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ORIGINAL RESEARCH PAPER

Validation of extraction methods for total RNA and miRNA from bovine blood prior to quantitative gene expression analyses

Andrea Hammerle-Fickinger · Irmgard Riedmaier · Christiane Becker · Heinrich H. D. Meyer · Michael W. Pfaffl · Susanne E. Ulbrich

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Abstract The benefit and precision of blood diagnosis by quantitative real-time PCR (qPCR) is limited by sampling procedures and RNA extraction methods. We have compared five different RNA extraction protocols from bovine blood regarding RNA and miRNA yield, quality, and most reproducible data in the qRT-PCR with the lowest point of quantification. Convincing results in terms of highest quantity, quality, and best performance for mRNA qPCR were obtained by leukocyte extraction following blood lysis as well as extraction of PAXgene stabilized blood. The best microRNA qPCR results were obtained for samples extracted by the leukocyte extraction method.

Keywords Blood · Bovine · miRNA · Molecular diagnosis · mRNA · qPCR

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A. Hammerle-Fickinger · I. Riedmaier · C. Becker · H. H. D. Meyer · M. W. Pfaffl · S. E. Ulbrich (✉)
Physiology Weihenstephan, Technische Universitaet Muenchen, Weihenstephaner Berg 3, 85354 Freising, Germany
e-mail: ulbrich@wzw.tum.de

Introduction

Quantitative real-time PCR (qPCR) has improved the efficiency for the quantification of gene expression levels revolutionizing molecular diagnosis (Bustin et al. 2005; Pfaffl 2001). Moreover, for quantitative assays not just mRNA but also RNAs with low-molecular weight**,** such as microRNAs (miRNAs), are of emerging importance in molecular diagnostic (Stefani and Slack 2008; Shivdasani 2006). Blood is a preferred tissue for diagnostic tests as it displays an accumulation of cellular information from all sections of the body and is easy to access. However, the quantification of gene expression in blood samples faces numerous challenges concerning the handling and preparation of the samples, which can negatively impact qPCR sensitivity and accuracy (Rainen et al. 2002).

Results of gene expression analyses are directly affected by RNA quantity and quality (Fleige et al. 2006). Factors such as blood collection procedure, RNA stabilization, RNA isolation and further downstream processes contribute to RNA integrity and consequently influence the quantitative RNA analysis (Fleige and Pfaffl 2006).

Different extraction methods are available for the isolation of mRNA and miRNA from blood. Depending on the extraction protocol used, a direct influence on RNA integrity and furthermore on the qPCR results is evident. Consequently, in this study five different mRNA and miRNA extraction methods

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were considered to compare efficiency, accuracy and sensitivity as critical points for performing qPCR diagnosis assays. To this end it was of interest to test which method showed the best mRNA and miRNA quality, with the highest yield of RNA and miRNA concentration and the lowest quantification cycle (Cq) with the most reproducible data in the qPCR results.

Materials and methods

Sample collection and storage

Blood samples were drawn from the jugular vein of five healthy, 24 month old Brown Swiss heifers. Animals belonged to the same herd and obtained the same feeding regime. PAXgene blood RNA tubes (BD, Germany) and Vacuette evacuated blood collection tubes (Greiner Bio-one, Frickenhausen, Germany) were used to collect samples from each animal. For all extraction methods samples were collected in triplicates at the same time point from each animal. All blood collection tubes were gently inverted five times for mixing directly after collection and before storage. EDTA tubes were immediately stored on ice until further process and PAXgene RNA tubes were incubated at room temperature for two hours and stored at −20°C. All experiments were conducted with permission from the local veterinary authorities and in accord with accepted standards of Humane Animal Care.

Sample processing and RNA extraction

For each RNA extraction method blood samples were collected in triplicates from 5 animals ($n = 15$). A summary of all extraction methods is shown in Table 1.

As cost and time-effective method total RNA was extracted from whole blood (WB) or from leukocytes obtained after alkaline lysis of erythrocytes (LY). Three other methods (LL, PI, PAX) were performed using commercially available kits for the extraction of mRNA and miRNA in two separated fractions.

