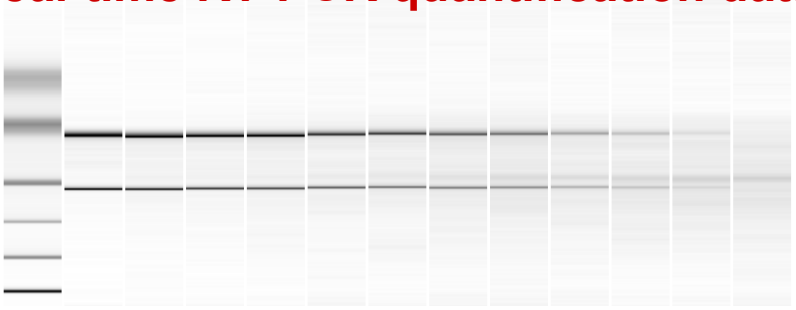
Influence of RNA integrity on real-time RT-PCR quantification data



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Germany

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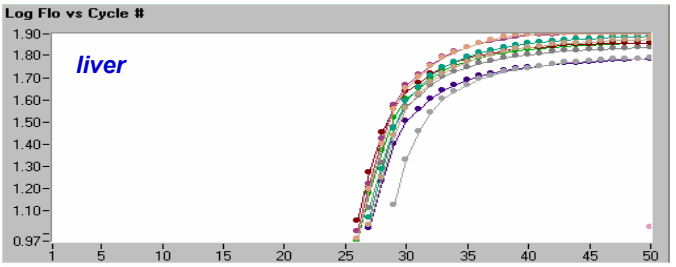
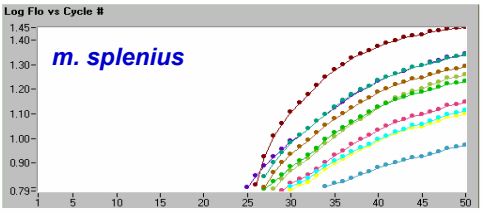
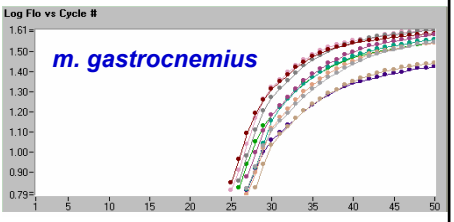
www.gene-quantification.info
TATAA.gene-quantification.info

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

IGF-1 mRNA amplification in three cattle tissues

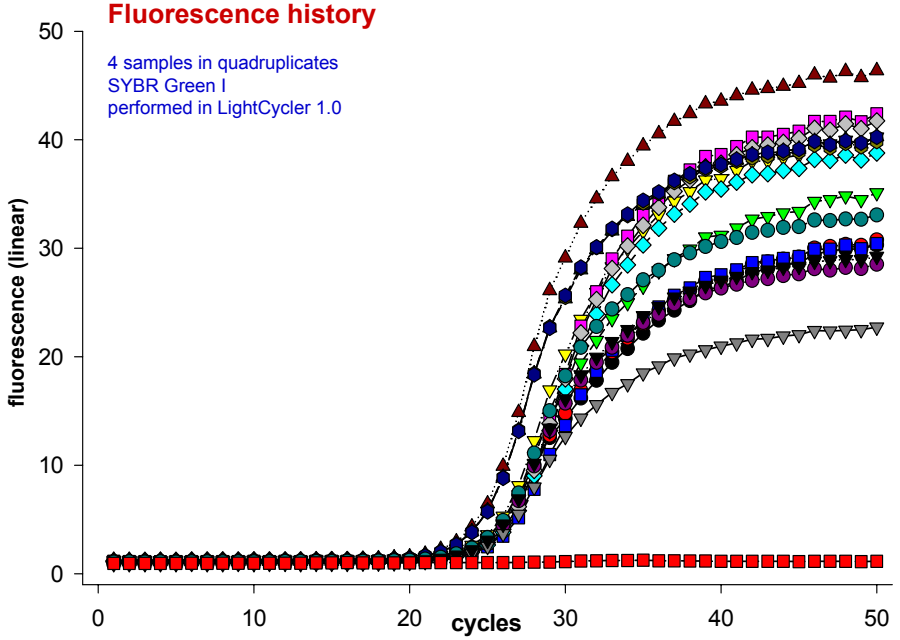






Fluorescence history

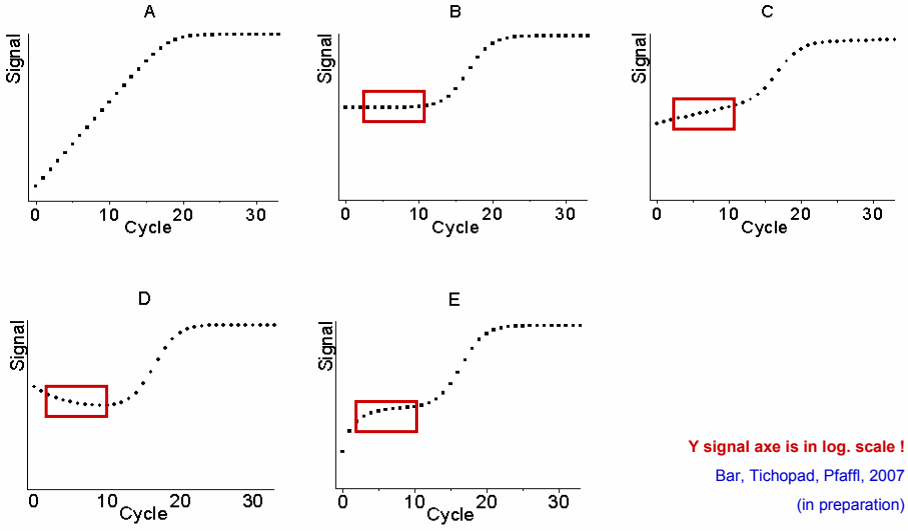
4 samples in quadruplicates
SYBR Green I
performed in LightCycler 1.0



Noise in real-time PCR !

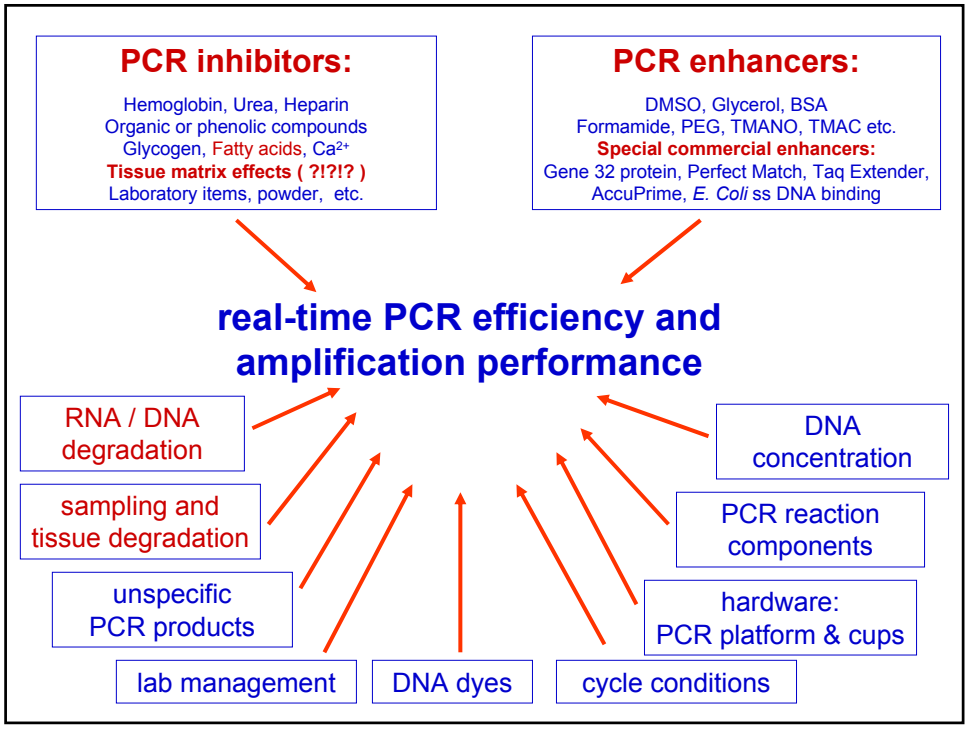
⇒ **Effect of different types of background on an amplification history**

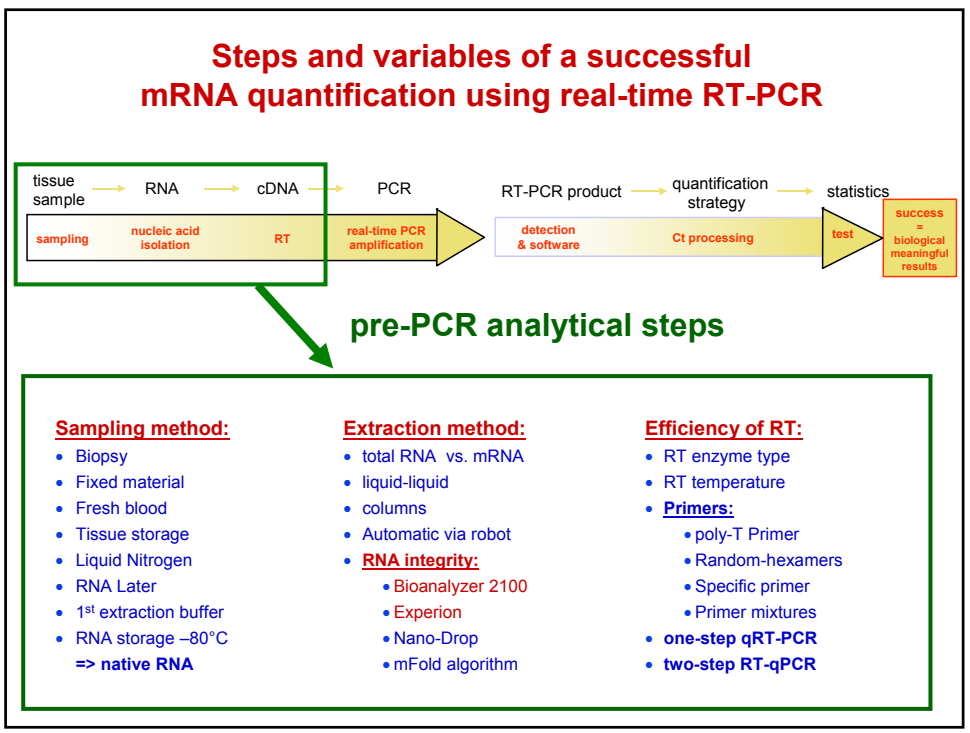
A. No background **B.** Constant background (Roche Biochemicals 1999) **C.** Linear rising background (Tichopad et al., 2000 & 2003) **D.** Decreasing non-linear background (Johnson et al., 2004) **E.** Increasing non-linear background (SoFar, Wilhelm et al., 2003).



