Development and initial evaluation of a miRNA system for forensically relevant body fluids using capillary electrophoresis

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ABSTRACT

Body fluid identification (BFID) can provide crucial information during the course of an investigation. In recent years, microRNAs (miRNAs) have shown considerable body fluid specificity, are able to be co-extracted with DNA, and their small size (18-22 nucleotides) make them ideal for analyzing highly degraded forensic samples.

In this study, we designed a new 8-marker system for BFID to differentiate between venous blood, menstrual blood, semen, and saliva using a capillary electrophoresis approach. This panel expands on a previously reported linear primer system [1] in order to incorporate additional miRNA markers by forming a comprehensive four-dye multiplex system.

The miRNA system was able to distinguish between venous blood, menstrual blood, and semen. The saliva primers were not specific to the miRNA targets and were removed from the multiplex. All STR amplifications from co-extracted DNA yielded complete DNA profiles.

INTRODUCTION

Determining the body fluid origin of a stain may provide probative information about the events that transpired during the commission of a crime. MiRNAs have shown tissue and cell-type specific expression and the stability necessary for a robust BFID system.

A common strategy for miRNA profiling systems is to analyze relative expression (ΔCT) values of various miRNAs compared to an endogenous reference gene using RT-qPCR. However, most instrumentation for qPCR can detect up to five different fluorescent dyes, which would limit the amount of markers that can be amplified simultaneously. Additional reactions would be required to analyze multiple markers, which increases sample consumption, the risk of contamination, cost of reagents, and time of analysis [2].

Van der Meer et al. [3] developed a method to co-analyze DNA (STRs) and miRNA with stem-loop primers using capillary electrophoresis. However, the use of stem-loop primers has been shown to result in PCR artifacts that may complicate interpretation. Li et al. [1] addressed these potential problems by designing a linear primer set composed of three primers working in conjunction to amplify specific miRNAs. While the linear primer system does address the technical challenges of the stem-loop primers, only one universal primer for one dye channel was designed and tested by Li et al. [1]. As novel markers are continually being discovered for BFID and normalization, it would be advantageous to expand the linear primer system to additional dye channels and incorporate more markers and reference markers.

RESULTS & DISCUSSION

Marker Selection and Primer Design

Two miRNA markers were chosen for each of menstrual blood, venous blood, semen, and saliva based on a consensus of candidate markers for body fluid identification in various published studies [1, 4-6]. To minimize non-specific binding, sequences previously designed by Lindblad-Toh et al. [7] and M13 sequences were used for the reverse transcription/universal paired binding sequence.

Body Fluid	Marker	miRNA Sequence	RT Primer	Specific Primer	Universal Primer
Venous Blood	miR-451	AAACCGUUACCAUUACUGAGUU	GTTCTTGCTGTCAACGATACGCTAC GTTTTCTTTTCTTTTAACTCAGT*	GTTCTTCTTTTTCTTTTTCTTTTTC AAACCGTTACCATT*	FAM'GTTCTTCTTTTTCTTTTT CTGCTGTCAACGATACGCTACG*
	miR-142-3	UGUAGUGUUUCCUACUUUAUGGA	GTTCTTGCTGTCAACGATACGCTAC GTTTTCTTTTCTTTTTCCATAAA	GTTCTTCTTTTTCTTTTTCTTTTTCTTTTT CTTGTAGTGTTTCCTAC	
Menstrual Blood	miR-141-3	UAACACUGUCUGGUAAAGAUGG	GTTCTTAACTGACTAAACTAGGTGCC TTTTCTTTTCTTTTCCATCTTT	GTTCTTCTTTTTCTTTTTCTT AACACTGTCTGGT	VIC'GTTCTTCTTTTTCTTTTTCT AACTGACTAAACTAGGTGCC
	miR-412	ACUUCACCUGGUCCACUAGCCGU	GTTCTT AACTGACTAAACTAGGTGCC TTTTCTTTTCTTTTACGGCTAG	GTTCTTCTTTTTCTTTTTCTTTTTCTTTTT CTACTTCACCTGGTCCA	
Semen	miR-891	UGCAACGAACCUGAGCCACUGA	GTTCTT ACGTCGTGAAAGTCTGACAA TTTTCTTTTCTTTTTCAGTGGC	GTTCTTCTTTTTCTTTTTCTTTTTCTTTTT CTTTTTGCAACGAACCTGA	NED'GTTCTTCTTTTTCTTTTTCT ACGTCGTGAAAGTCTGACAA
	miR-10	UACCCUGUAGAACCGAAUUUGUG	GTTCTT ACGTCGTGAAAGTCTGACAA TTTTCTTTTCTTTTCACAAATT	GTTCTTCTTTTTCTTTTTCTTTTTC TATGGGACATCTTCCG	
Saliva	miR-205	UCCUUCAUUCCACCGGAGUCUG	GTTCTTGTAAAACGACGGCCAG TTTTCTTTTCTTTCAGACTCC	GTTCTTCTTTTTCTTTTTCTTTTTCTTTT CCTTCATTCCACC	PET'GTTCTTCTTTTTCTTTTTCT GTAAAACGACGGCCAG
	miR-658	GGCGGAGGGAAGUAGGUCCGUUGGU	GTTCTTGTAAAACGACGGCCAGTTTT CTTTTCTTTTACCAACGG	GTTCTTCTTTTTCTTTTTGGCGG AGGGAAGTAGGT	

Table 1. Reverse transcription, specific, and universal primers sequences. (* primer was taken from Li et al.)