Extraction of total RNA from whole blood after blood collection (WB)

In a 2 ml centrifuge tube (Eppendorf) 350 µl of whole blood were added to 700 µl TriFast reagent (Peqlab Biotechnologie, Erlangen, Germany), mixed for 10 s and stored at −80°C. Total RNA was isolated by a standardized phenol based extraction method according to the manufacturer's instructions (for details see supplementary data).

Extraction of total RNA from leukocytes after lysis of erythrocytes (LY)

The isolation of leukocytes or white blood cells (WBC) from 9 ml whole blood collected in EDTA tubes was done by alkaline lysis of erythrocytes. The whole blood was diluted 1:1 (v/v) with lysis buffer (8.3 g NH_4Cl; 0.037 g Na-EDTA; 1 g KCl in 100 ml

Table 1 Summary of the five blood extraction methods evaluated. For the two phenol based extraction methods (LY and WB) total RNA was isolated in one fraction

Method applied	Equipment necessary before RNA extraction	Length of procedure before RNA extraction or storage (min)	RNA stabilization	Cell types isolated	RNA extraction methodology	Obtained mRNA and miRNA (fractions)
WB	Vortex mixer	5	No	RBC WBC	Phenol based extraction	1
LY	Swinging bucket centrifuge	40–50	No	WBC	Phenol based extraction	1
LL	–	5	No	WBC	mirVana isolation kit	2
PI	Swinging bucket centrifuge	10–20	No	RBC WBC	miRNeasy Mini isolation kit	2
PAX	–	120	Yes	RBC WBC	PAXgene blood RNA isolation kit	2

For LL, PI and PAX methods mRNA and miRNA were isolated in two separated fractions. WB, PI and PAX extraction methods sampling contain RBC and WBC and LY and LL extraction methods sampling just WBC

water pH 7.4) and centrifuged for 10 min in 50 ml reaction tubes at 1,000 rpm and 4°C. Supernatants were discarded, the cell pellet was resuspended twice in lysis buffer and centrifugation was repeated. The WBC were suspended in a vial containing 1 ml TriFast reagent and ceramic beads, subjected to mechanical homogenization in the MagNA Lyzer instrument (Roche) for 20 s at 6,000×*g* and stored at −80°C. Total RNA was extracted from samples according to the manufacturer's instructions (for details see supplementary data).

Extraction of mRNA and miRNA from leukocytes stabilized by LeukoLOCK (LL)

The isolation of the leukocyte population was done immediately after blood collection of 9 ml whole blood in an EDTA tube using the LeukoLOCK Total RNA Isolation System (Applied Biosystems). RNA-later (Applied Biosystems) was used to stabilize the total RNA of cells captured in the filter. The filters were kept on ice and then frozen at −20°C within 1 h after collection. The isolation of mRNA and miRNA in two different fractions was performed using the mirVana miRNA isolation Kit (Applied Biosystems) following the manufacturer's instructions (for details see supplementary data).

Extraction of mRNA and miRNA from leukocytes after blood fractionation (PI)

RNA was isolated from leukocytes obtained from the plasma interphase of coagulated centrifuged blood. EDTA stabilized blood (9 ml) was centrifuged for 10 min at 2,500×*g* and 4°C. The plasma was removed and the WBC were collected with a pipette (∼500 µl), suspended in a vial containing 700 µl Qiazol reagent (Qiagen) and stored at −80°C. The isolation of mRNA and miRNA in two different fractions (<200 nt and >200 nt, respectively) was performed using the miRNeasy Mini kit (Qiagen) following the manufacturer's instructions (for details see supplementary data).

Extraction of mRNA and miRNA from whole blood collected in PAXgene tubes (PAX)

The isolation of miRNA and mRNA was performed in two different fractions from blood samples collected in PAXgene blood RNA tubes (2.5 ml) (BD). For the extraction the PAXgene Blood RNA Kit (Qiagen) was used employing an amended version of the manufacturer's guidelines as previously described by (Kruhoffer et al. 2007) (for details see supplementary data).

RNA concentration and quality determination

Extracted amounts of total RNA, mRNA and miRNA were quantified using the NanoDrop 1000 (Peqlab Biotechnologie, Germany). RNA integrity and quality control were additionally measured for all samples via automated capillary electrophoresis using the Eukaryotic total RNA Nano assay on the 2100 Bioanalyzer (Agilent). The 2100 Bioanalyzer calculates the RNA integrity number (RIN) ranging from 1 being the most degraded profile to 10 being the most intact. In addition, RNA integrity was also evaluated by analysis of the electropherogram obtained. For the small RNA fraction the integrity of a sample can not be evaluated by a RIN number. In this case an analysis of the miRNA percentage in the small RNA fraction can be measured using the Small RNA Assay on the 2100 Bioanalyzer. The software calculates the miRNA percentage as a ratio of the miRNA concentration existent in total small RNA.

