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ELECTROPHORESIS

RESEARCH ARTICLE

Differentiation of five forensically relevant body fluids using a small set of microRNA markers

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Abstract

In forensic investigations, identifying the type of body fluid allows for the interpretation of biological evidence at the activity level. Over the past two decades, significant research efforts have focused on developing molecular methods for this purpose. MicroRNAs (miRNAs) hold great promise due to their tissue-specific expression, abundance, lack of splice variants, and relative stability. Although initial findings are promising, achieving consistent results across studies is still challenging, underscoring the necessity for both original and replication studies. To address this, we selected 18 miRNA candidates and tested them on 6 body fluids commonly encountered in forensic cases: peripheral blood, menstrual blood, saliva, semen, vaginal secretion, and skin. Using reverse transcription quantitative PCR analysis, we confirmed eight miRNA candidates (miR-144-3p, miR-451a, miR-205-5p, miR-214-3p, miR-888-5p, miR-891a-5p, miR-193b-3p, miR-1260b) with high tissue specificity and four (miR-203a-3p, miR-141-3p, miR-200b-3p, miR-4286) with lesser discrimination ability but still contributing to body fluid differentiation. Through principal component analysis and hierarchical clustering, the set of 12 miRNAs successfully distinguished all body fluids, including the challenging discrimination of blood from menstrual blood and saliva from vaginal secretion. In conclusion, our results provide additional data supporting the use of a small set of miRNAs for predicting common body fluids in forensic contexts. Large population data need to be gathered to develop a body fluid prediction model and assess its accuracy.

KEYWORDS
body fluid identification, expression analysis, forensic genetics, miRNA

1 | INTRODUCTION

There is a growing focus on methods for inferring the tissue composition of forensic evidentiary material. Body fluid identification may provide valuable information to optimize the handling of the stain in the laboratory, in addition to providing important elements regarding the way the trace was deposited and therefore for the reconstruction of the criminal activity.

Traditional serological techniques currently used in forensic casework rely on enzymatic activity or immunological features of proteins highly abundant in certain body fluids. These methods show several limitations, such as low specificity, a single-test approach leading to the

important trace consumption with labor-intensive procedures, and finally, a lack of targets for several forensically relevant body fluids, such as menstrual blood, nasal and vaginal secretion, and epithelial cells for contact traces [1].

In the past 20 years, many studies have searched among molecular tools for novel solutions for body fluid identification. The availability of methods for co-extracting RNA and DNA is an attractive solution for the optimal processing of limited sample amounts [2, 3]. RNA biomarkers hold great promise because of their tissue-specific expression pattern and the increased use in clinics for the noninvasive diagnosis of certain diseases, including cancer [4]. Conversely, epigenetic-based approaches and microbial trace analysis have been explored but remain less developed and technically more complex [5–8].

Sets of mRNA markers were studied to provide candidates for discriminating the forensically most relevant body fluids/tissues, that is, blood, semen, saliva, vaginal secretion, menstrual blood, and skin [1, 9–11]. The specificity and sensitivity of the identified mRNAs were also confirmed by various collaborative exercises [12, 13]. Several research groups implemented mRNA profiling for body fluid identification in casework [14–16]. Results from real samples show that the mRNA quantity and quality of specific targets are good in both post-mortem and aged crime scene stains [17], although some environmental factors, such as humidity and UV radiation [18, 19], may affect the level of gene expression [20, 21].

MicroRNAs (miRNAs) are another class of RNA that proved useful for body fluid identification. Similar to mRNAs, they are expressed in a tissue-specific manner, but, due to their short size (18–24 nucleotides in length) and binding of a protecting protein complex [22], miRNAs are more stable and might be preferred for samples that are environmentally exposed and heavily degraded [11]. These small, non-coding RNAs are regulators of gene expression at the posttranscriptional level by adjusting the mRNA decay of target molecules or their translational repression [23]. Interestingly, the expression of miRNAs was reported to be much higher than mRNAs [24], and finally, the lack of splice variants may also be an advantage.

Interestingly, studies aiming at differentiating tumors by RNA profiling have shown that about 200 miRNAs offer a better tumor classification than those obtained with several thousand mRNAs [25, 26], indicating that the miRNome may better correlate with cell type and status than the cellular transcriptome.

Although many studies have identified sets of miRNAs as biomarkers for inferring body fluids [27–33] (reviewed in Refs. [1, 11]), these reports suggest alternative small sets of targets for analysis, leading to little overlap in recommendations. Moreover, many proposed markers could not be confirmed with other techniques or when testing mock forensic traces [34–40]. Implementing miRNA expression profiling in routine casework needs further replication studies across laboratories using forensic standard methods to provide trustworthy results and possibly replace conventional methods.

To bridge this gap, we selected from previous works 18 miRNA candidates and tested them for the discrimination of six body fluids, comprising peripheral blood, menstrual blood, saliva, semen, vaginal secretion, and skin. To expedite the implementation of the method in our service laboratory, we have chosen to analyze stains deposited and stored similarly to forensic casework samples, in addition to using reverse transcription quantitative PCR (RT-qPCR) for marker quantification.

