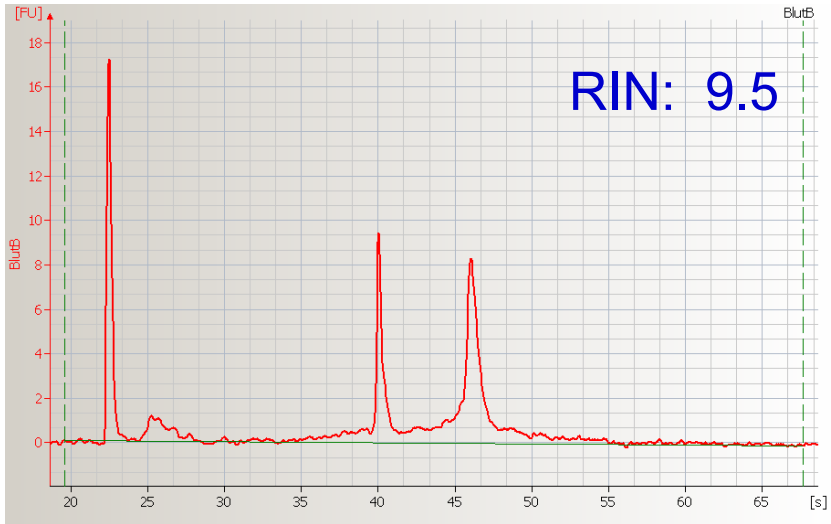
	External RNA molecules added[a]				
	10^6	10^5	10^4	10^3	
	Reverse transcription yield (%)[b]				Average[c]
MMLVH	22	50	48	(125)	40 ± 16
Omniscript	7.2	3.1	11.5	(66)	7.3 ± 4.2
AMV	0.4	0.6	4.9	(44)	2.0 ± 2.5
MMLV	32	49	50	(110)	44 ± 10
Improm-II	32	22	12	(98)	22 ± 10
cAMV	6.3	17	35	(88)	19 ± 15
ThermoScript	1.1	9.0	14	(46)	8.0 ± 6.6
SuperScript III	87	72	90	(43)	83 ± 10
Average	**24±29**	**28±26**	**33±29**	**78±32**	**28 ± 27**