Quantification of mRNA by qPCR analysis

Quantitative qPCR was performed to evaluate the influence of the different extraction methods on the mRNA expression. Therefore a two-step qPCR was performed where 0.5 µg of either total RNA or RNA >200 nt were reverse transcribed to cDNA in a 60 µl reaction volume containing 12 µl 5× Buffer (Promega, Mannheim, Germany), 3 µl Random Primers 50 mM (Invitrogen, Carlsbad, USA), 3 µl dNTPs 10 mM (Fermentas, St Leon-Rot, Germany) and 1 µl 200 U of MMLV H- Reverse Transcriptase (Promega). A negative control was added without enzyme for excluding DNA contamination. Reverse transcription was performed in a single reaction for each sample extracted.

Gene expression of 11 genes was quantified using the Mastercycler ep realplex (Eppendorf). The following primers were used for amplification: *18S rRNA* (for. 5′-AAGTCTTTGGGTTCCGGG, rev. 5′-GGACATCTAAGGGCATCACA), *ACTB*

(for. 5′-AACTCCATCATGAAGTGTGAC, rev. 5′-GATCCACATCTGCTGGAAGG), *H3F3A* (for. 5′-AC TGCTACAAAAGCCGCTC, rev. 5′-ACTTGCCTCC TGCAAAGCAC), *UBQ3* (for. 5′-AGATCCAGGA TAAGGAAGGCAT, rev. 5′-GCTCCACCTCCAGG GTGAT), *IL1B* (for. 5′-TTCTCT CCAGCCAACC TTCATT, rev. 5′-ATCTGCAGCTGGATGTTTCC AT), *CD14* (for. 5′-GCA GCC TGGAACAGTTT CTC, rev. 5′-TCCTCAAGCGTCAGTTCCTTG), *C3* (for. 5′-AAGTTCATCACCCACATCAAG, rev. 5′-CACTGTTTCTGGTTCTCCTC), *C1Q* (for. 5′-ATTG AAAGGCACCAAAGGC, rev. 5′-TTCTGGTACAC GTTCTCCTGG), *CSF1* (for. 5′-CTCCCTCTTGCC CAGAGAG, rev. 5′-ACGTCTTCCATCCCAGTG AC), *NFKB1* (for. 5′-CCCGAGGCTCTTTTTCAC AAG, rev. 5′-GTCTGGCAAGTACTGGAATTCC), *PTGS2* (for. 5′-GCCAGGGGAGCTACGACTA, rev. 5′-AAGGACAATGGGCATGAAACTGTG).

Quantitative PCR was performed using the 2.5 × 5 PRIME qPCR MasterMix Kit (5 PRIME, Hamburg, Germany). The predicted size of PCR products was verified by a high resolution agarose gel electrophoresis after ethidium bromide staining (data not shown). A negative control was included by measuring water instead of cDNA. Automated pipetting was done by the robot system epMotion 5075 (Eppendorf).

The following general real-time PCR protocol was employed: denaturation (95°C, 2 min), cycling program [40 cycles: 95°C denaturation (5 s); 60°C annealing (10 s); 68°C elongation (20 s)] and finally a melting curve analysis. The Cq value of a sample was set at the cycle number at which the fluorescence signal intersected with the threshold. This value was determined automatically by the CalQplex realplex software (Version 1.5, Eppendorf) and was significantly above the noise of the baseline.

Quantification of miRNA by qPCR analysis

Quantitative PCR of specific miRNA was performed to evaluate the integrity of small RNA (<200 nt) achieved by the different extraction methods. Therefore a two-step qPCR was performed where either 0.125 µg purified small RNA (LL, PI, PAX) or 0.5 µg total RNA (WB. LY) were first reverse transcribed to cDNA in a 10 µl reaction volume using the miScript Reverse Transcription Kit (Qiagen). As negative control enzyme was omitted for excluding DNA contamination of the RNA. Reverse transcription was performed in a single reaction for each sample extracted.

