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mRNA & microRNA integrity - the key to success

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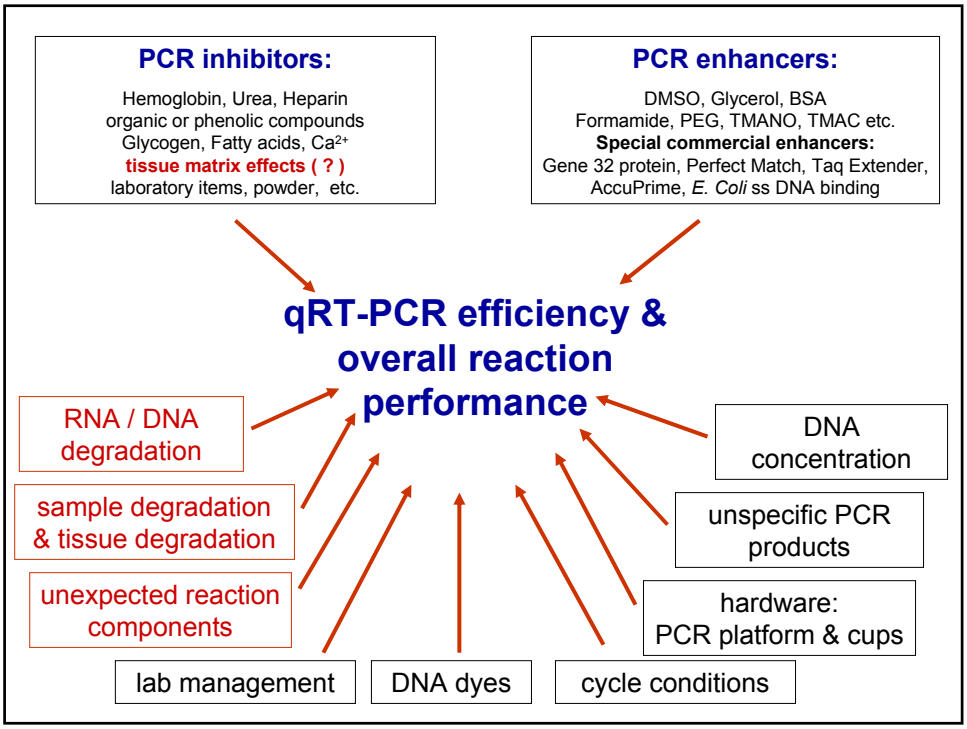
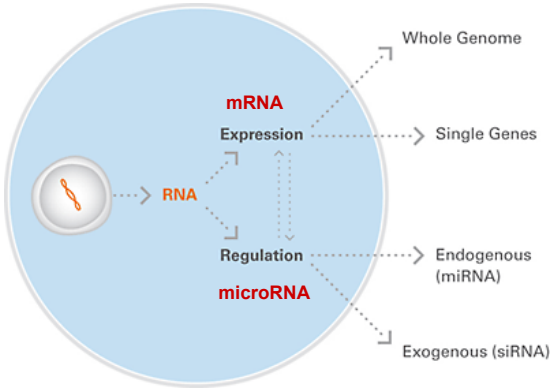




Q 1: Which is an appropriate quantification system?

Q 2: Impact on mRNA and/or microRNA "integrity" on real-time RT-PCR performance ?

Q 3: Impact of extraction procedure on overall RNA integrity ?

Q 4: Impact on quantitative result ?

Comparison of two platforms
Determination of RNA-Quality and RNA-Quantity
on the basis of their internal algorithms

RIN

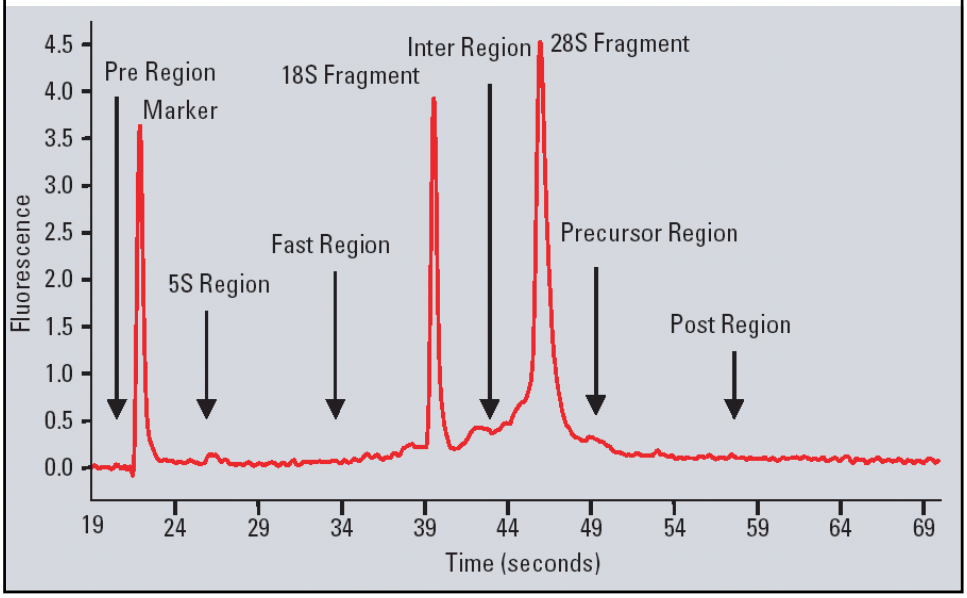
RNA Integrity Number

RQI

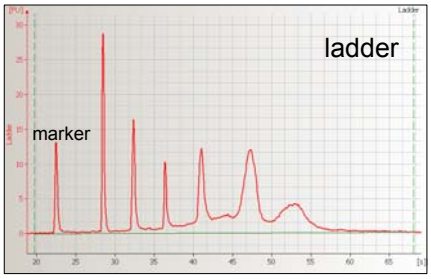
RNA Quality Indicator

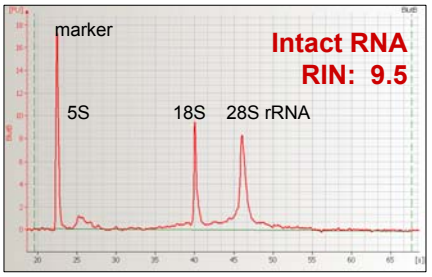
RNA quality control via. capillary electrophoresis

