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An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensically relevant body fluids

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ABSTRACT

Micro-RNA (miRNA) based analysis of body fluids and composition of complex crime stains has recently been introduced as a potential and powerful tool to forensic genetics. Analysis of miRNA has several advantages over mRNA but reliable miRNA detection and quantification using quantitative PCR requires a solid and forensically relevant normalization strategy.

In our study we evaluated a panel of 13 carefully selected reference genes for their suitability as endogenous controls in miRNA qPCR normalization in forensically relevant settings. We analyzed assay performances and variances in venous blood, saliva, semen, menstrual blood, and vaginal secretion and mixtures thereof integrating highly standardized protocols with contemporary methodologies and included several well established computational algorithms.

Based on these empirical results, we recommend normalization to the group of *SNORD24*, *SNORD38B*, and *SNORD43* as this signature exhibits the most stable expression levels and the least expected variation among the evaluated candidate reference genes in the given set of forensically relevant body fluids.

To account for the lack of consensus on how best to perform and interpret quantitative PCR experiments, our study's documentation is compliant to MIQE guidelines, defining the "minimum information for publication of quantitative real-time PCR experiments".

1. Introduction

RNA based analytical methods are on the rise in forensic molecular biology [1] and early international trial exercises for forensic RNA analysis have already been conducted [2–4]. The analysis of differential expression of mRNA may be used in forensic settings to identify body fluid components of mixed stains [5], to estimate wound or stain age [6,7], detect pregnancy [8], and to help discern the cause of death [9].

There are, however, drawbacks associated with the analysis of mRNA, e.g. its susceptibility to degradation and lack of specificity in the identification or discrimination of particular body fluids especially vaginal secretions [10,11]. Therefore, in addition, feasibility and practicability of forensic miRNA analysis [12] based on quantitative PCR (qPCR) is being assayed since recently by several groups [13–15].

Quantitative PCR is widely considered as the gold standard for the quantification of miRNA expression but for qPCR to deliver a reliable and biologically meaningful report of target molecule numbers an accurate and relevant normalization of non biological variances is essential [16–19]. A robust normalization strategy that is specific for a particular experimental setup should encompass an individual and evidence based selection of one or a group of reference genes [20–22]. Therefore, in the present study, we present a group of endogenous reference genes selected on the base of empirical evidence for the normalization of qPCR data from expression analysis of 13 preselected miRNAs in forensically relevant body fluids.

2. Material and methods

2.1. Adherence to the MIQE guidelines

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated 'essential' according to the MIQE guidelines [23] is reported, where applicable.

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2.2. Samples

Samples for each tested body fluid, i.e. venous blood, saliva, vaginal fluid, menstrual blood, and semen, were collected from healthy volunteers, after obtaining informed consent.

Venous blood was collected by venipuncture using dry vacutainer tubes and spotted onto sterile cotton swabs. For collection of saliva via buccal swab, donors were asked to abstain from eating, smoking, drinking and oral hygiene at least 30 min prior to sampling. Samples of semen-free vaginal secretion were collected by the female donors themselves using sterile stemmed cotton swabs. Menstrual blood samples were obtained by the female donors using tampons. Freshly ejaculated semen was provided in sealed Falcon tubes by male donors and dried onto sterile stemmed cotton swabs by the researcher immediately after receipt. All samples were dried at room temperature and processed for RNA extraction after 24 h.

2.3. RNA extraction and quantification

All surfaces, devices, and machines utilized in the extraction procedure were thoroughly cleaned using RNase-Zap® (Ambion, Austin, TX, USA) to remove ambient RNases and only RNase-free reagents and plastic consumables were used.

Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. Prior to extraction, samples (whole cotton tip or approximately 2 cm^2 of the tampon or blood stain) were cut into pieces and incubated with 350 μl Lysis/Binding Buffer at 56 °C for 1 h. Venous blood samples were additionally treated with RBC Lysis Solution (Qiagen, Hilden, Germany) to selectively lyse red blood cells prior to incubation. Spin baskets (Promega, Mannheim, Germany) were used to separate lysate and substrate by centrifugation at 13.000 × *g* for 1 min. Total RNA eluates were stored at −80 °C until further processing.

For removal of potential traces of genomic DNA, subsequent DNase I digestion was performed with the Turbo DNA-free™ Kit (Ambion), following the manufacturer's protocol. Total RNA concentration and quality, represented by the RNA integrity number (RIN) [24], were determined using the Quant-iT™ RNA Assay Kit on a Qubit fluorometer (both Invitrogen, Darmstadt, Germany) and the RNA 6000 Nano Kit on a Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively.