To analyze miRNA gene expression qPCR was performed using the miScript SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. For the qPCR reaction a further negative control was included by measuring one sample containing water instead of cDNA. Pipetting was done by the epMotion 5075 robot system (Eppendorf). The following general real-time PCR protocol was employed: denaturation (95°C, 15 min), cycling program [40 cycles: 94°C denaturation (15 s); 55°C annealing (30 s); 70°C elongation (30 s)], and melting curve analysis. The Cq value of each sample was determined automatically by the CalQplex realplex software (Eppendorf). The predicted size of PCR products was assessed via capillary electrophoresis in the Experion system (Bio-Rad Laboratories, Hercules, USA).

The following miRNAs were quantified: *MIR let-7a*, *MIR 27b*, *MIR 101*, *MIR 145*, *MIR 142*, *MIR 181a,* and *MIR 16*. The sequences of these miRNAs in various species are published at miRBase (http://microrna.sanger.ac.uk/sequences/). The primer homology to bovine was controlled with the "Basic Local Alignment Search Tool" (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and miRNA primer were commercially synthesized (Qiagen).

Data analysis and statistics

The statistical analysis was conducted using SigmaStat v. 3.0 software (SPSS Inc., Chicago, IL). For the relative quantification of mRNA the Cq values of the target genes (TG) were normalized against the chosen reference gene (RG) according to Livak and Schmittgen method (Schmittgen and Livak 2008). For mRNA and miRNA gene expression results means were compared by *t* test, two-way and three-way ANOVA. All pairwise multiple comparison procedures were done by the Holm-Sidak method. Results were considered as statistically significant at $P < 0.05$.

To analyze the relationship between the different extraction methods a principal component analysis (PCA) was employed using GenEx v. 4.3.6 (MultiD Analyses AB, Gothenburg, Sweden).

Results

RNA quantity

The amounts of total RNA, mRNA and miRNA measured varied with respect to the different extraction approach. The results are depicted in Table 2. Considering all five extraction methods of total RNA/mRNA a three-way ANOVA test was evaluated (Table 2).

RNA quality

Significant differences in the mean $A_{260/280}$ ratios and in the mean RIN values among the different methods were observed (Tables 2 and 3). For extraction of total RNA/mRNA the statistical analysis obtained by a three-way ANOVA is shown in Table 3. Electropherograms of total RNA from all samples of LY, PI, PAX, and LL extraction methods had a comparable profile, with no undefined peaks. By contrast, all samples of WB extraction method had electropherograms with unexpected peaks.

For the small RNA fraction it was not possible to quantify the relative amount of miRNA obtained for WB and LL methods. For LY, PI, and PAX the results obtained by a three-way ANOVA are shown in Table 3. In addition to these results, an analysis of each small RNA electropherogram showed that samples extracted by the LY and the PAX methods had a uniform profile with no unexpected peaks. For the other methods considered the electropherograms show unexpected peaks for all samples.

The results obtained for the determination of the mRNA and miRNA quality as mean RIN number ± SEM versus mean miRNA% ± SEM for each extraction method are summarized in Fig. 1. To obtain a high quality of mRNA and miRNA for the same sample the LY extraction method gives the best results.

qPCR results of mRNA quantification and data analysis

Different gene transcript abundances of mRNA were quantified showing the best mRNA qPCR performance for samples extracted by the LY and PAX methods. The results shown in Table 4 are expressed comparing the methods PI, WB, PAX, and LL to the LY method, which was considered to give the lowest Cq with the least standard error of the mean (SEM). For each extraction method a PCA was employed

Table 2 RNA quantity, RNA purity, RNA integrity number (RIN) of large RNA (>200 nt) small RNA (<200 nt)