Y signal axe is in log. scale !

Bar, Tichopad, Pfaffl, 2007
(in preparation)


PCR inhibitors:
Hemoglobin, Urea, Heparin
Organic or phenolic compounds
Glycogen, Fatty acids, Ca2+
Tissue matrix effects (?!?!?)
Laboratory items, powder, etc.
PCR enhancers:
DMSO, Glycerol, BSA
Formamide, PEG, TMANO, TMAC etc.
Special commercial enhancers:
Gene 32 protein, Perfect Match, Taq Extender,
AccuPrime, E. Coli ss DNA binding
real-time PCR efficiency and amplification performance
RNA / DNA degradation
sampling and tissue degradation
unspecific PCR products
lab management
DNA dyes
cycle conditions
DNA concentration
PCR reaction components
hardware: PCR platform & cups


Steps and variables of a successful mRNA quantification using real-time RT-PCR
tissue sample
RNA
cDNA
PCR
RT-PCR product
quantification strategy
statistics
sampling
nucleic acid isolation
RT
real-time PCR amplification
detection & software
Ct processing
test
success = biological meaningful results
pre-PCR analytical steps
Sampling method:
Biopsy
Fixed material
Fresh blood
Tissue storage
Liquid Nitrogen
RNA Later
1st extraction buffer
RNA storage –80°C
=> native RNA
Extraction method:
total RNA vs. mRNA
liquid-liquid
columns
Automatic via robot
RNA integrity:
Bioanalyzer 2100
Experion
Nano-Drop
mFold algorithm
Efficiency of RT:
RT enzyme type
RT temperature
Primers:
poly-T Primer
Random-hexamers
Specific primer
Primer mixtures
one-step qRT-PCR
two-step RT-qPCR

Determination the total RNA extraction efficiency

Bovine tissue samples:
liver, spleen, kidney,
caecum, colon, reticulum

tissue spike
with recombinant RNA
100 – 100,000 pMS1 RNA molecules / tube

total RNA extraction

RNA
OD_{260}

RT
reverse transcription

cDNA

qPCR

CP data analysis

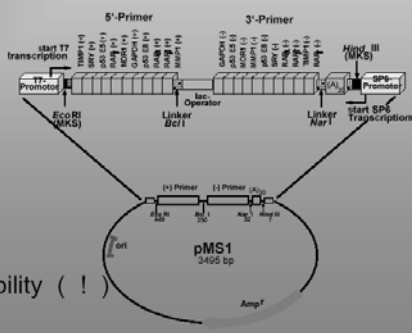
standard curve
with recombinant DNA
10 – 100,000 pMS1 DNA molecules / capillary

Roboscreen®
Gesellschaft für molekulare Biotechnologie mbH

Extraction Control Plasmid

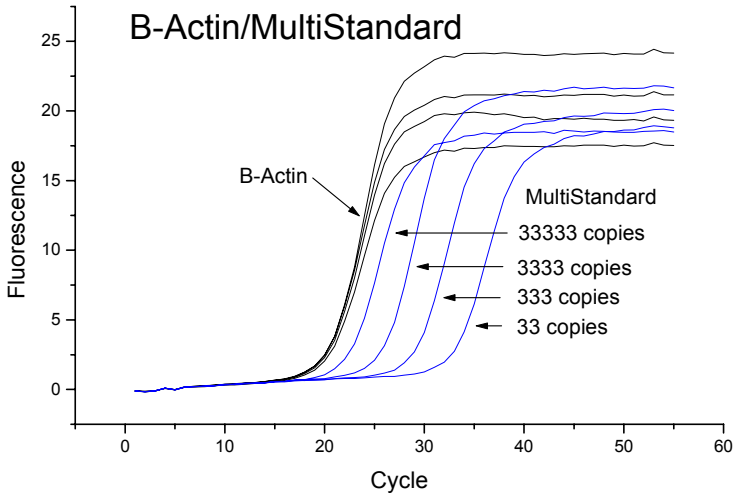
artificial synthetic DNA and RNA standard sequence

structure of the pMS1 standard



- ✓ minimal homology to any DNA or RNA target gene
- ✓ any contaminationcan can be excluded
- ✓ target compatible extraction efficiency
- ✓ exact kown copy numbers (!)
- ✓ guaranteed DNA and RNA stability (!)

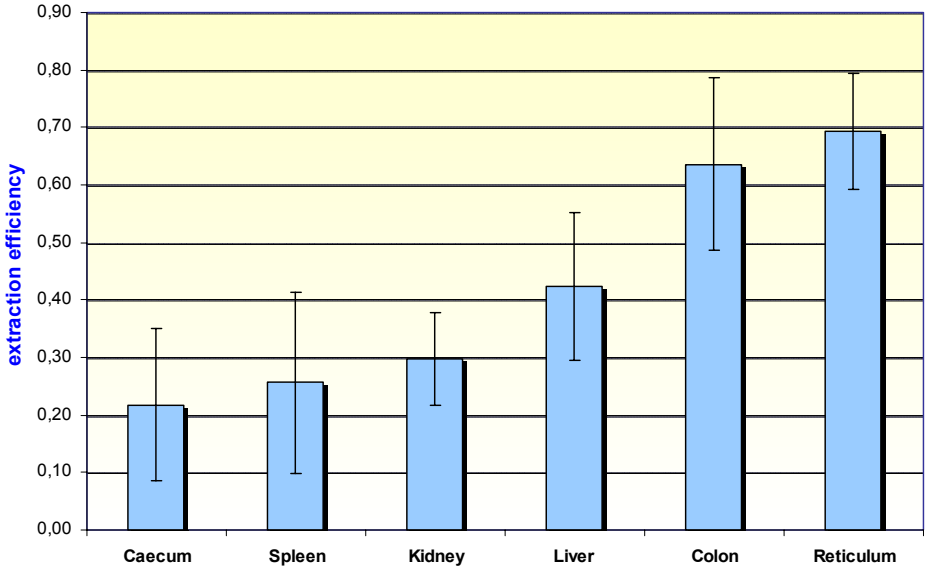
Absolut quantification with a known and exact defined recRNA reference **(RNA multi-standard pMS1)**