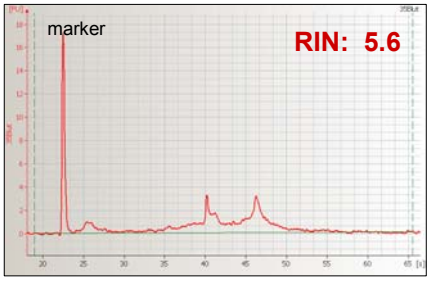
E-Gram & Electopherogram

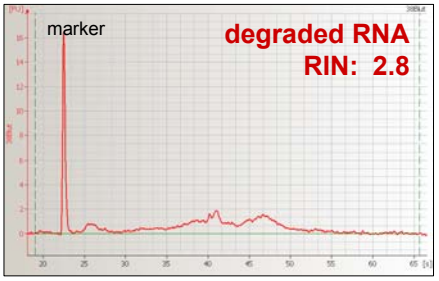


Various total-RNA qualities analysed in the Bioanalyzer 2100





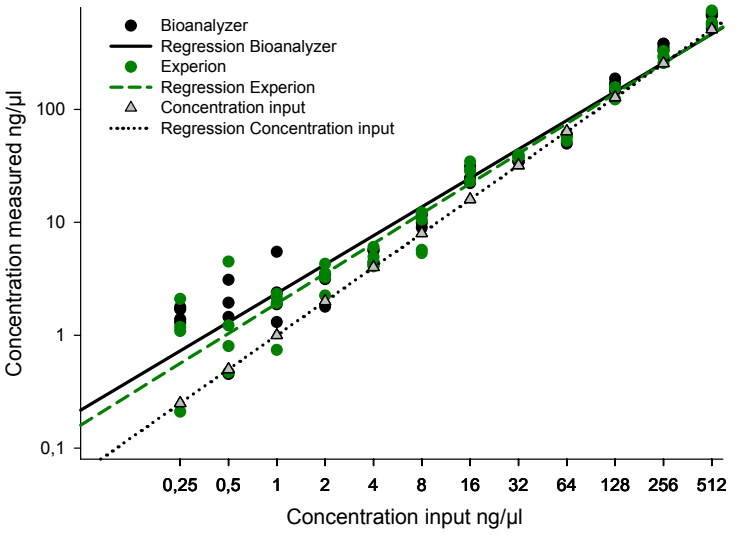




Fleige & Pfaffl, Mol Aspects Med 2006; Fleige, et al., Biotechnology Letters 2006

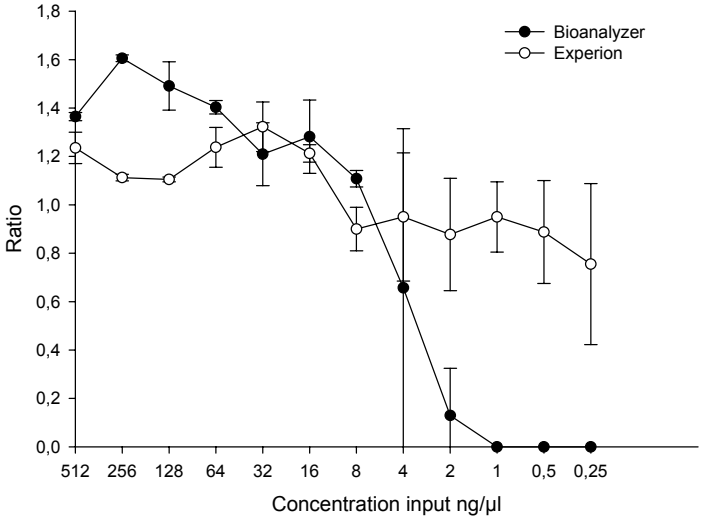
Linearity and sensitivity of quantification

