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MODERN PERSPECTIVES IN MOLECULAR IDENTIFICATION OF FORENSIC BODY FLUIDS: A COMPREHENSIVE REVIEW OF MRNA EXPRESSION ANALYSIS VIA RT-QPCR.

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ABSTRACT

The identification of forensic body fluids plays a critical role in reconstructing crime scenes and providing contextual evidence in criminal investigations. Traditional biochemical and serological methods, while useful for preliminary detection, often suffer from limitations in specificity, sensitivity, and reliability, particularly when dealing with degraded or mixed samples. In recent years, molecular approaches—especially messenger RNA (mRNA) expression analysis using reverse transcription quantitative polymerase chain reaction (RT-qPCR)—have emerged as powerful alternatives for accurate body fluid identification. This review provides a comprehensive overview of modern perspectives in mRNA-based forensic analysis, focusing on the biological basis of tissue-specific gene expression, the principles and workflow of RT-qPCR, and recent advancements in marker discovery and assay development. Specific mRNA markers associated with commonly encountered body fluids, including blood, semen, saliva, vaginal fluid, and menstrual blood, are discussed in detail, highlighting their diagnostic potential and forensic applicability. The review also examines the impact of environmental factors on RNA stability and outlines strategies to mitigate degradation, such as optimized extraction methods and short amplicon design. Furthermore, recent developments in multiplex assays, computational analysis, and machine learning approaches are evaluated for their role in improving accuracy and interpretation, particularly in complex and mixed samples. Validation studies and casework applications are also considered, demonstrating the growing acceptance and reliability of mRNA-based techniques in forensic practice. Despite ongoing challenges related to standardization and legal admissibility, the integration of mRNA profiling with emerging biomarkers such as microRNA and epigenetic markers offers promising future directions. Overall, mRNA expression analysis via RT-qPCR represents a significant advancement in forensic molecular biology, enhancing the specificity, sensitivity, and evidentiary value of body fluid identification and contributing to more accurate and scientifically robust criminal investigations.

Keywords: *Forensic body fluid identification; mRNA expression analysis; RT-qPCR; tissue-specific biomarkers; molecular forensics*

INTRODUCTION

Forensic science has long served as a cornerstone of modern criminal justice systems, providing objective, scientific evidence to reconstruct events, establish associations, and support judicial decision-making. Among its various sub-disciplines, the identification of biological materials—particularly body fluids—holds a uniquely critical position. Body fluids such as blood, semen, saliva, vaginal secretions, menstrual blood, and sweat are frequently encountered at crime scenes and can offer invaluable insights into the nature of a criminal act, the sequence of events, and the individuals involved. The ability not only to detect but also to accurately identify the specific origin of these fluids is therefore fundamental to forensic investigations. In this context, the field has witnessed a paradigm shift from traditional biochemical assays toward advanced molecular methodologies, with mRNA expression analysis via reverse transcription quantitative polymerase chain reaction (RT-qPCR) emerging as a particularly promising approach.

The importance of forensic body fluid identification lies in its capacity to provide contextual information that complements DNA profiling. While DNA analysis excels in individualization—linking biological material to a specific person—it does not inherently reveal the type of biological fluid from which the DNA originated. This distinction is crucial in many investigative and legal scenarios. For instance, the presence of blood may indicate violence or injury, whereas the detection of semen or vaginal fluid can be directly relevant in cases of sexual assault. Similarly, saliva traces may suggest activities such as biting, licking, or the sealing of envelopes. Therefore, the accurate identification of body fluids contributes not only to reconstructing criminal events but also to corroborating or refuting witness statements, strengthening evidentiary value, and enhancing the overall probative force of forensic findings.

Historically, the identification of body fluids has relied on a range of conventional biochemical and serological methods. These include presumptive and confirmatory tests based on enzymatic activity, protein detection, or chemical reactivity. For example, the Kastle–Meyer test has been widely used for the presumptive detection of blood through its peroxidase-like activity, while acid phosphatase tests have been employed for semen identification. Similarly, amylase-based assays have been used to detect saliva. Although these methods have proven useful for preliminary screening, they are often limited by issues of specificity, sensitivity, and susceptibility to false positives or false negatives. Environmental factors such as heat, humidity, microbial activity, and exposure to light can further compromise the reliability of these tests by degrading target molecules or altering chemical properties.