2 | MATERIALS AND METHODS

2.1 | Samples

Six samples for each body fluid, including peripheral/venous blood, saliva, menstrual blood, semen, vaginal secretion, and skin, were collected from healthy volunteers. Peripheral blood was collected by venipuncture using EDTA-containing tubes. Saliva samples were collected in sterile plastic tubes, and donors abstained from drinking, eating, smoking, and dental cleaning at least 30 min before collection. Semen samples were collected in a sterile plastic cup. Menstrual blood and semen-free vaginal secretions were collected by medical personnel during gynecological consultations using a disposable sterile plastic gynecological spoon (12M200-06, Mederen) and transferred to a sterile tube (Eppendorf). Skin contact traces were collected by wet swabbing the inner side of the arm 10 times. All liquid samples were stored at −20°C and transferred to the laboratory frozen. Upon reception, 25 µL of each sample were deposited on sterile forensic swabs (Copan 4N6FLOQSwabs, Copan), dried for 12 h, and stored at −80°C. Body fluids were collected from volunteers (age: 19–58 years old, African Americans and Caucasians) by the company Innovative Research (https://www.innov-research.com/) using procedures approved by the Centre Hospitalier Universitaire Vaudois and Université de Lausanne institutional review board. Written informed consent was obtained from all participants.

2.2 | Marker selection

Eighteen miRNA markers and five reference genes were selected based on previously published results (Table 1 and references herein).

TABLE 1 Candidate microRNAs (miRNAs).

Body fluid	Marker name	miR Base Accession ID	GeneGlobe ID #	References
Blood (Bld)	miR-144-3p	MIMAT0000436	YP00204754	[33, 35, 36, 38, 45]
Blood (Bld)	miR-451a	MIMAT0001631	YP02119305	[27, 36, 39, 45–49]
Saliva (Sal)	miR-203a-3p	MIMAT0000264	YP00205914	[27, 36, 38–40, 45, 49]
Saliva (Sal)	miR-205-5p	MIMAT0000266	YP00204487	[27, 36, 39, 40, 48, 49]
Semen (Spe)	miR-888-5p	MIMAT0004916	YP00206024	[9, 36, 45]
Semen (Spe)	miR-891a-5p	MIMAT0004902	YP00204220	[9, 33, 36–38, 40, 45, 46, 48]
Menstrual blood (Mbld)	miR-214-3p	MIMAT0000271	YP00204510	[9, 36, 40, 46]
Menstrual blood (Mbld)	miR-141-3p	MIMAT0000432	YP00204504	[48]
Menstrual blood (Mbld)	miR-200b-3p	MIMAT0000318	YP00206071	[31, 37]
Menstrual blood (Mbld)	miR-412-3p	MIMAT0002170	YP00204460	[48, 49]
Menstrual blood (Mbld)	miR-3120-3p	MIMAT0014982	YP02118957	[50]
Vaginal secretion (Vag)	miR-193b-3p	MIMAT0002819	YP00204226	[50]
Vaginal secretion (Vag)	miR-1260b	MIMAT0015041	YP02106579	[45, 46, 30]
Vaginal secretion (Vag)	miR-4286	MIMAT0016916	YP02112356	[39]
Vaginal secretion (Vag)	miR-372-3p	MIMAT0000724	YP00204137	[49]
Vaginal secretion (Vag)	miR-124-3p	MIMAT0000422	YP00206026	[9, 36, 38, 39, 45, 49]
Vaginal secretion (Vag)	miR-654-3p	MIMAT0004814	ZP00001998	[30, 36]
Skin	miR-3139	MIMAT0015007	ZP00000587	[39, 46]
Housekeeping	5S rRNA	–	YP00203906	[34, 45, 51]
Housekeeping	RNU1A1	–	YP00203909	[34]
Housekeeping	miR-320a-3p	MIMAT0000510	YP00206042	[51]
Housekeeping	miR-103a-3p	MIMAT0000101	YFI0450028	[34, 36]
Housekeeping	RNU6	-	YP02119464	[27, 34, 36, 40, 49, 50–53]

2.3 | RNA extraction and quantification

Potential ambient RNases were removed from all surfaces and equipment using RNaseZap (Merck). The laboratory space for RNA experiments was a separate room. The miRNeasy Micro Kit (Qiagen AG) was used for RNA extraction according to the manufacturer's instructions. For all biological samples, the tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to the QIAzol solution (50 mM TCEP-QIAzol) as recommended to optimize the sperm RNA-extraction process. RNA was eluted in 30 µL RNase-free water. The concentration of the total RNA was determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). DNase treatment on extracted RNA samples was not performed as in previous studies [37], and our preliminary tests indicated no interference of genomic DNA with miRNA detection.

2.4 | cDNA synthesis

All RT reactions were performed using the miRCURY LNA RT kit (Qiagen AG) according to the manufacturer's instructions. Reactions were performed using 20 ng of total RNA using a VeritiPro thermocycler (Thermo Fisher Scientific) with the following cycling conditions: 42°C for 60 min, 95°C for 5 min, and 4°C hold. The spike-in template UniSp6 was used as recommended positive control RT. After RT, the cDNA was either directly used in qPCR or stored at −20°C for a maximum of 5 weeks.