total RNA input vs. RNA concentration measured

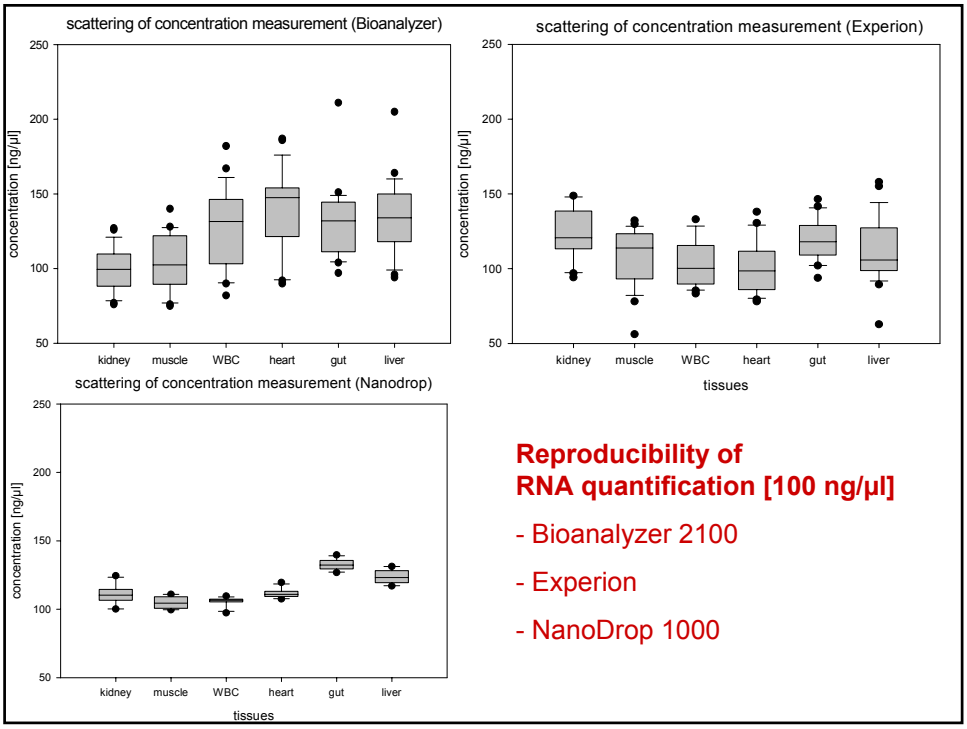


Pfaffl, Fleige, Riedmaier; BBE 2008

Linearity of 28S/18S rRNA ratio



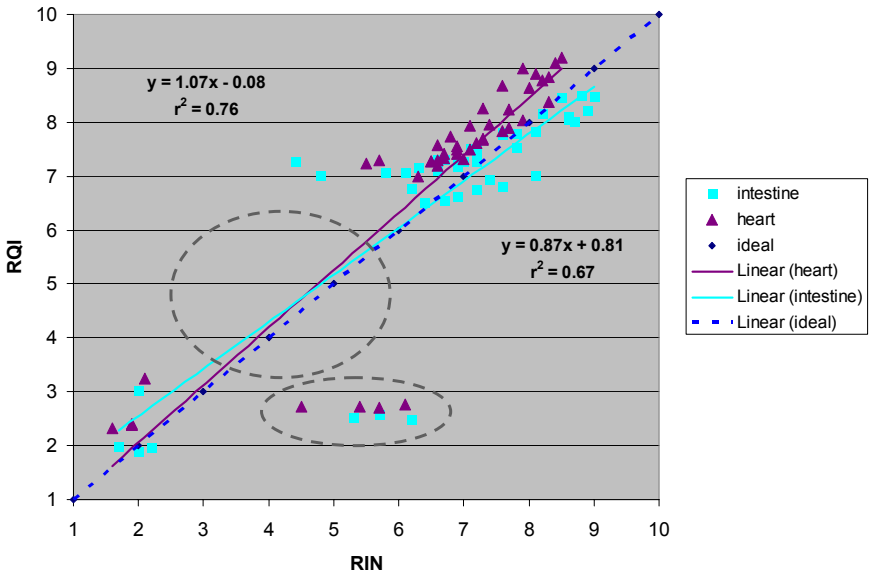
Pfaffl et al., BBE 2008



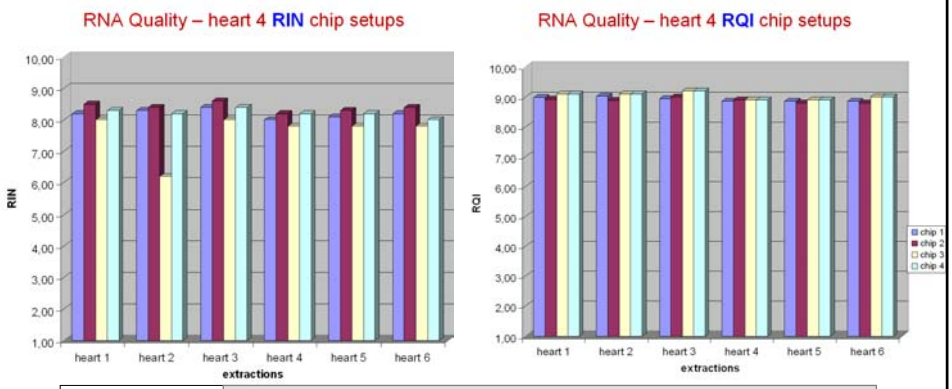
Reproducibility of RNA quantification [100 ng/µl]

- Bioanalyzer 2100
- Experion
- NanoDrop 1000

RNA integrity – 11 degradation steps in heart and intestine tissue

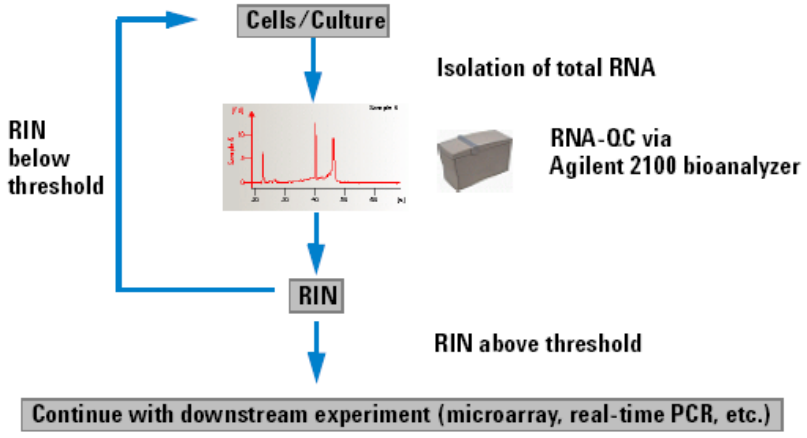


Influence of tissue (n=6), extraction (n=6), and chip setup (n=4) on RIN and RQI value



	p-value			
	RIN		RQI	
	extraction	chip run	extraction	chip run
kidney	<0.001	0.304	<0.001	0.925
muscle	<0.001	0.877	<0.001	0.170
blood	<0.001	0.163	<0.001	0.294
heart	<0.001	0.135	<0.001	0.530
intestine	<0.001	0.379	<0.001	0.900
liver	<0.001	0.052	<0.001	0.960

