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ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2026;15(3):1242-8

Evaluating stability of miR-891a and miR-10a markers in semen samples to various conditions

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Received 16 April 2026; Accepted 15 June 2026
Available online 15 August 2026 with doi: 10.5455/medscience.2026.04.078

Abstract

MicroRNAs (miRNAs) have emerged as promising biomarkers for body fluid identification in forensic biology because of their small size and tissue-specific patterns. Their relative stability compared with other RNA molecules makes them potential alternatives in cases where DNA-based analysis is limited by degradation or insufficient sample quantity. The aim of this study is to evaluate the stability of miR-10a and miR-891a in semen samples exposed to environmental conditions relevant to forensic casework. Semen samples obtained from five healthy male volunteers were deposited onto 100% cotton fabric and exposed to four environmental conditions: room temperature, burial in soil, submersion in lake water, and washing at 90°C with detergent. Total RNA was isolated using a spin-column-based extraction kit, followed by cDNA synthesis and quantitative real-time PCR (RT-qPCR) analysis. Relative expression levels were normalized using RNU6b as a reference gene and evaluated using the $2^{-\Delta\Delta Ct}$ method. miR-891a showed greater relative stability than miR-10a under the tested conditions. Increased miRNA degradation was observed in samples exposed to high temperature and detergent, and miRNA recovery was not achieved in these samples was not possible. Although condition-related differences were observed, statistical analysis did not show significant overall effect of environmental exposure on miRNA stability ($p > 0.05$). miR-891a may represent a promising biomarker for semen identification in forensic casework. Nevertheless, the results should be interpreted cautiously, as recovery was limited under extreme exposure conditions. Additional studies with larger sample sizes are required to further evaluate the applicability of these markers in degraded samples.

Keywords: Semen, miRNA, forensic biology, degradation, body fluid identification

Introduction

In forensic science, biological samples collected from crime scenes play a fundamental role in clarifying criminal events and identifying perpetrators. Body fluids such as blood, saliva, semen, urine, and vaginal secretions are among the most frequently encountered forms of biological evidence. Proper identification of these materials is crucial for determining the type and timing of the incident, as well as clarifying the relationship between the victim and the perpetrator [1].

Semen is one of the most important biological evidence in sexual assault investigations. In routine casework, semen detection relies on methods such as the acid phosphatase (AP) test, prostate-specific antigen (PSA) test, and microscopic examination [2,3]. However, these tests may occasionally yield false-positive or false-negative results and may also disruption DNA integrity in trace samples. For these reasons, RNA-based molecular approaches have gained increasing attention in forensic biology in recent years [4,5].

Ribonucleic acid (RNA) molecules can be used for determining the origin of biological samples due to their tissue-specific expression profiles [5]. Among RNA species, microRNAs (miRNAs) are particularly well-suited as forensic biomarkers

CITATION
Kurt AC, Ercan B, Holumen N, et al. Evaluating stability of miR-891a and miR-10a markers in semen samples to various conditions. Med Science. 2026;15(3):1242-8.

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DOI: 10.5455/medscience.2026.04.078

because of their small size (~22 nucleotides), high stability, and resistance to degradation. miRNAs regulate gene expression at the post-transcriptional level, play essential roles in cellular processes, and exhibit distinct tissue-specific expression patterns [6,7]. Previous research has shown that certain miRNAs are highly expressed in specific body fluids, making them valuable for body fluid identification [8,9]. In semen, miR-10a and miR-891a have been found at significantly higher levels than in other body fluids and have therefore been proposed as semen-specific molecular biomarkers [9–15]. Nevertheless, the stability of these miRNAs under various environmental conditions and their ability to remain stable under crime scene conditions have not been sufficiently investigated.

The aim of this study is to evaluate the stability of miR-10a and miR-891a in semen samples exposed to different environmental conditions, to determine changes in their expression levels during degradation, and to contribute to next-generation RNA-based approaches in forensic biology. Consequently, it is anticipated that miRNA profiling methods may provide reliable support in cases where DNA analysis is limited or the samples are degraded.