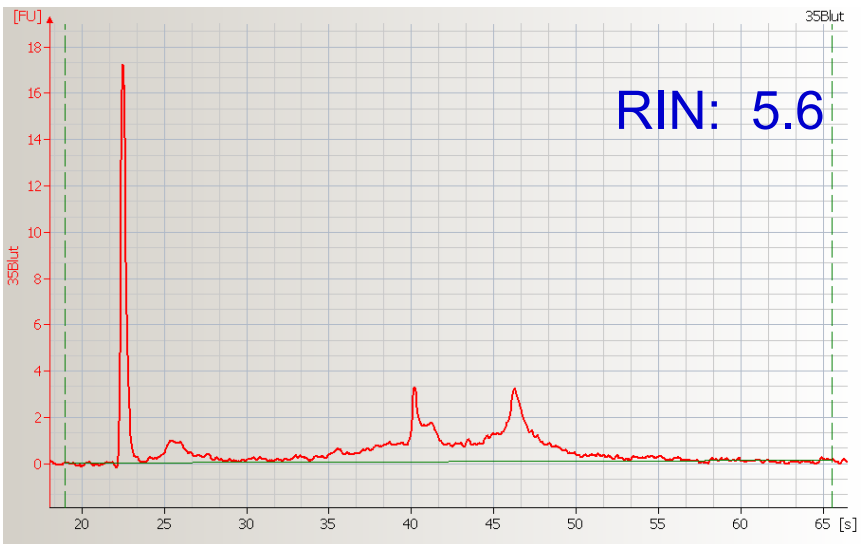
Stahlberg et al., Clin Chem. 50(9) 2004

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

total RNA extracted bovine WBC analysed in Bioanalyzer 2100

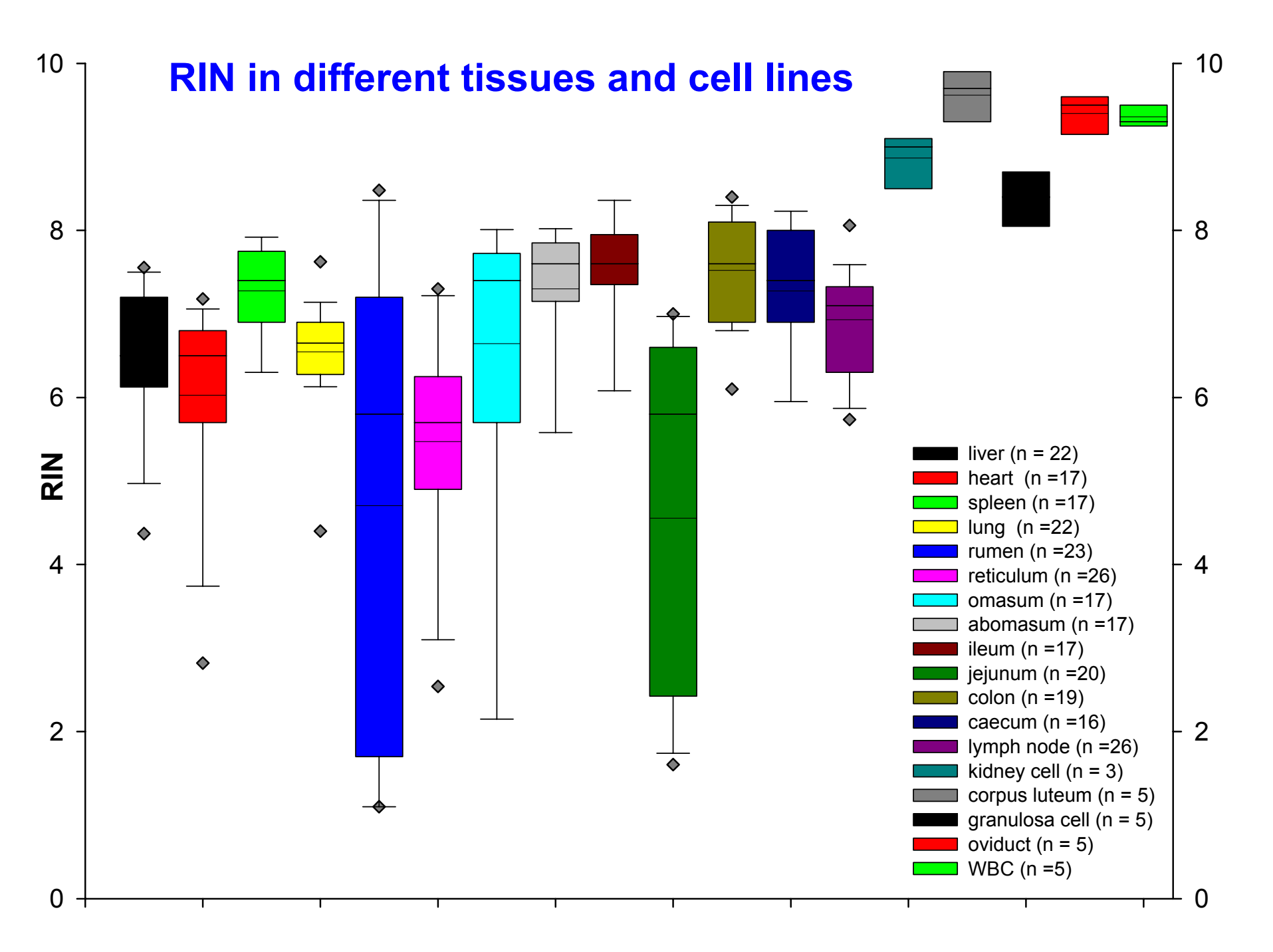




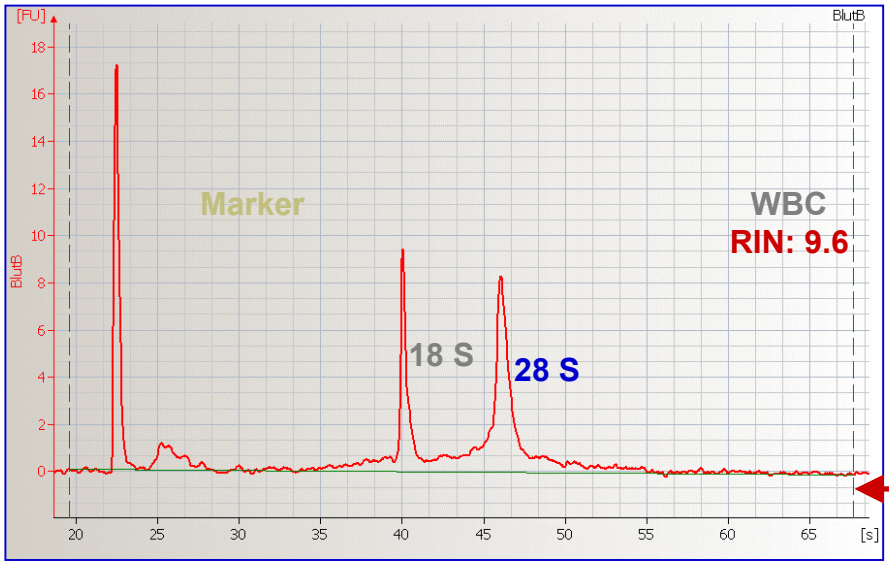




S. Fleige, V. Walf, S. Huch, MW. Pfaffl, 2005


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

analyzed in Bioanalyzer

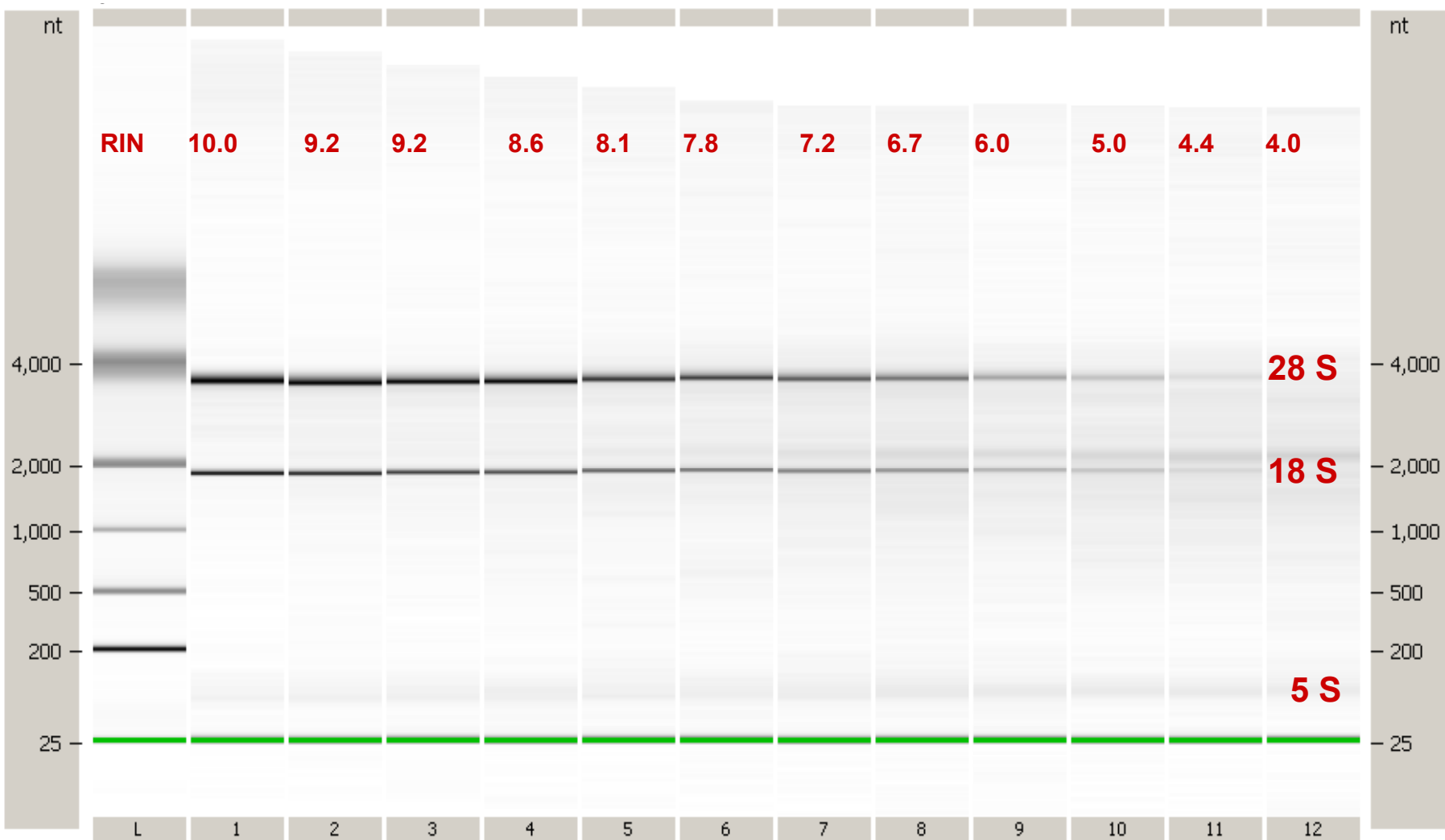
Degradation