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

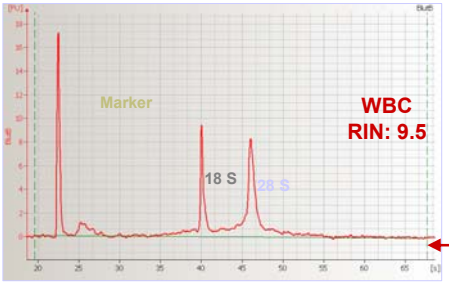


Q 1: Impact on **mRNA integrity** on real-time RT-PCR performance ?

Q 2: Impact on **microRNA integrity** on real-time RT-PCR performance ?

Q 3: Impact on quantitative result ?

Degradation scale



total RNA extracted from bovine tissues

analyzed in Bioanalyzer

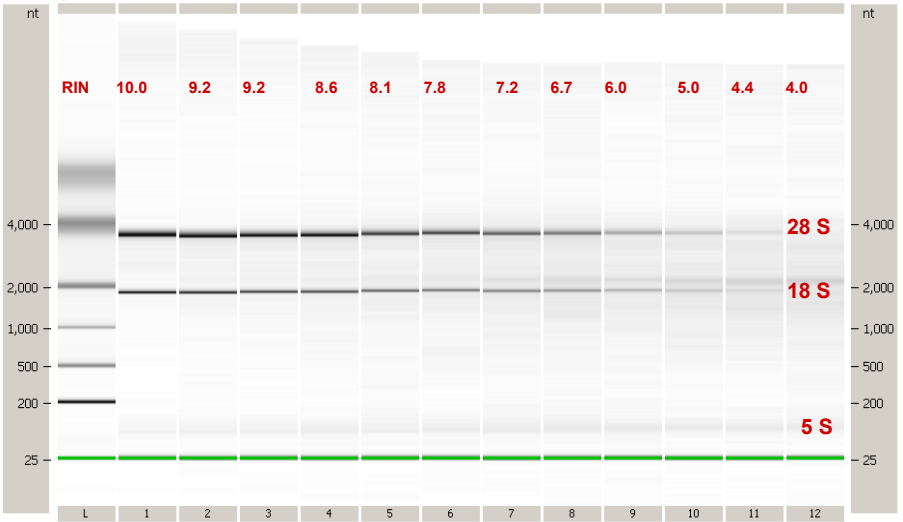
artificial degradation

degradation scale of mixtures between good and bad samples

analyzed in Bioanalyzer

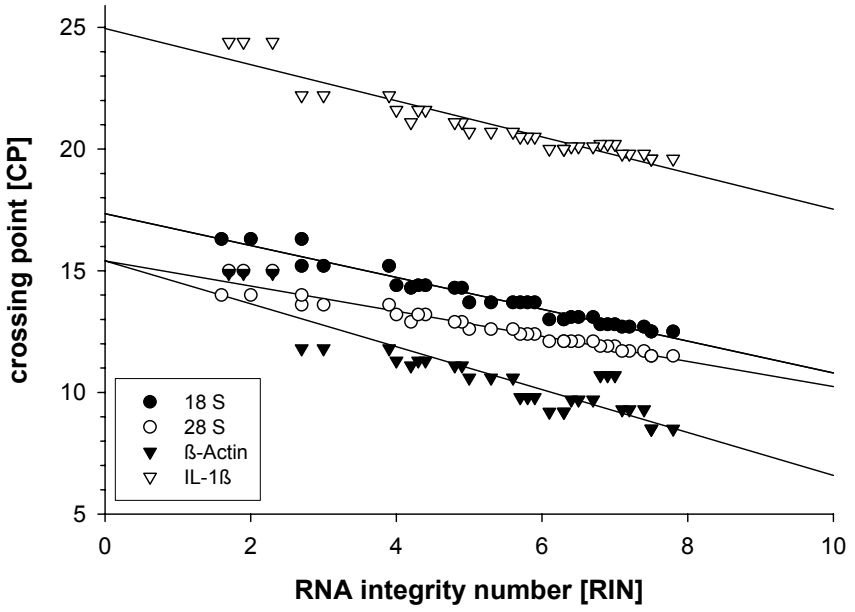
Fleige, et al., Biotechnolgy Letters 2006