2.4. Preparation of samples

Individual samples were diluted to 2 ng/μl based on quantification results and were used as single samples only or additionally for the preparation of a pooled sample per body fluid by combining identical volumes of diluted sample. Five individual samples per body fluid were examined. Pooled samples for blood and saliva consisted of 10 donor samples, while those for vaginal secretion, menstrual blood and semen consisted of five donor samples. Further, a mixture of all five body fluids was prepared for efficiency determination experiments containing identical volumes of the above mentioned pooled samples.

2.5. Selection of candidate reference genes

A panel of 13 potential reference genes was selected based on a literature survey, mainly focusing on reference genes previously used in forensic miRNA analyses and the manufacturer's recommended control panel [13–15,25–29]. The selected panel encompassed *hsa-miR-93-5p*, *hsa-miR-191-5p*, *RNU6-1*, *RNU6-2*, *SNORA66*, *SNORA74A*, *SNORD7*, *SNORD24*, *SNORD38B*, *SNORD43*, *SNORD44*, *SNORD48* and *SNORD49A* (Supplementary Table 1).

2.6. Reverse transcription qPCR (RT-qPCR)

Complementary DNA (cDNA) was synthesized using target-specific stem-loop primers (Supplementary Table 1) and the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Weiterstadt, Germany), as per manufacturer's protocol. Each 15 μl reaction volume contained 10 ng total RNA, 1X RT primers, 50 U MultiScribe™ reverse transcriptase, 1 mM dNTPs, 3.8 U RNase inhibitor, and 1X reverse transcription buffer. Reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Besides extraction negative and H_2O controls, we employed RT(−)-controls to control for potential contamination with genomic DNA. For efficiency determination experiments, reverse transcriptions of the mixture containing all body fluids were conducted twice. RT reaction products were stored at −20 °C.

QPCR reactions were performed using target-specific TaqMan® Assays (Supplementary Table 1) and the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies) as per manufacturer's protocol: 1.3 μl of the appropriate RT reaction product were added into a 20 μl reaction volume, containing 1X TaqMan Universal PCR Master Mix and 1X specific TaqMan® Assay. All sample-assay combinations were run in triplicates for the pooled samples and in duplicates for the individual samples, respectively. The internal PCR control from the Quantifiler® Human DNA Quantification Kit (Life Technologies) was used as an inter plate calibrator. PCR cycling conditions consisted of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and were performed on an ABI Prism 7500 (Life Technologies). Data collection was performed during the 60 °C step by the SDS software version 1.2.3 (Life Technologies). Along with the C_q-values calculated automatically by the SDS software (threshold value = 0.2, baseline setting: cycles 3–15) raw fluorescence data (R_n-values) were exported for further analyses.

2.7. Data analysis and software based selection of endogenous reference genes

The LinRegPCR program version 2012.3 [30] was employed to compute C_q-values and amplification efficiencies from R_n-values. The arithmetic mean values of amplification efficiencies per triplicate repeats were used in further analysis, with efficiencies outside 5% of the group median being excluded from mean efficiency calculation. For C_q calculation, a common threshold value was set to $-0.7\ \log_{10}$(fluorescence). C_q-values deviating more than one cycle from the triplicate median were excluded from subsequent pre-processing. For comparison, amplification efficiencies were computed analogously using the Real-time PCR Miner algorithm [31].

Analysis of qPCR data including pre-processing was then performed using the GenEx software version 5.3 (multiD Analyses, Göteborg, Sweden) into which LinRegPCR and SDS spread sheet exported data was imported, respectively. Pre-processing of qPCR encompassed the following steps in the given order: interplate calibration, efficiency correction, and averaging of technical qPCR replicates.

To evaluate gene expression stability, we applied the following algorithms: NormFinder [32], geNorm [22], both implemented in the GenEx software, and the Excel-based BestKeeper [33]. NormFinder takes intra- and inter-group variances into account and provides a stability value per gene as a direct measure for the estimated expression stability, indicating the systematic error introduced when using the respective gene for normalization. Moreover, it is possible to assess the optimal number of reference genes by means of the

accumulated standard deviation. GeNorm calculates and compares a so called gene stability measure (M-value) of all candidate genes, selecting an optimal pair of reference genes by stepwise exclusion of the gene with the highest M-value. BestKeeper uses pair wise correlation analysis of the C_q-values of all pairs of candidate reference genes to determine the most stable gene. Calculations were performed separately for pooled samples per body fluid and for individual body fluid samples.