degradation scale of mixtures between good and bad samples

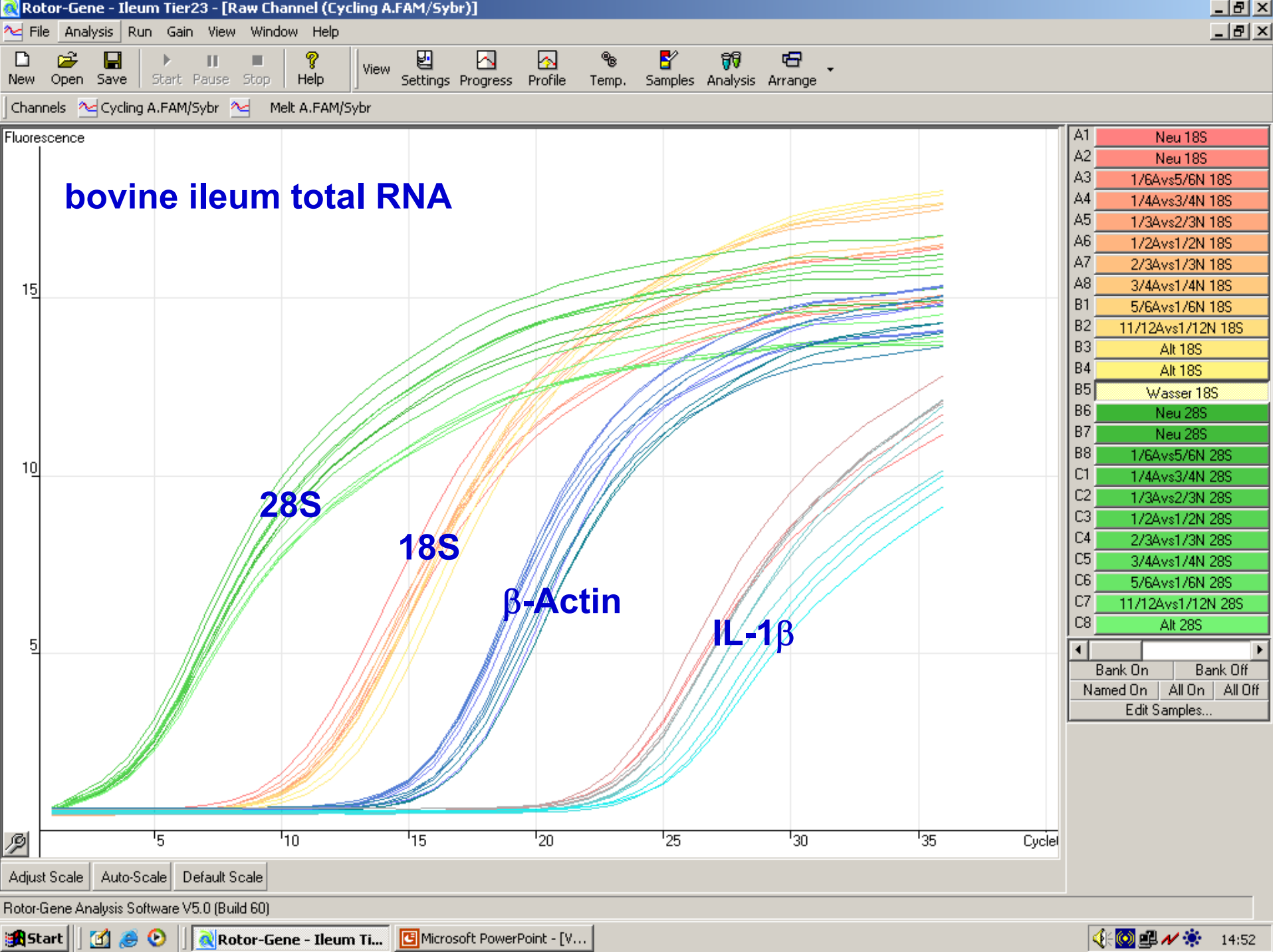
analyzed in Bioanalyzer

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

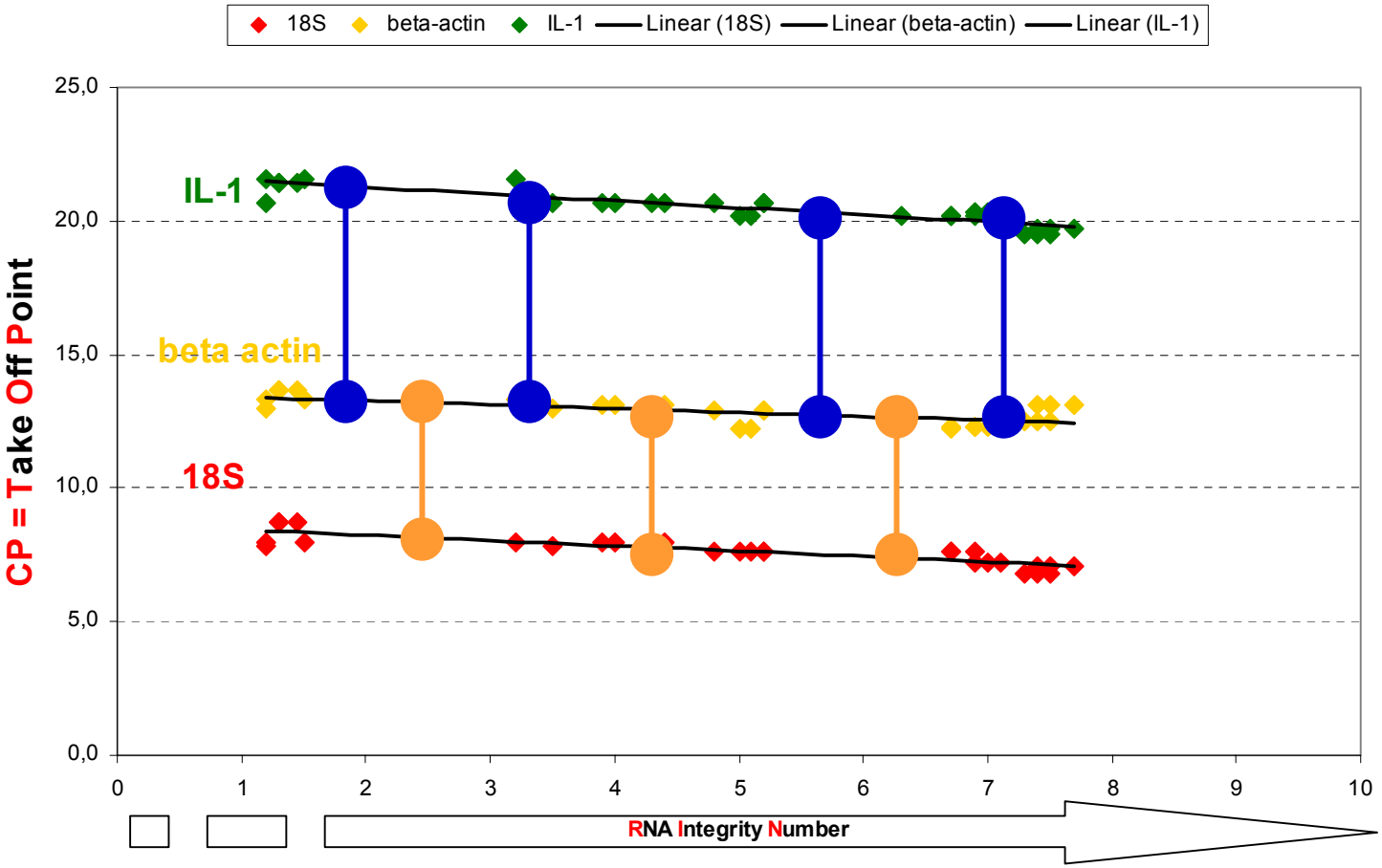
Degradation of tissue extracted total RNA



The intensity of bands decreases with increasing 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

bovine ileum: CP = TOP



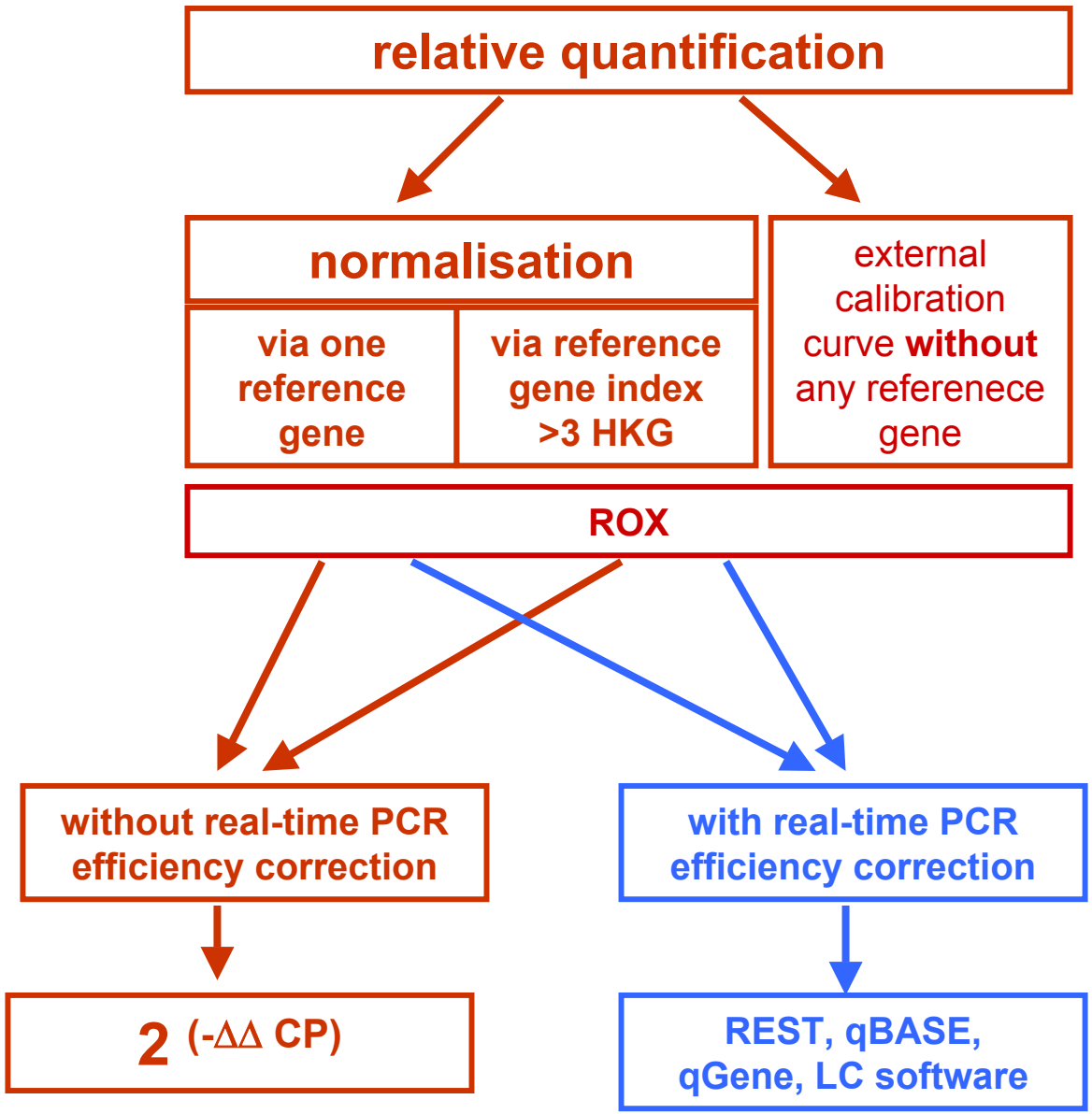
$y = -0{,}2583x + 21{,}787$
$R^2 = 0{,}8119$