Degradation of extracted total-RNA



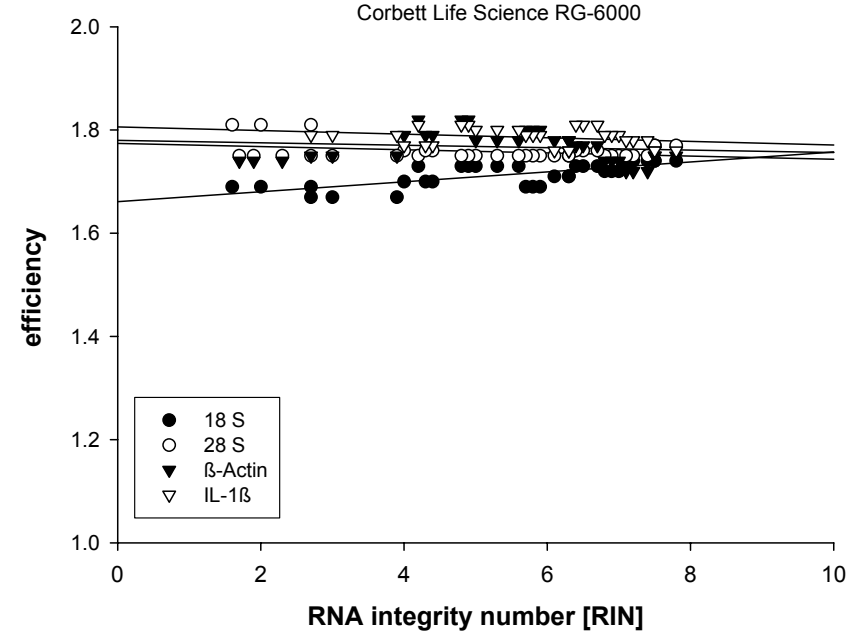
The intensity of bands decreases with increasing total-RNA degradation

Impact of RNA degradation on Cq value => mRNA quantification



Impact of RNA degradation on qPCR efficiency

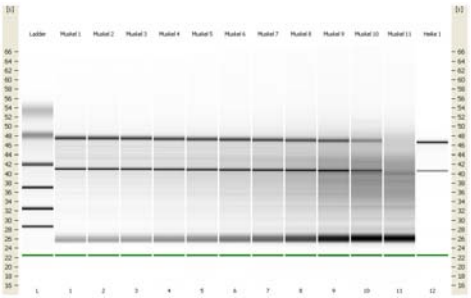
Corbett Life Science RG-6000



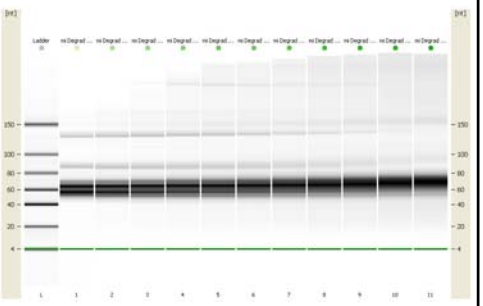
Effect of degradation in muscle RNA
in long RNA (> 200 nt) and small RNA (< 200 nt) fractions

11 artificial UV degradation steps
"one-tube" extraction procedure (Qiagen)

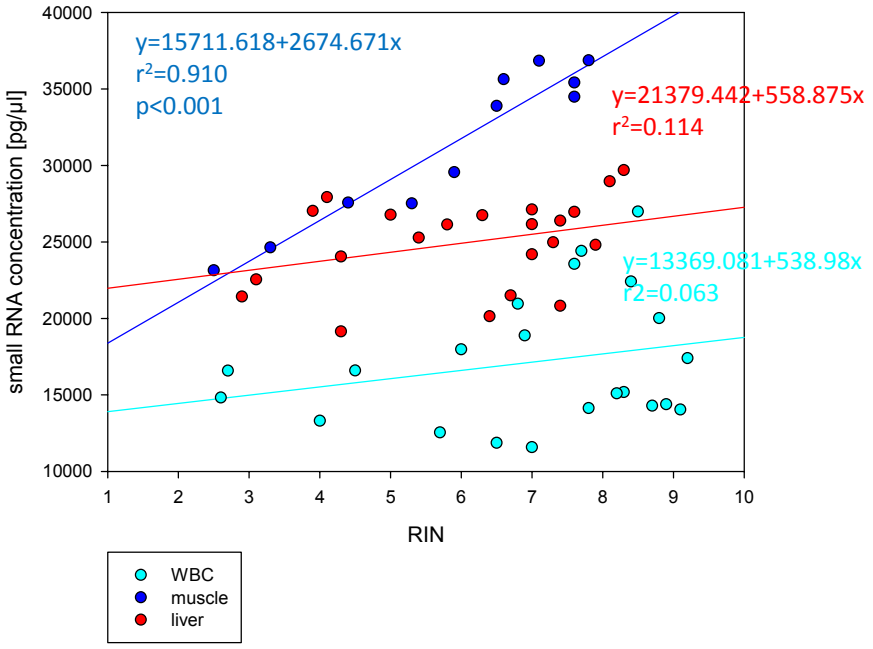
analysis of muscle long RNA
11 degradation steps on "NANO eukaryote RNA chip"



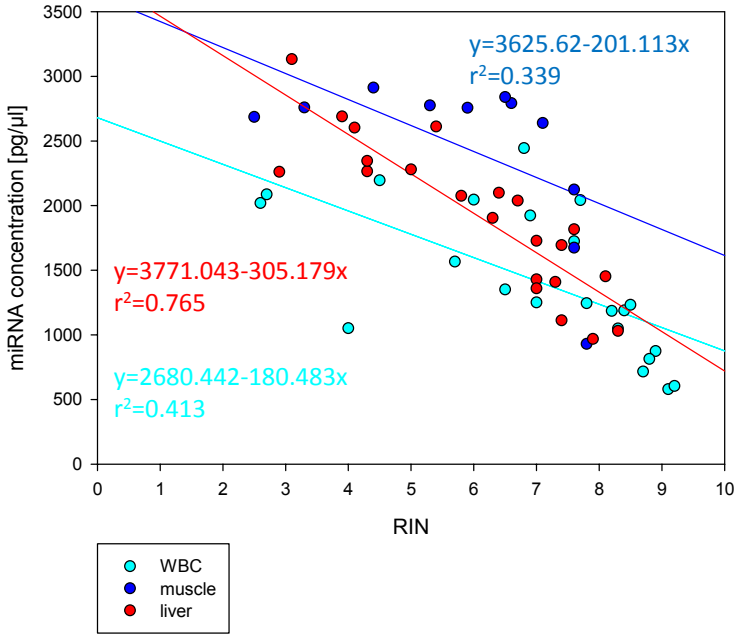
analysis of muscle small RNA
11 degradation steps on "Small RNA chip"



