NOFOR
NOVEL FORENSIC RESEARCH

DOI: 10.5455/NOFOR.2025.09.06

NOFOR. 2025;4(2):54-63

Review Article

The role of micrornas in forensic genetics and a comparative efficiency analysis of isolation methods from body fluids

Fatma Ebru Bekiroglu, Nazli Holumen, Emel Hulya Yukseloglu

İstanbul University-Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine, İstanbul, Türkiye

Received February 12, 2025; Accepted July 17, 2025; Available online August 15, 2025

Abstract

This review aims to evaluate the role of microRNAs (miRNAs) as molecular biomarkers in forensic genetics and to comparatively analyze the efficiency of different miRNA isolation methods from various body fluids. A systematic literature review was conducted following PRISMA guidelines. Publications published between 2010 and 2025 were screened in PubMed, Scopus, and Google Scholar databases using the keywords "microRNA", "forensic genetics", "RNA isolation", and "body fluids". Fifty-three peer-reviewed studies met the inclusion criteria and were evaluated in terms of yield, purity, cost, and applicability of the isolation method. The comparative analysis included phenol–chloroform, column-based, magnetic bead-based, and hybrid protocols. The reviewed studies revealed that while phenol–chloroform methods provided the highest RNA yield, column-based and hybrid systems ensured superior purity and reproducibility. Magnetic bead–based techniques were advantageous for automation and reduced contamination risk. The optimal isolation method varied depending on the biological fluid type. miRNAs exhibit high stability and diagnostic potential in forensic applications such as body fluid identification, age estimation, and post-mortem interval determination. Selecting isolation methods optimized for specific biological matrices enhances analytical reliability and facilitates the integration of miRNA-based analyses into routine forensic workflows.

Keywords: microRNA, forensic genetics, RNA isolation, comparative analysis

INTRODUCTION

The need for reliable molecular biomarkers in forensic genetics has increased as the limits of conventional Short Tandem Repeat (STR) analyses become more apparent. In this context, microRNAs (miRNAs) have emerged as valuable alternatives due to their stability, tissue-specific expression, and regulatory roles in gene expression [1].

miRNAs are small non-coding RNAs, about 18–25 nucleotides long, and remain stable in various cellular and extracellular environments, a feature that supports their forensic usefulness [2]. Their multistep biogenesis, from primary to mature forms, further contributes to their robustness and analytical value [3].

Because miRNA expression differs across fluids such as blood, saliva, and semen, they provide practical advantages for body fluid identification and for interpreting mixed or degraded biological traces [4,5]. These properties also support their growing use in applications such as age estimation and evaluating environmental influences on evidence.

This review summarizes the biomarker potential of miRNAs and compares commonly used isolation techniques—phenol–chloroform extraction, column-based methods, magnetic bead systems, and hybrid approaches—based on yield, purity, processing time, and cost [6]. Appropriate method selection is essential to obtain reliable results and ensure the validity of forensic interpretations [5].

CITATION
Bekiroglu FE, Holumen N, Yukseloglu EH. The role of micrornas in forensic genetics and a comparative efficiency analysis of isolation methods from body fluids. NOFOR. 2025;4(2):54-63. DOI: 10.5455/NOFOR.2025.09.06

Corresponding Author: Fatma Ebru Bekiroglu, İstanbul University-Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine, İstanbul, Türkiye
Email: fatmaebru.bekiroglu@ksbu.edu.tr

DOI: 10.5455/NOFOR.2025.09.06

Data Sources and Search Strategy

This review followed PRISMA recommendations for systematic literature searches. Publications from 2010 to 2025 were screened in PubMed, Scopus, and Google Scholar using the keywords "microRNA," "forensic genetics," "RNA isolation," and "body fluids." Only peer-reviewed articles in English were considered.

The search identified 168 records. After removing duplicates and screening titles and abstracts, 60 full texts were examined. A total of 53 studies met all criteria and were included. The overall selection steps are summarized in Figure 1.

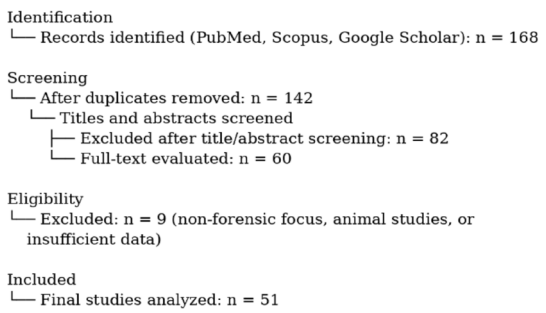

Figure 1. PRISMA flow summary; The figure outlines the literature selection process, from database search to final inclusion, based on PRISMA 2020 standards

Inclusion and Exclusion Criteria

Inclusion criteria:

- Studies published between 2010–2025 addressing microRNA isolation in forensic, biomedical, or diagnostic contexts.
- Research comparing yield, purity, or reproducibility of miRNA extraction methods.
- Experimental or review papers involving body fluids such as blood, semen, saliva, milk, or plasma.
- Exclusion criteria:
- Non-English papers, conference abstracts, or non–peer-reviewed preprints.
- Studies limited to animal samples, cell lines, or unrelated biomedical topics.
- Papers without quantitative measures such as RNA yield or purity ratios [5,7].