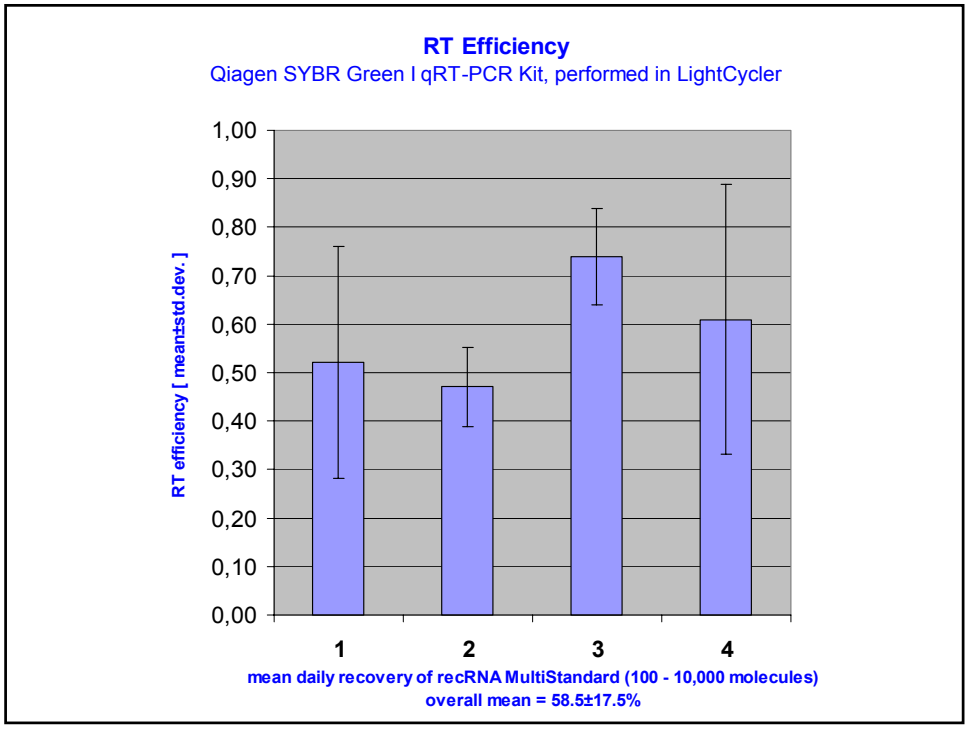


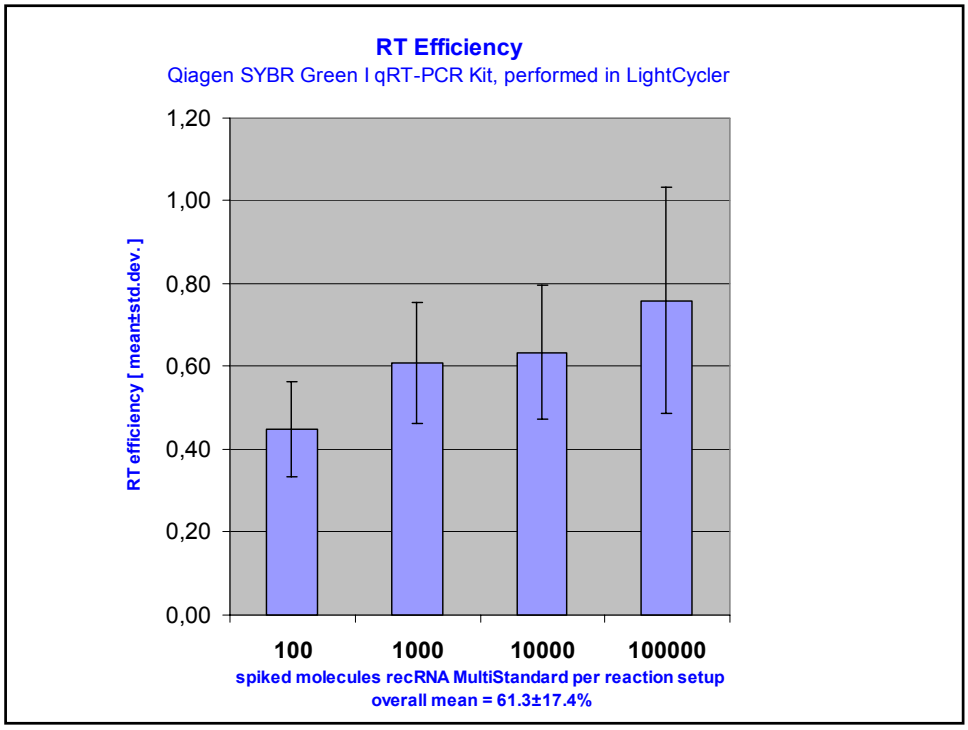
Stahlberg et al., Clin Chem. 50(9) 2004

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
RT efficiency [mean±std.dev.]
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
1
2
3
4
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
RT efficiency [mean±std.dev.]
1,20
1,00
0,80
0,60
0,40
0,20
0,00
100
1000
10000
100000
spiked molecules recRNA MultiStandard per reaction setup
overall mean = 61.3±17.4%

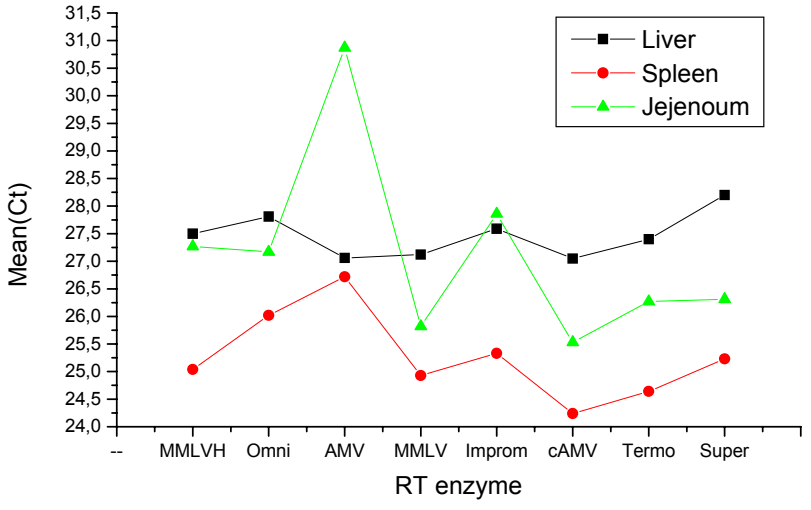
Table

Table 1. Absolute reverse transcription yields for RNA MultiStandard.

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

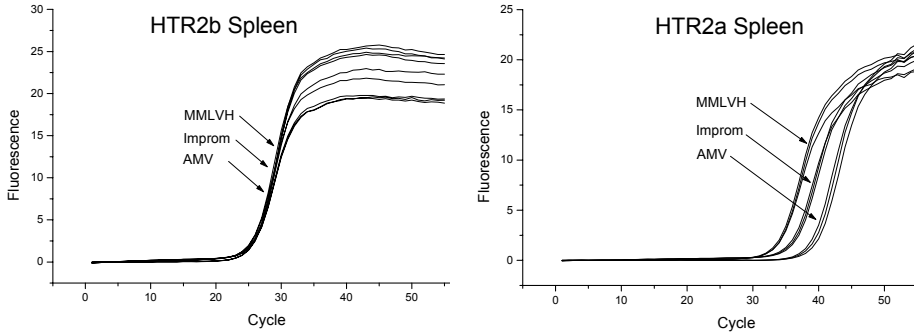
	External RNA molecules added[a]				
	10^6	10^5	10^4	10^3	
	Reverse transcription yield (%)[b]				Average[c]
MMLVH	22	50	48	(125)	40 ± 16
Omniscript	7.2	3.1	11.5	(66)	7.3 ± 4.2
AMV	0.4	0.6	4.9	(44)	2.0 ± 2.5
MMLV	32	49	50	(110)	44 ± 10
Improm-II	32	22	12	(98)	22 ± 10
cAMV	6.3	17	35	(88)	19 ± 15
ThermoScript	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**

RT enzyme and *"tissue background matrix"* affect the RT efficiency



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

RT efficiency depends on enzyme and gene



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

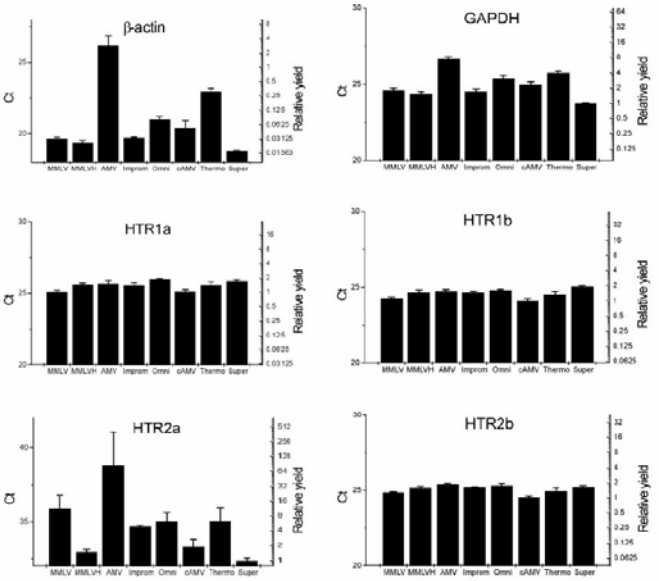


Fig. 1. QPCR Ct values reflecting the amounts of cDNA produced by the reverse transcriptases, with total RNA from spleen as input material.

Error bars indicate SD of samples run in quadruplicate. Yields relative to the least efficient reverse transcriptase, expressed in number of cDNA copies (assuming 100% PCR efficiency), are indicated by the *right-hand y axis*. The reverse transcriptases are as follows: (*left* to *right*) MMLV, MMLVH, AMV, Improm-II (*Improm*); Omniscript (*Omni*), cAMV, ThermoScript (*Thermo*), and SuperScript III (*Super*).