2.5 | Real-time quantitative PCR

Real-time qPCR was performed using the QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific) with the miRCURY SYBR Green PCR kit (Qiagen AG) according to the manufacturer's instructions, using 3 µL of 30-fold diluted template cDNA. The real-time PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 56°C for 1 min. All sample-assay combinations were run in triplicate technical replicates. QuantStudio Design & Analysis software v 1.5.2 was used to collect and analyze the data. The primer sequences are shown in (https://www.qiagen.com/ at "mircury-lna-mirna-pcr-assays"). A Ct value greater than 35 (average of three technical replicates) was considered to indicate nonspecific amplification and negative results. A qPCR negative control was set up by using nuclease-free water as a template. The efficiency values for the targets measured were all between 95% and 100%. Inter-run calibrator (IRC) samples represented by a pool of the cDNAs of all 30 samples were included, as recommended by Hellemans et al. [41]. The evaluation of IRC values indicated negligible differences between assays (data not shown).

2.6 | Data analysis

The qPCR data set was corrected with the software LinRegPCR (https://www.gear-genomics.com/rdml-tools/) [42]. LinRegPCR calculates Ct and PCR efficiency values based on fluorescent amplification curves. Next, delta CT (ΔCt) was calculated as follows: ΔCt = Ct(target miRNA) − Ct (reference RNA). The Ct (reference RNA) was assigned as the geometric mean of the expression of three (5S RNA, RNU1A1, miR-320a-3p) endogenous reference RNAs. For all not detected miRNAs (Ct above 35), a value of Ct 40 was used for the calculations. The determination of the most suitable reference genes was carried out by using the global mean normalization algorithm geNorm [43]. geNorm is implemented in the reference gene validation software qBasePLUS (Biogazelle), which calculates a gene stability measure (*M*-value) for each candidate.

Box plots were generated using the open-source software BoxPlotR (http://shiny.chemgrid.org/boxplotr/). Note that a low value of ΔCt for a miRNA means that it is highly expressed in the sample. Principal component analysis (PCA) and unsupervised hierarchical clustering visualized by a heat map were generated using the open-source software ClustVis (http://biit.cs.ut.ee/clustvis/) [44].

3 | RESULTS

3.1 | Validation of the endogenous reference genes

Special care was taken in the selection and validation of multiple reference genes for the normalization of the RT-qPCR results. Five reference genes, 5S rRNA, RNU1A1, miR-103a-3p, U6, and miR-320a-3p, most endorsed by previous studies, were included in all RT-qPCR experiments to obtain normalized expression data; this means that all samples of each body fluid were tested for the complete set of endogenous references. Reference RNAs retained for normalization purposes were selected among those showing positive results in all tested body fluids and similar expression levels among individuals and body fluids. Moreover, the stability of the endogenous reference targets, evaluated by *M*-value (geNorm) (Figure S1), indicated three genes below the value of 2. No *M*-values could be calculated for miR-103a-3p and U6 that were therefore not considered for normalization. The reference U6 showed low expression levels with Ct values close to or above 30 for all tissues except vaginal secretion. The other four RNAs showed very similar expression values across individuals and body fluids except for miR-103a-3p, which was less expressed in saliva, menstrual blood, vaginal secretion, and skin. The final panel of reference genes included one miRNA (same RNA type as the selected discriminating markers), miR-320a-3p, and two other very commonly used RNAs: 5S rRNA and RNU1A1.

3.2 | Selection of miRNA candidates

First, markers were investigated for RT-qPCR amplification efficiency by using a pool of six cDNA samples per body fluid and six serial dilutions of fivefold. Of the 18 candidates (Table 1), 2 miRNAs selected for discriminating menstrual blood (miR-3120-3p and miR-412-3p), 3 miRNAs selected for vaginal secretions (miR-372-3p, miR-124-3p, and miR-654-5p), and the one selected for skin (miR-3169) either showed no expression or too weak values (Ct > 35)

in the specific body fluid they should discriminate. The miRNA markers that were not suitable for our detection system were discarded. Because no other potential miRNA candidate for skin could be selected from previous studies, the analysis of this tissue was not continued in this study.

3.3 | Comparison of miRNA expression among five body fluids

The relative abundance of each miRNA was normalized with three validated endogenous genes (5S rRNA, RNU1A1, and miR-320a-3p), and the expression level was quantified by the ΔCt method. Figures 1 and 2 show the results for 12 miRNAs, each analyzed across body fluids and individuals. Note that, because of the way ΔCt values are calculated, smaller values correspond to higher miRNA expression.

As previously reported, none of the miRNA candidates were found to be expressed exclusively in one tissue. Several markers showed a high degree of body fluid specificity in the expression level as reported in the literature: miR-888-5p and miR-891a-5p were highly expressed only in semen (Figure 1), miR-214-3p showed a higher expression level in menstrual blood (Figure 2), and miR-193b-3p and miR-1260b were highly expressed in vaginal secretion (Figure 2). Two miRNAs highly expressed in blood (miR-144-3p and miR-451a) (Figure 1) were also highly expressed in menstrual blood. Their use in combination with the miR-214-3p specific for menstrual blood should enable the discrimination between blood and menstrual blood. Similarly, miR-205-5p was more expressed in saliva but also in vaginal secretion (Figure 1); therefore, in combination with the two miRNAs specific for vaginal secretion (miR-193b-3p and miR-1260b), it has the potential for saliva inference. Finally, unsatisfactory results were obtained for the four remaining miRNAs: miR-203a-3p, a candidate for saliva, was more expressed in vaginal secretion (Figure 1), miR-141-3p and miR-200b-3p, candidates for menstrual blood, were also highly expressed in several other body fluids (Figure 2), and miR-4286, a candidate for vaginal secretion, appeared less specific than the other two candidates (Figure 2).