3. Results

Quantity and integrity of total RNA varied notably among samples of the same body fluid as well as between groups with RIN values generally ≤4 (Table 1). Overall, saliva samples exhibited the lowest (total RNA concentration: 3.6–25.3 ng/μl; RIN: n.d.–1.8) and vaginal secretion samples the highest overall values (total RNA concentration: 41.9–257 ng/μl; RIN: 2.9–4).

RT(−)-controls for *RNU6-1* and *SNORD48* showed scarce unspecific amplification and these markers were excluded from further analyses (data not shown). The negative controls were negative for all candidate genes when extracted from stemmed cotton swabs, while extracts from tampons and sterile cotton swabs produced a weak unspecific signal for *miR-93* and *miR-191* (difference to C_q-values of mixture >15 C_q, data not shown).

Table 1
Total RNA quantity and integrity per sample.

Body fluid	Sample number	Gender	Total RNA concentration (ng/μl)	RIN	Additional information
Blood	1	F	8.7	2	
	2	F	12.6	2.4	
	3	M	14.3	1.1	
	4	M	15.2	1.6	
	5	M	15.6	1.2	
	6*	F	18.1	1.8	
	7*	M	19.8	1.3	
	8*	M	19.9	1	
	9*	F	20.8	1.3	
	10*	F	23.8	2.4	
Menstrual blood	1*	F	24.8	2.6	Day 3 of menstruation
	2*	F	27.5	1.8	Not specified
	3*	F	37.2	2.4	Day 4 of menstruation
	4*	F	52.0	2.7	Not specified
	5*	F	150.0	2.6	Day 3 of menstruation
Saliva	1	F	3.6	1	
	2*	F	6.5	1.1	
	3	M	8.2	1	
	4*	F	8.4	1	
	5	M	9.0	1	
	6	F	11.2	1	
	7*	M	12.5	1.2	
	8*	F	16.1	n.d.	
	9*	M	17.8	1.1	
	10	M	25.3	1.8	
Semen	1*	M	11.4	1	
	2*	M	16.0	1	
	3*	M	16.4	2.1	
	4*	M	18.3	1.2	
	5*	M	38.4	2.2	
Vaginal secretion	1*	F	41.9	2.9	Day 10 after menstruation
	2*	F	51.5	3.4	Day 20 after menstruation
	3*	F	85.5	2.9	Not specified
	4*	F	110.0	3.3	Not specified
	5*	F	257.0	4	Day 17 after menstruation

RIN RNA integrity number; *F* female, *M* male; *n.d.* not detectable; *sample used for individual analyses.

Table 2
Amplification efficiencies of candidate reference genes calculated by LinRegPCR software and Real-time PCR Miner algorithm, respectively.

Gene symbol	Amplification efficiency of mixture			
	LinRegPCR		Real-time PCR Miner	
	Mean[a]	SD	Mean[b]	SD
miR-191	1.78	0.016	0.82	0.005
miR-93	1.82	0.017	0.87	0.018
RNU6-2	1.84	0.005	0.87	0.004
SNORA66	1.82	0.010	0.93	0.011
SNORA74A	1.88	0.015	0.96	0.010
SNORD24	1.84	0.028	0.92	0.013
SNORD38B	1.89	0.030	0.98	0.015
SNORD43	1.84	0.035	0.92	0.031
SNORD44	1.61[c]	0.006	–	–
SNORD49A	1.83	0.024	0.92	0.026
SNORD7	1.78[c]	0.028	–	–

SD, standard deviation; –, not computed.
[a] Efficiencies are given as values between 1 and 2, with 2 representing an amplification efficiency of 100%.
[b] Efficiencies are given as values between 0 and 1, with 1 representing an amplification efficiency of 100%.
[c] Excluded from study.

3.1. Amplification efficiency

Amplification efficiency per amplicon was derived from the mixture containing all five body fluids, including two distinct RT-reactions and qPCR triplicates into the computation. Mean efficiencies per amplicon computed with LinRegPCR ranged from 89% (*SNORD38B*) to 61% (*SNORD44*) (Table 2). Due to its grossly outlying amplification efficiency, *SNORD44* was excluded from further analyses. *SNORD7* was excluded since an additional examination of the amplification efficiencies in the pooled samples revealed considerable variation between body fluids ranging from 88% in menstrual blood to 59% in semen (data not shown).

Mean efficiencies per amplicon computed with Real-time PCR Miner ranged from 98% (*SNORD38B*) to 82% (*miR-191*) (Table 2).

3.2. Determination of most suitable reference genes

3.2.1. Pooled samples

A first examination of the most suitable reference genes using LinRegPCR spread sheet exported data for the pooled samples of each body fluid type was performed including the remaining nine candidate genes (Supplementary Table 2).