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

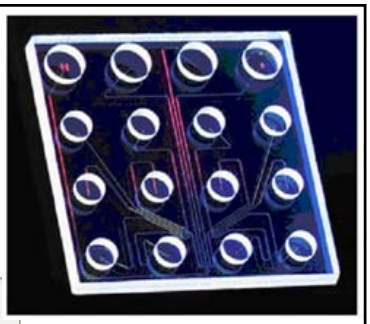
RNA integrity => RIN => CP

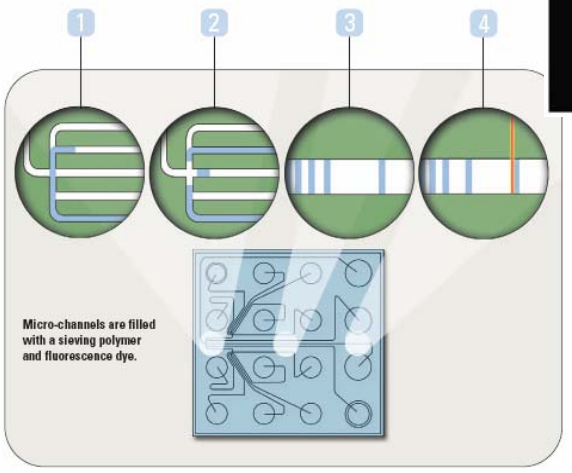
Bioanalyzer 2100, Agilent Technologies

Bioanalyzer 2100

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

Agilent 2100 Bioanalyzer RNA chip



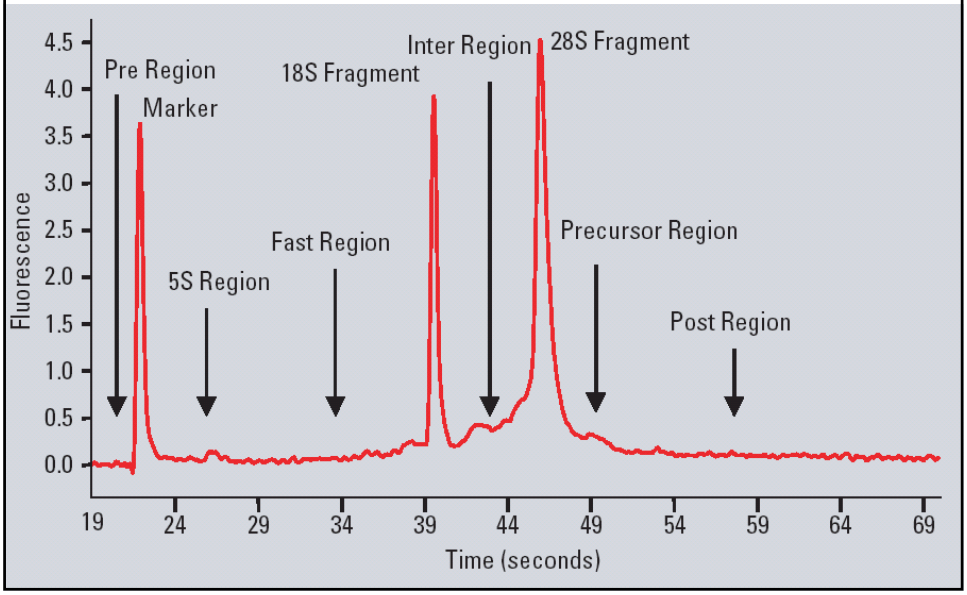


2 The sample is injected into the separation channel.

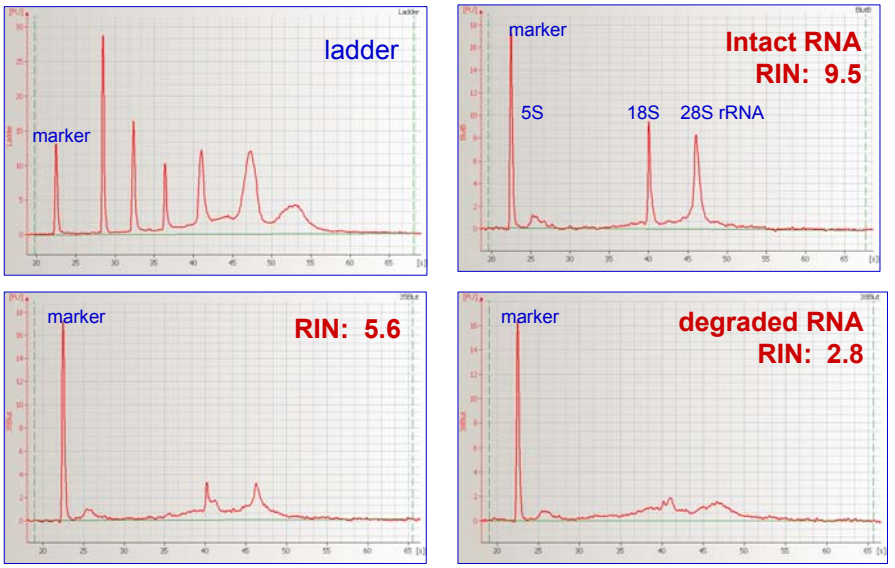
3 Sample components are electrophoretically separated.

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

Agilent Bioanalyzer 2100

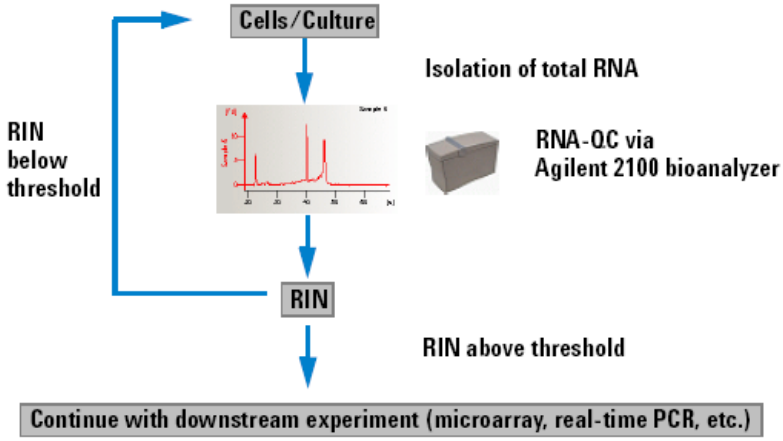


Various total-RNA qualities analysed in the Bioanalyzer 2100



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:

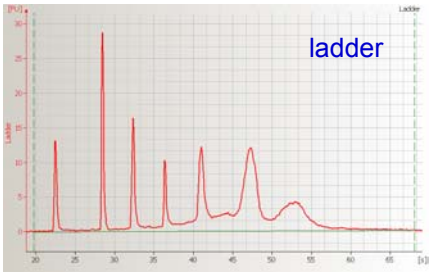


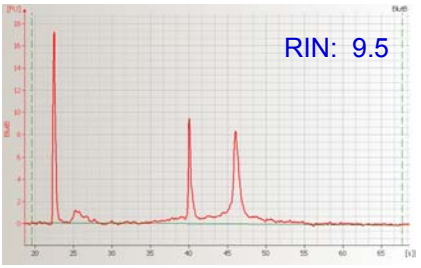
Q: Impact of RNA integrity on the qRT-PCR performance ?

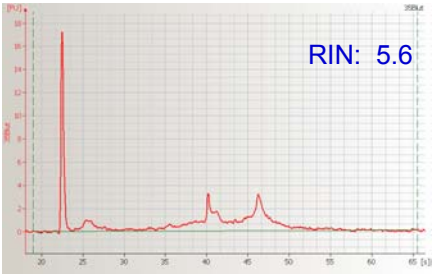
Q: Impact on physiological result ?

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

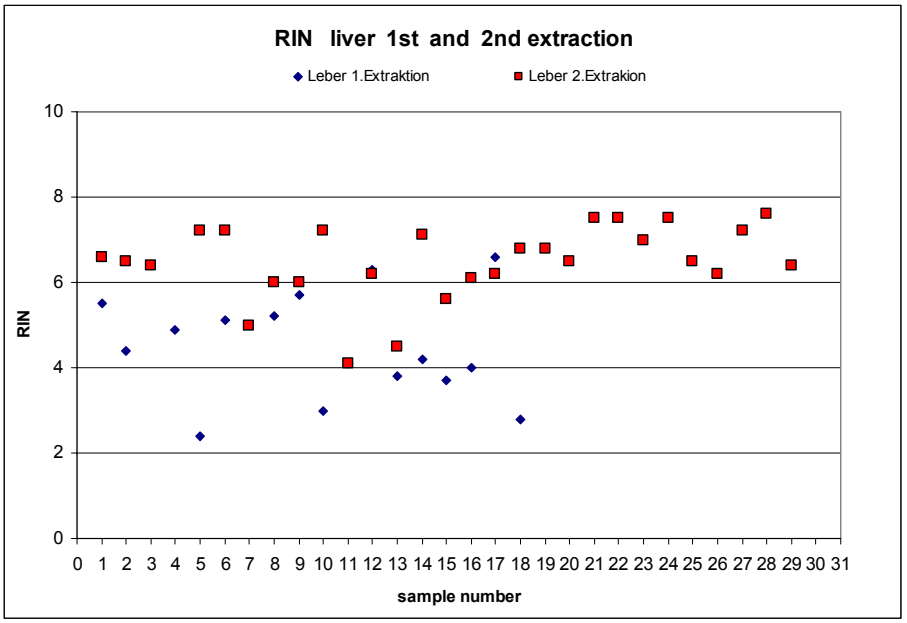
total RNA extracted bovine WBC analysed in Bioanalyzer 2100