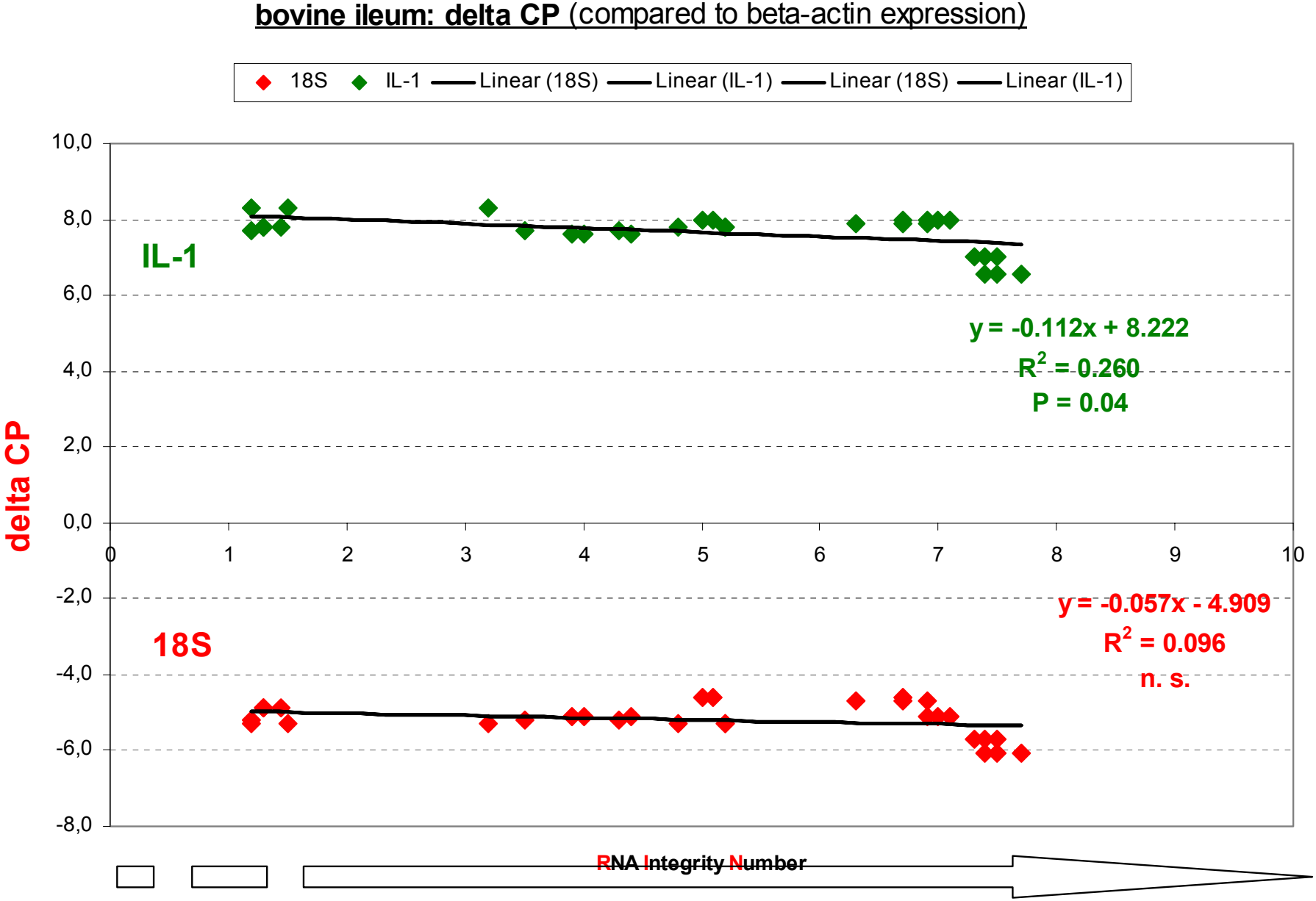
$y = -0{,}1461x + 13{,}565$
$R^2 = 0{,}4903$

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

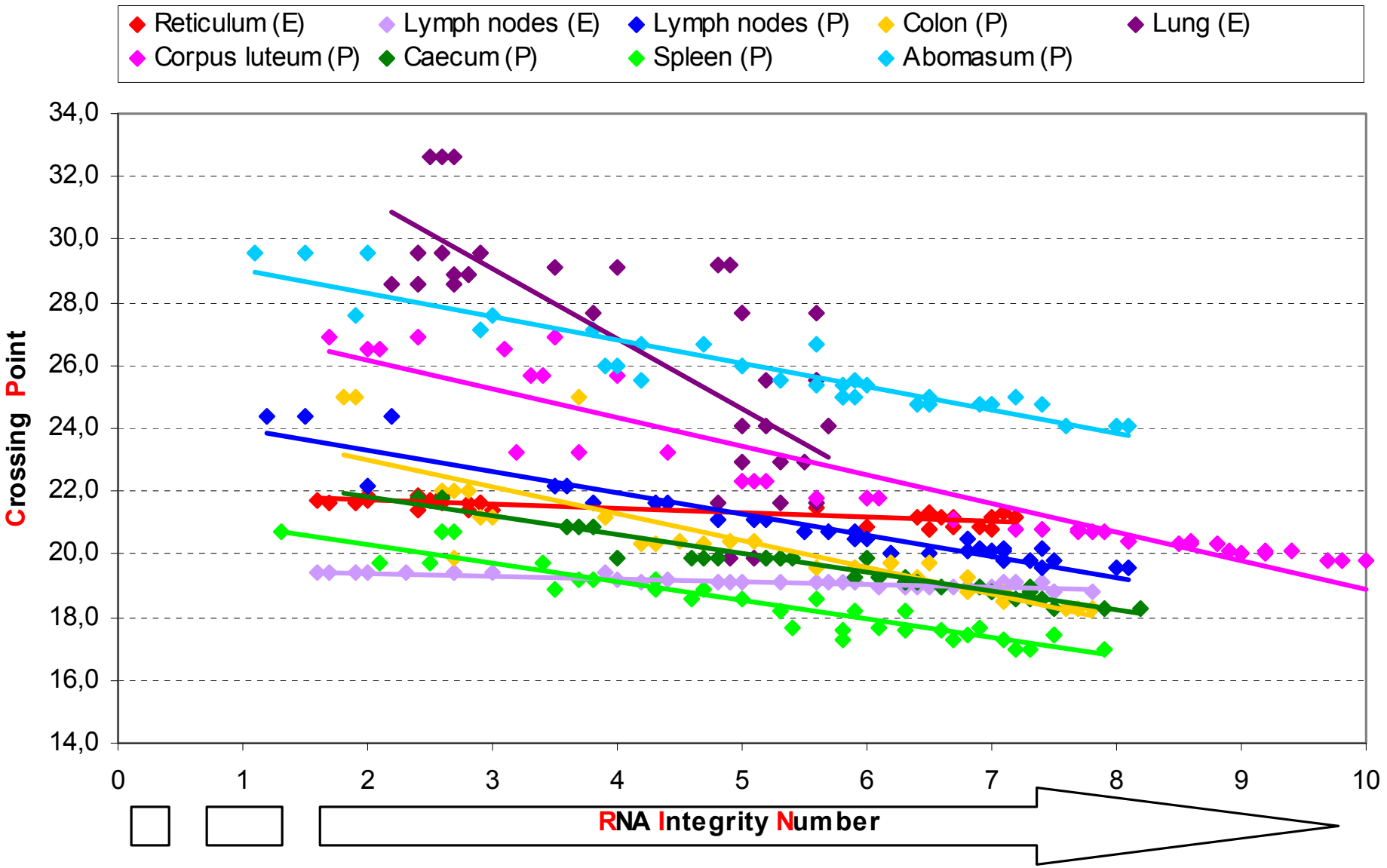
gene	level of significance
Interleukin-1beta	P < 0.001
beta-Actin	P < 0.01
18S	P < 0.001