Data Extraction and Analysis

For each study, the sample type, extraction technique, commercial kit, and performance indicators (yield, A260/280, A260/230) were recorded. Statistical comparisons were performed using one-way ANOVA ($p<0.05$). Findings were summarized narratively and grouped by isolation method and sample type [8]. Additional comparative tables are provided in Tables 1-5.

Applications of miRNAs in Forensic Genetics

miRNAs have gained attention in forensic genetics due to their stability, tissue-specific expression, and regulatory roles in gene expression [9]. Rocchi et al. (2021) reported that these molecules support several forensic applications, including body fluid identification, age estimation, and post-mortem interval (PMI) evaluation [10].

Identification of Body Fluid Sources

Distinct miRNA expression patterns across body fluids provide a practical framework for determining the origin of biological traces. For instance, miR-205 is strongly associated with saliva, whereas miR-10b is predominantly detected in semen, making them useful fluid-specific markers [1,4].

Arat and Kaya Akyüzlü (2024) emphasized that miRNAs offer valuable discriminatory power, particularly in mixed or degraded samples frequently encountered in forensic casework [11]. Wang et al. (2013) showed that a panel of four miRNAs can distinguish body fluids with an accuracy of up to 95% [12,13]. Similarly, an in silico study by Çevik and Güzel Tanoğlu (2025) confirmed the high discriminatory potential of saliva-related miRNAs [14].

Together, these findings demonstrate that miRNA profiles form a reliable molecular basis for body fluid identification in forensic investigations.

Individual Identification and Age Estimation

Although miRNAs are not yet specific enough for direct individual identification, several age-related miRNAs—such as miR-34a, miR-181a, and miR-21—exhibit expression patterns that correlate with biological aging [15,16]. These changes can be incorporated into regression-based models to estimate an individual's age range with reasonable accuracy [17].

While still developing, this molecular approach may serve as a useful complement to traditional anthropological or skeletal assessments, especially when physical remains are limited or degraded.

Estimation of Post-Mortem Interval (PMI)

The post-mortem stability of miRNAs has generated interest in their use as molecular indicators of PMI. Several studies report time-dependent expression changes in cardiac miRNAs, including miR-1 and miR-133a, supporting their potential as molecular timing markers [8].

Pasaribu et al. (2023) reviewed findings from 18 studies and showed that miRNAs from the liver, skeletal muscle, and peripheral blood display measurable shifts in expression as PMI progresses [18]. Compared with morphological or entomological techniques, these molecular patterns may offer more consistent estimates, particularly when environmental conditions limit traditional approaches. Overall, current evidence suggests that miRNA-based profiling could strengthen PMI estimation in forensic practice.

DOI: 10.5455/NOFOR.2025.09.06

miRNA Isolation from Various Body Fluids

Because miRNAs are present in low quantities and are prone to nuclease degradation, the efficiency of the isolation step is critical in forensic workflows. Reported extraction strategies vary widely in yield, purity, and susceptibility to contamination. The most widely used methods include phenol–chloroform extraction, column-based purification, magnetic bead systems, and hybrid protocols that combine multiple steps [2,19].

General Protocols

Selecting an appropriate isolation method depends on the biological matrix—such as blood, saliva, or semen—as well as sample volume and planned downstream analyses. This choice is particularly important in forensic settings, where samples may be limited, degraded, or contain PCR inhibitors. Reducing processing steps and maximizing RNA recovery increase the likelihood of obtaining usable results.

miRNA quality is usually evaluated through RNA concentration (ng/µL), purity ratios [A260/A280 and A260/A230), and assessments of RNA integrity performed by electrophoresis or microfluidic systems. These metrics guide both method selection and the reliability of subsequent analyses.

Detailed Evaluation of Phenol-Chloroform-Based Methods

Phenol–chloroform extraction separates RNA through organic phase partitioning and has long been valued for its ability to recover high yields, including low-abundance miRNAs. Despite this advantage, the method involves multiple manual steps and relies on volatile, hazardous chemicals, which limit its suitability for routine forensic laboratories. Its hands-on complexity and operator-dependent variability also increase the risk of contamination and inconsistent results.

Conventional Phenol-Chloroform Extraction: This classical approach uses acidic phenol and chloroform to separate RNA into the aqueous phase, followed by ethanol or isopropanol precipitation to recover the RNA. Although it can produce high-quality RNA, the procedure requires repeated centrifugation and careful handling of unstable reagents, making it impractical for high-throughput forensic workflows [20].

TRIzol-Based Extraction: TRIzol combines phenol and guanidinium thiocyanate, enabling cell lysis and RNase inhibition in a single step. It is effective across a wide range of sample types; however, the multiple pipetting steps and phase transitions increase the chance of RNA loss, especially in limited forensic samples. Modified TRIzol protocols have shown improved performance in lipid-rich or viscous matrices such as plasma and brain tissue [21,22].

QIAzol-Based Extraction: QIAzol, a phenol-containing reagent similar to TRIzol, is designed for samples with high lipid or viscosity levels. Studies on human milk and neural tissues report improved yields where standard methods underperform. Nonetheless, purity ratios (A260/A280 and A260/A230) vary considerably between matrices, highlighting the need for sample-specific optimization—an essential consideration in forensic genetics where reproducibility is critical [4].

Proteinase K-Assisted Extraction: Proteinase K digestion helps break down proteins and improves RNA release, particularly in protein-dense fluids like semen. When combined with organic extraction, this pre-treatment significantly increases RNA recovery. Precise temperature and incubation control are essential, as suboptimal conditions can reduce RNA integrity, affecting downstream analytical reliability [23,24].