Another significant limitation of conventional approaches is their inability to provide definitive and simultaneous identification of multiple body fluids, particularly in mixed or degraded samples. Crime scene evidence is rarely pristine; biological traces are often present in minute quantities, intermixed, or subjected to adverse environmental conditions. Under such circumstances, traditional biochemical assays may fail to distinguish between closely related fluids or may produce ambiguous results. Furthermore, many of these methods are destructive in nature, consuming portions of the sample that could otherwise be used for downstream DNA analysis. This poses a critical challenge in forensic casework, where the preservation of evidence is paramount.

In response to these limitations, the field of forensic biology has increasingly turned to molecular techniques that exploit the genetic and transcriptomic characteristics of biological materials. Among these, mRNA-based approaches have garnered considerable attention due to their potential to achieve high specificity in body fluid identification. Messenger RNA (mRNA) molecules are transcribed from DNA and reflect gene expression patterns that are often tissue- or cell-type specific. This means that certain mRNA markers are uniquely or preferentially expressed in particular body fluids, enabling their identification based on molecular signatures. For example, specific transcripts such as hemoglobin beta (HBB) are associated with blood, while protamine genes (PRM1, PRM2) are indicative of semen.

The application of reverse transcription quantitative polymerase chain reaction (RT-qPCR) has further enhanced the utility of mRNA analysis in forensic contexts. RT-qPCR involves the conversion of mRNA into complementary DNA (cDNA) through reverse transcription, followed by the amplification and quantification of target sequences in real time. This technique offers several advantages, including high sensitivity, the ability to detect low-abundance transcripts, and quantitative capabilities that allow for the assessment of gene expression levels. Moreover, RT-qPCR can be designed for multiplex analysis, enabling the simultaneous detection of multiple markers corresponding to different body fluids within a single reaction. This is particularly advantageous when dealing with mixed samples, as it allows for comprehensive profiling without excessive consumption of the evidence.

The emergence of mRNA-based body fluid identification represents a significant advancement in forensic science, aligning with broader trends toward molecular precision and evidence-based methodologies. However, it is not without its challenges. RNA is inherently less stable than DNA and is more susceptible to degradation by environmental factors and enzymatic activity. This raises important considerations regarding sample collection, storage, and processing. Additionally, the interpretation of mRNA profiles requires careful validation to ensure reliability, reproducibility, and admissibility in legal proceedings. Despite these challenges, ongoing research and technological innovations continue to refine and optimize mRNA-based approaches, reinforcing their potential as a robust tool in forensic investigations.

In recent years, the integration of molecular techniques into forensic workflows has been further supported by advances in instrumentation, bioinformatics, and standardization efforts. The development of validated marker panels, improved extraction protocols, and standardized operating procedures has contributed to the growing acceptance of mRNA analysis in forensic laboratories. Furthermore, comparative studies have demonstrated the superiority of molecular methods over traditional assays in terms of specificity and sensitivity, particularly in complex or degraded samples. As a result, mRNA expression analysis via RT-qPCR is increasingly being recognized as a complementary technique that enhances the evidentiary value of forensic investigations.

From a broader perspective, the transition toward molecular forensic methods reflects a shift in the epistemological foundations of forensic science—from reliance on observable biochemical reactions to the interrogation of underlying genetic and transcriptomic information. This shift is not merely technical but also conceptual, emphasizing precision, reproducibility, and scientific rigor. It aligns with contemporary demands for robust forensic evidence that can withstand scrutiny in court and contribute to fair and accurate judicial outcomes.

In light of these developments, this review aims to provide a comprehensive examination of modern perspectives in molecular identification of forensic body fluids, with a particular focus on mRNA expression analysis via RT-qPCR. It will explore the biological basis of mRNA markers, the methodological principles underlying RT-qPCR, recent advances in marker discovery and validation, and the practical challenges associated with forensic implementation. By synthesizing current knowledge and highlighting future directions, this review seeks to contribute to the ongoing evolution of forensic science and to support the adoption of more precise and reliable methods for body fluid identification.