To conclude, eight miRNAs show good tissue specificity, whereas four appear less useful for discriminating body fluids.

3.4 | PCA and clustering analysis

To test if this set of differentially expressed miRNAs could identify a unique expression profile characteristic of each body fluid, the whole data set of ΔCt values was analyzed by PCA and clustering analysis visualized by a heat map. The PCA analysis (Figure 3) showed the first two components explained more than 80% of the variance of the data set. The five body fluid clusters were identified with no outliers or overlapping. Unsupervised hierarchical clustering of miRNA expressions visualized by a heatmap (Figure 4) also indicated high correlation within the samples of the same body fluid for all five tissues, which identified separate groups. One saliva sample showing a very low signal across all RNA markers did not cluster with the saliva samples.

3.5 | PCA and clustering analysis of a blind test

A blind sample assessment was performed in which five new trace evidences, representing each body fluid, were simulated and analyzed for the subset of eight miRNAs (miR-144-3p, miR-451a, miR-205-5p, miR-214-3p, miR-888-5p, miR-891a-5p, miR-193b-3p, and miR-1260b) (Figures 5 and 6). The PCA results show a correct clustering of the test samples with an overall loss of resolution, probably due to the elimination of the four less specific markers (miR-203a-3p, miR-141-3p, miR-200b-3p, and miR-4286) (Figure 5). The clustering from the heatmap is also correct (Figure 6), except for the non-classified saliva sample, as before.

These two common unsupervised learning techniques (PCA and clustering) show that patterns of differentially expressed miRNAs characteristic of each body fluid exist, suggesting the presence of distinctive miRNA profiles that may be used for body fluid inference. Note that the number of samples used here is not sufficient to build a model to assign a probability of tissue type to each sample. The procedure of testing a single unknown trace lacks sample variability, which is necessary to evaluate the discrimination power of the proposed panel, leading to a potential overestimation of the marker set's effectiveness.

4 | DISCUSSION

In this study, we further validated a small set of 12 miRNA markers to effectively discriminate among five common body fluids in forensic casework. The quantitative evaluation of marker levels was performed using the gold standard method of RT-qPCR, and biological samples of peripheral blood, menstrual blood, saliva, semen, and vaginal secretion were deposited in trace amounts and dried to simulate real forensic traces.

Previously, small sets of miRNAs for body fluid discrimination have been proposed [28, 32, 36–39, 45]; these studies mainly focus on discriminating the five main body fluids,