Relative Quantification in real time qRT-PCR

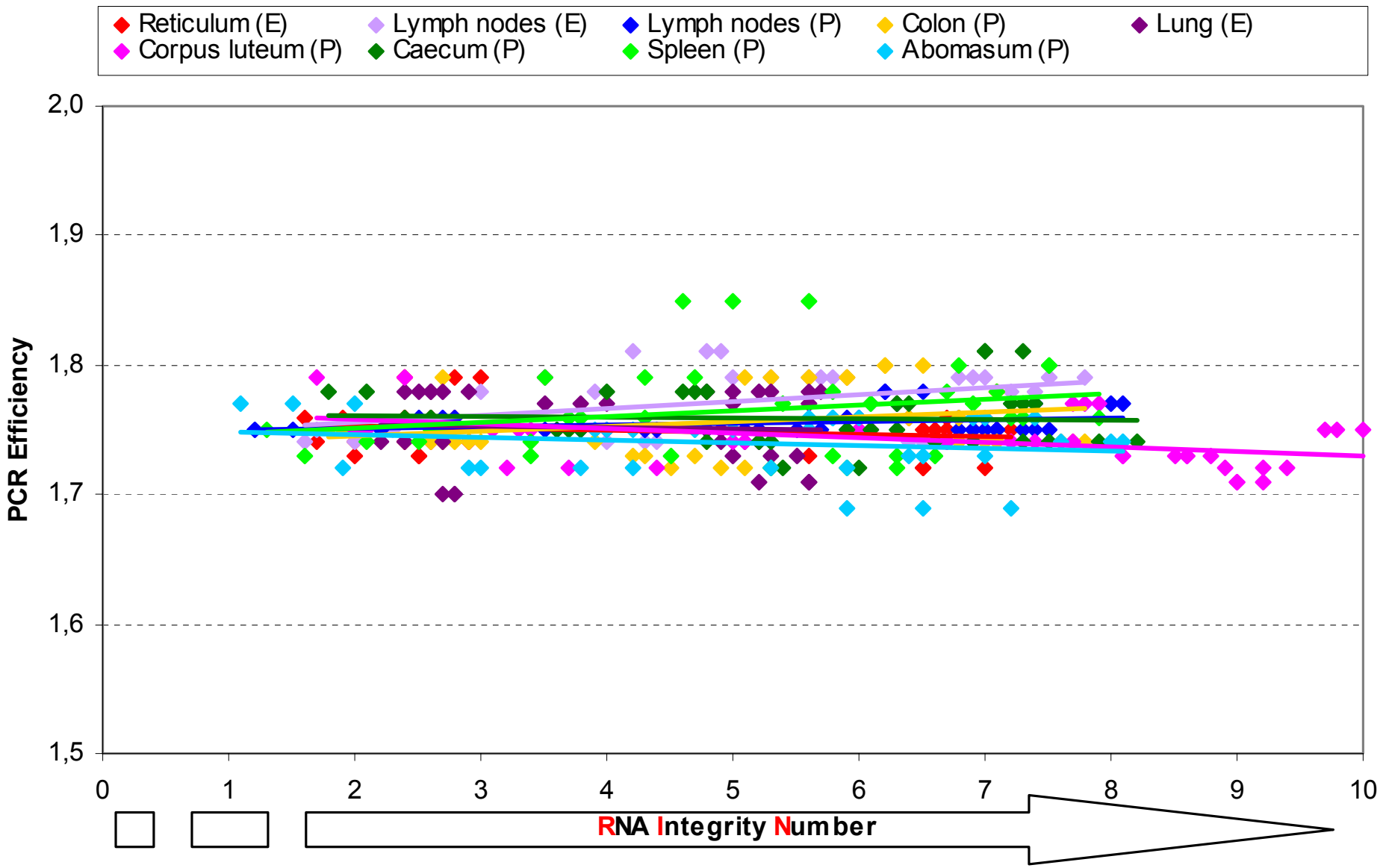


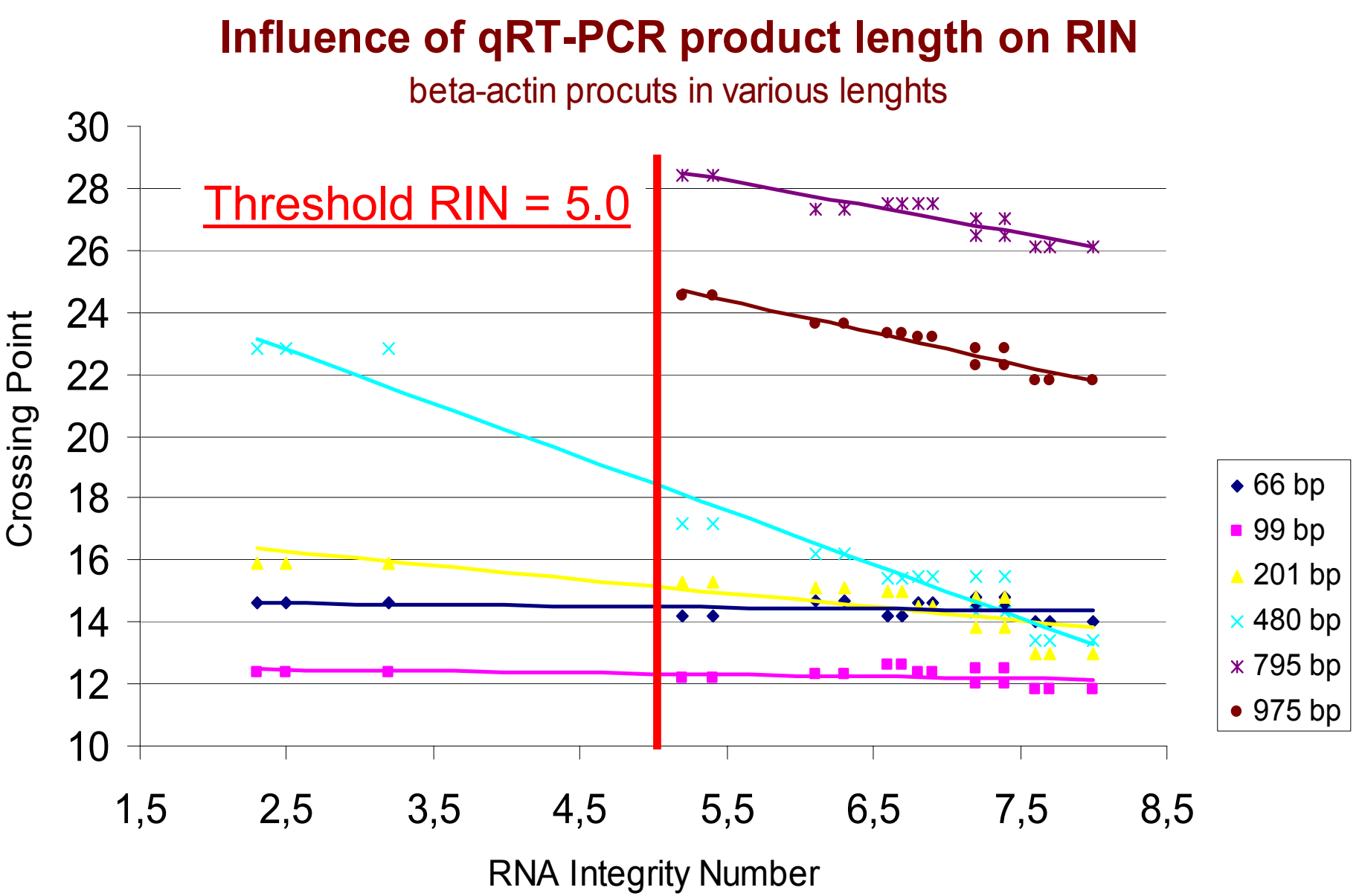

bovine ileum: delta CP (compared to beta-actin expression)
18S
IL-1
Linear (18S)
Linear (IL-1)
Linear (18S)
Linear (IL-1)
10,0
8,0
6,0
4,0
2,0
0,0
-2,0
-4,0
-6,0
-8,0
delta CP
IL-1
y = -0.112x + 8.222
R2 = 0.260
P = 0.04
18S
y = -0.057x - 4.909
R2 = 0.096
n. s.
0
1
2
3
4
5
6
7
8
9
10
RNA Integrity Number