According to NormFinder *SNORD38B* was the most stable gene with a stability value of 0.3049 standard deviations, followed by *SNORA66*, *SNORD24* and *SNORD43* (Supplementary Fig. 1, upper panel). The least stable gene was *miR-93* with a stability value of 2.4782. The simultaneously calculated accumulated standard deviation was lowest (0.2731) when the use of two reference genes was assumed (Supplementary Fig. 1, lower panel).

Analyzed with geNorm, the most stable pair of genes was *SNORD49A & SNORD24*, with an M-value of 0.3066, followed by *SNORD43* and *SNORA66*, while *miR-93* was the least stable gene with an M-value of 1.6026 (Supplementary Fig. 2).

The only candidate gene considered as stable (standard deviation of C_q-values <1.0) by BestKeeper was *RNU6-2* with a standard deviation of 0.83. The remaining values ranged between 1.17 (*SNORD43*) and 3.25 (*miR-93*) (Supplementary Fig. 3).

Subsequently, candidate reference gene data put out by the three algorithms, respectively, were transformed into consecutively numbered ranks with 1 representing the most and 9 the least stable gene (Supplementary Table 3A) and a comprehensive gene stability ranking was attained by calculation of the arithmetic mean ranking value per gene. In this comprehensive ranking *SNORA66* and

Table 3
Mean C_q-values and standard deviation per candidate reference gene of five individual body fluid samples after pre-processing (amplification efficiency and C_q-values as per LinRegPCR software).

Body fluid	*miR-191*	*miR-93*	*RNU6-2*	*SNORA66*	*SNORA74A*	*SNORD24*	*SNORD38B*	*SNORD43*	*SNORD49A*
Blood	15.92 ± 0.22	16.39 ± 0.49	24.90 ± 0.73	24.76 ± 0.32	26.21 ± 0.44	22.53 ± 0.57	25.17 ± 0.53	23.43 ± 0.56	21.97 ± 0.76
Menstrual blood	18.28 ± 2.97	19.08 ± 3.94	26.35 ± 0.87	26.91 ± 3.09	27.34 ± 1.94	23.61 ± 1.50	27.24 ± 1.36	23.75 ± 0.84	23.18 ± 1.07
Saliva	21.38 ± 0.38	22.31 ± 0.51	27.47 ± 0.89	26.33 ± 1.48	30.31 ± 1.61	24.79 ± 0.85	28.55 ± 0.76	24.23 ± 0.29	24.06 ± 1.03
Semen	20.57 ± 0.28	22.69 ± 0.44	28.00 ± 1.32	29.82 ± 1.83	31.18 ± 1.12	28.76 ± 1.59	31.34 ± 1.70	28.10 ± 1.30	28.53 ± 1.70
Vaginal secretion	21.95 ± 0.65	24.23 ± 1.55	26.16 ± 0.62	29.37 ± 2.75	28.46 ± 1.67	24.90 ± 0.96	29.14 ± 1.02	24.89 ± 0.40	23.93 ± 0.38

SNORD43 were top ranked, closely followed by *SNORD24* and *SNORD38B*. The least stable genes were *miR-93* and *miR-191*.

Analyzing the SDS software spread sheet exported data corrected with the amplification efficiencies as per Real-time PCR Miner (Supplementary Table 4) resulted in comparable ranking orders (Supplementary Table 3B and Supplementary Figs. 4–6).

3.2.2. Individual samples

Analyses for determination of the most suitable reference genes were simultaneously computed for a set of five individual samples per body fluid (Table 3 and Supplementary Tables 5 and 6).

LinRegPCR spread sheet exported data analyzed with NormFinder designated *SNORD38B* as the most stable gene with a stability value of 0.5730 standard deviations, followed by *SNORD24* and *SNORA74A* (Fig. 1, upper panel). The least stable gene was *miR-93* with a stability value of 2.0461. The simultaneously calculated accumulated standard deviation was lowest (0.4117) when the use of eight reference gene was assumed (Fig. 1, lower panel).

According to geNorm *SNORD24* & *SNORD49A* was the most stable pair with an M-value of 0.7183, followed by *SNORD43* and *SNORD38B*, while *miR-93* was the least stable gene with an M-value of 1.7195 (Fig. 2).

None of the candidate genes was considered stable by the BestKeeper algorithm, with the value of *RNU6-2* being close to the cut-off 1.08, however. The remaining values ranged between 1.37 for *SNORD43* and 2.93 for *miR-93* (Fig. 3).