Material and Methods

To minimize inter-individual variation, semen samples were collected from five healthy male volunteers aged 25–35 years. As smoking is known to influence semen composition, only non-smoking individuals were included. All samples were collected after a 72-hour period of sexual abstinence. Ethical approval for the study was obtained from the İstanbul University–Cerrahpaşa Ethics Committee on 18 January 2023 (Ethical approval number: E-83045809-604.01.01-593273). To ensure experimental consistency, only normospermic samples were included in the study. Semen analysis was performed by light microscopy to assess sperm count, morphology and motility and samples meeting the World Health Organization criteria [16] were selected for inclusion. All semen samples were stored at −80°C until experimental analysis. For sample preparation, 100% white cotton fabric was used. The fabric was cut into twenty pieces measuring 5 × 5 cm, and 200 µL of semen was applied to each piece. After air-drying at room temperature for 24 hours, the samples were assigned to four different degradation conditions. Five replicated were prepared for each condition, as presented in Table 1.

Table 1. Sample groups and corresponding environmental conditions used in the study

Environmental condition	Sample groups
Incubation at room temperature for 20 days	O1-O2-O3-O4-O5
Submersion in lake water for 20 days	S1-S2-S3-S4-S5
Burial in soil at a depth of 15 cm	T1-T2-T3-T4-T5
Washing in a household washing machine at 90°C with powdered detergent for 1 hour	Y1-Y2-Y3-Y4-Y5

Samples in the O group were kept at room temperature (20–25°C) for 20 days and served as the control group (n = 5).

For the T group, samples were buried in slightly moist soil was collected from the garden of İstanbul University–Cerrahpaşa Institute of Forensic Sciences and Legal Medicine. The samples were placed at a depth of 15 cm in glass beakers and kept at room temperature for 20 days (n = 5).

For the S group, samples were submerged in lake water collected from Büyükçekmece Lake. The water was placed in 250 mL glass beakers, and the samples were kept at the bottom of the containers at room temperature for 20 days (n = 5).

For the Y group, samples were washed on the same day in a standard household washing machine at 90°C with powdered detergent for approximately 1 hour and subsequently air-dried at room temperature for 20 days (n = 5).

RNA extraction from fabric samples was performed using the spin-column method by Zymo research, Quick-RNATM MiniPrep Plus Kit. Briefly, the fabric materials were cut into small sections and incubated in 400µL DNA/RNA Shield for approximately 10 minutes, followed by centrifugation to transfer cellular material into the liquid phase. For each sample, 15µL Proteinase K, 30µL PK Digestion Buffer, and 10µL DDT (Dichlorodiphenyltrichloroethane) were added, and the mixture was incubated at room temperature for 30 minutes to ensure cell lysis. Following RNA extraction, cDNA was synthesized using ABM miRNA All-In-One cDNA Synthesis Kit. This method provides polyadenylation- based reverse transcription with Poly-A Polymerase and oligo d(T) primers and it is used according to manufacturer's instructions. The reaction mixture was then incubated in a thermal cycler at 37°C for 30 minutes, 50°C for 15 minutes, 85°C for 5 minutes, and 4°C for 1 minute, after which the tubes were placed on ice. Target miRNA regions were amplified by RT-PCR system using specific primers. Amplification was monitored in real time with a fluorescent dye that binds nonspecifically to double-stranded cDNA. Cycle threshold (Ct) values were obtained for each sample and used in the subsequent analyses.

For quantitative analysis, forward primer sequences for miR-891a and miR-10a were selected based on the miRBase database and primers are listed as Table 2. RNU6b was used as the reference gene and relative expression levels were normalized to RNU6b [17,18]. qPCR was performed with an initial enzyme activation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute.

DOI: 10.5455/medscience.2026.04.078

Table 2. Sequence information for the target miRNAs

Target miRNA	Sequence	miRBase Accession Number
miR-819a	UGCAACGAACCUGAGCCACUGA	MIMAT0004902
miR-10a	UACCCUGUAGAUCCGAAUUUGUG	MIMAT0000253

Results

Ct values obtained from the qPCR analysis are presented in Table 3. For each sample, ΔCt values were calculated using the formula ΔCt = Ct(miRNA) – Ct(RNU6b), and these values were used for statistical analysis All statistical analyses were performed using IBM SPSS Statistics 22. Samples with Ct values ≥ 35 were excluded from the analysis. In addition, since no amplifiable miRNA was detected from the washed (Y) group, this group was also excluded from the statistical evaluation. Data distribution was assessed using the Shapiro–Wilk test, together with p-values and Skewness–Kurtosis values ($p > 0.05$). Differences in miRNA ΔCt values between groups were analyzed using One-Way ANOVA and Tukey's post hoc test.