Extraction method	Total volume (ml)	RNA (>200 nt)		
		µg/ml blood	260/280	RIN
LY	9	4.44 ± 0.30	2.02 ± 0.01	9.45 ± 0.04
PI	9	4.53 ± 0.58	1.95 ± 0.01	7.37 ± 0.90
WB	0.35	14.32 ± 1.10	1.71 ± 0.01	4.96 ± 0.58
PAX	2.5	1.68 ± 0.14	2.01 ± 0.02	8.87 ± 0.14
LL	9	3.10 ± 0.23	2.10 ± 0.01	7.04 ± 0.53
Extraction method	Total volume (ml)	RNA (<200 nt)		
		µg/ml blood	µg/ml blood	µg/ml blood
LY	9	–	–	–
PI	9	0.34 ± 0.04	0.34 ± 0.04	0.34 ± 0.04
WB	0.35	–	–	–
PAX	2.5	1.51 ± 0.18	1.51 ± 0.18	1.51 ± 0.18
LL	9	8.68 ± 0.73	8.68 ± 0.73	8.68 ± 0.73

For the two phenol based extraction methods (LY and WB) total RNA was isolated and therefore no absolute quantification of small RNA using the NanoDrop 1000 could be evaluated, only the relative estimation of the Bioanalyzer. Results are presented as the mean values ± SEM for triplicate samples from five animals

NA for not available

Table 3 Three-way ANOVA results of analysis of RNA characteristics

Source of variation	ANOVA statistics				
	df	SS	MS	F	P
RNA yield					
Extraction (LY, PI, WB, PAX and LL)	4	1502.09	375.52	76.33	<0.001
Animal	4	74.20	18.55	3.77	0.013
Replicates	2	1.85	0.92	0.18	0.829
RNA integrity number (RIN)					
Extraction (LY, PI, WB, PAX and LL)	4	185.35	46.34	15.84	<0.001
Animal	4	24.56	6.14	2.10	0.104
Replicates	2	8.11	4.05	1.38	0.265
RNA purity ($A_{260/280}$)					
Extraction (LY, PI, WB, PAX and LL)	4	1.50	0.37	250.51	<0.001
Animal	4	0.015	0.0039	2.61	0.053
Replicates	2	0.00039	0.00019	0.13	0.877
miRNA yield					
Extraction (PI, PAX and LL)	2	1165685.68	582842.84	185.52	<0.001
Animal	4	92742.58	23185.64	7.38	0.001
Replicates	2	7950.95	3975.47	1.26	0.309
miRNA (%)					
Extraction (LY, PI and PAX)	2	5244.40	2622.20	12.25	<0.001
Animal	4	626.97	156.74	0.73	0.583
Replicates	2	140.93	70.46	0.32	0.724

DF degrees of freedom, *SS* sum of squares, *MS* mean squares, *F* F-statistic test and *P* probability value

Significant differences are considered if $P < 0.05$

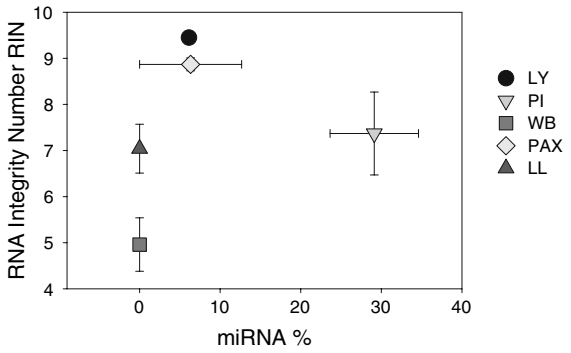

Fig. 1 Quality of mRNA versus miRNA. Quality of mRNA and miRNA (2100 Bioanalyzer, Agilent) and the RIN number and percentage of miRNA of small RNA obtained. For each extraction method mean RIN number ± SEM and mean miRNA ± SEM [%] were calculated and compared. miRNA [%] of WB and LL methods were not quantified due to total RNA extraction

using the expression values (Cq) of all quantified genes as the initial variable. Results are shown in Fig. 2a.

Tight cluster in the scatter plot show that the relationship between the samples highly correlated among themselves and spread clusters show a high variance between the samples. Therefore LY show the best results followed by PAX, PI, WB, and LL which shows the most spread cluster respectively.

Expression data were analyzed by relative quantification (ΔCq) for each extraction method. The mRNA quality expressed as RIN values showed minor influence on both Cq and ΔCq of the samples extracted by LY, PAX, and WB methods (Fig. 3). The mRNA quality of the samples extracted by PI and LL showed a high influence on the Cq and the ΔCq of specific target genes.

qPCR results of miRNA quantification and data analysis

The qPCR results of miRNA quantification show a tendency to obtain lower Cq for the samples extracted by the LY and the WB method followed by PI, PAX, and LL (Table 4). When comparing the reproducibility expressed by the SEM values the best results were obtained for the samples extracted by the LY method and the worst by the LL method.