Molecular Basis of mRNA Expression in Body Fluid Identification

The transition from conventional biochemical assays to molecular approaches in forensic science has been driven by the need for greater specificity, sensitivity, and reliability in body fluid identification. Among these molecular strategies, mRNA expression analysis has emerged as a particularly powerful tool due to its capacity to reflect tissue- and cell-type–specific gene activity. Unlike DNA, which remains largely constant across different tissues of an individual, messenger RNA (mRNA) provides a dynamic snapshot of gene expression, thereby enabling the

discrimination of biological fluids based on their unique transcriptional profiles. Contemporary forensic research has focused extensively on exploiting these molecular signatures through advanced technologies such as reverse transcription quantitative polymerase chain reaction (RT-qPCR), with an emphasis on improving marker specificity, stability, and interpretative robustness.

Biological Rationale for Tissue-Specific mRNA Markers

The fundamental principle underlying mRNA-based body fluid identification is the concept of differential gene expression. In multicellular organisms, although all somatic cells contain the same genomic DNA, gene expression is tightly regulated, resulting in the production of distinct sets of mRNA transcripts in different tissues. These transcriptional differences are governed by regulatory mechanisms such as transcription factors, epigenetic modifications, and cellular microenvironments, which collectively determine the functional identity of each tissue type.

In forensic applications, this biological specificity is harnessed by identifying mRNA markers that are either uniquely expressed or significantly upregulated in particular body fluids. For instance, hemoglobin subunit beta (HBB) and glycophorin A (GYPA) transcripts are strongly associated with peripheral blood due to their roles in erythrocyte function. Similarly, semen-specific markers such as protamine 1 (PRM1), protamine 2 (PRM2), and semenogelin 1 (SEMG1) are expressed during spermatogenesis and are highly abundant in seminal fluid. Saliva, on the other hand, is characterized by the expression of genes such as statherin (STATH) and histatin 3 (HTN3), which are involved in oral homeostasis.

Recent advances have expanded the repertoire of validated mRNA markers through high-throughput transcriptomic analyses, including RNA sequencing (RNA-Seq). These studies have enabled the identification of novel, highly specific markers and the development of multiplex panels capable of simultaneously detecting multiple body fluids. Importantly, current research emphasizes the selection of markers with minimal cross-reactivity and consistent expression across individuals, thereby enhancing the reliability of forensic interpretations. Furthermore, the integration of bioinformatics tools has facilitated the systematic evaluation of candidate markers, allowing for the optimization of marker panels based on sensitivity, specificity, and forensic applicability.

Stability and Degradation Considerations

Despite its advantages, the forensic application of mRNA analysis is inherently challenged by the relative instability of RNA molecules. Unlike DNA, mRNA is more susceptible to degradation due to its single-stranded structure and the ubiquitous presence of ribonucleases (RNases) in the environment. Factors such as temperature, humidity, ultraviolet radiation, microbial activity, and the age of the biological stain can significantly affect RNA integrity, thereby influencing the accuracy of mRNA-based identification. Contemporary research has addressed these challenges by investigating the stability profiles of specific mRNA markers under various environmental conditions. Studies have demonstrated that certain transcripts exhibit greater resilience to degradation, making them more suitable for forensic applications. For example, housekeeping genes and short amplicon targets tend to be more stable and are often incorporated into marker panels as internal controls. Additionally, the use of optimized RNA extraction methods and preservation techniques, such as the application of RNA stabilizing agents and immediate sample drying, has been shown to mitigate degradation effects.

Another important development is the design of RT-qPCR assays targeting short mRNA fragments, typically ranging from 70 to 150 base pairs. Shorter amplicons are more likely to remain intact in degraded samples, thereby improving detection rates in challenging forensic scenarios. Moreover, advances in quantitative analysis allow for the assessment of RNA quality through metrics such as the RNA integrity number (RIN) or amplification efficiency, enabling forensic scientists to evaluate the suitability of samples prior to analysis.Recent studies have also explored the use of alternative RNA species, such as microRNA (miRNA) and circular RNA

(circRNA), which exhibit greater stability than mRNA due to their structural characteristics. While mRNA remains the primary focus of many forensic protocols, these emerging biomarkers are increasingly being considered as complementary tools, particularly in cases involving highly degraded samples.