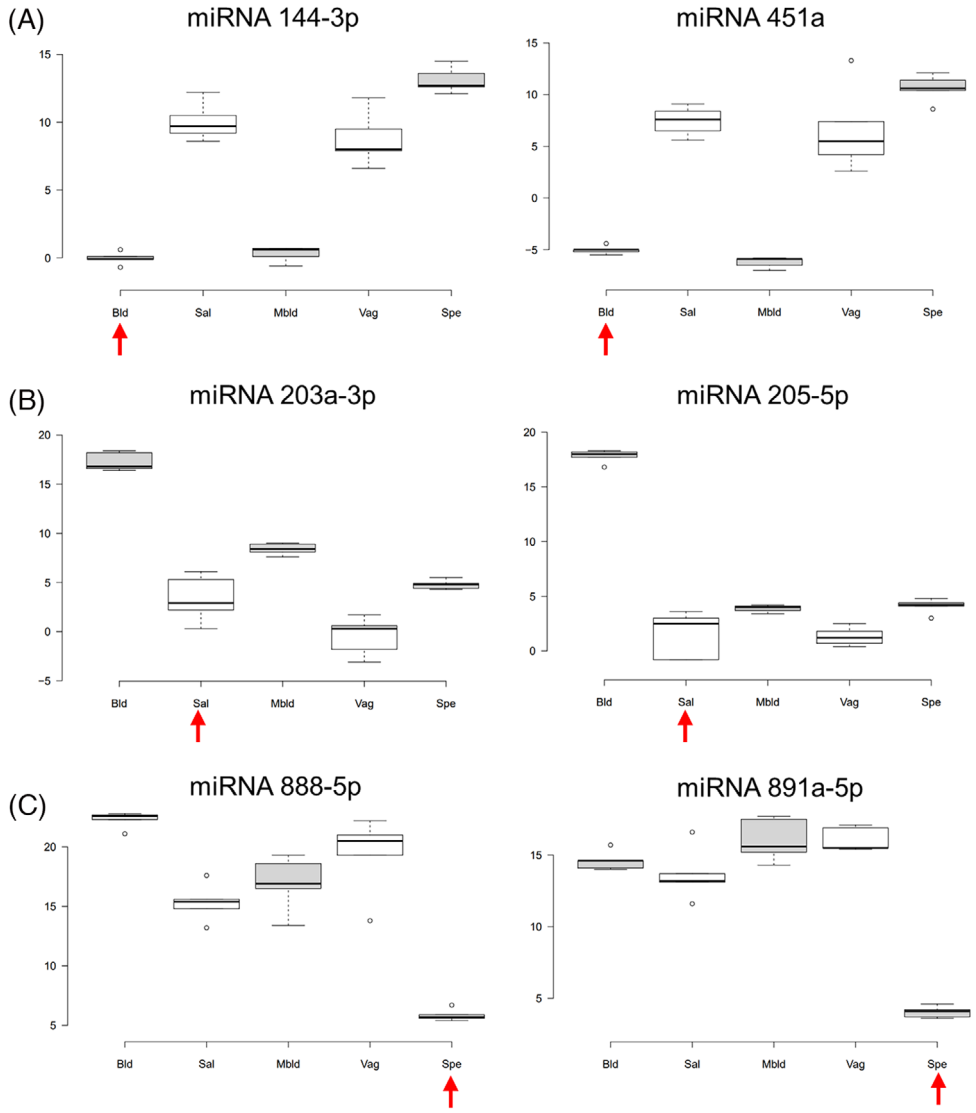

FIGURE 1 Normalized expression patterns (ΔCt values) of six microRNA (miRNA) markers evaluated in five individuals for each body fluid. Markers miRNA-114-3p and miRNA-451a were initially selected as peripheral blood-specific (A), miRNA-203a-3p and miRNA-205-5p for saliva (B), and miRNA-888-5p and miRNA-891a-5p for semen (C). Normalization was performed using validated endogenous controls RNU1A1, 5S rRNA, and miRNA-320a-3p. Results are depicted in box plots presenting the median with interquartile range, lower (25%), and upper (75%) quartiles. Whiskers indicate the maximum/minimum values without outliers. Outliers are presented as circles: peripheral blood (Bld), saliva (Sal), menstrual blood (Mbld), vaginal secretion (Vag), and semen (Spe). Red arrows indicate the body fluid expected to be discriminated by the specific miRNA based on previously published data.

peripheral blood, menstrual blood, saliva, semen, and vaginal secretion, sometimes with the inclusion of skin [28, 39] or the omission of menstrual blood [45].

Overall preceding results, based on interrogating four to 15 markers, showed that not all tissues can be qualitatively distinguished based on the most expressed miRNAs unless a sophisticated probabilistic discrimination model is used. Semen and blood can be easily discriminated across most reports; instead, distinguishing venous blood from menstrual blood, and saliva from vaginal secretion, appeared challenging [28, 30, 32, 33, 38, 45].

Compared to these studies, our data show a surprisingly marked differential expression of several miRNA markers among the body fluids (Figure 3, a complete set of 12 miRNAs), which may enable a statistically robust evaluation of the results once a larger database is gathered. Although PCA analysis and hierarchical clustering revealing distinct patterns of separate categories are promising, they do not fully guarantee the confirmation of results when testing a larger sample size. Yet, if the observed patterns persist with a large training set, this would provide the basis for a simple interpretation model.