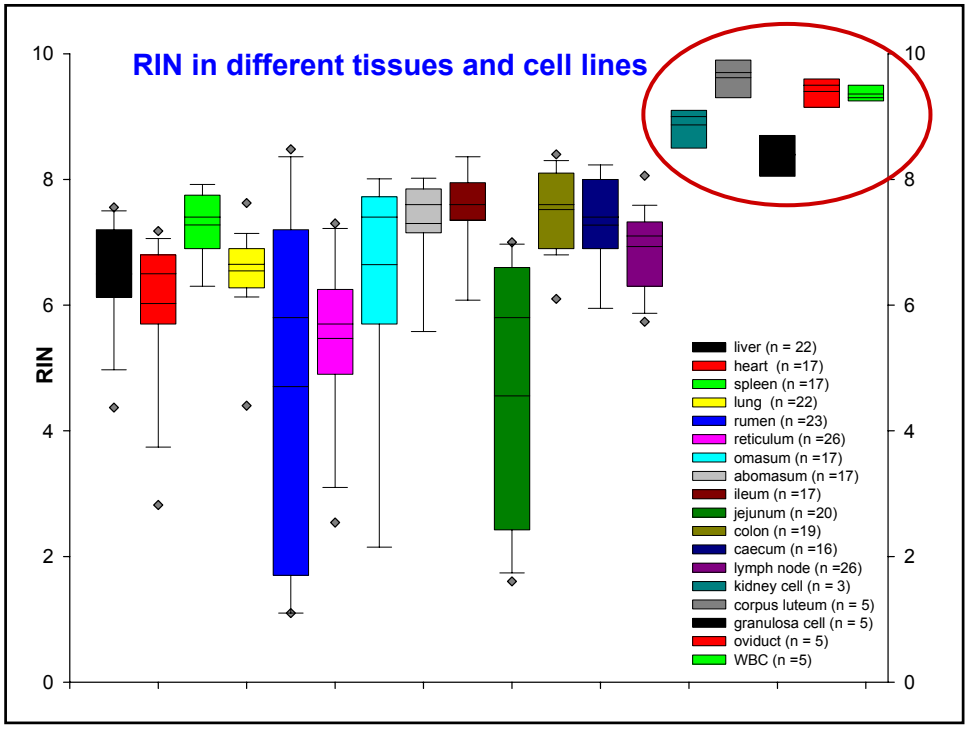


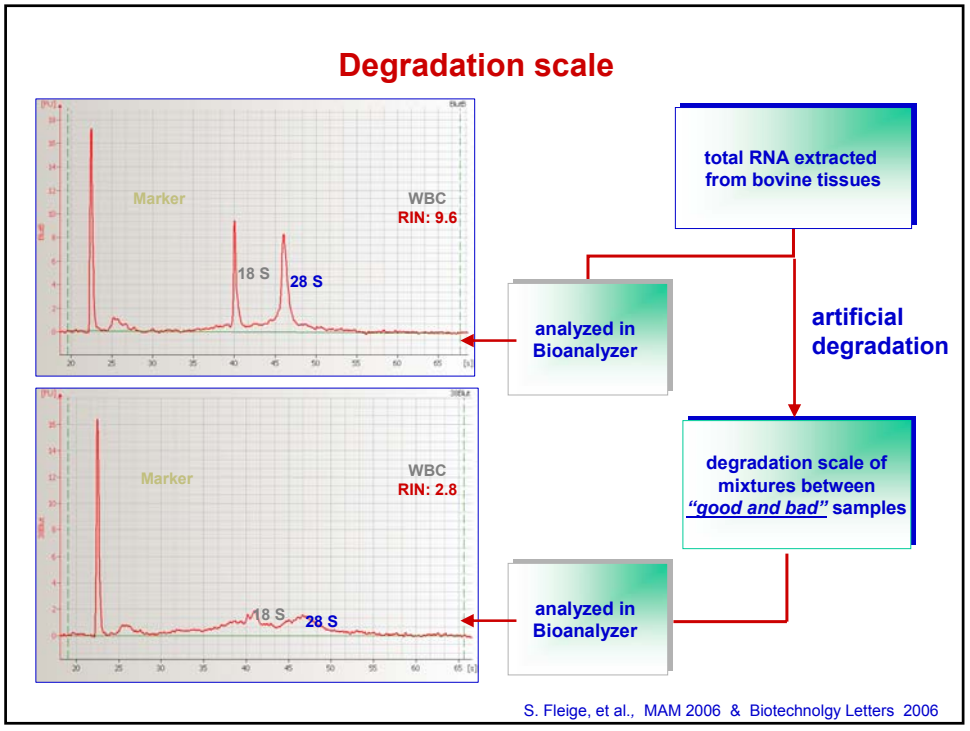




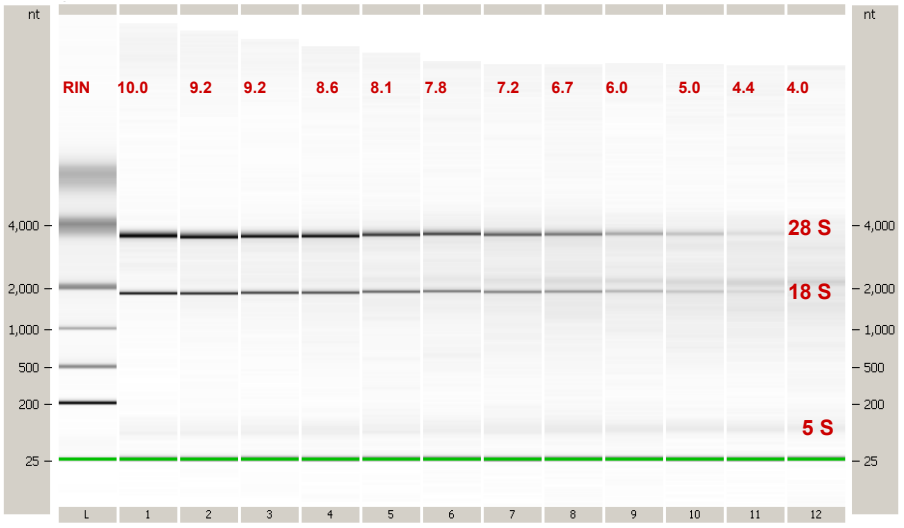
S. Fleige, et al., MAM 2006 & Biotechnolgy Letters 2006



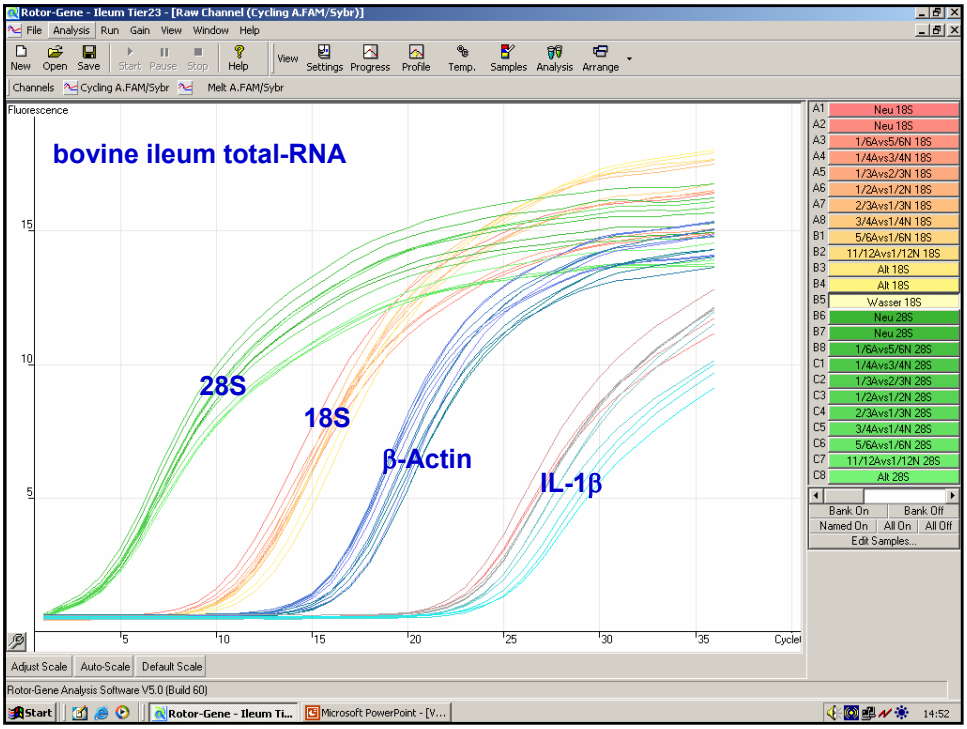

RIN in different tissues and cell lines
RIN
10
8
6
4
2
0
liver (n = 22)
heart (n =17)
spleen (n =17)
lung (n =22)
rumen (n =23)
reticulum (n =26)
omasum (n =17)
abomasum (n =17)
ileum (n =17)
jejunum (n =20)
colon (n =19)
caecum (n =16)
lymph node (n =26)
kidney cell (n = 3)
corpus luteum (n = 5)
granulosa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

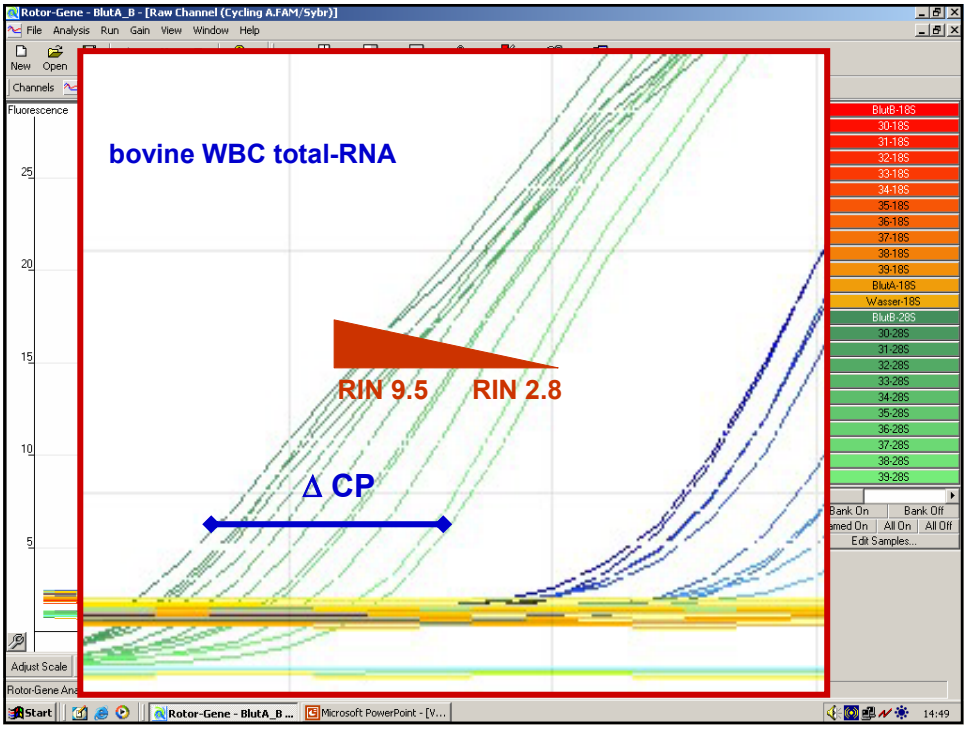

Degradation scale
Marker
WBC
RIN: 9.6
18 S
28 S
Marker
WBC
RIN: 2.8
18 S
28 S
total RNA extracted from bovine tissues
analyzed in Bioanalyzer
artificial degradation
degradation scale of mixtures between "good and bad" samples
analyzed in Bioanalyzer
S. Fleige, et al., MAM 2006 & Biotechnolgy Letters 2006

