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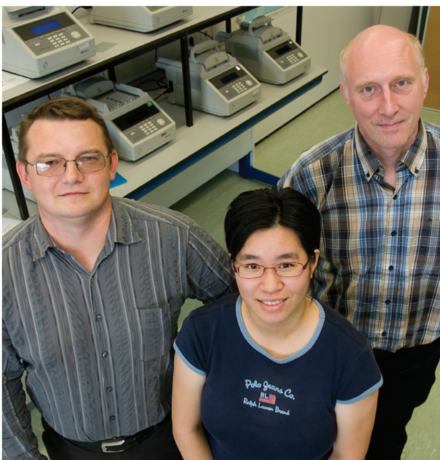
Applied Biosystems® TaqMan® MicroRNA Assays help researchers validate miRNA markers for forensic body fluid identification



Dmitry Zubakov, PhD, and researchers in the Department of Forensic Molecular Biology, in collaboration with the group of Erik Wiemer, PhD, at the Department of Medical Oncology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, the Netherlands, have discovered and validated potential microRNA (miRNA) markers for identification of venous blood and semen, two key body fluids in forensic casework. This article describes the use by Dr. Zubakov and colleagues of Applied Biosystems real-time PCR technologies and highly sensitive TaqMan® MicroRNA Assays to develop potential miRNA markers for identification of body fluid types in forensic applications.

From left to right: Dmitry Zubakov, PhD, research scientist, and Ying Choi, research assistant, of the Department of Forensic Molecular Biology, alongside Erik Wiemer, PhD, associate professor at Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, the Netherlands.

Featured products

Ambion® *mirVana*™ miRNA Isolation Kit

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Ambion® TURBO DNA-*free*™ Kit

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Applied Biosystems® TaqMan® MicroRNA Assays

Applied Biosystems® TaqMan® Universal PCR Master Mix, No AmpErase® UNG

Applied Biosystems® 7300 Real-Time PCR System

Importance and challenges of identifying body fluids in forensic casework

In forensic casework, correctly identifying body fluids such as blood, semen, or saliva is important for reconstructing a crime scene. Analysis of stains or biological fluids recovered during an investigation may provide potential sources of DNA that can be used to identify the donor of the fluid or stain [1]. However, body fluid samples can be difficult to work with, as often only limited quantities of sample are available at crime scenes. Moreover, samples may have been exposed to conditions that contribute to the degradation of RNA, and may yield only minute amounts of low-quality genetic material.

Traditional serological or immunological methods of identifying body fluids can be costly in terms of time and labor, and require large amounts of sample. Moreover, these protein-based assays have varying degrees of sensitivity and specificity [2]. To be suitable for forensic casework, a body fluid identification assay must:

- Demonstrate a high degree of specificity for each body fluid
- Permit parallel analysis of the different biological fluids being tested
- Be performed in a timely and labor-efficient manner
- Be sufficiently sensitive

Real-time PCR assays for body fluid identification

Recent studies have shown that mRNA profiling of tissue-specific gene transcripts from forensic samples has successfully identified body fluids and satisfies the criteria for an assay suitable for forensic casework [1]. However, use of mRNA for body fluid identification assays presents forensic scientists unique challenges related to the kind and quality of samples encountered in casework. For example, although mRNAs have been shown to be tissue-specific, RNA is susceptible to degradation by heat, humidity, and UV light. In addition, typical forensic assays employ some biomarkers that have amplicon sizes of more than 250 bases, resulting in assay failure in highly degraded samples [2]. The risk of physical degradation of mRNA requires use of very short amplicons for successful PCR detection. An alternative strategy would be to employ short RNA biomarkers instead of mRNA [2].

MicroRNAs as markers for body fluids

MicroRNAs (miRNAs) belong to a class of small (18–22 nucleotide) noncoding RNA molecules that regulate gene expression. A number of miRNAs have been implicated in embryonic development, cell proliferation, and differentiation, as well as in the pathogenesis of a number of human diseases. Several recent studies have revealed tissue-specific expression patterns of miRNAs [1].