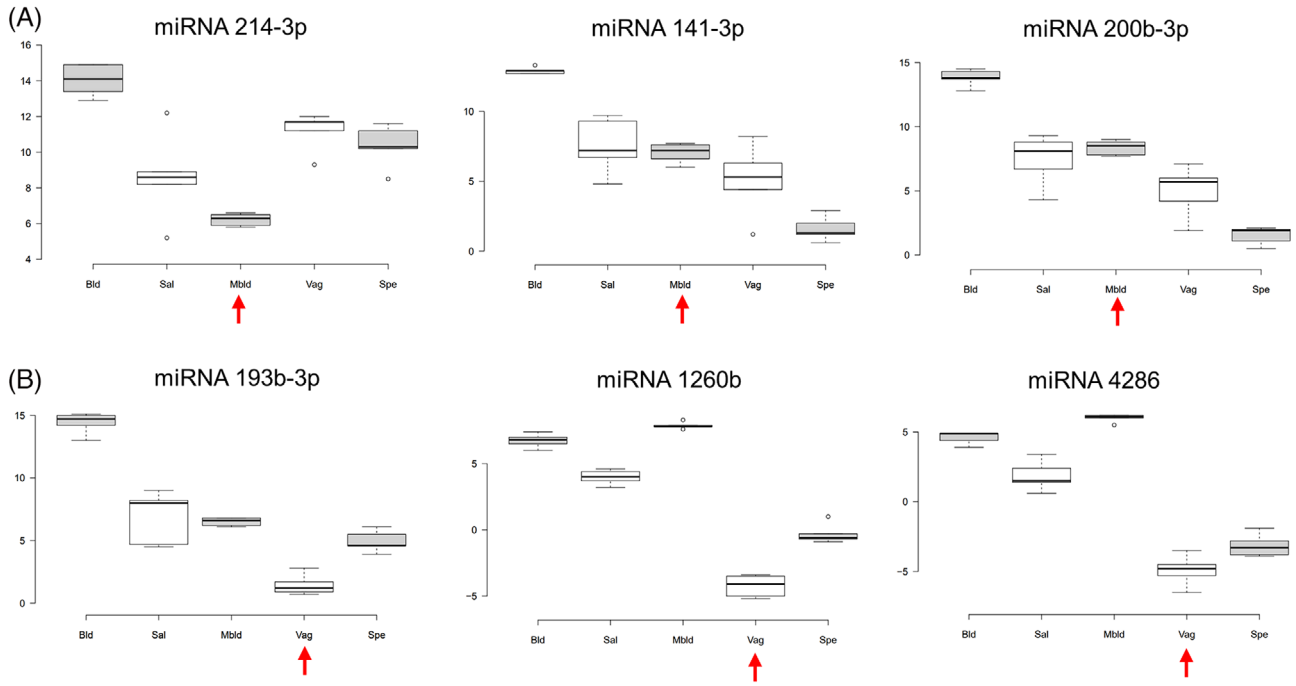

FIGURE 2 Normalized expression patterns (ΔCt values) of six microRNA (miRNA) markers evaluated in five individuals for each body fluid. Markers miRNA-214-3p, miRNA-141-3p, and miRNA-200b-3p were initially selected as menstrual blood specific (A), miRNA-193b-3p, miRNA-1260b, and miRNA-4286 for vaginal secretion (B). Normalization was performed using validated endogenous controls RNU1A1, 5S rRNA, and miRNA-320a-3p. Results are depicted in box plots presenting the median with interquartile range, lower (25%) and upper (75%) quartiles. Whiskers indicate the maximum/minimum values without outliers. Outliers are presented as circles: peripheral blood (Bld), saliva (Sal), menstrual blood (Mbld), vaginal secretion (Vag), and semen (Spe). Red arrows indicate the body fluid expected to be discriminated by the specific miRNA based on previously published data.

We believe that the strength of our data resides in some key experimental setups: (1) The preamplification step used in other studies [32, 39] was avoided to keep the full quantitative aspect of the assay; (2) the use of three endogenous reference genes, including one of the same type (miRNAs), for normalization of the Ct values; and finally, (3) the use of more than one marker specific for each body fluid.

Besides the successful validation of most of the miRNA candidates considered, some (six markers) were instead

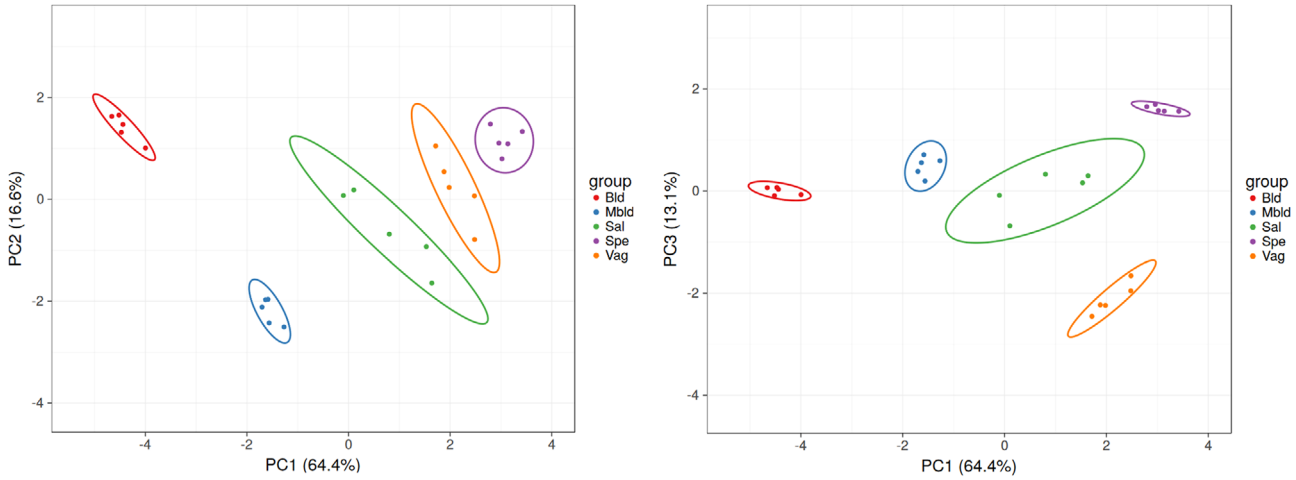

FIGURE 3 Principal component analysis (PCA) of ΔCt values of 25 samples, representing 5 body fluids, using 12 microRNA (miRNA) markers: peripheral blood (Bld), saliva (Sal), menstrual blood (Mbld), vaginal secretions (Vag), and semen (Spe).