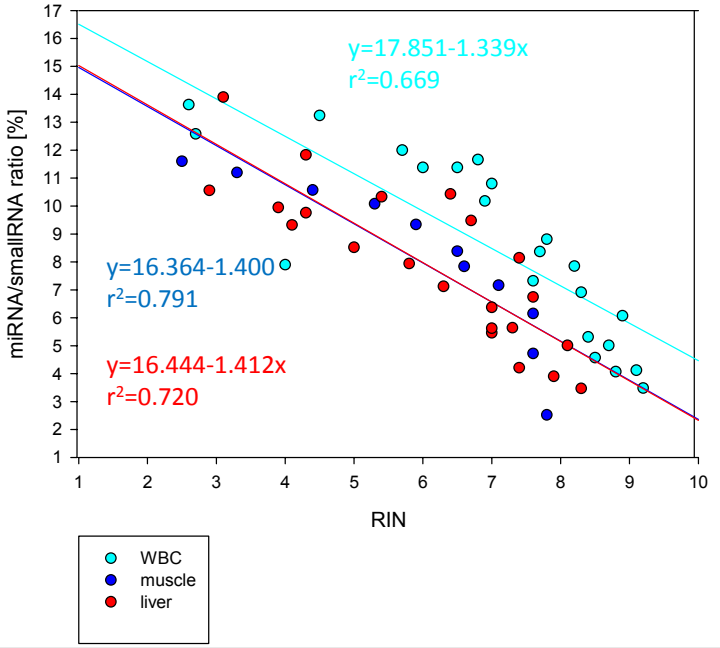
Influence on RIN value on small RNA quantity [pg/µl]

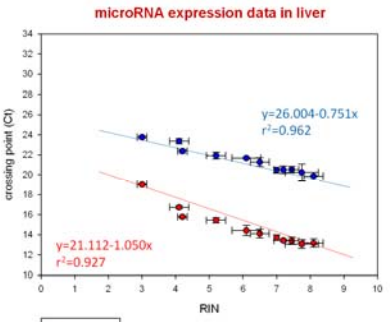


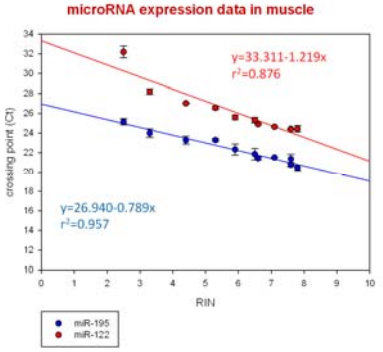
Influence on RIN value on microRNA quantity [pg/µl]

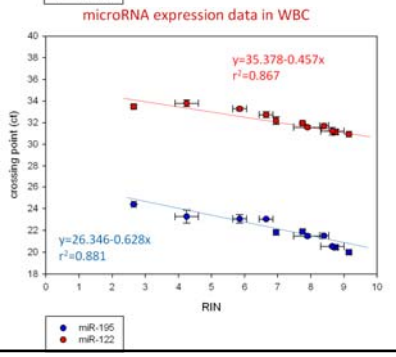


Influence on RIN value on microRNA quantity [as % of small RNA]



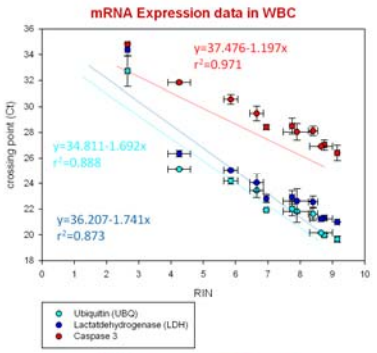


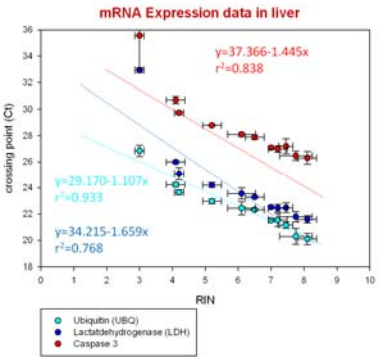


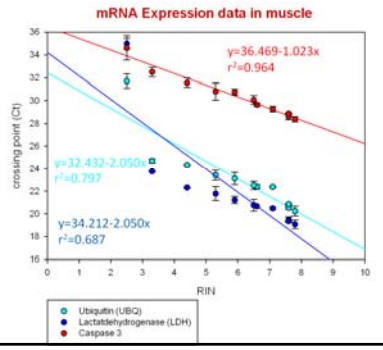


average slope = - 0.81

n = 6 p < 0.001







average slope = - 1.58

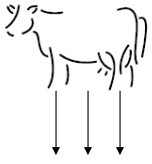
n = 9 p < 0.001

Which is the best blood sampling method for quantitative mRNA & microRNA analysis?