Types of Body Fluids and Associated Gene Expression Profiles

The successful application of mRNA-based identification relies on the characterization of distinct gene expression profiles associated with different body fluids. Each fluid type contains a unique cellular composition, which in turn determines its transcriptomic signature. Contemporary forensic research has identified and validated a range of mRNA markers for the most commonly encountered body fluids in criminal investigations.Blood is one of the most frequently analyzed fluids and is characterized by the presence of erythroid-specific transcripts such as HBB and ALAS2 (aminolevulinate synthase 2). These markers are highly abundant and provide strong signals even in small or degraded samples. Semen, another critical fluid in forensic casework, is identified באמצעות markers such as PRM1, PRM2, and TGM4 (transglutaminase 4), which are specific to spermatozoa and seminal plasma. The high expression levels of these genes contribute to the sensitivity of semen detection.Saliva is typically identified through markers such as STATH, HTN3, and MUC7 (mucin 7), which are expressed in salivary glands and play roles in oral lubrication and antimicrobial defense. Vaginal fluid identification relies on markers such as CYP2B7P1 and MUC4, which are associated with epithelial cells of the vaginal mucosa. Menstrual blood, which presents a more complex composition due to the presence of both blood and endometrial tissue, is identified markers such as matrix metalloproteinases (MMP7, MMP11) and other endometrial-specific transcripts.

In recent years, there has been growing interest in expanding the range of identifiable fluids to include skin, sweat, and nasal secretions. Skin identification, for example, has been explored through markers such as loricrin (LOR) and keratin genes, while sweat-specific markers remain an area of ongoing research. The development of comprehensive multiplex panels that incorporate markers for multiple fluids has further enhanced the practicality of mRNA analysis in forensic workflows.Current trends also emphasize the importance of quantitative interpretation of gene expression data. Rather than relying solely on the presence or absence of specific markers, researchers are increasingly utilizing expression ratios and statistical models to improve discrimination between fluid types, particularly in mixed samples. Machine learning approaches are also being explored to classify gene expression patterns and enhance the accuracy of body fluid identification.

RT-qPCR Technology in Forensic Applications

The integration of reverse transcription quantitative polymerase chain reaction (RT-qPCR) into forensic biology has marked a decisive shift toward highly sensitive, specific, and quantitative molecular diagnostics. In the context of forensic body fluid identification, RT-qPCR serves as a cornerstone technique for analyzing mRNA expression patterns, enabling the discrimination of biological fluids based on their tissue-specific transcriptional signatures. Contemporary forensic research and practice increasingly rely on RT-qPCR due to its robustness, adaptability to low-template samples, and compatibility with multiplex assay designs. This section outlines the fundamental principles of RT-qPCR, details the forensic workflow from sample collection to data interpretation, and highlights its major advantages in modern applications.

Principles of Reverse Transcription Quantitative PCR

RT-qPCR is a two-step or one-step molecular process that combines reverse transcription of RNA into complementary DNA (cDNA) with real-time quantitative PCR amplification. The foundational concept lies in converting inherently unstable RNA molecules into more stable cDNA, which can then be exponentially amplified and quantitatively monitored during PCR cycles.In the reverse transcription phase, the enzyme reverse transcriptase synthesizes cDNA from mRNA

templates using sequence-specific primers, oligo(dT) primers, or random hexamers. This step is critical because the efficiency of cDNA synthesis directly affects downstream quantification accuracy. In forensic contexts, where RNA is often degraded or present in trace amounts, optimized reverse transcription protocols are essential to maximize yield and preserve representativeness of gene expression.The subsequent quantitative PCR phase involves the amplification of target cDNA sequences using DNA polymerase in the presence of fluorescent reporters. Two main detection chemistries are commonly employed: intercalating dyes such as SYBR Green, which bind to double-stranded DNA, and sequence-specific probes such as TaqMan probes, which provide higher specificity through hybridization to target sequences. As amplification progresses, fluorescence increases proportionally to the amount of PCR product generated, allowing real-time monitoring.Quantification is typically achieved באמצעות the cycle threshold (Ct) value, which represents the PCR cycle at which fluorescence exceeds a predefined threshold. Lower Ct values indicate higher initial template abundance. In forensic applications, Ct values are interpreted in relation to reference genes and marker panels to determine the presence and relative expression of body fluid–specific transcripts. Current approaches often incorporate normalization strategies using housekeeping genes to account for variability in RNA input and amplification efficiency.