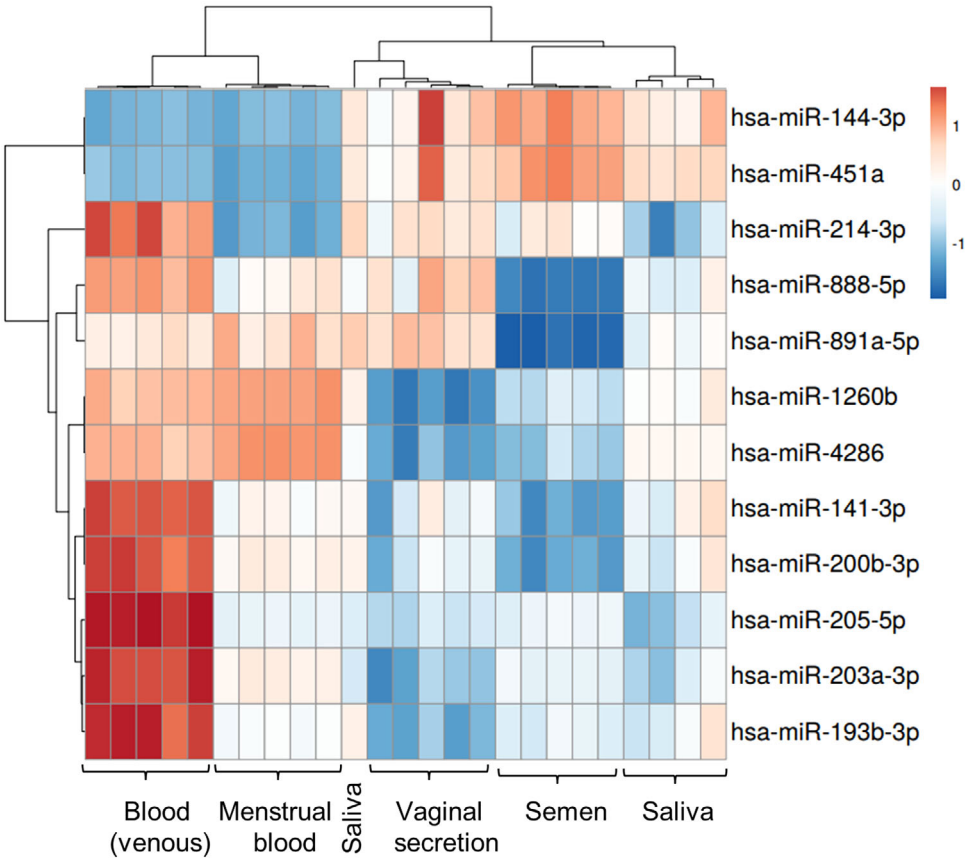

FIGURE 4 Heatmap and two-dimension hierarchical clustering based on the normalized expression levels of 12 microRNAs (miRNAs). Each column represents a sample; each row represents a miRNA.

not detected in the specific body fluid, and few markers also appeared not to contribute to the discrimination process. These are the candidates for menstrual blood, miR-141-3p and miR-200b-3p, which, in addition to being positive for semen, do not distinguish menstrual blood from vaginal secretion. This difficulty, previously reported, is due to the similar composition of all the fluids of the late secretory phase of the uterine cycle [37]. Luckily, we could confirm miR-214-3p for discriminating menstrual blood from blood and all other fluids [32, 36, 46]. Like other studies, we report difficulties in discriminating saliva and vaginal secretion [30, 32, 36] with their specific markers; yet, the complete miRNA profile generates a tissue-specific signature, which finally enables the discrimination of the two fluids.

With this study, we confirm an important feature of miRNAs for forensic applications: On one side, the absence of markers exclusive to one tissue, and on the other, the presence of miRNA expression signature specific to forensically relevant body fluids. Finally, the selection of only one marker for skin tissue, which did not work in our hands, led us to discard this important type of trace, which will require an extensive evaluation of markers capable of distinguishing all body fluids composed of epithelial cells, including saliva and vaginal secretion [39].

5 | CONCLUDING REMARKS

Researchers in the field of body fluid identification using miRNA profiling advocated for validating standardized forensic procedures, to avoid variation in the results due to different methods and devices for miRNA extraction, RT, quantitation, and marker sets. Our work contributes to the search for an optimal marker set and analytical conditions, including normalization procedures needed for the implementation of this approach into routine casework.

Further research based on a larger number of samples per body fluid is needed to assess the robustness and provide the statistical significance of the results presented here after considering the individual variability in miRNA expression. Finally, forensic validation typically includes traces exposed to various environmental, biological conditions and mixtures. miRNAs are expected to be stable to environmental factors; however, the possible changes in miRNA gene expression due to specific diseases or non-physiological conditions should be investigated [27].