Analyzing the SDS software spread sheet exported data corrected with the amplification efficiencies as per Real-time PCR Miner resulted in comparable results and the same ranking orders (Supplementary Figs. 7–9).

The comprehensive ranking following both computations designated *SNORD24* followed by *SNORD38B* and *SNORD43* as the best suitable reference genes in the given single sample set (Table 4).

4. Discussion

For qPCR to deliver reliable and biologically meaningful results an accurate and relevant normalization of non biological variances is essential [16–19]. Non-biological variances can include variations in PCR efficiency, amount of starting material by sample-to-sample variation, RNA integrity, RT efficiency and cDNA sample loading [34–36]. This has to be accounted for especially when, as in most forensic settings, samples have been obtained from different individuals, different body fluids and different time courses. In this study we applied highly standardized protocols starting with the handling, storage and extraction of samples to minimize the external variances. To compensate for internal non-biological variances, the use of endogenous reference genes is essential.

As the so called "housekeeping genes", like *ACTB* and *GAPDH*, that were commonly used as reference genes for normalization in numerous studies have long been shown to be differentially expressed under many different experimental conditions [37–43], which has only recently been confirmed in a forensic setting [44], they should not any longer be used uncritically for normalization purposes. Consequently, reference genes intended for qPCR normalization in a given experimental setting have to be selected beforehand and then based on their empirically proven suitability. This is particularly important for miRNA analysis as a general agreement on methodological standardization of qPCR in miRNA quantification has not been achieved yet. The aim of our study was therefore to present a reliable and empirically derived reference framework for normalization of qPCR data in the analysis of miRNA expression in realistic representations of five forensically relevant body fluids. The selection of 13 candidates for the starting panel of reference genes was based upon a literature survey [13–15,25–27] and several criteria such as a relatively constant and highly abundant expression across a large number of tissues and cell lines [28].

Another important aspect that has to be accounted for in qPCR data analysis is PCR efficiency. Samples from different tissues are known to exhibit different PCR efficiencies caused by variations in RT and PCR due to inhibitors and by variations on the total RNA fraction pattern extracted. It has been shown that omission of correction for differential PCR efficiencies [45] introduces bias in the expression results [21,46–48] and thus, a separate determination of qPCR efficiency for each performed transcript is necessary [16,18,49]. There is as yet no consensus as to which of several algorithms presented so far for determining C_q-values and PCR efficiencies from raw fluorescent data is best suited. However, Ruijter et al. recently published a first comprehensive benchmark study of the evaluation of nine qPCR analysis methods [50] and the LinRegPCR method [30] was top ranked for precision and resolution and also showed high linearity without introducing excessive bias [50]. This software was shown to underestimate efficiencies compared to those determined by standard curve analysis, though. To account for this we did not apply the commonly used standard efficiency criteria (90–110% of efficiency), but accepted efficiencies down to 70% as calculated per LinRegPCR software.

In addition, we employed the Real-time PCR Miner method for efficiency computation [31], which was highly ranked in the benchmark study by Ruijter et al. as well but was shown to rather overestimate efficiencies compared to those determined by standard curve analysis [30]. This algorithm determines the cycle threshold for each sample dynamically based on its reaction kinetics. As the composition of analyzed stains is usually unknown in a forensic setting, we decided to take a more robust approach by employing the C_q-values determined with the SDS software.

The results of our efficiency calculations employing these two distinct algorithms indeed reflect their differences in efficiency estimation as demonstrated by Ruijter et al. [30] with Real-time PCR Miner reporting higher and LinRegPCR reporting lower efficiency values, respectively. To investigate whether these divergent estimates of efficiency influence the algorithmic identification of most stable reference genes we performed two separate computations, based on values given by LinRegPCR and Real-time PCR Miner. The reference gene rankings extracted from LinRegPCR data or Real-time PCR Miner data are very similar thus