Recent advancements in RT-qPCR technology include digital PCR adaptations, improved probe chemistries, and enhanced thermal cycling instruments, all of which contribute to increased sensitivity and reproducibility. Additionally, the adoption of standardized guidelines, such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), has strengthened methodological rigor and data transparency in forensic research.

Workflow: Sample Collection → RNA Extraction → Amplification → Analysis

The forensic application of RT-qPCR follows a multi-stage workflow, each step requiring careful optimization to ensure the integrity and reliability of results, particularly given the challenging nature of forensic samples.

Sample Collection and Preservation:

The process begins at the crime scene, where biological evidence is collected using sterile techniques to prevent contamination. Swabs, cutting tools, or adhesive tapes may be used depending on the substrate and nature of the stain. Immediate drying and proper storage—often at low temperatures—are critical to minimize RNA degradation. Contemporary protocols emphasize the use of RNA stabilization reagents and protective transport conditions to preserve transcript integrity.

RNA Extraction:

Following collection, total RNA is isolated from the sample using commercially available extraction kits or standardized laboratory protocols. This step involves cell lysis, removal of proteins and contaminants, and purification of RNA. In forensic settings, extraction methods must be optimized for low-input and potentially degraded samples. Magnetic bead–based extraction systems and silica column technologies are commonly used due to their efficiency and compatibility with automation. DNase treatment is often incorporated to eliminate genomic DNA contamination, ensuring that subsequent amplification reflects true RNA-derived signals.

Reverse Transcription and Amplification:

Extracted RNA is then subjected to reverse transcription to generate cDNA, followed by quantitative PCR amplification. Modern forensic assays frequently employ multiplex RT-qPCR, in which multiple target genes are amplified simultaneously within a single reaction. This approach conserves sample material and enables comprehensive profiling of multiple body fluids. Reaction conditions, including primer design, annealing temperatures, and cycle parameters, are meticulously optimized to prevent non-specific amplification and ensure reproducibility.

Data Analysis and Interpretation:

The final stage involves the analysis of amplification curves, Ct values, and expression

patterns. Specialized software is used to process fluorescence data and determine the presence or absence of specific markers. In contemporary practice, interpretation often threshold-based decision models, expression ratios, or probabilistic frameworks to classify body fluids. For mixed samples, deconvolution strategies are applied to identify contributions from multiple sources. Increasingly, statistical and machine learning models are being integrated to enhance interpretative accuracy and reduce subjectivity.Quality control measures are implemented throughout the workflow, including the use of positive and negative controls, internal reference genes, and replication of assays. These safeguards are essential to ensure the reliability of results, particularly in legal contexts where forensic evidence must withstand rigorous scrutiny.

Advantages: Sensitivity, Specificity, and Multiplexing Capability

One of the most significant advantages of RT-qPCR in forensic applications is its exceptional sensitivity. The technique is capable of detecting minute quantities of RNA, making it well-suited for trace evidence analysis. Even highly degraded samples can yield detectable signals when short amplicon designs and optimized protocols are employed. This sensitivity is particularly valuable in cases involving limited or compromised biological material.Specificity is another critical strength of RT-qPCR. Through careful selection of primers and probes targeting tissue-specific mRNA markers, the technique can distinguish between closely related body fluids with high accuracy. The use of probe-based detection systems further enhances specificity by requiring sequence-specific hybridization, thereby reducing the likelihood of false-positive results. Contemporary marker panels are rigorously validated to minimize cross-reactivity and ensure consistent performance across diverse populations and environmental conditions.

Multiplexing capability represents a transformative feature of RT-qPCR in forensic science. By enabling the simultaneous amplification of multiple targets within a single reaction, multiplex assays allow for comprehensive analysis of several body fluids without excessive sample consumption. This is particularly advantageous in forensic casework, where sample quantity is often limited. Advances in fluorophore chemistry and instrument design have expanded the number of detectable targets, facilitating the development of highly informative marker panels.In addition to these core advantages, RT-qPCR offers rapid turnaround times, quantitative output, and compatibility with automated platforms, all of which contribute to its practicality in forensic laboratories. The technique's scalability and standardization potential further support its integration into routine workflows.