Table 3. Ct values of the target miRNAs in the study samples

Sample	RNU6b Ct	RNU6b average Ct	miR-10a Ct	miR-10a average Ct	miR-891a Ct	miR-891a average Ct
O1	30.71		35.00		35.00	
O2	30.83		31.13		38.23	
O3	37.08	31.84	36.28	32.79	40.00	35.21
O4	28.59		30.00		33.71	
O5	32.00		31.55		34.10	
S1	32.46		35.00		35.00	
S2	29.43		29.19		35.48	
S3	36.90	32.54	35.03	33.20	36.83	35.17
S4	30.69		33.75		34.15	
S5	33.24		33.04		34.40	
T1	31.92		35.00		35.00	
T2	28.44		29.99		33.84	
T3	39.17	32.75	36.28	32.90	37.06	34.70
T4	32.09		31.00		33.90	
T5	32.11		32.80		33.72	
Y1	35.00		35.00		35.00	
Y2	37.06		38.87		37.91	
Y3	40.00	36.58	36.42	35.90	40.00	36.58
Y4	35.00		35.00		35.00	
Y5	34.77		34.23		35.00	

Statistical Results

Differences among environmental conditions were assessed separately for each miRNA using one-way ANOVA. The results of the statistical analyses are presented in Table 4.

According to the results of the one-way ANOVA and Tukey's post hoc test, no statistically significant differences were observed between thee environmental conditions ($p > 0.05$), indicating that these conditions did not significantly affect miRNA stability.

DOI: 10.5455/medscience.2026.04.078

Table 4. Comparison of miR-10a and miR-891a under different environmental conditions and corresponding p-values

miRNA	Compared conditions	P value
miR-10a	O-T	0.971
	O-S	0.729
	T-S	0.733
miR-891a	O-T	0.381
	O-S	0.562
	T-S	0.759

Fold Change Findings

In studies involving multiple experimental conditions, Ct and ΔCt values alone may not be sufficient to fully interpret relative miRNA expression. Therefore, fold change values were calculated using the 2−ΔΔCT method, and the results are presented in Figure 1 and Figure 2.

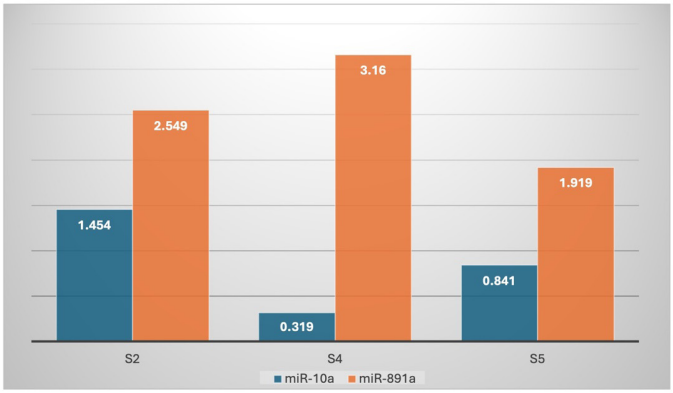

Figure 1. Fold change values of miR-10a and miR-891a in the S group

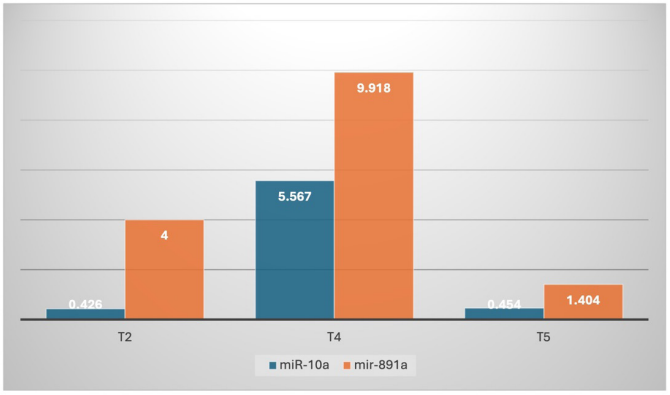

Figure 2. Fold change values of miR-10a and miR-891a in the T group

ΔΔCT values were calculated according to the formula:

$$\Delta\Delta CT = \Delta CT \text{ (experimental group)} - \Delta CT \text{ (control group)}$$