In this article, we describe how Dr. Dmitry Zubakov and colleagues from Erasmus University Medical Center used microarray analysis and real-time PCR assays to profile expression patterns of 718 miRNAs in five forensically relevant body fluids. The researchers identified two potential miRNA markers for venous blood and two others for semen that were not prone to degradation in samples stored for one year. Essential to this finding was the validation of miRNA markers using Applied Biosystems® real-time PCR technology that included highly sensitive TaqMan® MicroRNA Assays capable of detecting miRNAs from subpicogram amounts of total RNA. According to Dr. Zubakov, the ability of TaqMan® Assays to identify relevant miRNA markers from minute quantities of aged samples holds great promise for their inclusion in future forensic methods for identifying body fluids.

Sample preparation
- **Invitrogen™ TRIzol® Reagent or Ambion® *mir*Vana RNA Isolation Kit***
  - -Extract total RNA from patient samples and controls
- **Ambion® TURBO DNA-*free*™ Kit**
  - -Remove potential traces of DNA

***Ambion® *mir*Vana RNA Isolation Kit not used in study by Zubakov et al., but recommended by LifeTechnologies**

Microarray analysis
- Evaluate expression profiles of miRNAs using LNA™ modified oligonucleotides (Exiqon) as capture probes
- Select subsets of miRNAs that best characterize each body fluid based on expression analysis by PAM software

Reverse transcription
- **TaqMan® MicroRNA Reverse Transcription Kit**
  - Generate cDNA

Note: In analysis of samples degraded over time, each reaction contained either single or multiple (pentaplex) stem-loop miRNA-specific primers.

Real-time PCR validation of miRNA markers
- **Applied Biosystems® TaqMan® MicroRNA Assays**
- **Applied Biosystems® TaqMan® Universal PCR Master Mix, No AmpErase® UNG**
- **Applied Biosystems® 7300 Real-Time PCR System**
  - Load and run TaqMan® MicroRNA Assays (45 cycles in triplicate) and evaluate expression levels of candidate miRNA markers

Figure 1. Method for analysis of miRNA markers for body fluid types.

Method for identifying miRNA markers for body fluids

Five kinds of body fluids (saliva, venous blood, menstrual blood, semen, and vaginal secretion) were collected from three male and three female volunteers with informed consent [1]. Figure 1 shows a method for miRNA marker analysis that includes RNA isolation and analysis of small quantities of low-quality RNA sample.

TaqMan® Assays validate miRNA markers for semen and blood

In this study, Dr. Zubakov and colleagues used microarrays to survey miRNA expression levels in five different kinds of body fluids (semen, blood, saliva, menstrual blood, and vaginal secretion). They differentiated between fluid types based on expression levels of selected miRNAs. The researchers then evaluated a subset of these candidate markers for expression levels in target fluids. They then applied variance-based (PAM software scores) and fold-change metrics to assess changes in differential expression levels between target and nontarget fluids. Based on these calculations, the researchers chose the 14 most promising candidate markers (two to four for each of the five body fluids) for validation by real-time PCR.

To validate if expression levels of miRNA markers identified using microarray analysis were the best candidates for characterizing each of the body fluid types, the researchers performed real-time PCR in triplicates, using Applied Biosystems® TaqMan® MicroRNA Assays and Applied Biosystems® TaqMan® Universal PCR Master Mix, No AmpErase® UNG, run on an Applied Biosystems® 7300 Real-Time PCR System (45 cycles). Relative expression levels of candidate miRNA markers in different body fluids were quantified using the ΔC_t method; the average expression levels of three reference small RNAs (RNU24, RNU44, and RNU48) were used for normalization.

The level of agreement between gene expression data obtained using microarray analysis and real-time PCR was highly dependent on the body fluid type analyzed. Expression patterns of candidate miRNA markers for semen and venous blood were highly concordant between the two approaches. Three miRNAs (miR-20a, miR-106a, and miR-185) in venous blood and four (miR-135a, miR-10a, miR-507, and miR-943) in semen showed high expression levels relative to all other body fluids tested.

A fifth miRNA marker, miR-891a, was first dismissed as a candidate marker for semen, based on microarray hybridization

data. However, real-time PCR analysis with TaqMan® MicroRNA Assays revealed that miR-891a was highly and exclusively expressed in semen, confirming literature data on miR-891a showing it to be present only in epididymal tissue. The microarray data, however, revealed that miR-891a is expressed at a higher level in vaginal secretion than in semen. Northern blotting using a biotinylated LNA™ probe targeting miR-891a showed the miRNA expressed in semen and not in vaginal secretion samples (Figure 2). According to the researchers, the erroneous result from microarray analysis may be attributed to cross-hybridization with RNA species from bacterial and/or fungal RNA present in total RNA extracts from vaginal samples that were analyzed at the same time as semen samples. Alternatively, incomplete processing of immature miRNA in vaginal samples may also account for the microarray hybridization data.