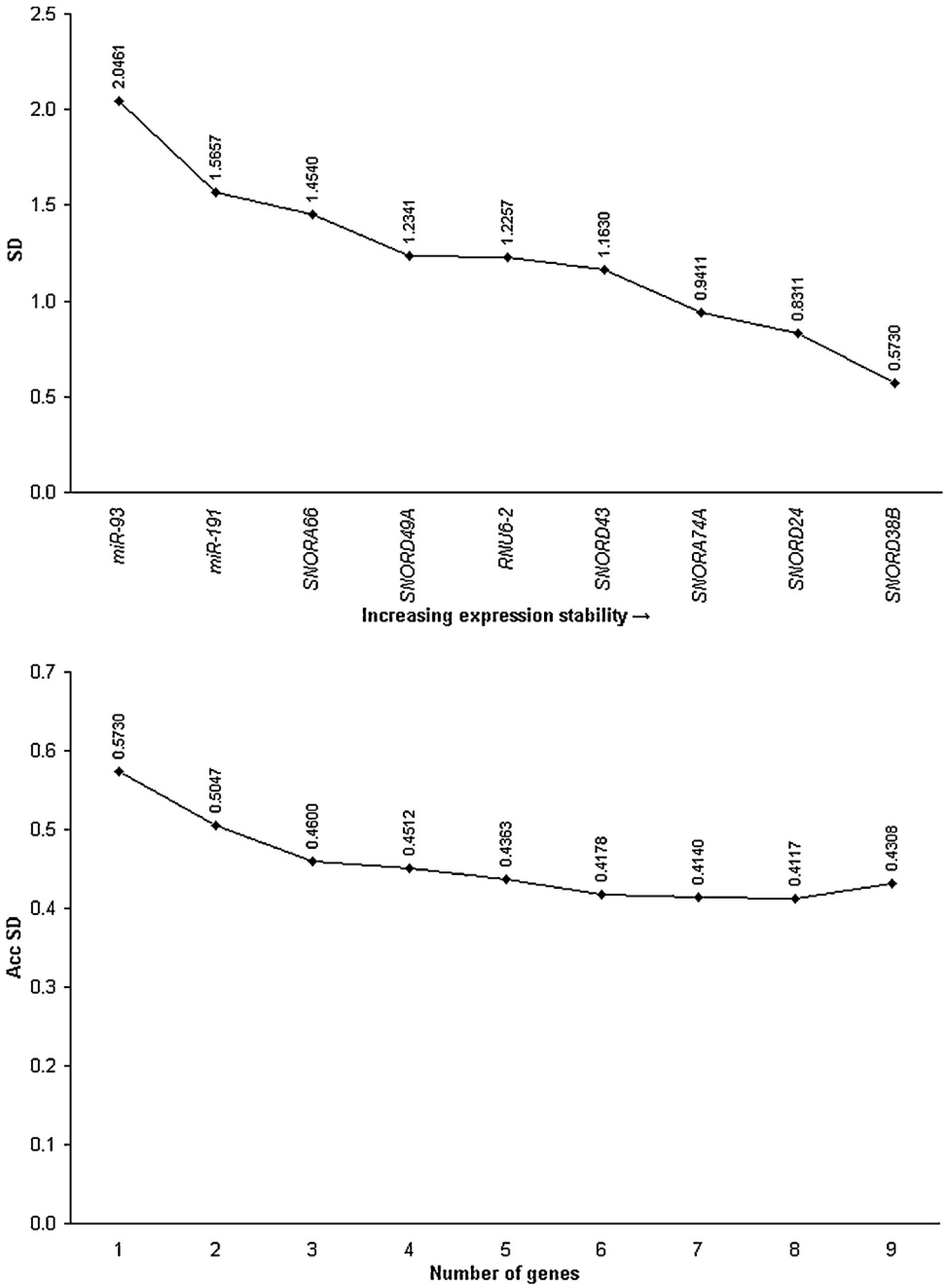

Fig. 1. NormFinder data analysis of the nine candidate genes in individual body fluid samples.
(upper panel) Gene expression stability values of genes – from least (*left*) to most stable (*right*). (lower panel) Determination of the optimal number of reference genes by computation of accumulated standard deviation values. Amplification efficiency and C_q-values as per LinRegPCR software.

suggesting that the influence of the difference between these algorithms on reference gene selection is negligible.

We used the amplification efficiencies as computed from a mixture of all five body fluids for all subsequent calculations. This is probably the most conservative approach since it is usually unknown which and how many different types of body fluids and of how many individuals are present in a given casework stain (e.g. sexual assault crimes). In addition, a computation of amplification efficiencies in pooled samples per body fluid was performed, however, to assess the variation between the distinct body fluids and resulted in the exclusion of *SNORD7* from further analyses.

Analogous to algorithms for the calculation of PCR efficiency, there is as yet no consensus as to which of several present algorithms performs best in identifying the most suitable endogenous reference out of a set of candidate genes. We therefore employed three well established and commonly used methods – NormFinder, geNorm, and BestKeeper – and reported both the results for each algorithm and in combination as described by Wang et al. [51]. The notably different ranking resulting from BestKeeper might be due to the fact, that this algorithm uses C_q-values directly, while geNorm and NormFinder transform imported C_q-values to relative quantities for stability calculations.