Column-Based miRNA Isolation Methods

Silica membrane–based column systems are widely used for miRNA extraction because they offer standardized workflows, ease of use, and reproducible results. RNA binds to the silica matrix under chaotropic conditions and is subsequently washed and eluted, producing clean nucleic acids suitable for downstream analyses. These methods are particularly useful for low-volume forensic samples, where limiting RNA loss is critical [25].

Li and Kowdley (2012) showed that column-based methods provide strong reproducibility and maintain RNA integrity in low-biomass materials such as serum. Kits such as miRNeasy (Qiagen) and mirVana (Ambion) remain common choices in both clinical diagnostics and forensic settings due to their reliable performance [26].

Evaluation of Major Column-Based Kits

miRNeasy (Qiagen): This kit isolates both small and large RNA through silica membrane binding. Its high purity and sensitivity, especially in serum and plasma, make it a frequent choice for forensic applications [13].

mirVana (Ambion): mirVana begins with an organic solvent–based lysis step before RNA binding to silica membranes. Although it provides high-quality RNA, the multiple centrifugation steps add complexity and extend handling time, which may increase variability in forensic workflows [27].

Promega ReliaPrep™ System: ReliaPrep offers a rapid, user-friendly protocol and performs well in complex matrices such as human milk. However, some studies report lower overall yields, which can be a limitation when dealing with low-template forensic samples [15].

Total Exosome RNA & Protein Isolation Kit (Thermo Fisher): This kit is designed for simultaneous isolation of RNA and proteins from exosomes in body fluids. It performs particularly well for exosomal miRNAs—an emerging category of forensic biomarkers. However, its requirement for adequate sample volume can be problematic in degraded or limited forensic specimens [5,28].

PureLink™ miRNA Isolation Kit (Invitrogen): PureLink targets small RNA recovery from complex matrices, including plasma and human milk. It consistently produces high-purity RNA

DOI: 10.5455/NOFOR.2025.09.06

based on A260/280 and A260/230 ratios, although overall RNA yield can vary depending on sample type and input quantity [6].

NucleoSpin® miRNA Kit (Macherey-Nagel): NucleoSpin enables parallel isolation of total and small RNA from diverse sample sources and is appreciated for its affordability and ease of use. Nonetheless, some studies report reduced yields in dilute fluids like saliva or plasma, which may limit its forensic utility in trace samples [7].

Magnetic Bead-Based Isolation Methods

Magnetic bead–based extraction systems have gained traction as an efficient and automation-friendly option for miRNA isolation. These methods use magnetizable particles coated with nucleic acid–binding surfaces, allowing RNA to bind selectively while contaminants are washed away under a magnetic field. The result is a rapid, clean isolation process that works well even when sample volumes are small—an important advantage in forensic casework.

Their key strengths include short processing times, reduced manual handling, minimal contamination risk, and strong compatibility with automated platforms. These features improve workflow reproducibility and biosafety, making bead-based systems increasingly valuable for downstream applications such as RT-qPCR and next-generation sequencing in both forensic and clinical settings [16].

Chen et al. (2020) evaluated a two-step magnetic bead protocol and reported extraction efficiencies approaching 91% for exosomal miRNAs in plasma, demonstrating the method's strong performance in complex matrices [29]. These findings support the use of magnetic bead systems as a practical and reliable alternative to more labor-intensive manual extraction methods, particularly when automation is needed.

Hybrid Extraction Methods

Hybrid strategies combine components of conventional and modern extraction techniques to improve both yield and purity. These methods are especially useful in forensic work, where samples may be degraded, scarce, or contain inhibitors.

TRIzol + miRNeasy Combination: This approach begins with TRIzol lysis, followed by silica column purification using the miRNeasy system. The pairing of TRIzol's strong lysis efficiency with the high-purity output of column methods produces consistently higher-quality RNA, particularly in challenging materials such as serum and human milk [8].

Liquid–Liquid Extraction + Solid-Phase Cleanup: In this workflow, the aqueous phase from phenol–chloroform extraction is further purified on a silica column. It performs well in protein-rich fluids such as seminal plasma. Additives like Proteinase K, glycogen, or linear acrylamide can improve yields, although studies indicate that exosome pre-isolation does not provide significant added benefit for low-abundance forensic samples [30].

TRIzol Plus Liquid–Liquid Extraction Method: Developed by Zununi Vahed et al. (2016), this protocol begins with TRIzol lysis, removes large RNAs via potassium acetate, and selectively precipitates small RNAs—including miRNAs—using lithium chloride. It has shown strong performance in FFPE samples and viscous fluids and is noted for being cost-effective and adaptable for laboratories that do not rely on commercial kits [31].

Other Methods

Several alternative miRNA extraction strategies have been described for difficult or highly contaminated biological matrices, although these approaches are not yet standard in forensic workflows. Many of these methods focus on modifying reagent composition or adjusting extraction steps to improve recovery from samples with low RNA content or high levels of inhibitors.

Non-Standard Chemical Protocols: Procedures such as lithium chloride precipitation, PEG-assisted extraction, and glass fiber–based filtration have been applied successfully to challenging fluids including bile, milk, and fecal material [32]. Because these matrices often contain substantial debris or enzymatic inhibitors, they typically require pre-processing steps—such as high-speed centrifugation, filtration, or differential ultracentrifugation—to reduce contaminants before the actual extraction begins [33].