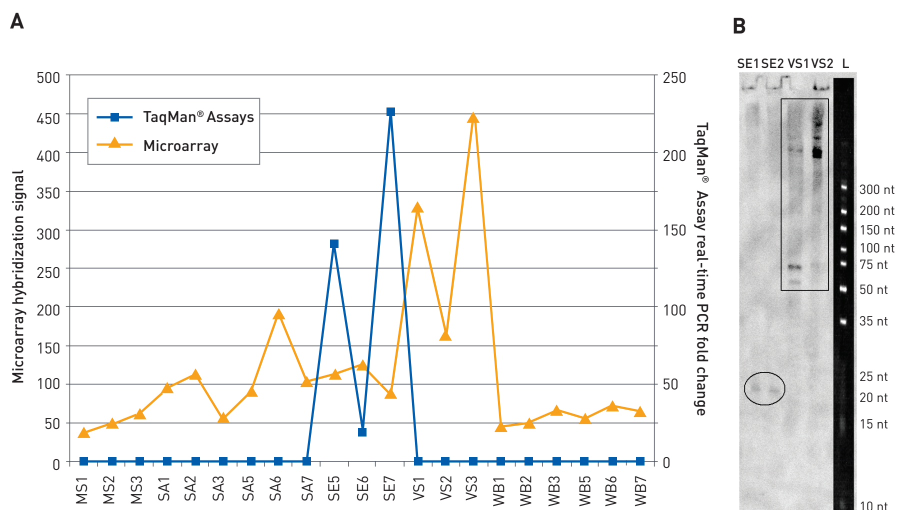

Figure 2. Expression profile for miRNA marker miR-891a. (A) Expression of miR-891a according to microarray and real-time PCR analysis using TaqMan® MicroRNA Assays on five body fluids—menstrual blood (MS), saliva (SA), semen (SE), vaginal secretion (VS), venous blood (WB)—from several individuals. **(B)** Northern blot of total RNA from semen and vaginal secretion samples, hybridized with miR-891a–specific LNA™ oligonucleotide. L = size ladder.

Of all the candidate miRNA markers, the researchers chose two blood (miR-144 and miR-185) and two semen (miR-135a and miR-891a) miRNA markers for sensitivity testing because they appeared most abundant and most specifically expressed according to analyses using TaqMan® Assays. These four markers served as the best candidate miRNA markers for blood and semen in future forensic applications. The researchers were unable to identify reliable miRNA markers for the three other body fluids (saliva, menstrual blood, and vaginal secretion).

A robust method for identifying body fluid markers

To evaluate the stability of the candidate blood and semen miRNA markers, the researchers tested how long-term storage of body fluid samples would affect the ability of the TaqMan® MicroRNA Assays to detect these miRNAs in the samples. The same blood and semen samples that were used in the initial experiments were aged for 1 year under laboratory conditions (relatively constant humidity and ambient temperature, no UV exposure, and dust free). A comparison of real-time PCR results from fresh and 1-year-old samples plotted in parallel revealed strong similarities in expression profiles and absolute expression levels (Figure 3). The researchers reported that not only could the miRNAs be easily detected in experimentally aged body fluid samples, but