Recent developments in RT-qPCR–based forensic analysis reflect a broader trend toward increased precision and integration with other molecular approaches. For example, the combination of mRNA profiling with DNA typing enables simultaneous individualization and body fluid identification from a single sample. Efforts are also underway to harmonize protocols across laboratories, establish standardized marker panels, and develop validated commercial kits for forensic use.Moreover, the incorporation of advanced data analysis methods, including machine learning algorithms, is enhancing the interpretative power of RT-qPCR results. These approaches allow for more accurate classification of complex samples and reduce reliance on subjective thresholds. The ongoing refinement of assay design, coupled with improvements in instrumentation, continues to expand the applicability of RT-qPCR in forensic science.

Advances and Applications of mRNA-Based Body Fluid Identification

The last decade has witnessed substantial progress in mRNA-based forensic methodologies, positioning transcriptomic analysis as a mature and increasingly operational tool in body fluid identification. Driven by innovations in molecular biology, assay design, and computational analysis, current research has moved beyond proof-of-concept studies toward validated, multiplexed systems capable of addressing real forensic challenges. The application of mRNA profiling—primarily through RT-qPCR—has expanded in scope, accuracy, and forensic relevance, particularly in the identification of specific body

fluids and the interpretation of complex casework samples.Contemporary advancements in mRNA-based body fluid identification have largely been shaped by high-throughput transcriptomic technologies and improved marker discovery strategies. RNA sequencing (RNA-Seq) has played a pivotal role in identifying novel tissue-specific transcripts with high discriminatory power. Unlike earlier approaches that relied on a limited number of candidate genes, RNA-Seq enables comprehensive profiling of the transcriptome, facilitating the discovery of highly specific and abundantly expressed markers for each body fluid. Recent studies have focused on refining marker panels by selecting genes with minimal inter-individual variability and negligible cross-reactivity. This has led to the development of robust multiplex assays capable of simultaneously detecting multiple body fluids within a single reaction. These panels often include internal reference genes and degradation-resistant targets to ensure reliability under forensic conditions.Another important development is the integration of statistical and computational models into mRNA analysis. Machine learning algorithms, such as support vector machines and random forests, are increasingly being used to classify expression profiles and improve the accuracy of body fluid identification. These approaches are particularly valuable when dealing with mixed samples or borderline expression levels, حيث traditional threshold-based interpretation may be insufficient.

In addition, recent research has explored the combination of mRNA markers with other molecular biomarkers, such as microRNA (miRNA), DNA methylation patterns, and protein markers, to create multi-modal identification systems. These hybrid approaches aim to overcome the limitations of single-marker systems and enhance robustness in degraded or environmentally challenged samples.The development of commercially viable kits and standardized protocols is another notable trend. Several forensic laboratories are now moving toward validated, ready-to-use RT-qPCR kits that incorporate optimized primer/probe sets and streamlined workflows. This shift toward standardization is essential for ensuring reproducibility and facilitating the admissibility of mRNA-based evidence in court.

Identification of Specific Body Fluids

One of the most significant applications of mRNA profiling is the precise identification of individual body fluids based on their unique gene expression signatures. Current research has established well-characterized marker sets for the most commonly encountered forensic fluids:

Blood:

Blood identification relies on erythroid-specific transcripts such as *HBB*, *ALAS2*, and *GYPA*. These markers are highly abundant and provide strong signals even in degraded samples. Recent studies have also investigated additional markers to distinguish peripheral blood from menstrual blood, addressing a critical forensic challenge.

Semen:

Semen is one of the most reliably identified fluids באמצעות mRNA analysis due to the high expression of sperm-specific genes such as *PRM1*, *PRM2*, *SEMG1*, and *TGM4*. These markers exhibit exceptional specificity and sensitivity, making semen identification highly robust, even in trace amounts or mixed samples.

Saliva:

Saliva identification is typically based on markers such as *STATH*, *HTN3*, and *MUC7*. While these markers are generally reliable, recent research has focused on improving specificity due to occasional cross-reactivity with other epithelial tissues. The inclusion of multiple markers in multiplex panels has significantly enhanced accuracy.

Vaginal Fluid:

Markers such as *CYP2B7P1*, *MUC4*, and *HBD1* are commonly used for vaginal fluid identification. Current studies emphasize the need to account for physiological variations, such as hormonal cycles and microbiome influences, which may affect gene expression profiles.

Menstrual Blood:
Menstrual blood presents a complex mixture of peripheral blood and endometrial tissue. Specific markers such as *MMP7*, *MMP11*, and other endometrial-associated genes have been identified to distinguish it from peripheral blood. Recent advances have improved the reliability of this differentiation, which is crucial in sexual assault investigations.