Exosomal RNA Isolation Approaches: Extraction of miRNAs from extracellular vesicles, particularly exosomes, has been performed using workflows such as differential ultracentrifugation, size-exclusion chromatography, or microfluidic separation. These methods can provide highly enriched RNA fractions but also come with drawbacks, including long processing times, specialized equipment requirements, and increased operational complexity [34].

Use of DNA Extraction Protocols for Small RNA Recovery: Interestingly, several studies have noted that some DNA extraction kits—both silica column and magnetic bead formats—retain small RNAs during purification. This allows the recovery of miRNAs and genomic DNA from the same sample, a significant advantage when working with limited or irreplaceable forensic material [35].

Extracellular RNA Isolation from Biofluids: In serum and plasma, techniques such as precipitation, membrane filtration, affinity-based purification, and ultracentrifugation have been used to isolate extracellular miRNAs. Commercial systems like ExoQuick and miRCURY often yield satisfactory recovery, whereas ultracentrifugation tends to produce the highest-purity RNA fractions [36,37].

Next-Generation Isolation Platforms: Emerging technologies—including microfluidic devices and antibody-coated magnetic systems—are being evaluated for their potential to enrich low-abundance miRNAs with greater selectivity and throughput. These platforms may support future forensic workflows that require high sensitivity and scalable processing [6].

A summary of the commercial isolation kits referenced in this review is provided in Table 1.

Table 1. Summarizes the types of isolation kits used, classification of the methods, their suitability for experimental purposes, and the primary evaluation criteria

Reference source	Commercial kit name	Biological sample type	Forensic diagnostic utility	Matrix composition	Advantages	Limitations
Chen et al. (2020) [29]	2MBB	Plasma	Magnetic bead-based method	Magnetic bead platform	High purity; specific RNA binding; suitable for automation	Long protocol duration
Li and Kowdley (2012) [26]	miRNeasy	Serum	Column-based method	Silica membrane column	High reproducibility; recovery of high-quality RNA	Reduced RNA yield in certain samples; may require high input volume
Li and Kowdley (2012) [26]	mirVana	Serum	Column-based method	Silica membrane column	High reproducibility; recovery of high-quality RNA	Multi-step protocol may be time-consuming and increase user-dependent variability
Li and Kowdley (2012) [26]; Rocchi et al. (2021) [10]	Promega ReliaPrep™	Plasma, serum, breast milk	Silica column + Proteinase K	Hybrid method	High yield; strong lysis capacity; compatible with complex matrices such as breast milk	Risk of protein contamination; requires multiple processing steps
Xu et al. (2022) [6]; Rocchi et al. (2021) [10]	PureLink miRNA	Plasma, tissue	Silica membrane column	Column-based method	Easy to implement; suitable for high-throughput applications	Low RNA yield reported in certain sample types
Rocchi et al. (2021) [10]; Glynn ve O'Leary (2018) [38]	NucleoSpin	Serum, plasma	Silica column	Column-based method	Cost-effective; simple protocol	Reduced RNA yield in aqueous or dilute samples
Rekker et al. (2014) [36]	ExoQuick	Plasma, cell culture supernatant	PEG-based precipitation	Polymer-based precipitation method	Membrane-supported selective isolation; high RNA purity	Expensive; long protocol duration
El-Khoury et al. (2016) [42]; Rekker et al. (2014) [36]	miRCURY Exosome Kit	Blood, cell culture, organ tissue	Membrane filtration + precipitation	Hybrid method (membrane filtration + precipitation)	High yield; compatible with vacuum-based workflows	Contains phenol; toxic and time-consuming
Roy et al. (2020) [22]; Zununi Vahed et al. (2016) [31]; Glynn et al. (2020) [17]	TRIzol	Blood, brain, liver	Phenol–chloroform	Organic extraction method	Efficient lysis; prevents DNA contamination	Phenol toxicity requires careful handling; laborious
Rocchi et al. (2021) [10]; Xu et al. (2022) [6]	QIAzol	Serum, plasma	Phenol–chloroform derivative	Organic extraction method	High purity RNA from complex fluids such as plasma	Lower yield compared to TRIzol
Eldh et al. (2012) [28]	Total Exosome RNA & Protein Isolation Kit	Plasma	Exosome purification with organic solvents	Exosome precipitation + organic extraction	Isolates exosome-specific RNA with high purity	May require protocol customization for different matrices

DOI: 10.5455/NOFOR.2025.09.06

DOI: 10.5455/NOFOR.2025.09.06

Comparison of miRNA Isolation Methods from Different Body Fluids

Different extraction protocols vary substantially in their RNA yield, purity, and stability, largely because each biological fluid has its own biochemical profile. Kit chemistry and workflow complexity further influence performance. This section summarizes comparative findings from the literature and highlights method preferences for specific specimen types.

Evaluation of RNA Purity, Concentration, and Degradation Rates

Multiple studies have assessed isolation efficiency using metrics such as total RNA concentration, A260/280 and A260/230 ratios, and replicate consistency.

An early multicenter comparison by Eldh et al. (2012) found that the miRCURY protocol produced the highest RNA concentrations, whereas RNeasy and TRIzol + Cleanup achieved a more balanced outcome between yield and purity (Table 2) [28].

Zununi Vahed et al. (2016) reported that a potassium acetate + 2.5M LiCl workflow yielded the best overall purity for urine samples. Although extraction with 0.4M LiCl produced higher RNA amounts, purity was markedly lower—limiting its applicability in downstream analyses (Table 1) [31].