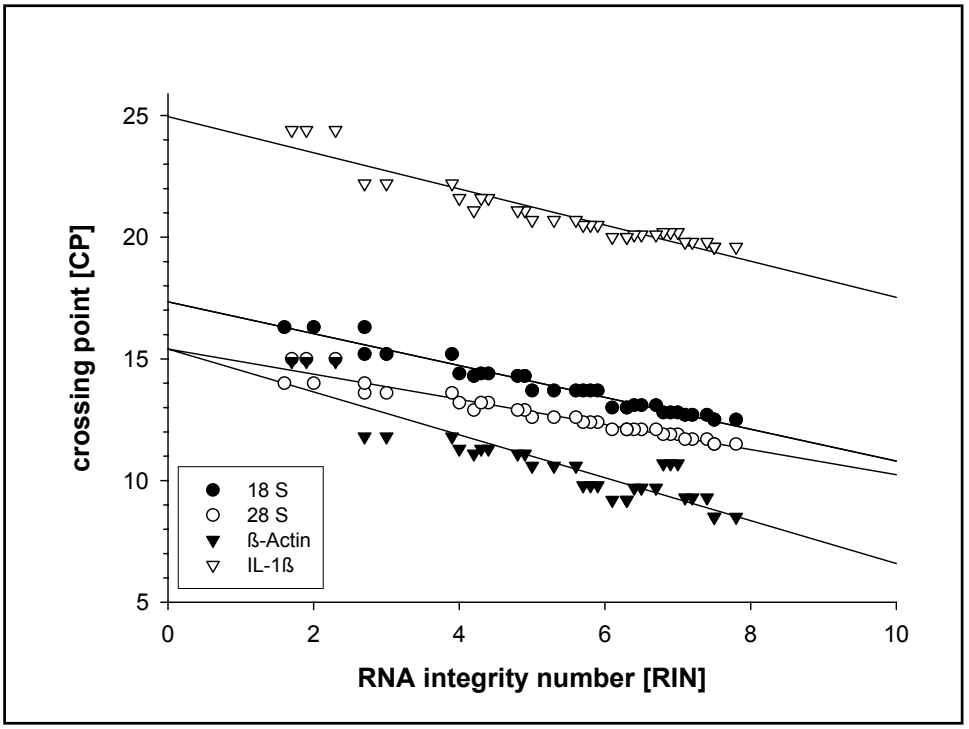
Degradation of extracted total-RNA



The intensity of bands decreases with increasing degradation




Rotor-Gene - BlutA_B - [Raw Channel (Cycling A.FAM/Sybr)]
File Analysis Run Gain View Window Help
New Open
Channels
Fluorescence
bovine WBC total-RNA
RIN 9.5
RIN 2.8
Δ CP
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


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

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)

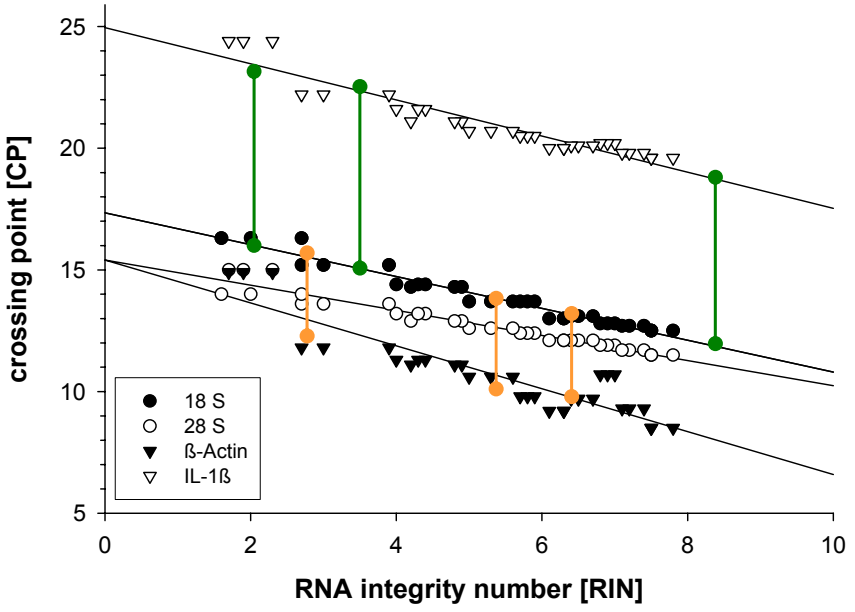
Relative quantification of gene expression

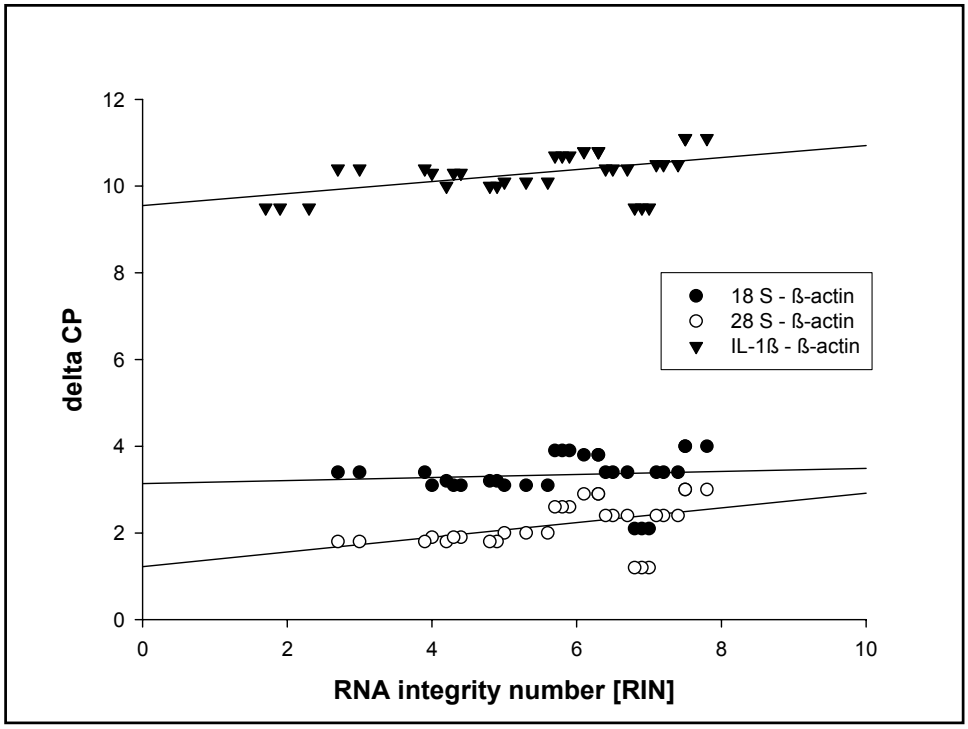
$$\Delta CP = CP_{\text{target gene}} - CP_{\text{refence gene}}$$

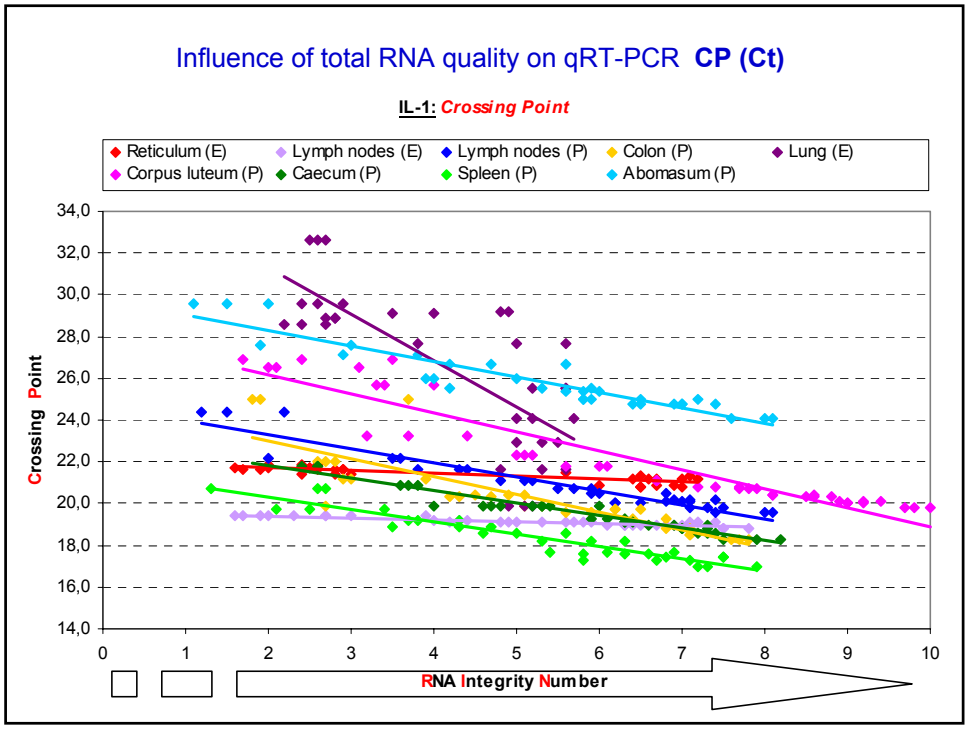
$$\text{expression ratio} = 2^{-[\Delta CP\ \text{treatment} - \Delta CP\ \text{control}]}$$