- Blood Sampling — Which method is the best and most consistent?
- Methods for mRNA & microRNA extraction:

5 animals

3 sampling



n =15

WB: Whole blood (350µl) (n=15)			Phenol based extraction method of total RNA
LY: Whole blood (9ml) (n=15)	→	WBC	
LL: Whole blood (9ml) (n=15)	→ WBC →		LeukoLOCK + mirVana isolation Kit of mRNA and miRNA in two fractions
PI: Whole blood (9ml) (n=15)	→ WBC →		miRNeasy Mini isolation Kit of mRNA and miRNA in two fractions
PAX: Whole blood (2,5ml) (n=15)	→		PAXgene Blood RNA isolation Kit of mRNA and miRNA in two fractions

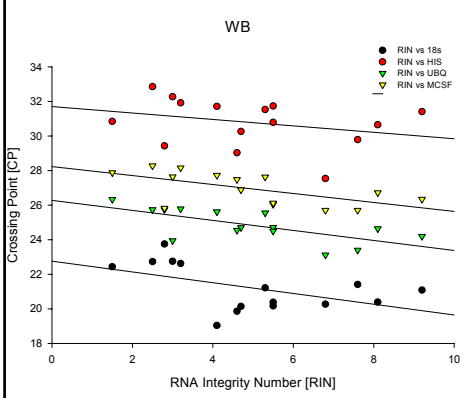
Results

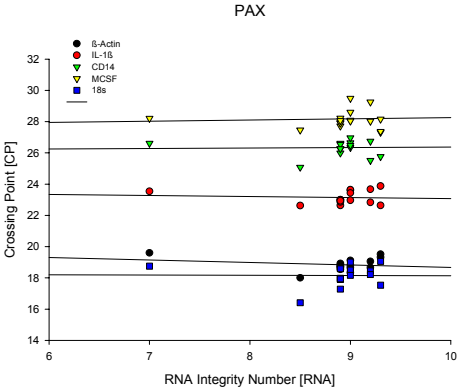
RNA Integrity & RNA yields

extraction method	total volume [ml]	RNA (> 200 nt)			RNA (< 200 nt)		
		µg/ml blood	260/280	RIN	µg/ml blood	260/280	miRNA %
LY	9	4.44±0.30	2.02±0.01	**9.45±0.04**	---	---	6.13 ± 0.27
PI	9	4.53±0.58	1.95±0.01	7.37±0.90	0.34±0.42	1.23±0.03	29.13±5.48
WB	0.35	14.32±1.10	1.71±0.01	4.96±0.58	---	---	NA
PAX	2.5	1.68±0.144	2.01±0.02	**8.87±0.14**	1.51± 0.18	1.18±0.10	6.33±1.74
LL	9	3.10±0.23	2.10±0.01	7.04±0.53	8.68 ± 0.73	1.57±0.04	NA

Results

qRT-PCR results and data analysis for WB and PAX method





Mean mRNA expression levels [Cq±sem]					
	LY	**PI**	**WB**	**PAX**	**LL**
18S rRNA	**17.28±0.16**	20.37±0.51***	21.22±0.350***	18.15±0.180	*22.24±0.956****
β-Actin	18.98±0.10	19.28±0.20	20.59±0.184**	**18.85±0.110**	*22.33±0.807****
UBQ	**22.26±0.20**	23.29±0.22**	24.84±0.243***	22.52±0.126	*24.97±0.507****
Histone	30.00±0.21	**27.98±0.53****	30.79±0.360	28.92±0.473	*33.13±0.625****
IL-1β	**22.34±0.09**	*27.67±0.41****	25.42±0.123***	23.15±0.111	25.96±0.704***
CD14	25.74±0.20	**25.37±0.13**	27.11±0.146***	26.34±0.147*	*27.75±0.406****
C3	**26.36±0.22**	27.80±0.23***	28.59±0.186***	27.29±0.179*	*28.63±0.501****
C1q	25.9±0,13	**24.99±0.14*****	26.34±0.198*	25.99±0.117	*27.44±0.305****
MCSF	28.26±0.14	**25.53±0.19*****	26.94±0.241***	28.17±0.149	*29.3±0.317****
NFκB	**24.77±0.11**	25.61±0.11*	26.09±0.075***	25.19±0.214	*26.78±0.486****
COX2	28.13±0.61	**25.43±0.96****	27.47±0.794	*31.04±0.552***	30.29±0.870*

Numbers in **bold** are equivalent to the **lowest mean Cq ± sem values** considering each gene and the different extractions;
Numbers in *italics* are equivalent to *the highest mean Cq ± sem values* considering each gene and the different extraction;
Significance comparing all methods in relation to the best method * for P<0.5 ** for P< 0.01 and *** for P<0.001 (n=15)

Mean miRNA expression levels [Cq±sem]					
	LY	**PI**	**WB**	**PAX**	**LL**
miR16	18.14±0.15	19.98±1.26	**15.59±0.24***	18.11±0.59	*24.81±0.85***
miR let7 a	**18.07±0.15**	21.16±1.21**	18.19±0.15	21.48±0.40***	*29.67±0.61***
miR 142	23.94±0.13	25.96±0.61**	25.77±0.30**	*26.15±0.64***	**23.24±0.56**
miR 181	22.59±0.11	25.02±1.24*	**21.41±0.23**	26.19±0.56***	*28.50±0.72****
miR 27b	**22.56±0.18**	25.55±0.67***	22.88±0.32	26.01±0.80***	*29.67±0.75****
miR 101	26.39±0.16	24.33±0.67***	**23.91±0.33*****	26.28±0.91	*27.57±0.62**
miR 145	**28.41±0.22**	30.72±0.66***	28.85±0.26	30.10±0.63*	*36.85±0.56****