In order to ensure reliable normalization and comparison, samples with Ct values ≥35 were excluded from the fold change analysis. In addition, samples from the Y group was excluded because no amplifiable miRNA was recovered. Fold change comparisons for the S and T groups were performed relative to the O group, which served as the reference condition.

Fold change analysis showed that miR-891a generally exhibited greater relative expression than miR-10a in samples exposed to lake water and soil conditions. In sample S2, both miRNAs were upregulated relative to the control group, with a greater increase observed for miR-891a. In sample S4, miR-891a expression increased by approximately 3.16-fold, whereas miR-10a showed an approximately 3.13-fold decrease compared with the control group similarly, in sample S5, miR-891a remained upregulated, while miR-10a was expressed lower level than the control.

A similar pattern was observed in the soil-exposed samples. In sample T2, miR-891a showed a marked increase in expression, whereas miR-10a was downregulated relative to control group. The highest fold change among all analyzed samples was observed in T4, where both miR-10a and miR-891a were upregulated, with a more pronounced increase in miR-891a. Overall, miR-891a showed increased expression relative to the control group, whereas miR-10a showed an overall decrease. These findings indicate that miR-891a was more stable than miR-10a under tested conditions.

Discussion

With advancements in technology, alternative approaches for the identification of body fluids have gained increasing interest, and RNA molecules—known for their tissue-specific characteristics and ability to be co-extracted with DNA—have emerged as promising candidates. The first RNA type investigated for body fluid identification was mRNA. However, due to its long molecular structure, mRNA is prone to environmental degradation. In contrast, microRNAs (miRNAs) are more resistant to degrading conditions and nuclease activity because they are shorter in length and protected within protein complexes [19]. In a study by Mayes et al., the effects of environmental conditions on semen mRNA (PRM1) and semen miRNA (miR-891a) were compared, demonstrating that miR-891a is considerably more stable than PRM1, particularly under extreme heat and humidity [20]. Similarly, Bamberg et al. evaluated semen mRNA/miRNA expression in body fluids and diluted samples; although mRNAs performed better in diluted samples, miRNAs were shown to be much more successful molecules in degraded samples [21]. For a biomarker to be ideal in body fluid identification, its stability under various environmental conditions must be thoroughly examined and supported by an established database. Tong et al. reported that miRNAs remained detectable in semen samples stored for one year, with an approximate 80% success rate, even at low total RNA concentrations (0.01 ng) [18]. In a study examining storage conditions and sample age, Lancia et al. emphasized that degradation of semen miRNAs was influenced more by environmental exposure than by time itself, and confirmed that miR-10a and miR-891a were effective discriminators of semen even in aged or mixed samples [22]. Other studies involving

DOI: 10.5455/medscience.2026.04.078

degraded or trace biological materials consistently indicate that miRNAs can be detected even in harsh environmental conditions and hold significant potential for forensic applications [14,20,23–25]. Based on the literature, miR-10a and miR-891a were selected as target markers in the present study. miR-10a has been shown to be abundantly expressed in semen and proposed as a reliable biomarker for semen identification [9,11,22,26,27]. Numerous studies have also confirmed miR-891a as one of the most ideal semen-specific markers, consistently found at much higher concentrations in semen than in other body fluids [12,13,15,26,28–31]. In our study, semen samples were applied to 100% white cotton fabric under four scenarios simulating crime scene conditions. To achieve optimal-quality isolation of semen miRNAs, a fabric type containing the least amount of PCR-inhibitory substances, such as dyes and chemical additives, was chosen.