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

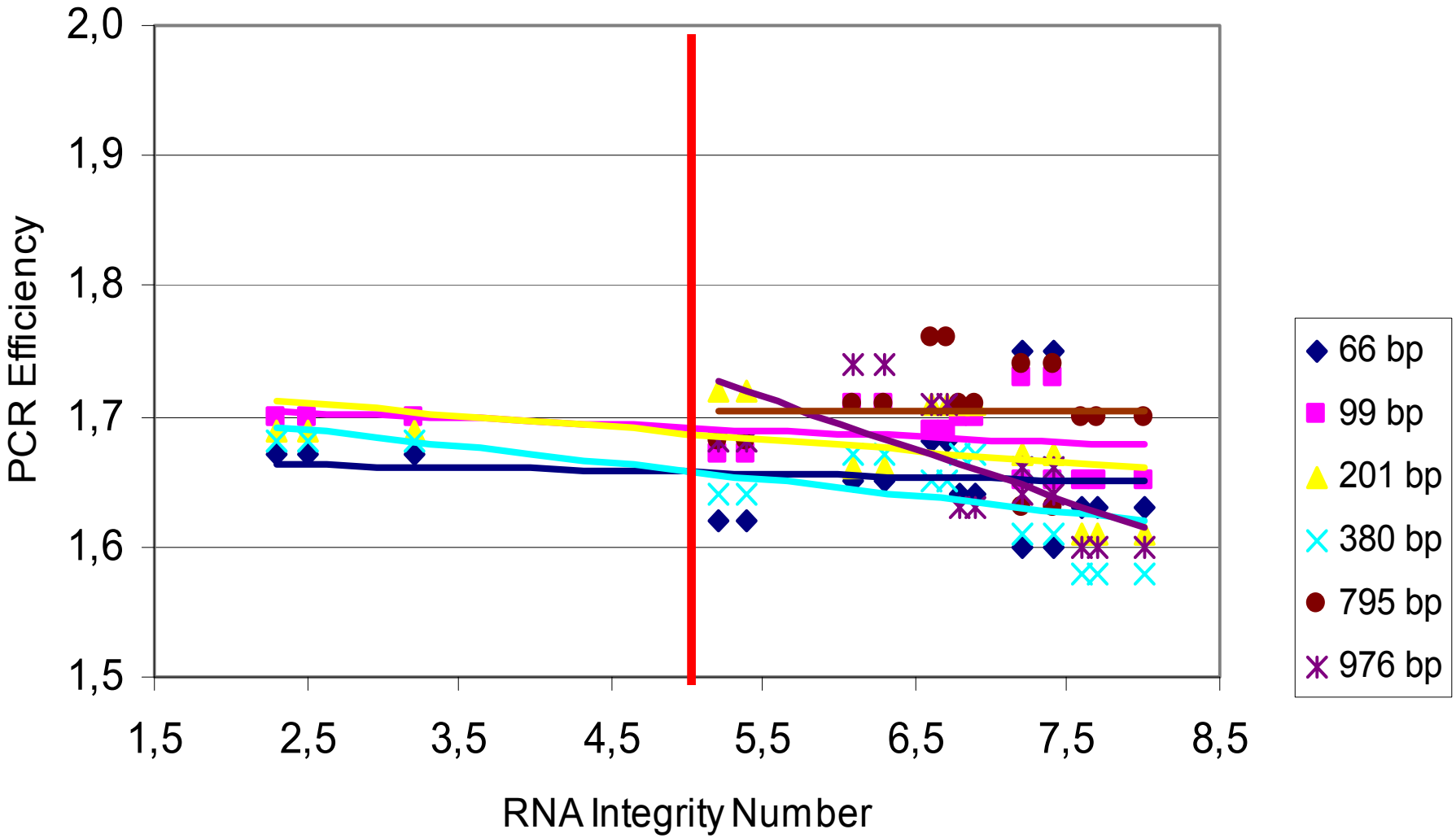


Influence of total RNA quality on qRT-PCR **efficiency**

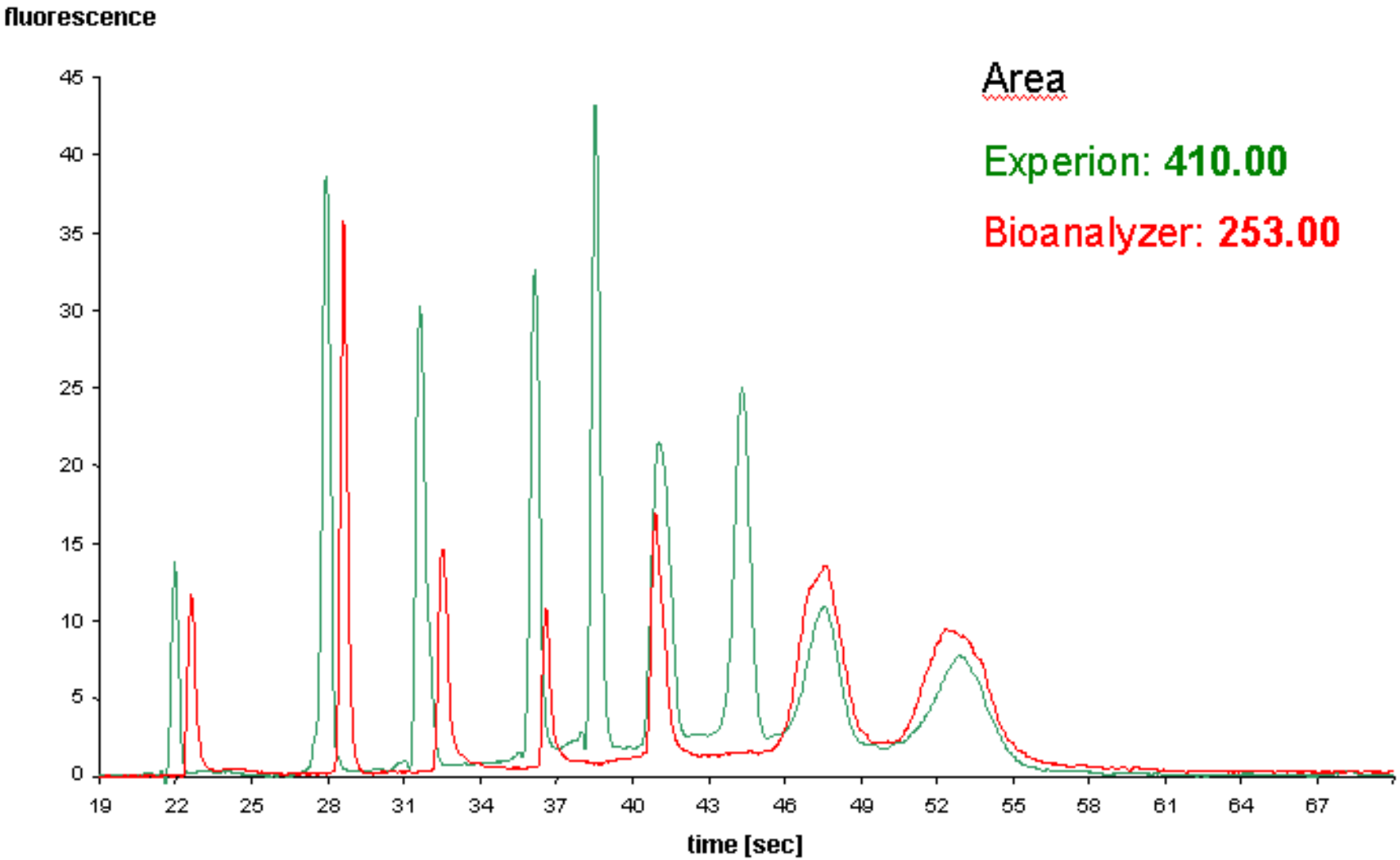



Influence of qRT-PCR product length on RIN
beta-actin procuts in various lenghts
Threshold RIN = 5.0
Crossing Point
30
28
26
24
22
20
18
16
14
12
10
1,5
2,5
3,5
4,5
5,5
6,5
7,5
8,5
RNA Integrity Number
66 bp
99 bp
201 bp
480 bp
795 bp
975 bp