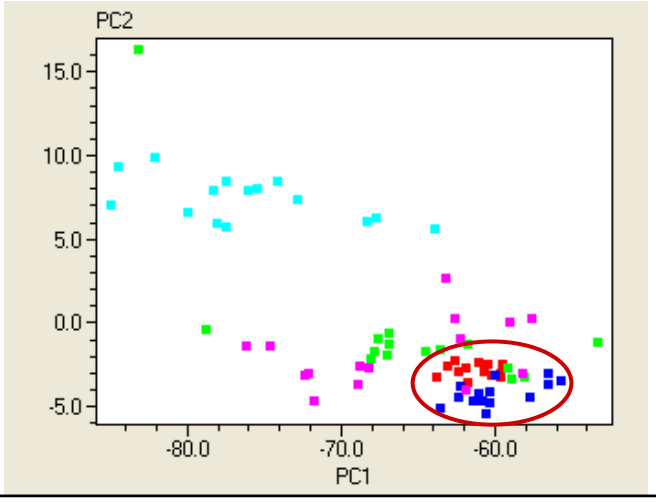
Numbers in **bold** are equivalent to the **lowest mean Cq ± sem values** considering each gene and the different extractions;
Numbers in *italics* are equivalent to *the highest mean Cq ± sem values* considering each gene and the different extraction;
Significance comparing all methods in relation to the best method * for P<0.5 ** for P< 0.01 and *** for P<0.001 (n=15)

	ANOVA statistics				
Source of Variation	**n / df**	**SS**	**MS**	**F**	**P**
RNA Yield					
Extraction method	5 / 4	1502.095	375.524	76.335	<0.001
Animal	5 / 4	74.203	18.551	3.771	0.013
Replicates	3 / 2	1.857	0.929	0.189	0.829
RNA Integrity Number (RIN)					
Extraction method	5 / 4	185.359	46.340	15.844	<0.001
Animal	5 / 4	24.563	6.141	2.100	0.104
Replicates	3 / 2	8.110	4.055	1.386	0.265
RNA Purity ($A_{260/280}$)					
Extraction method	5 / 4	1.501	0.375	250.519	<0.001
Animal	5 / 4	0.0157	0.00392	2.616	0.053
Replicates	3 / 2	0.000395	0.000197	0.132	0.877
miRNA Yield					
Extraction method	5 / 4	1165685.689	582842.844	185.523	<0.001
Animal	5 / 4	92742.585	23185.646	7.380	0.001
Replicates	3 / 2	7950.950	3975.475	1.265	0.309
[%] miRNA					
Extraction method	5 / 4	5244.400	2622.200	12.255	<0.001
Animal	5 / 4	626.978	156.744	0.733	0.583
Replicates	3 / 2	140.933	70.467	0.329	0.724

PCA = Principle component analysis

for small RNA < 200 nt

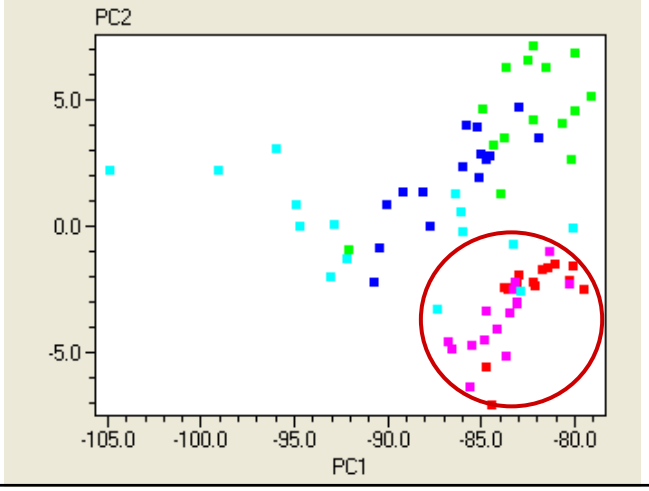
(PCA) has been conducted with all **microRNA target genes** and showed differences between the different extraction groups. LY (red dots) show the best results followed by WB (dark blue dots), PAX (pink dots), PI (green dots) and LL (light blue dots) which shows the most spread cluster.



PCA = Principle component analysis

for messenger RNA > 200 nt

PCA has been conducted with all mRNA target genes and showed differences between the different extraction groups. LY (red dots) show the best results followed by PAX (pink dots), PI (green dots), WB (dark blue dots) and LL (light blue dots) which shows the most spread cluster.



Summary & Conclusion

- Capillary electrophoresis is a perfect tool to measure RNA integrity:
  - Bioanalyzer 2100 => **RIN** algorithm
  - Experion => **RQI** algorithm
  - 18S/28S ratio is not appropriate for RNA quality control
- For RNA quantification Nano-Drop is recommended !
- qRT-PCR performance is dependent on **total-RNA quantity & quality !**
- RNA quality (RIN / RQI value) is highly **tissue dependent !**
  - good RIN [8-10] for cell cultures and WBC
  - lower RIN [5-8] for solid tissues, requiring more homogenization during extraction
  - RIN / RQI higher than 5 is recommended *(Fleige & Pfaffl, MAM 2006)*
- **mRNA & microRNA integrity** in an ONE tube extraction method:
  - mRNA & microRNA quality is extraction method dependent
  - mRNA & microRNA quality is highly reproducible within extraction replicates
- Effects of RNA integrity on **qRT-PCR results** in mRNA and microRNA quantification:
  - high significant influence on mRNA quantification (higher slope!)
  - high significant influence on microRNA quantification (lower slope!)
  - minor influence on amplification efficiency
  - relative quantification using an internal reference gene, performing the ΔCP approach, can partly circumvent the RNA integrity problematic for mRNA *(Fleige & Pfaffl, MAM 2006)*
  - Appropriate controls or database for microRNA reference genes are missing!

Thank you team !

Thank you for your attention !