O'Leary and Glynn (2018) compared three commercial kits across several body fluids. Their data indicated that miRNeasy generated the highest RNA yield overall, while a modified mirVana protocol performed best with saliva. Each experiment included ten technical replicates (Table 2) [38].

Table 2. Comparative Analysis of Total RNA Yield and Purity Across Seven Isolation Methods [28, 38]

Method	RNA yield (ng/10^6 cells)	A260/280 ratio	A260/230 ratio	Technical replicates (n)	Comment
Trizol®	24.1±8.3	2.1	1.8	3–4	Limited recovery of small RNAs
Trizol® + Cleanup	41.3±6.6	2.2	2.1	3–4	Improved yield and purity
RNeasy®	82.8±27.1	2.3	2.6	3–4	Among the highest purity values
Modified RNeasy®	75.7±27.6	1.9	1.2	3–4	Lower purity observed
miRNeasy	13.0±7.7	1.9	1.5	3–4	Overall RNA yield was low
miRCURY™	107.7±25.7	2.0	2.0	3–4	Highest total RNA yield
mirVana™	33.1±17.7	1.9	1.8	3–4	High yield of small RNA species

Spectrophotometric and fluorometric assessments continue to guide forensic decision-making. TRIzol protocols often produce strong yields but frequently show reduced A260/230 ratios due to residual organic contaminants [39]. Column-based systems typically yield cleaner RNA but at the expense of lower total recovery, a pattern echoed across several comparative studies [40].

Williams et al. (2015) emphasized that, particularly in forensic contexts where sample quantity is limited, the isolation method chosen can directly alter quantitative outcomes and downstream interpretation [41]. El-Khoury et al. (2016) further noted that miRCURY delivers high purity but may underperform with small input volumes due to column saturation, whereas miRNeasy captures more small RNA species but with slightly reduced purity [42].

Finally, even standardized protocols show noticeable inter-laboratory variation, reinforcing the need for harmonized isolation procedures in both forensic and clinical molecular settings [43].

Experimental Design

Comparative studies evaluating miRNA isolation methods depend heavily on using a consistent experimental setup. Most investigations process the same biological material—often serum—side-by-side with several extraction protocols and then compare RNA yield, purity ratios, and RT-PCR performance. Typically, 3–5 technical replicates are included, and statistical differences are assessed using independent t-tests or one-way ANOVA [44]. The use of internal reference miRNAs is also essential, as it reduces technical variation and supports reliable normalization [25].

In the experimental data reviewed here, differences in RNA concentration and purity across methods were analyzed using one-way ANOVA, with statistical significance defined as $p<0.05$.

Selection of the Most Efficient Isolation Method According to Body Fluid Type

Comparative findings from the included studies highlight that miRNA isolation efficiency is strongly dependent on the biochemical characteristics of each fluid.

Mercadal et al. (2020) examined six EV isolation techniques for semen. Their results showed that the miRCURY 1500×g protocol produced the highest RNA yield, while ultracentrifugation remained the benchmark for purity (Table 3) [45].

For human milk, Ahlberg et al. (2021) tested five column-based kits. Promega ReliaPrep™ generated the highest RNA concentrations, whereas the Sigma-Aldrich mirPremier™ kit achieved superior purity metrics (Table 4) [20].

Urbizu et al. (2023) evaluated saliva collection systems combined with column-based kits. The NucleoSpin + Oragene pairing yielded the most RNA, while miRNeasy produced samples with the lowest levels of DNA contamination (Table 5) [40].

DOI: 10.5455/NOFOR.2025.09.06

Table 3. Among the compared miRNA isolation protocols from urine specimens, the KCH_3COOH + LiCl 2.5M method demonstrated superior performance in terms of yield and purity [31, 45]

Method	RNA Yield (ng/µL)	A260/280 ratio	A260/230 ratio	Technical replicates (N)	Comment
KCH_3COOH + LiCl 2.5M + Ethanol	231±5.9	1.84	3.85	3	Best purity and yield
LiCl 0.4M + Ethanol	1382±3.4	1.71	0.70	3	Highest yield but poor purity
LiCl 8M	657±21	1.43	0.32	3	Low yield and low purity
PEG 4000	~25.7 Ct	-	-	3	Low RNA yield based on Ct value
PEG 6000	~28.1 Ct	-	-	3	Lowest PCR efficiency

Table 4. miRNA isolation efficiency from distinct body fluids was compared using three commercial kits, with the number of technical replicates specified [20, 38]

Body Fluid	Method	Mean RNA yield (ng/µL)	Technical replicates (n)	Comment
Venous blood	miRNeasy	29.3±6.4	10	Highest yield
Venous blood	miRVana	4.5±1.5	10	Lowest yield
Venous blood	Modified miRVana	14.8±18.2	10	High variation
Menstrual blood	miRNeasy	232.2±188.6	10	High yield, variable
Menstrual blood	miRVana	56.4±53.4	10	Moderate yield
Menstrual blood	Modified miRVana	53.6±61.5	10	High inconsistency
Semen	miRNeasy	83.8±101.5	10	Large variation
Semen	miRVana	13.6±10.3	10	Low yield
Semen	Modified miRVana	16.8±9.8	10	More consistent
Saliva	miRNeasy	65.7±50.3	10	Good yield
Saliva	miRVana	5.9±4.7	10	Very low yield
Saliva	Modified miRVana	67.5±47.3	10	Best saliva RNA yield
Vaginal material	miRNeasy	331.6±226.0	10	Highest overall yield
Vaginal material	miRVana	111.9±68.5	10	Moderate yield
Vaginal material	Modified miRVana	110.3±58.8	10	Comparable outcome