PCR efficiency in dependence of RIN



Comparison of Experion & Bioanalyzer 2100



Comparison of 18S/28S rRNA ratio

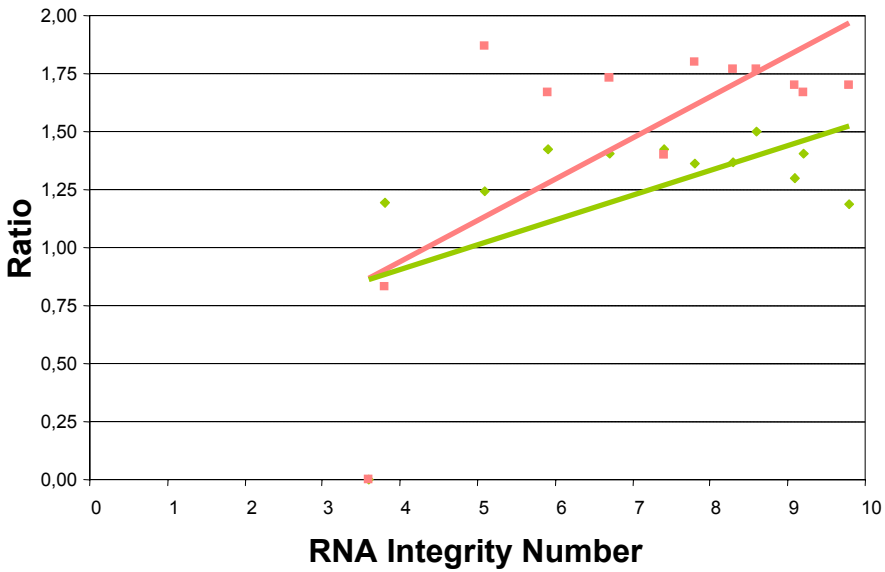
Experion & Bioanalyzer 2100

Bioanalyzer 2100

y = 0.177x + 0.2346
R2 = 0.47

Experion

y = 0.107x + 0.475
R2 = 0.32

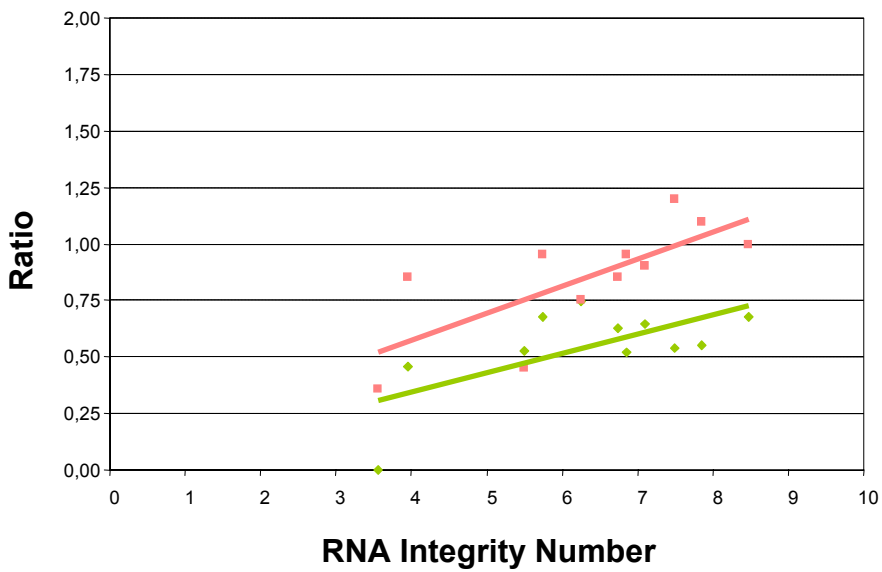


Bioanalyzer 2100

y = 0.1201x + 0.092
R2 = = 0.53

Experion

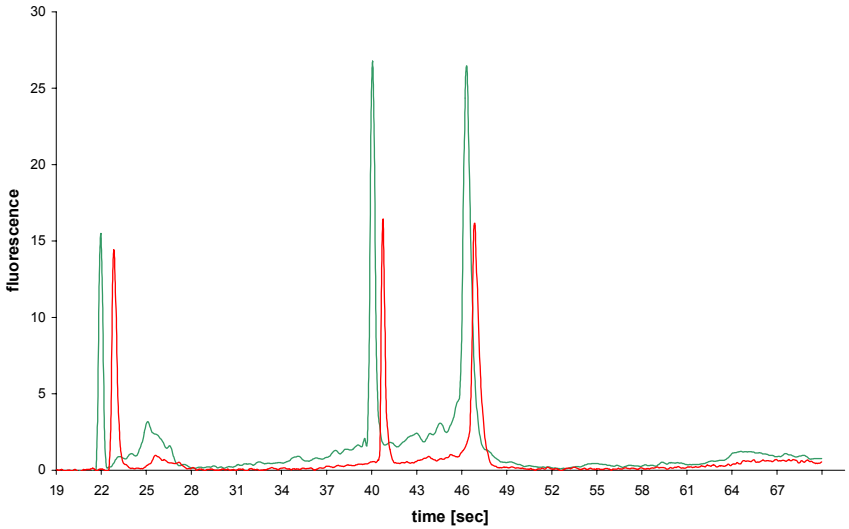
y = 0.085x + 0.005
R2 = 0.43



200 ng total RNA analysed **50 ng**

Run performance

Experion & Bioanalyzer 2100

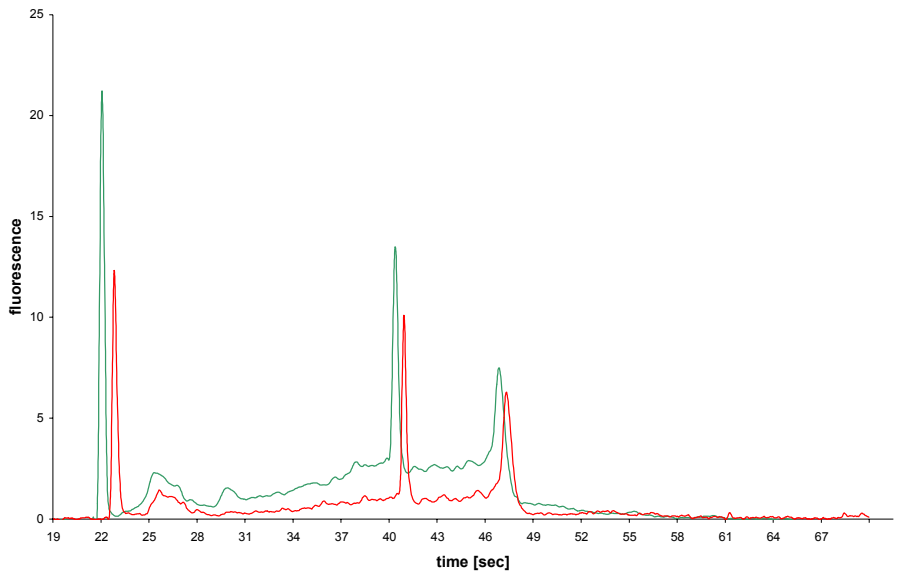


Experion: 165.34 [71.47 ng/µl]
Ratio [28S/18S]: **0.93**

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

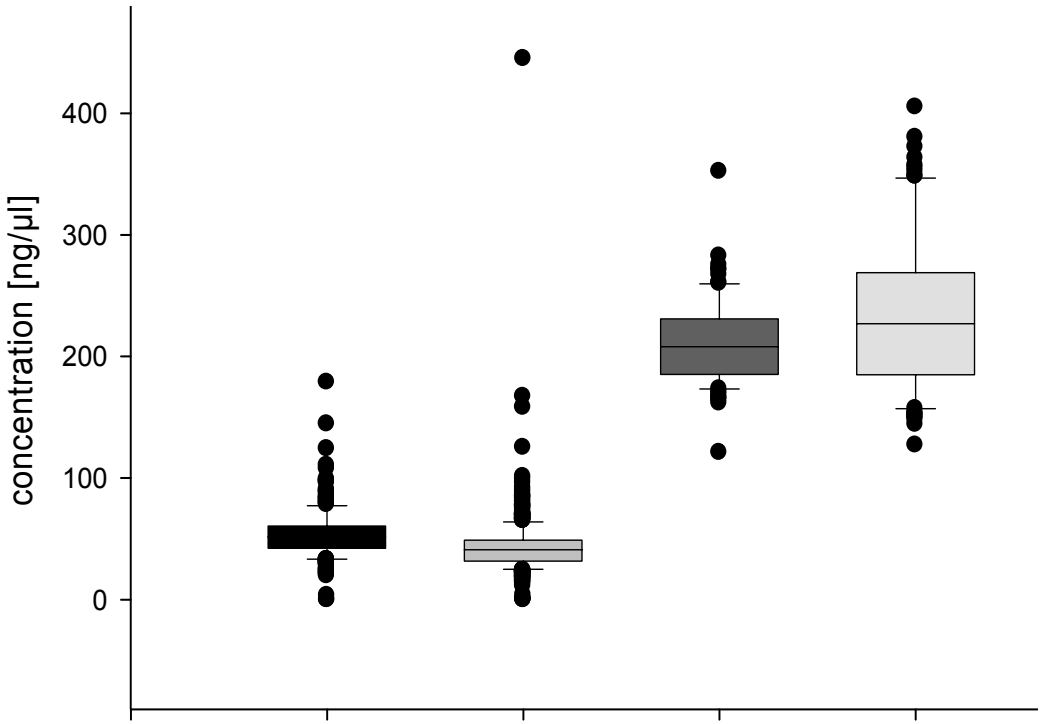
Experion & Bioanalyzer 2100

A: Experion (50 ng/µl)

B: Bioanalyzer (50 ng/µl)

C: Experion (200 ng/µl)

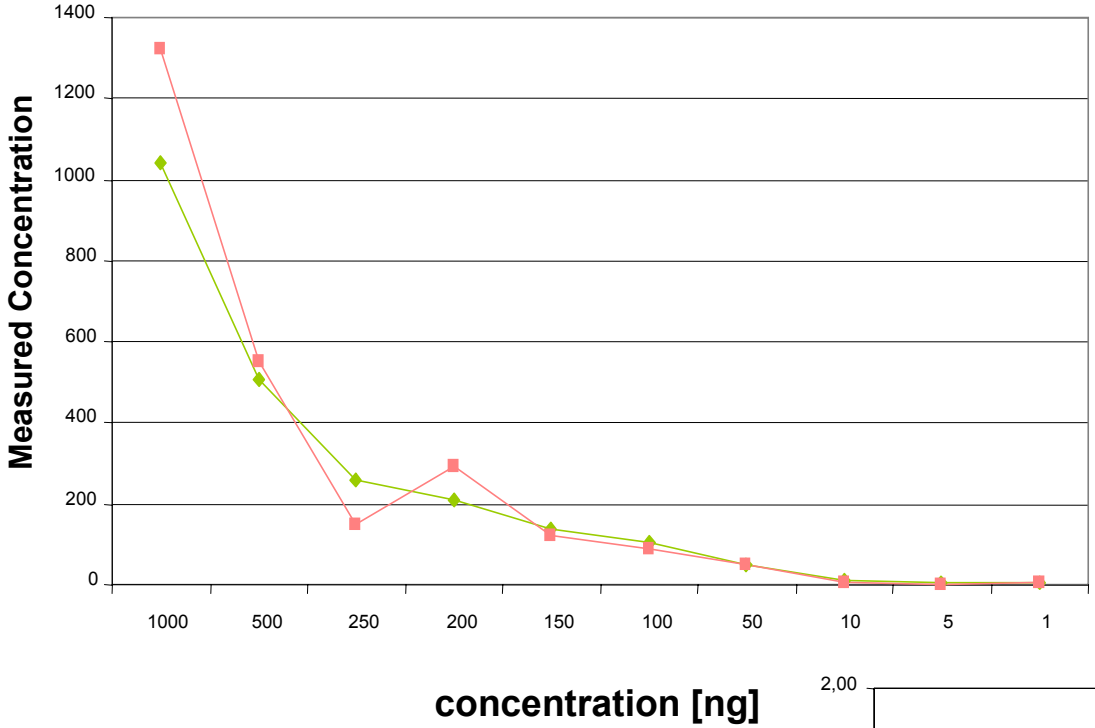
D: Bioanalyzer (200 ng/µl)

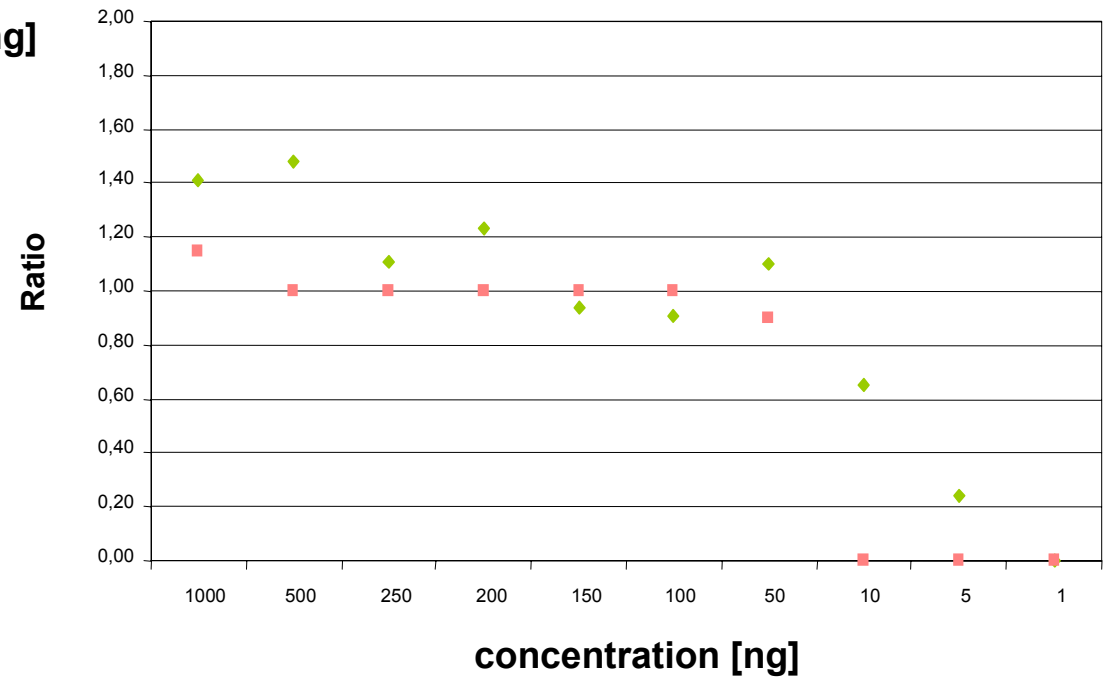


	A	B	C	D
mean [ng]	54.2	43.4	211.1	235.8
CV [%]	39.1	57.1	14.7	27.4
	n = 207		n = 171	

Sensitivity

Experion & Bioanalyzer 2100





Summary and Conclusions

- Total RNA Extraction efficiency is highly variable [CV >50%]
- Total RNA Extraction is very tissue dependent [20% to 70% extraction efficiency]
- RT efficiency is highly enzyme dependent [<10% for AMV, 50-85% for MMLV H-]
- RT is very sensitive [~ 30% day-to-day variations]
- RT is dependent of the abundancy [40% for low- and 75% for high abundant genes]
- PCR performance is dependent on total RNA quantity and quality
- RNA quality (=RIN) is highly tissue dependent:
  - good RIN [8-10] for single cells like cell cultures and WBC
  - Lower RIN [5-8] for solid tissues, requiring more homogenisation
- Influence of RNA quality on qRT-PCR results:
  - Minor influence on classical qRT-PCR products under 200 bp
  - RIN threshold of RIN = 5 for longer qRT-PCR products over 400 bp
  - NO influence on amplification efficiency
  - Relative quantification can circumvents the RIN problematic
- Tools to measure RNA integrity:
  - Advantages of Bioanalyzer 2100: RIN algorithm and better 18S/28S ratio
  - Advantages of Experion: more sensitivity and less variability

=> Pre-analytical steps are HIGHLY variable and replicates should be done here and NOT later during PCR reaction.

Thank you team !

Thank you for your attention !