Previous researches has shown that fabric type can significantly affect both RNA yield and quality [32–35]. RNA extraction was performed using a spin-column-based kit following the manufacturer's protocol. For RNA recovery from fabric, incubation time in DNA/RNA Shield solution was extended. After isolation, the RNA quantity and quality of the samples were found to be low; therefore, the samples were incubated following the addition of 10 µL of DDT to enhance cell lysis. Since the samples incubated with DDT showed a significant increase in RNA amount and purity, the same procedure was applied to the other samples. Samples with Ct values ≥ 35 were considered highly degraded [11,36,37]. Based on mean Ct values, samples stored at room temperature (O group) represented the least degraded condition. The reference gene (RNU6b) was more conserved than the target miRNAs. Among the O-group samples, miR-891a exhibited higher average Ct values (lower expression) than miR-10a. The O-group samples in this study were stored for 20 days at room temperature in a dark, ventilated environment. Compared with other studies, miRNA stability under these conditions was lower than expected. In the lake water-exposed (S group) and soil-exposed (T group) samples, miR-10a showed lower mean Ct values than miR-891a; however, fold change analysis demonstrated that miR-891a had a relative advantage under these conditions. Lake water and soil typically contain numerous microorganisms and nucleases capable of degrading nucleic acids, yet degradation in our samples was lower than we expected. The degradation rate of nucleic acids in water exposed samples depends on factors such as temperature, microorganism density, pH level, salinity, and water movement. In the samples washed at 90°C in a washing machine (Y group), both the target miRNAs and the reference gene showed Ct values greater than 35, indicating that the target miRNAs could not be detected. During the washing process, a bleach-free powder detergent containing Sodium Lauryl Sulfate as the active ingredient was used. Genetic material in biological stains is known to undergo severe degradation during washing due to exposure to high temperature and detergent. Study conducted by Mayes et al. reported detectable miR-891a in washed semen stains but noted a significant decrease in miRNA concentration with increasing washing temperature [20]. Other studies examining washed fabrics consistently report that temperatures ≥ 60°C cause highly destructive effects on genetic material, causing body fluid characteristics to be lost as forensic evidence [34,38].

A limitation of this study is the relatively small sample size. As this project was carried out within the scope of a Master's thesis, the number of samples was constrained by budget limitations. Future studies with larger cohorts are needed to validate these preliminary findings.

Despite this sample size limitation, our study offers valuable insights and contributions to the field of forensic sciences. While miRNAs are not yet part of routine forensic laboratory practice, this research addresses a critical gap in body fluid identification: the stability of biological markers under varying environmental stressors. By evaluating how semen-specific markers (miR-891a and miR-10a) with stand diverse degradation conditions, this study provides essential baseline data for future forensic workflows. Among the two markers evaluated, miR-891a appeared significantly more promising due to its resilience, marking it as a strong candidate for forensic application. Continued research, larger validation studies, and the establishment of standardized analytical protocols may fully support the future integration of these miRNAs into routine forensic practice. However, further investigation remains necessary before the forensic utility of miR-891a can be definitively established.

Conclusion

The present findings contribute to the growing evidence that miRNAs may serve as useful biomarkers for body fluid identification in forensic science. At the same time, the performance of these markers should be interpreted cautiously under adverse environmental conditions, where recovery may be limited. Compared with previous reports, the overall degree of degradation observed in this study appeared higher than expected, underscoring the importance of carefully evaluating the stability of each marker under different exposure conditions.

Although miRNAs are not yet part of routine forensic laboratory practice, continued research, larger validation studies, and the establishment of standardized analytical protocols may support their future use in forensic applications. Among the two markers evaluated, miR-891a appeared more promising; however, further investigation is needed before its forensic utility can be fully established.

Ethical Approval

Ethics committee approval was received from İstanbul University-Cerrahpaşa Clinical Research Ethics Committee with E-83045809-604.01.01-593273 numbered and 18.01.2023 dated.

Patient Consent

All volunteers were informed about the purpose and procedures of the study and written informed consent was obtained prior to sample collection. No identifying personal information is included in this manuscript.

Conflict of Interest
The authors confirm the absence of any conflicts associated with this work.

Funding
This study was supported by İstanbul University-Cerrahpaşa Scientific Research Projects (BAP) (project number 37534).

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DOI: 10.5455/medscience.2026.04.078

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