Table 5. Comparison of six different EV isolation protocols applied to semen samples in terms of RNA yield [40, 45]

Isolation Method	RNA yield (ng/µL)	A260/280 ratio	A260/230 ratio	Technical replicates (n)	Comment
Ultracentrifugation (UC)	22.39±7.79	1.78±0.17	0.60±0.40	5	Reference method, medium quality
ExoGAG 1500×g	8.17±2.55	1.76±0.05	0.13±0.05	2	Low purity
ExoGAG 3500×g	12.29±8.60	1.79±0.08	0.47±0.40	4	Acceptable quality
ExoQuick Ultra A 3000×g	4.27±0.94	1.53±0.04	0.39±0.18	2	Lowest yield
miRCURY Cell/Urine/ Cerebrospinal Fluid (CSF) 1500×g	31.03±2.94	1.63±0.11	0.40±0.26	4	Highest yield, low purity
miRCURY Cell/Urine/CSF 10,000×g	3.65±3.04	1.17±0.01	0.10±0.01	2	Very poor quality

DOI: 10.5455/NOFOR.2025.09.06

Given the biological diversity of fluids—differences in lipid content, protein density, and enzyme activity—method selection must be matrix-specific. TRIzol LS performs well with lipid-rich matrices such as milk, whereas column-based systems (mirVana, miRNeasy) are widely favored for plasma [46]. In protein-dense fluids like semen, adding a Proteinase K pretreatment step has been shown to improve recovery [47].

Glynn and O'Leary (2018) further demonstrated that each biological fluid exhibits characteristic miRNA signatures, reinforcing the need for extraction methods tailored to the specimen type [38].

Table 6. Comparison of five column-based kits used for RNA isolation from human milk in terms of yield and purity (20).

Kit	Total RNA (ng/µL)	miRNA (ng/µL)	A260/280 ratio	A260/230 ratio	Technical replicates (n)	Comment
Promega ReliaPrep™	28.30 (7.68–52.65)	12.75 (3.35–56.65)	1.94	0.52	10	Highest quality and yield
Zymo Quick-RNA MicroPrep	20.30 (8.47–28.45)	10.60 (3.72–34.70)	1.35	0.32	10	Low A260/280 ratio
Norgen Total RNA	23.10 (10.70–34.05)	8.40 (3.99–45.35)	1.48	0.24	8	Moderate yield, low purity
Norgen Single Cell	6.20 (0.02–6.89)	1.92 (1.02–22.92)	1.53	0.14	8	Lowest yield
Sigma-Aldrich mirPremier™	14.16 (3.97–38.25)	3.99 (0.85–37.95)	2.20	1.48	10	Best purity, low yield

Table 7. RNA yield and DNA contamination levels obtained from saliva samples using different sample collectors and column-based kit combinations [40]

Combination	Total RNA (ng)	Yield (ng/µL)	A260/280 (%)	DNA Contamination
Oragene + miRNeasy	2636	4.39	60	No
Oragene + miRVana	2365	4.73	60	Yes
Oragene + NucleoSpin	5003	5.56	60	Yes
50 mL Tube + miRNeasy	575	2.00	80	No
50 mL Tube + miRVana	2914	5.83	80	Yes
50 mL Tube + NucleoSpin	825	2.28	80	Yes
Salimetrics + miRNeasy	402	2.00	50	No
Salimetrics + miRVana	1311	2.82	50	Yes
Salimetrics + NucleoSpin	340	1.22	50	Yes

Comparative Analysis

Comparative findings across the literature show that no single protocol consistently outperforms others for miRNA isolation. The most effective method depends on the biological matrix and the demands of the downstream analysis. Column-based systems generally provide higher purity and straightforward operation, whereas TRIzol-based protocols are known for producing greater RNA yields. Magnetic bead–based methods offer reduced contamination risk and work efficiently with automated platforms. Hybrid approaches, which combine organic extraction with silica purification, often achieve a useful balance between purity and yield and are particularly effective for complex or inhibitor-rich samples [47,35].

Advances in downstream analytical tools—especially digital PCR and high-sensitivity microarrays—further improve the reliability of miRNA detection after isolation, allowing accurate measurements even from low-template forensic samples [48].

This review compared the main miRNA isolation strategies used in forensic genetics and evaluated their strengths across different biological matrices. Although phenol–chloroform protocols consistently produce high RNA yields, their dependence on hazardous chemicals, operator skill, and extended processing steps reduces their suitability for routine forensic workflows [44,46].

Column-based systems, on the other hand, provide more reproducible purity and are easier to standardize between laboratories. Their primary drawbacks are the higher cost and reliance on commercial reagents, which may limit feasibility in low-resource forensic units [25]. Magnetic bead–based techniques represent an appealing alternative because they

minimize contamination and can be integrated with automated systems, although differences in bead chemistry and the need for specialized equipment remain significant challenges [35].

Hybrid protocols—particularly combinations of TRIzol extraction followed by silica-based purification—showed the most balanced performance, yielding both high purity and high RNA recovery [47]. However, the heterogeneity of study designs, measurement platforms, and sample storage conditions highlights the ongoing need for harmonized validation standards.