A major challenge for the establishment of a solid normalization strategy in forensic settings is posed by the nature of forensically relevant samples e.g. body fluids containing multiple different types of cells, as well as by the potentially complex composition of typical forensic stain evidence that may include several body fluids in vastly different proportions. Also, sampling and storage

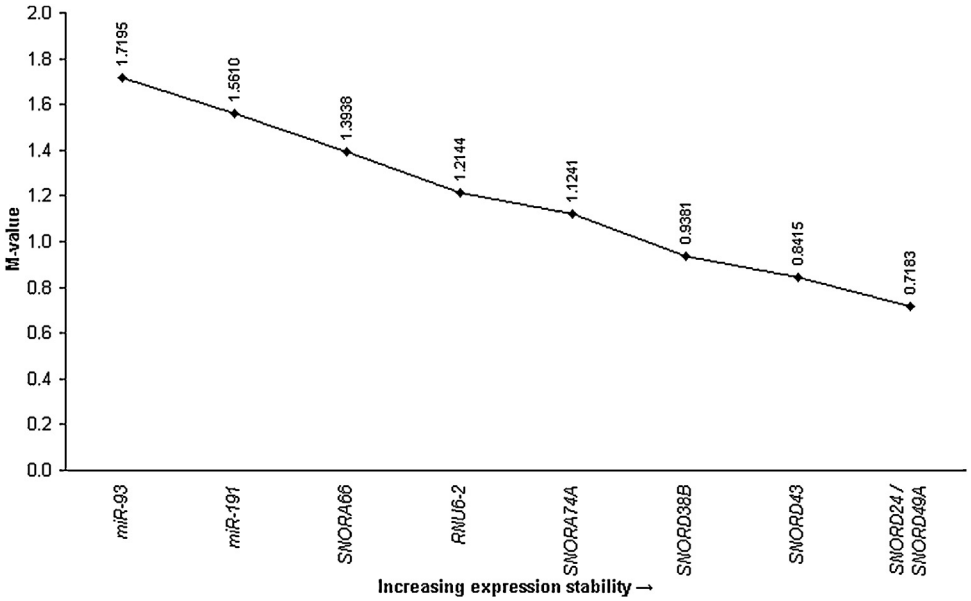

Fig. 2. GeNorm data analysis of the nine candidate genes in individual body fluid samples.
GeNorm proceeds by calculation of the gene stability measure (M-value) per gene – from least (*left*) to most stable (*right*); determination of the optimal pair of reference genes by stepwise exclusion of the gene with the highest M-value. Amplification efficiency and C_q-values as per LinRegPCR software.

conditions of evidential material will often not be optimized for potential RNA analysis and thus result in low total RNA concentration and integrity. Therefore, to mimic realistic forensic casework we used dried body fluid stains in our study instead of fresh, less compromised samples. The weak signal detected in negative controls of *miR-93* and *miR-191* can most probably be explained by the type of sample, too, since tampons and cotton swabs were not declared DNA-/RNA-free as was the case for stemmed cotton swabs. However, with C_q-values for these extraction controls being so high and far off those from the actual samples (>15 C_q), the respective candidates had not to be excluded from analyses.

Another important difference to previously published studies on miRNA normalization [26] is, again owing to the forensic scope of this study, that the evaluated samples do not represent two conditions of the same tissue or cell type (e.g. healthy/cancerous or treated/untreated) but up to five distinct body fluids. The more types of body fluids are included in a mixture, the higher the variances of expression values for any one reference gene are expected to be. It was therefore unlikely in the first place to identify one or even a group of reference genes that exhibit no or very low expression variances between samples.

We further took into account that the composition of a stain encountered in forensic casework is usually unknown in terms of