Table 4 Gene expression was determined for each mRNA and miRNA transcript

Mean expression levels					
	LY	PI	WB	PAX	LL
18S rRNA	**17.28 ± 0.16**	20.37 ± 0.51***	21.22 ± 0.35***	18.15 ± 0.18	*22.24 ± 0.95****
ACTB	18.98 ± 0.10	19.28 ± 0.20	20.59 ± 0.18**	**18.85 ± 0.11**	*22.33 ± 0.80****
UBQ3	**22.26 ± 0.20**	23.29 ± 0.22**	24.84 ± 0.24***	22.52 ± 0.12	*24.97 ± 0.50****
H3F3A	30.00 ± 0.21	**27.98 ± 0.53****	30.79 ± 0.36	28.92 ± 0.47	*33.13 ± 0.62****
IL1B	**22.34 ± 0.09**	*27.67 ± 0.41****	25.42 ± 0.12***	23.15 ± 0.11	25.96 ± 0.70***
CD14	25.74 ± 0.20	**25.37 ± 0.13**	27.11 ± 0.14***	26.34 ± 0.14*	*27.75 ± 0.40****
C3	**26.36 ± 0.22**	27.80 ± 0.23***	28.59 ± 0.18***	27.29 ± 0.17*	*28.63 ± 0.50****
C1Q	25.9 ± 0.13	**24.99 ± 0.14*****	26.34 ± 0.19*	25.99 ± 0.11	*27.44 ± 0.30****
CSF1	28.26 ± 0.14	**25.53 ± 0.19*****	26.94 ± 0.24***	28.17 ± 0.14	*29.3 ± 0.31****
NFKB1	**24.77 ± 0.11**	25.61 ± 0.11*	26.09 ± 0.07***	25.19 ± 0.21	*26.78 ± 0.48****
PTGS2	28.13 ± 0.61	**25.43 ± 0.96****	27.47 ± 0.79	*31.04 ± 0.55***	30.29 ± 0.87*
MIR 16	18.14 ± 0.15	19.98 ± 1.26	**15.59 ± 0.24***	18.11 ± 0.59	*24.81 ± 0.85****
MIR let7a	**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 value considering each gene and the different extractions. Numbers in italic are equivalent to the highest Cq ± SEM value

Significance comparing all methods in relation to the best method (LY) is given as: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$

Additionally with the use of a PCA analysis for each extraction method the relationship between all genes measured was determined (Fig. 2b). For the samples obtained by LY it can be observed that the group arranges tightly showing a better clustering than WB, PAX, PI, and LL, respectively.

Discussion

With the purpose to evaluate blood sampling, handling, and preparation effects this study compared RNA yield, quality profiles, and gene expression performances by different extraction methods.

Regarding RNA yield and considering the initial volume of blood applied in each method the highest concentration of total RNA extracted is obtained by the LY method. We found no significant differences between the replicates assuring optimal labor reproducibility. However, the mean yields obtained significantly varied from animal to animal. This can be explained by the individual leukocyte number which changes during estrous cycle and exposure to stress (Ahmadi et al. 2006).

Small RNA yield quantification did not show reproducible results for the samples extracted by the PAX, PI, and LL methods. A cause for that may be the extraction protocol used to isolate total RNA in two fractions (mRNA + miRNA). Probably the conditions of the filter membrane were not perfectly optimized for binding small miRNA resulting in the shown high variance.

RNA quality by means of RIN number showed the best results for the samples extracted by the LY method, followed by PAX, PI, LL and WB. Thus, with the LY method it is possible to extract the most intact RNA for further expression analysis. It should be noted that WB and LY extraction methods differ with respect to the presence of red blood cells in the samples. This may contribute substantially to the lowest RNA quality of WB, as red blood cells could