Beyond isolation efficiency, the forensic value of miRNAs also lies in their tissue-specific expression patterns, which support the identification of biological traces even when samples are degraded [49]. Despite this potential, the absence of internationally recognized guidelines for miRNA extraction, quantification, and result interpretation continues to restrict their acceptance as forensic evidence [50].

Limitations and Future Directions

This review has several limitations.

First, the included studies used different protocols, instruments, and analytical criteria, which can affect comparability and overall reproducibility.

Second, publication bias is possible, as many studies report optimized or successful outcomes.

Third, restricting the search to English-language publications may have excluded relevant regional or local research.

Finally, although isolation efficiency was compared across studies, a quantitative meta-analysis could not be performed due to inconsistent reporting of standardized metrics.

Future work should focus on:

1. Developing internationally accepted guidelines for forensic miRNA isolation and validation.
2. Incorporating automation and next-generation sequencing into forensic workflows.
3. Conducting comparative studies using low-template, aged, or environmentally compromised samples.
4. Evaluating chain-of-custody, judicial admissibility, and quality assurance frameworks for RNA-based evidence.

CONCLUSION

miRNAs represent stable and tissue-specific molecular markers with considerable promise for forensic applications, including body fluid identification, age estimation, and post-mortem interval analysis [45]. Findings from this review show that no single isolation protocol is universally optimal; the most effective approach depends on the biological matrix and the requirements of downstream analyses. Establishing standardized extraction procedures and robust quality-control measures will be essential for integrating miRNA-based assays into routine forensic practice. With such developments, miRNA analysis has the potential to transition from an experimental technique to a reliable evidentiary tool that strengthens the interface between molecular biology and forensic medicine [51].

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

REFERENCES

1. Roest HP, IJzermans JNM, van der Laan LJW. Evaluation of RNA isolation methods for microRNA quantification in a range of clinical biofluids. BMC Biotechnol. 2021;21:48.
2. Wijenayake S, Eisha S, Tawhidi Z, et al. Comparison of methods for pre-processing, exosome isolation, and RNA extraction in unpasteurized bovine and human milk. PLoS One. 2021;16:e0257633.
3. Hitit M, Kurar E, Güzeloğlu A. MikroRNA biyogenezi. Harran Univ Vet Fak Derg. 2015;4:1-6.
4. Sidekli Ö, Korkmaz Ağaoğlu Ö. Kantitatif RT-PCR (RT-qPCR) ile mikroRNA (miRNA) ekspresyon profillemesi. Erciyes Univ Vet Fak Derg. 2021;18:48-56.
5. Jiawei S, Zhi C, Kewei T, Xiaoping L. Magnetic bead-based adsorption strategy for exosome isolation. Front Bioeng Biotechnol. 2022;10:942077.
6. Xu D, et al. MicroRNAs in extracellular vesicles: sorting mechanisms, diagnostic value, isolation, and detection technology. Front Bioeng Biotechnol. 2022;10:948959.
7. Turgut AO, Korkmaz Ağaoğlu Ö. MikroRNA ekspresyon profillemesinde yaygın kullanılan normalizasyon yaklaşımları. Erciyes Univ Vet Fak Derg. 2022;19:152-9.
8. Smela D, Zelinkova E, Rehulka P, et al. Phenol/chloroform-free TiO2-based miRNA extraction from cell lysate. Int J Mol Sci. 2022;23:8848.
9. Albayrak İG. miRNA'lar: biyogenezi, analiz yöntemleri ve biyobelirteç potansiyeli. Van Sag Bil Derg. 2022;15:95-102.
10. Rocchi A, Chiti E, Maiese A, et al. MicroRNAs: an update of applications in forensic science. Diagnostics. 2021;11:32.
11. Arat Ö, Kaya Akyüzlü D. Vücut sıvılarının kimliklendirilmesinde miRNA çalışmalarının potansiyel önemi: Güncel bir bakış. J For Med. 2024;38:80-92.
12. Wang Z, Luo H, Pan X, et al. Body fluid mixtures: resolution using forensic microRNA analysis. Forensic Sci Int Genet. 2013;7:127-31.
13. Salim H, Pero-Gascon R, Pont L, et al. A review of sample preparation for purification of microRNAs and analysis by mass spectrometry methods. Microchem J. 2022;182:107849.
14. Çevik FE, Güzel Tanoğlu E. Adli genetikte tükürük sıvılarındaki mikroRNA'ların in siliko analizi. Adli Tıp Dergisi. 2025;39:86-93.
15. Lai JJ, Chau ZL, Chen SY, et al. Exosome processing and characterization approaches for research and technology development. Adv Sci. 2022;9:2103222.
16. Dikme R. MikroRNA'lar. In: Kafadar H, editor. Sağlık & Bilim 2022: Güncel Tıp-1. İstanbul: Efe Akademi; 2022:229-43.
17. Glynn CL. Potential applications of microRNA profiling to forensic investigations. RNA. 2020;26:1-9.

18. Pasaribu RS, Auerkari EI, Suhartono AW, Auerkari P. A small RNA, microRNA as a potential biomolecular marker to estimate post mortem interval in forensic science: a systematic review. Int J Legal Med. 2023;137:1313-25.

19. Potla P, Ali SA, Kapoor M. A bioinformatics approach to microRNA-sequencing analysis. Osteoarthr Cartil Open. 2021;3:100131.