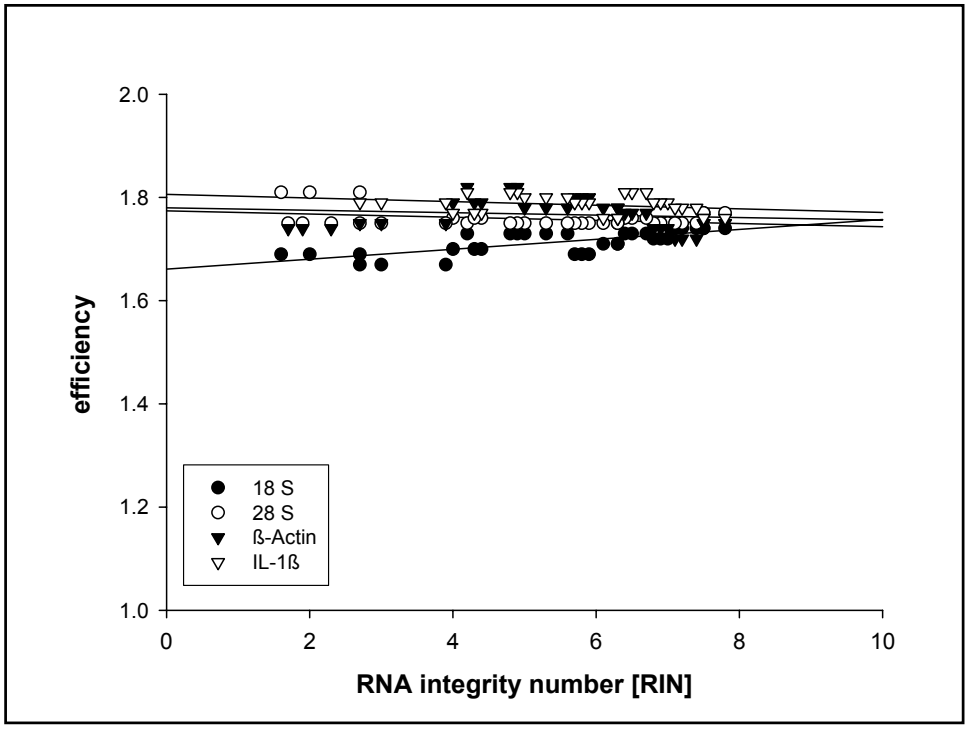
$$\text{expression ratio} = 2^{-\Delta\Delta CP}$$

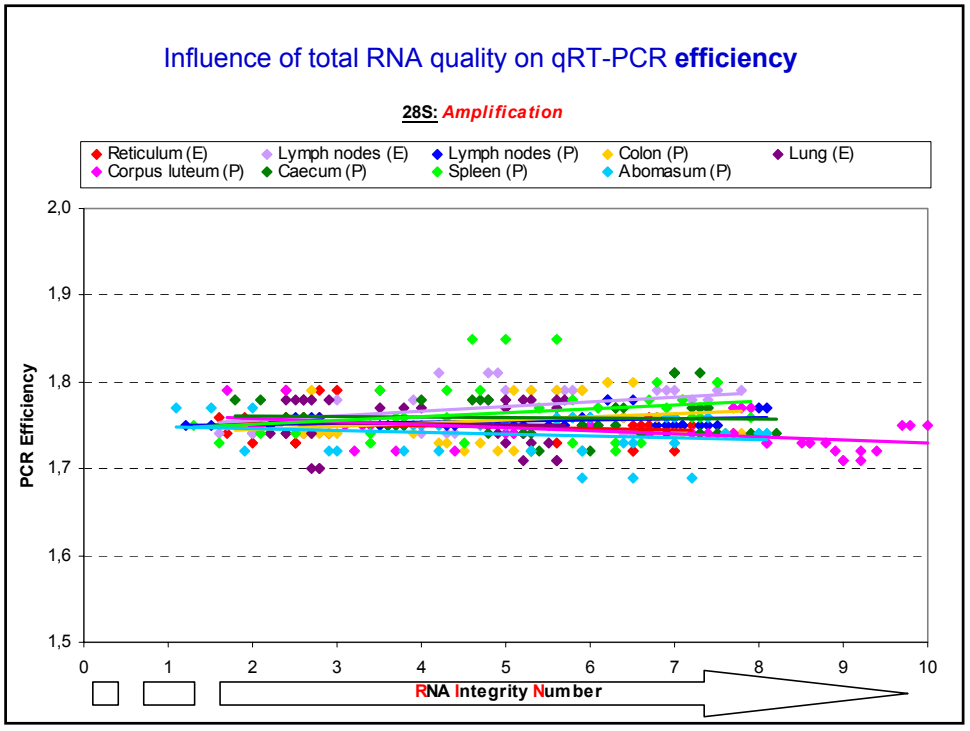
Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{[-\text{delta deltaC(T)}]}$ method.
Methods, 2001 ***25(4):*** *402-408.*



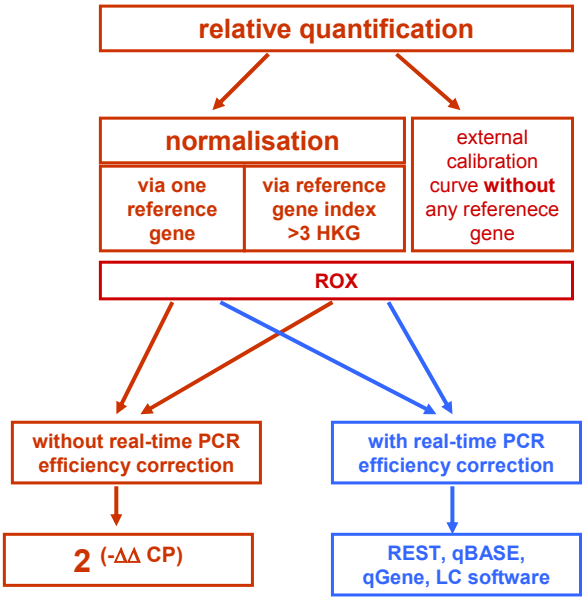

delta CP
RNA integrity number [RIN]
18 S - ß-actin
28 S - ß-actin
IL-1ß - ß-actin
0
2
4
6
8
10
12


Influence of total RNA quality on qRT-PCR CP (Ct)
IL-1: Crossing Point
Reticulum (E)
Lymph nodes (E)
Lymph nodes (P)
Colon (P)
Lung (E)
Corpus luteum (P)
Caecum (P)
Spleen (P)
Abomasum (P)
Crossing Point
RNA Integrity Number
34,0
32,0
30,0
28,0
26,0
24,0
22,0
20,0
18,0
16,0
14,0
0
1
2
3
4
5
6
7
8
9
10


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

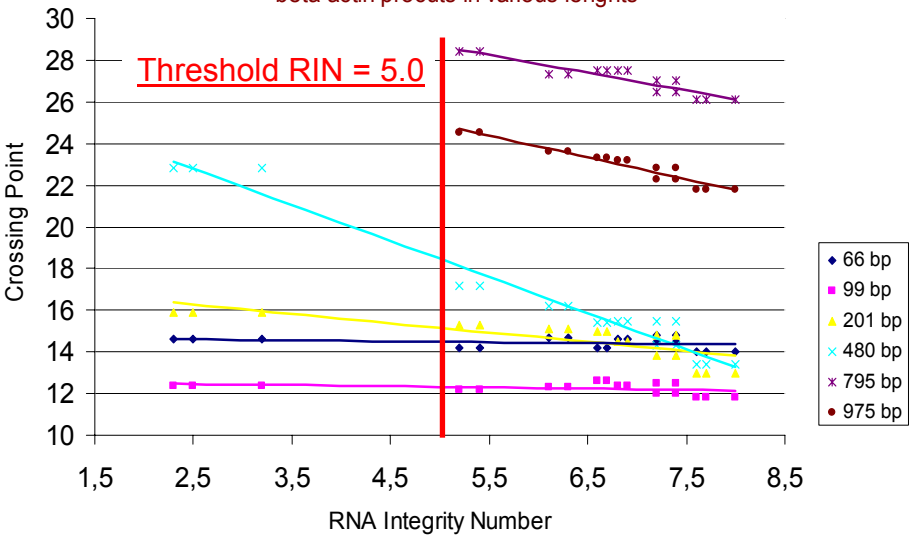

Influence of total RNA quality on qRT-PCR efficiency
28S: Amplification
Reticulum (E)
Lymph nodes (E)
Lymph nodes (P)
Colon (P)
Lung (E)
Corpus luteum (P)
Caecum (P)
Spleen (P)
Abomasum (P)
2,0
1,9
1,8
1,7
1,6
1,5
PCR Efficiency
0
1
2
3
4
5
6
7
8
9
10
RNA Integrity Number

Relative Quantification in real time qRT-PCR using an internal control for normalisation