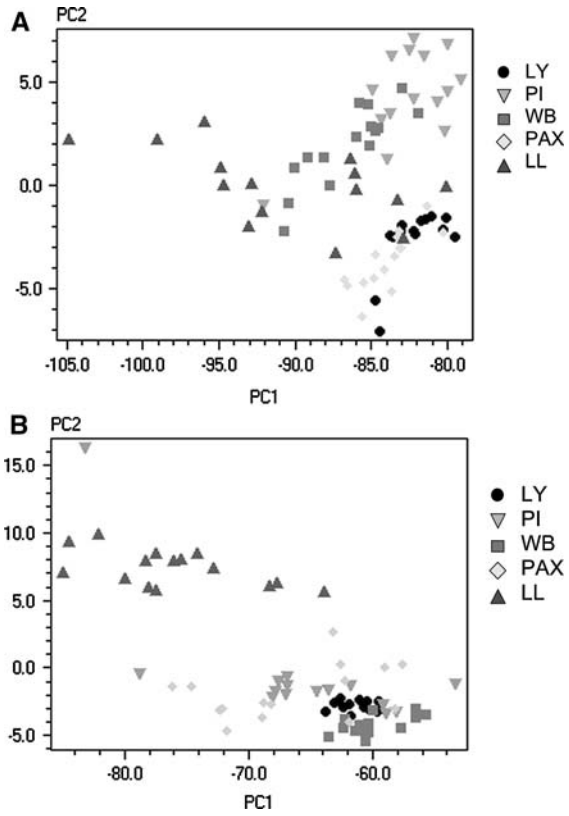

Fig. 2 Principal components analysis (PCA). PCA conducted with all mRNA (Fig. 2a) and miRNA (Fig. 2b) genes measured as the initial variables and reduced to two principal components in two dimensions. As reference gene *18S rRNA* was used. LY (*black dots*) show the best results as samples cluster the most, followed by PAX, PI, WB and LL. The latter (*grey upright triangles*) show the most spread cluster indicating a high variance between samples

interfere in gene expression quantification assays (Fleige et al. 2006; Wright et al. 2008; Feezor et al. 2004; Fleige and Pfaffl 2006).

The precision of molecular assays depends in large part on the quality of the resulting extracted RNA and on the reproducibility of the sample collection. To test the precision of the results, we examined the Cq standard error of mean (SEM) for the different extraction methods. Therefore differentially abundant genes were measured and results show a tendency to obtain the lowest Cq within the most reproducible data for the LY method.

Even though in the PAX method all blood cells are included in RNA extraction, no statistically significant difference was obtained for most of the target genes measured comparing to LY method containing only WBC. For the LY method, blood handling or processing procedures could improve the risk of inducing changes in gene expression. It was shown that storage time of blood in EDTA tubes influence gene induction (Rainen et al. 2002). This induction could result in gene activation and for this reason the LY method could have shown the lowest Cq values. Since there is no significant difference between both methods, it is possible to assume that the length of LY procedure before RNA extraction or storage is not relevant for the genes measured in this study.

In contrast, the WB method does not show high RNA quality, but its gene expression results can be successfully normalized by a reference gene (Fig. 3b). It has been shown that normalization by an internal reference gene can decrease sample preparation derived effects on the quantitative results for mRNA (Fleige and Pfaffl 2006; Wittwer et al. 1997).

In addition, miRNA quality was evaluated and a comparison of miRNA versus mRNA qualities for each extraction was drawn. The results suggest that the LY method is the best option to get a high mRNA and miRNA quality from the same extraction. Likewise for miRNA quantification the LY method often showed the most reproducible data. Besides the LY, the WB method shows accurate results and the other three methods PI, PAX and LL did not show reproducible results for miRNA quantification. Although most methods tested worked well for mRNA extraction, some adjustments in the protocols may be done to obtain better miRNA quality. There is a tendency to obtain the best miRNA results for the samples where total RNA was extracted in one fraction. Thus to improve the results, one possibility would be to adjust the PAX, PI and LL protocols and extract total RNA in one fraction.

Moreover the PI method showed reproducibility complications in all variables considered in this study. A reason can be the inaccurate form of WBC collection from the interphase between plasma and red blood cells. In addition it has to be considered that the LL system is optimized to stabilize and extract RNA from human blood which differs from bovine blood with respect to its cellular composition. Thus, the results obtained show that this system may be improved prior to the use with bovine blood.