20. Ahlberg E, Jenmalm MC, Tingö L. Evaluation of five column-based isolation kits and their ability to extract miRNA from human milk. J Cell Mol Med. 2021;25:7973-9.

21. Sriram H, Khanka T, Kedia S, et al. Improved protocol for plasma microRNA extraction and comparison of commercial kits. Biochem Med (Zagreb). 2021;31:030705.

22. Roy D, Tomo S, Modi A, et al. Optimising total RNA quality and quantity by phenol-chloroform extraction method from human visceral adipose tissue. MethodsX. 2020;7:101113.

23. Sauer E, Reinke AK, Courts C. Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR. Forensic Sci Int Genet. 2016;22:139-46.

24. Precazzini F, Detassis S, Imperatori AS, et al. Measurement methods for the development of microRNA-based tests for cancer diagnosis. Int J Mol Sci. 2021;22:1176.

25. Chorley BN, Atabakhsh E, Doran G, et al. Methodological considerations for measuring biofluid-based microRNA biomarkers. Crit Rev Toxicol. 2021;51:264-282.

26. Li Y, Kowdley KV. Method for microRNA isolation from clinical serum samples. Anal Biochem. 2012;431:69-75.

27. Skryabin GO, Vinokurova SV, Elkina NV, et al. Comparison of methods for microRNA isolation from extracellular vesicles obtained from ascitic fluids. Biochemistry (Moscow). 2022;87:1354-66.

28. Eldh M, Lötvall J, Malmhäll C, Ekström K. Importance of RNA isolation methods for analysis of exosomal RNA. Mol Immunol. 2012;50:278-86.

29. Chen S, Shiesh SC, Lee GB, Chen C. Two-step magnetic bead-based techniques for immunocapture of extracellular vesicles and quantification of microRNAs. PLoS One. 2020;15:e0229610.

30. Eikmans M, D H Anholts J, Blijleven L, et al. Optimization of microRNA acquirement from seminal plasma and identification of diminished seminal microRNA-34b. Int J Mol Sci. 2020;21:4089.

31. Zununi Vahed S, Barzegari A, Rahbar Saadat Y, et al. A microRNA isolation method from clinical samples. BioImpacts. 2016;6:25-31.

32. Kupec T, Bleilevens A, Iborra S, et al. Stability of circulating microRNAs in serum. PLoS One. 2022;17:e0268958.

33. Li L, Piontek KB, Kumbhari V, et al. Isolation and profiling of microRNA-containing exosomes from human bile. J Vis Exp. 2016;112:54036.

34. Sidhom K, Obi PO, Saleem A. A review of exosomal isolation methods. Int J Mol Sci. 2020;21:6466.

35. Lewis CA, Layne T, Seashols SJ. Detection of microRNAs in DNA extractions for forensic biological source identification. J Forensic Sci. 2019;64:1361-7.

36. Rekker K, Saare M, Roost AM, et al. Comparison of serum exosome isolation methods for microRNA profiling. Clin Biochem. 2014;47:135-8.

37. Filant J, Nejad P, Paul A, et al. Isolation of extracellular RNA from serum/plasma. Methods Mol Biol. 2018;1740:43-57.

38. Glynn CL, O'Leary KR. Investigating the isolation and amplification of microRNAs for forensic body fluid identification. J Forensic Res. 2018;7:187-94.

39. Biskup E, Schejbel L, Oliveira DNP, Høgdall E. Test of an improved DNA and RNA purification protocol. Separations. 2022;9:324.

40. Urbizu A, Arnaldo L, Beyer K. Obtaining miRNA from saliva—comparison of sampling and purification methods. Int J Mol Sci. 2023;24:2386.

41. Williams G, Uchimoto ML, Coult N, et al. Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. Forensic Sci Int Genet. 2015;14:94-100.

42. El-Khoury V, Pierson S, Kaoma T, et al. Assessing cellular and circulating miRNA recovery. Sci Rep. 2016;6:19529.

43. Gandham S, Su X, Wood J, et al. Technologies and standardization in research on extracellular vesicles. Trends Biotechnol. 2020;38:1066-98.

44. Martins TS, Vaz M, Henriques AG. Comparative studies addressing exosome isolation methods from body fluids. Anal Bioanal Chem. 2023;415:1239-63.

45. Mercadal M, Herrero C, López-Rodrigo O, et al. Impact of extracellular vesicle isolation methods on downstream miRNA analysis in semen. Int J Mol Sci. 2020;21:5949.

46. exRNAQC Consortium. Blood collection tube and RNA purification method recommendations for extracellular RNA transcriptome profiling. Nat Commun. 2025;16:4513.

47. Grunt M, Failla AV, Stevic I, et al. A novel assay for exosomal and cell-free miRNA isolation and quantification. RNA Biol. 2020;17:425-40.

48. Mathew R, Mattei V, Al Hashmi M, Tomei S. Updates on the current technologies for microRNA profiling. MicroRNA. 2020;9:17-24.

49. Parker VL, Cushen BF, Gavriil E, et al. Comparison and optimisation of microRNA extraction from plasma of healthy pregnant women. Mol Med Rep. 2021;23:258.

50. Mompeón A, Ortega-Paz L, Vidal-Gómez X, et al. Disparate miRNA expression in serum and plasma of patients with acute myocardial infarction. Sci Rep. 2020;10:5373.

51. Boga I, Prasad SV, Deka D, et al. A study on total RNA including microRNA isolation from cell lines by different methods. Int J Exp Res Rev. 2022;29:94-104.