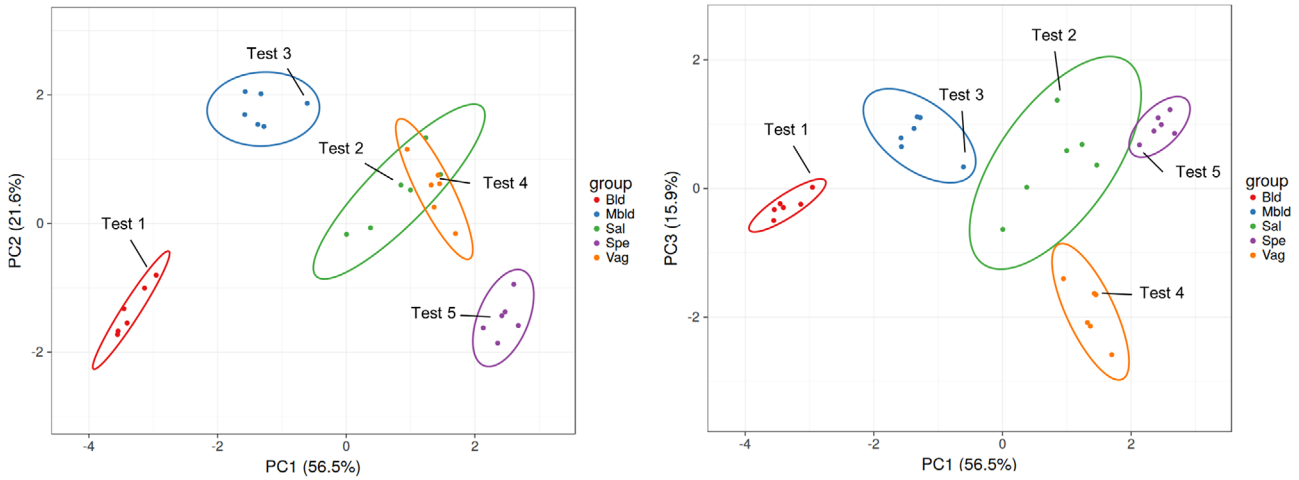

FIGURE 5 Principal component analysis (PCA) of ΔCt values of 25 plus 5 test samples, representing the 5 body fluids. The microRNA (miRNA) markers used are eight (miR-144-3p, miR-451a, miR-205-5p, miR-214-3p, miR-888-5p, miR-891a-5p, miR-193b-3p, and miR-1260b), four (miRNA-203a-3p, miRNA-141-3p, miRNA-200b-3p, and miRNA-4286) were not included: peripheral blood (Bld), saliva (Sal), menstrual blood (Mbld), vaginal secretions (Vag), and semen (Spe); Test 1 (Bld), Test 2 (Sal), Test 3 (Mbld), Test 4 (Vag), and Test 5 (Spe).

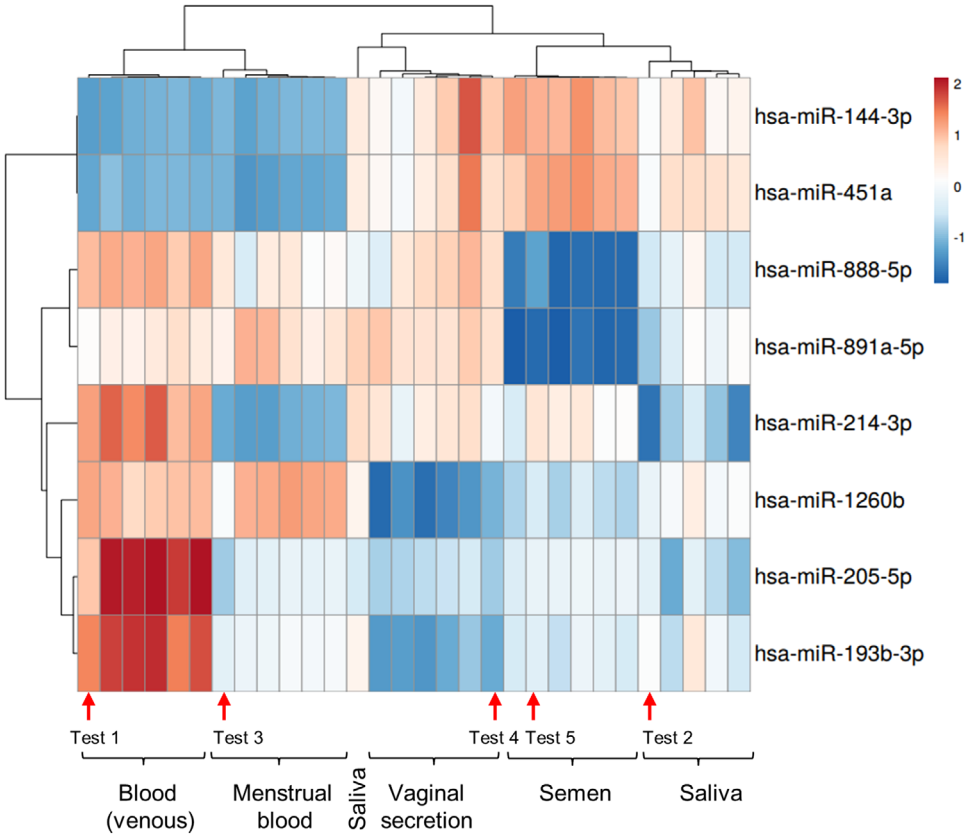

FIGURE 6 Heatmap and two-dimension hierarchical clustering based on the normalized expression levels of eight microRNAs (miRNAs). Each column represents a sample; each row represents a miRNA. Arrows indicate the five test samples, one for each body fluid. The miRNA markers used are eight (miR-144-3p, miR-451a, miR-205-5p, miR-214-3p, miR-888-5p, miR-891a-5p, miR-193b-3p, and miR-1260b); four (miRNA-203a-3p, miRNA-141-3p, miRNA-200b-3p, and miRNA-4286) were not included.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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