In summary, the results of this study show that the LY method has a lot of advantages over the other

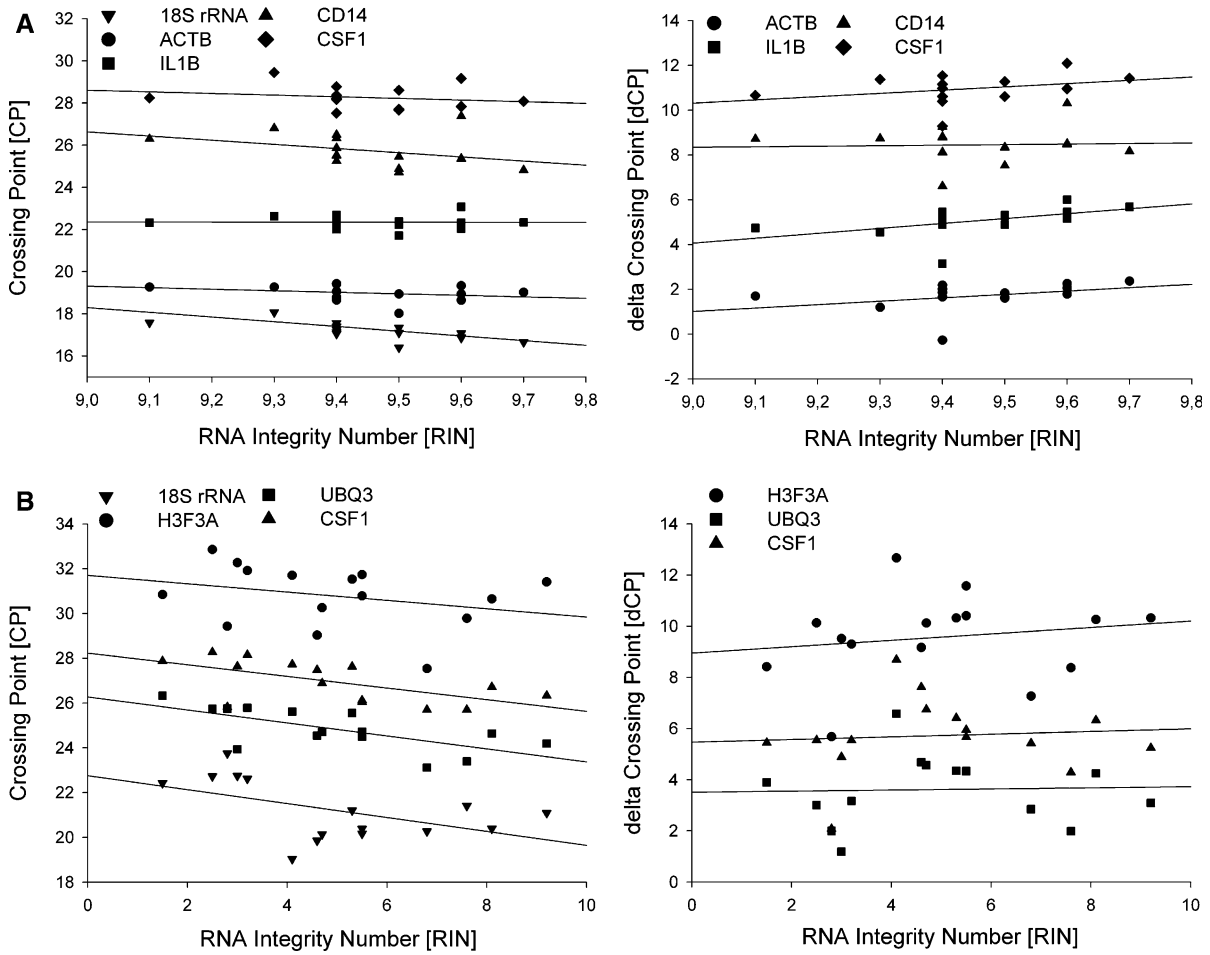

Fig. 3 Influence of the RNA integrity (RIN) on the Crossing Point (CP) and on the delta CP. Normalized expression results obtained for the LY method (Fig. 3a) and for the WB method (Fig. 3b). On the right side graphs the relation between RNA degradation to the amplified product can be observed. On the left side graphs by normalizing the target genes to a reference gene (*18S rRNA)* the effect of RNA integrity could be decreased

methods tested. For optimal results, blood samples must be processed as fast as possible after collection to avoid possible gene activation in WBC.

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