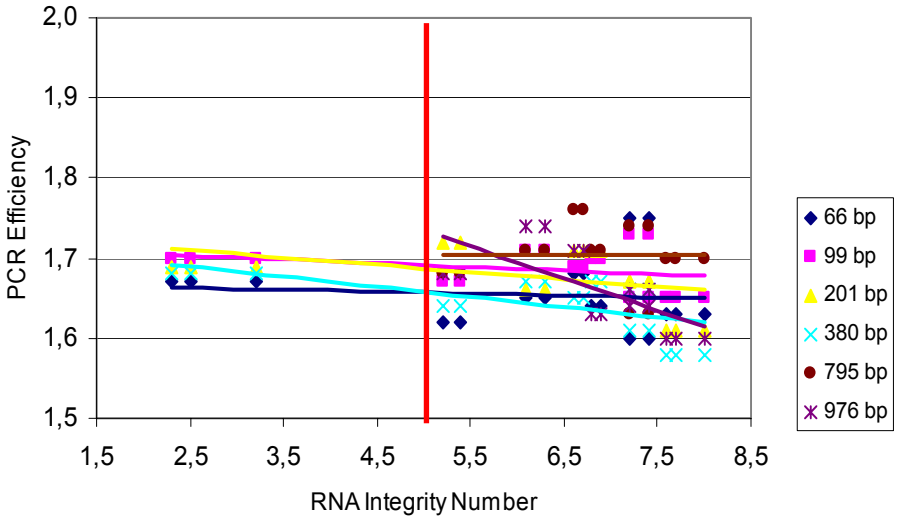


Influence of qRT-PCR product length on RIN

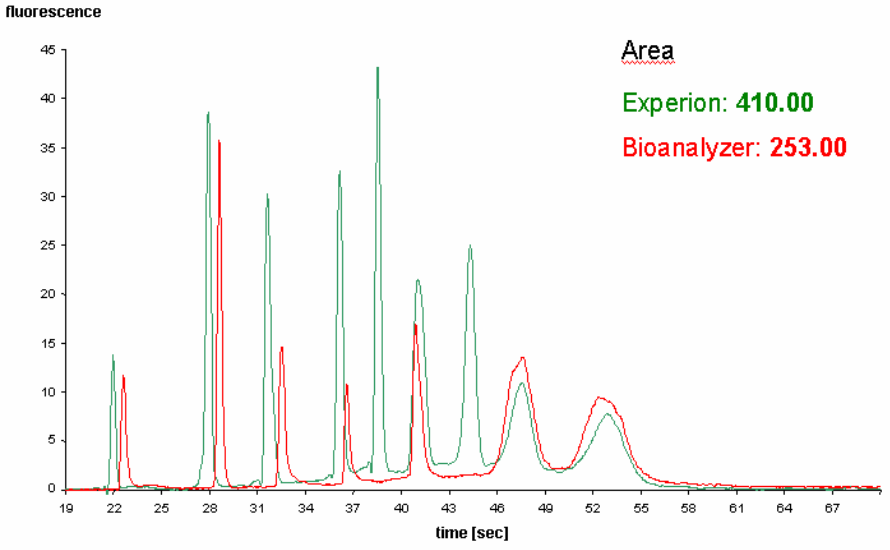
beta-actin procuts in various lenghts



PCR efficiency in dependence of RIN



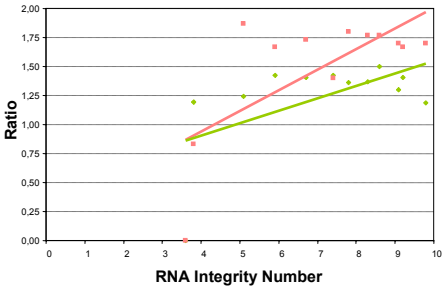
Comparison of Experion & Bioanalyzer 2100



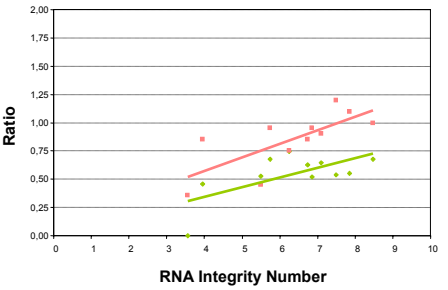
Comparison of 18S/28S rRNA ratio

Experion & Bioanalyzer 2100

Bioanalyzer 2100	Experion
y = 0.177x + 0.2346	y = 0.107x + 0.475
r^2 = 0.47	r^2 = 0.32



Bioanalyzer 2100	Experion
y = 0.1201x + 0.092	y = 0.085x + 0.005
r^2 = 0.53	r^2 = 0.43



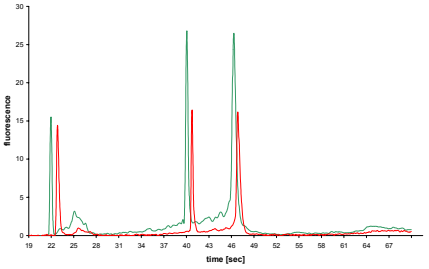
200 ng
n = 171

total RNA analysed

50 ng
n = 207

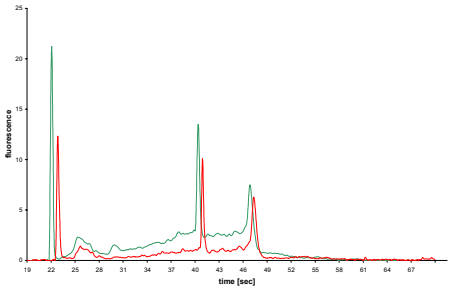
Run performance

Experion & Bioanalyzer 2100



Experion: 165.34 [71.47 ng/µl]
Ratio [28S/18S]: **0.93**
RIN: n.a.
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**
RIN: n.a.
Ladder Area: **----**

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

Variability

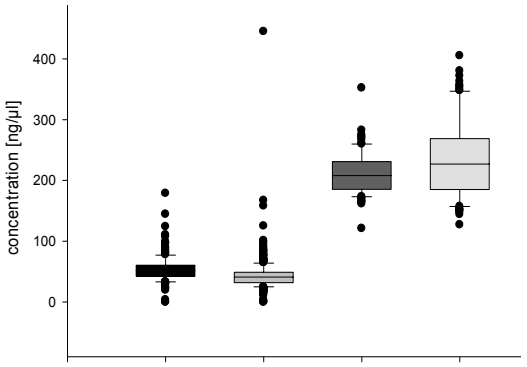
Experion & Bioanalyzer 2100

A: Experion (50 ng/µl)

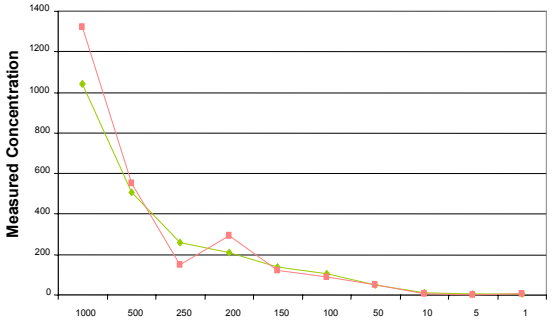
B: Bioanalyzer (50 ng/µl)

C: Experion (200 ng/µl)

D: Bioanalyzer (200 ng/µl)

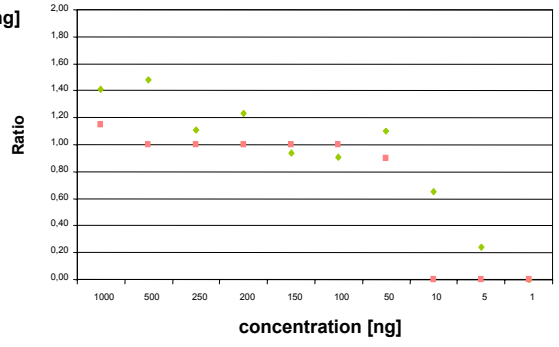


	A	B	C	D
mean [ng]	54.2	43.4	211.1	235.8
CV [%]	39.1	57.1	14.7	27.4
	n = 207 (50 ng)		n = 171 (200 ng)	



Sensitivity

Experion & Bioanalyzer 2100



Part 1 - Summary and Conclusion

- 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 mRNA abundancy [40% for low- and 75% for high abundant genes]

Part 2 - Summary and Conclusion

- qRT-PCR performance is dependent on **total-RNA quantity and quality !**
- RNA quality (= RIN value) 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 homogenization during extraction
- Total RNA classification using the RIN:
  - RIN > 8: perfect total-RNA
  - 5 < RIN < 8: good RNA **=> RIN threshold = 5**
  - RIN < 5: RNA quality is highly questionable
- Effects 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
  - minor influence on amplification efficiency
  - relative quantification using an internal control gene, performing the ΔCP approach, can partly circumvent the RIN problematic
- Tools to measure RNA integrity:
  - Bioanalyzer 2100 => Advantages in **RIN algorithm** & *"better"* 18S/28S ratio
  - Experion => Advantages in more sensitivity and less variability
  - mFOLD software => future studies !

Part 3 - Summary and Conclusion

CONCLUSION:

Pre-PCR analytical steps (sampling, extraction and reverse transcription) are HIGHLY VARIABLE and replicates should be done at the pre-PCR analytical level and not during later PCR reaction !

References:

- Fleige S. and Pfaffl M. W. (2006) **RNA integrity and the effect on the real-time qRT-PCR performance.** Molecular Aspects of Medicine **(27):**126-139
- Simone Fleige, Vanessa Walf, Silvia Huch, Christian Prgomet, Julia Sehm & Michael W. Pfaffl (2006) **Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR.** Biotechnology Letters **(28):** 1601-1613
- Mueller, O., Lightfoot, S., Schroeder, A., (2004) **RNA Integrity Number (RIN) – Standardization of RNA Quality Control.** Agilent Application Note, Publication 5989-1165EN, 1-8.
- Lightfood, S. (2002) **Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis, and UV spectrometry.** Agilent Application Note, Publication Number 5988-7650EN.
- Livak, K.J., Schmittgen, T.D., (2001) **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\text{Delta Delta C(T)})}$ Method.** Methods **25**, 402-408
- MW. Pfaffl (2001) **A new mathematical model for relative quantification in real-time RT-PCR.** Nucleic Acids Research 2001 **29 (9):** e45

Web resources:

- **http://www.gene-quantification.info/**
- **http://RNA-integrity.gene-quantification.info/**
- **http://relative.gene-quantification.info/**
- **http://REST.gene-quantification.info/**

Thank you team !

Thank you for your attention !