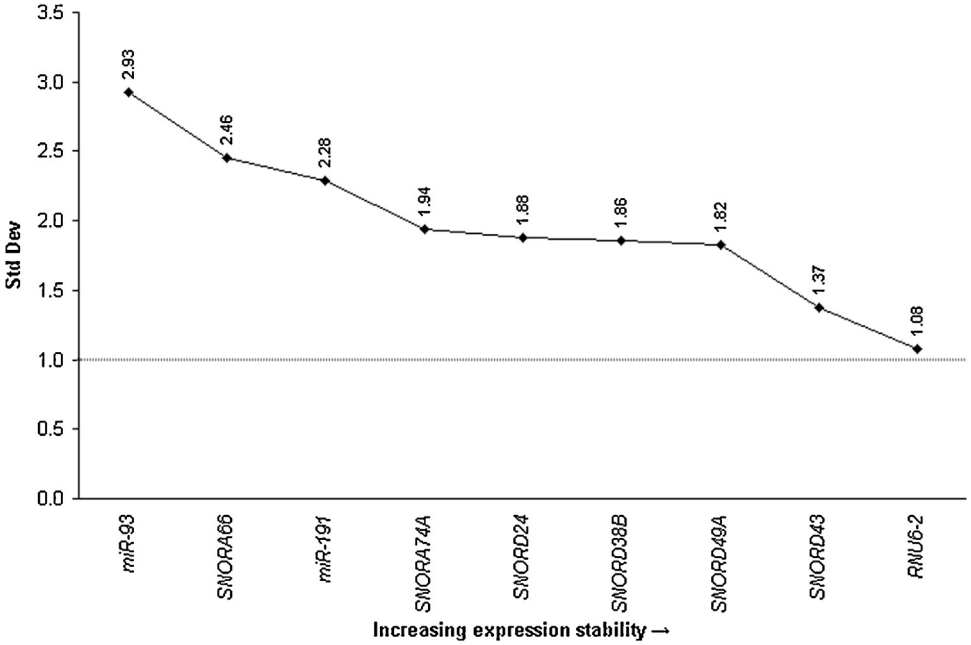

Fig. 3. BestKeeper data analysis of the nine candidate genes in individual body fluid samples.
BestKeeper proceeds by pair wise correlation analysis of the C_q-values of all pairs of candidate reference genes – from least (*left*) to most stable (*right*). Amplification efficiency and C_q-values as per LinRegPCR software.

Table 4
Comprehensive ranking order of the candidate reference genes for individual body fluid samples, derived by integrating rankings of NormFinder, geNorm, and BestKeeper. Computations with the LinRegPCR software derived values and computations with amplification efficiency as per Real-time PCR Miner algorithm and C_q-values as per SDS software resulted in identical ranking orders.

Ranking order	NormFinder	geNorm	BestKeeper	Comprehensive ranking (mean rank value)
1	*SNORD38B*	*SNORD24 & SNORD49A*	*RNU6-2*	*SNORD24* (2.67)
2	*SNORD24*		*SNORD43*	*SNORD38B/SNORD43* (3.00)
3	*SNORA74A*	*SNORD43*	*SNORD49A*	
4	*SNORD43*	*SNORD38B*	*SNORD38B*	*SNORD49A* (3.33)
5	*RNU6-2*	*SNORA74A*	*SNORD24*	*RNU6-2* (4.00)
6	*SNORD49A*	*RNU6-2*	*SNORA74A*	*SNORA74A* (4.67)
7	*SNORA66*	*SNORA66*	*miR-191*	*SNORA66* (7.33)
8	*miR-191*	*miR-191*	*SNORA66*	*miR-191* (7.67)
9	*miR-93*	*miR-93*	*miR-93*	*miR-93* (9.00)

the types of body fluids present and the number of individual contributors, respectively.

In our view it was the most conservative approach to compromise on the recommendation of a group of reference genes for a normalization procedure that assumes that all relevant types of body fluid may be present in a given sample. It is possible though, to apply a more specific normalization strategy if, for whatever reason, the composition of a stain is less unknown, if e.g. the presence in the stain of one body fluid can reliably be excluded. This would have to be validated in the given setting.

For an initial screening of suitable reference genes, we used pooled samples in which the expected inter-individual differences are counterbalanced. This analysis indicated *SNORA66* and *SNORD43* to be stable reference genes followed by *SNORD24* and *SNORD38B*.

Subsequent comprehensive computations were performed with five individual samples per body fluid, hence taking into account biological variation. As expected, blood samples showed low inter-individual differences in all markers. Higher standard deviations between individual samples were present in semen, vaginal secretion and menstrual blood, whereas the high standard deviation values in the latter can be attributed to outlying values in a single sample.

As in the initial screening, *SNORD24*, *SNORD38B* and *SNORD43* were again the top ranked markers. *SNORA66*, however, appears to show large inter-individual differences resulting in a high mean rank value, and was therefore excluded from the recommended set of reference genes.

We are aware of the relatively small sample size and aim to further assess the presented set of reference genes in terms of its value for data normalization in future studies in which miRNA candidates for the identification of body fluids will have to be validated with the presented normalization strategy.

5. Conclusion

Herein we analyzed 13 potential reference genes and empirically determined *SNORD24*, *SNORD38B* and *SNORD43* to be the most stable endogenous reference genes for a reliable normalization of qPCR data from forensic miRNA expression analysis of body fluids in a set of body fluid samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.03.011.

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