Other Fluids (Emerging Research):
Ongoing research is expanding the scope of identifiable fluids to include skin (via keratin and loricrin genes), sweat, and nasal secretions. Although these areas are still under development, preliminary findings suggest that mRNA profiling can be extended to a broader range of biological materials.
A key advancement in this domain is the development of **multiplex RT-qPCR assays** that combine markers for multiple fluids into a single test. This not only conserves sample material but also enables simultaneous detection and differentiation, which is particularly valuable in mixed or limited samples.

Casework Applications and Validation Studies
The transition of mRNA-based methods from research to operational forensic casework has been supported by extensive validation studies and pilot implementations. Contemporary validation efforts focus on key parameters such as sensitivity, specificity, reproducibility, robustness, and resistance to environmental degradation.Studies have demonstrated that mRNA profiling can successfully identify body fluids in a wide range of forensic scenarios, including aged stains, environmentally exposed samples, and mixed biological evidence. For example, controlled degradation studies have shown that carefully selected mRNA markers remain detectable after exposure to heat, humidity, and UV radiation, albeit with reduced expression levels. These findings underscore the importance of marker selection and assay design in ensuring forensic applicability.
In practical casework, mRNA analysis has been applied in sexual assault investigations, violent crime reconstruction, and trace evidence analysis. The ability to identify the type of body fluid present at a crime scene provides critical contextual information that complements DNA profiling. For instance, distinguishing between saliva and vaginal fluid can help reconstruct the nature of physical interactions, while identifying menstrual blood can clarify the origin of biological stains.Validation studies have also addressed the performance of mRNA assays in mixed samples, which represent a common challenge in forensic investigations. Multiplex RT-qPCR panels, combined with quantitative and statistical analysis, have demonstrated the ability to detect multiple fluids within a single sample and estimate their relative contributions. This capability is particularly valuable in complex cases involving multiple individuals or overlapping biological evidence.From a legal perspective, the admissibility of mRNA-based evidence depends on rigorous validation, standardization, and documentation. Recent efforts have focused on establishing guidelines for assay validation, quality control, and reporting standards. Organizations and forensic laboratories are increasingly adopting standardized protocols to ensure consistency and reliability across jurisdictions.
Furthermore, inter-laboratory studies and proficiency testing have been conducted to evaluate the reproducibility of mRNA-based methods. These studies have generally reported high concordance rates, supporting the robustness of RT-qPCR–based body fluid identification. However, ongoing efforts are needed to harmonize methodologies and address remaining challenges related to interpretation and reporting.

Conclusion
The integration of mRNA expression analysis via RT-qPCR into forensic body fluid identification represents a transformative advancement that bridges the gap between molecular biology and forensic investigation. This review has demonstrated that, unlike traditional biochemical methods, mRNA-based approaches provide superior specificity and sensitivity by exploiting tissue-specific gene expression profiles, enabling accurate identification of body fluids such as blood, semen, saliva, vaginal fluid, and menstrual blood. The underlying molecular rationale,

supported by advances in transcriptomics, has facilitated the development of highly selective markers and robust multiplex assays capable of analyzing even degraded or mixed samples. Furthermore, the structured RT-qPCR workflow—from sample collection and RNA extraction to amplification and data interpretation—ensures systematic and reproducible analysis, while technological innovations continue to enhance efficiency and reliability. Recent research developments, including the incorporation of bioinformatics tools and machine learning models, have significantly improved the interpretation of complex expression patterns, thereby strengthening forensic conclusions. Validation studies and practical casework applications confirm that mRNA profiling is not merely a theoretical tool but an increasingly operational method with strong evidentiary value. Although challenges such as RNA instability, environmental degradation, and the need for standardization persist, ongoing methodological refinements and the exploration of complementary biomarkers, such as microRNA and epigenetic signatures, indicate a promising trajectory for the field. Ultimately, mRNA-based body fluid identification via RT-qPCR contributes to a more precise, reliable, and contextually informative forensic framework, enhancing the ability of investigators to reconstruct events and supporting the delivery of justice. As forensic science continues to evolve toward molecular precision, this approach is poised to become a standard component of advanced forensic practice.

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