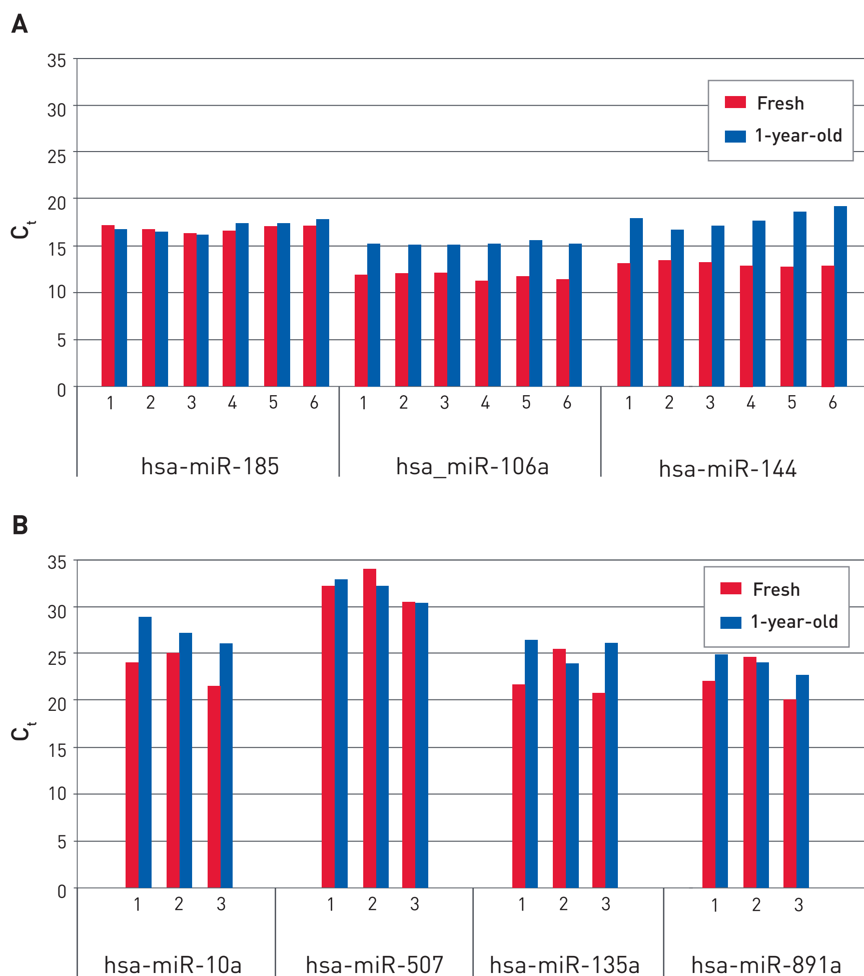

Figure 3. Stability of blood and semen miRNA markers. A comparison of TaqMan® Assay profiles of miRNA markers for **(A)** six venous blood samples (markers: hsa-miR-185, hsa-miR-106a, hsa-miR-144) and **(B)** three semen samples (markers: hsa-miR-10a, hsa-miR-507, hsa-miR-135a, hsa-miR-891a) in fresh body fluids and 1-year-old stains.

also the absolute levels did not diminish in older samples. Overall, they detected almost no effect of degradation over time on the stability of the miRNA markers tested [1].

Single-cell detection sensitivity

To establish the sensitivity of the TaqMan® MicroRNA Reverse Transcription Kit for detecting candidate blood and semen miRNA markers, the researchers prepared 10-fold serial dilutions of total RNA isolated from fresh venous blood and semen samples and performed real-time PCR using 20ng–2pg of RNA and TaqMan® MicroRNA Assays. All four markers were detected in the target body fluid using the lowest amount of total RNA (2 pg) for cDNA synthesis. This corresponds to as little as 0.1 pg RNA in single PCR tube, or the amount of total RNA in a single cell. Based on these results, the authors of the study expect these semen and blood miRNA markers to be useful for most forensic cases. According to Dr. Zubakov, the high sensitivity of TaqMan® MicroRNA Assays makes them potentially valuable tools for future forensic applications.

Possibility to detect miRNA markers for other body fluids

Another feature of TaqMan® MicroRNA Assays that makes them a potentially valuable technology for identifying body fluids in forensic casework is the unique stem-loop primers used for reverse transcription. These primers enable TaqMan® MicroRNA Assays to specifically detect only mature miRNA molecules. In fact, the authors of this study noted that their inability to identify reliable miRNA markers for the other three body fluids analyzed (saliva, menstrual blood, and vaginal secretion) may be related to the lack of specificity in the hybridization methods they used. The LNA™ capture probes used in the microarray may not discriminate between mature miRNA and homologous RNA molecules, whereas stem-loop primers are strictly specific to short miRNAs. This is important because bacteria and fungi naturally contaminate these other three body fluids. TaqMan® MicroRNA Assays, with their high specificity for mature miRNA molecules, may be less affected by these contaminating RNAs. For this reason, the authors noted that future screening of candidate miRNA markers for saliva, vaginal secretion, and menstrual blood employing highly specific TaqMan® MicroRNA Assay technology may reveal promising markers for these kinds of body fluids [1].

References

1. Zubakov D, Boersma AWM, Choi Y et al. (2010) MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. *Int J Legal Med* Feb 10 ePub:1–10.
2. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. *Anal Biochem* 387:303–314.

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