Multiplex Reactions

In the initial multiplex reactions, it was found that the saliva primers amplified genomic DNA and were removed from the multiplex system at this time. The cross-reactivity of miR-141-3 (menstrual blood marker) with semen has not been previously reported.

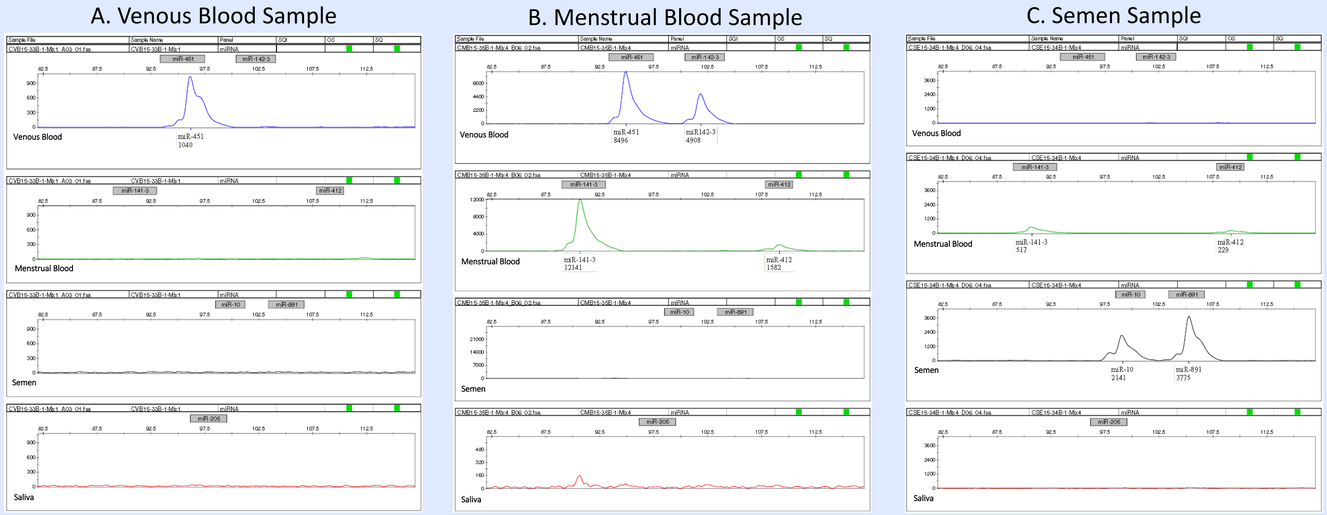

Figure 1. Electropherograms from miRNA multiplex reactions. A. Peaks miR-451 observed for venous blood. B. Peaks miR-451, miR-142-3, miR-412 and miR-141-3 observed for menstrual blood. C. Peaks miR-141-3, miR-10, miR-412, and miR-891 observed for semen.

REFERENCES

1. Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y. A strategy for co-analysis of microRNAs and DNA. Forensic Science International: Genetics. 2014;12:24-9.
2. van der Meer DJ, Williams GA. Performing body fluid identification with microRNAs using capillary electrophoresis. Forensic Science International: Genetics Supplement Series. 2015;5:e592-e4.
3. van der Meer D, Uchimoto ML, Williams G. Simultaneous Analysis of Micro-RNA and DNA for Determining the Body Fluid Origin of DNA Profiles. Journal of Forensic Sciences. 2013;58(4):967-71.
4. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Analytical Biochemistry. 2009;387(2):303-14.
5. Zubakov D, Boersma AWM, Choi Y , van Kuijk PF, Wiemer EAC, Kayser M. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. International Journal of Legal Medicine. 2010;124(3):217-26.
6. Li Z, Bai P, Peng D, Long B, Zhang L, Liang W. Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Science International: Genetics Supplement Series. 2015.
7. Lindblad-Toh K, Winchester E, Daly MJ, Wang DG, Hirschhorn JN, Laviolette J-P, et al. Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. Nature genetics. 2000;24 (4):381-6.

MATERIALS AND METHODS

Sample Collection Samples of venous blood, menstrual blood, semen, and saliva (n=5) were collected with Sam Houston State University IRB approval (#2015-09-26124).

DNA/RNA co-extraction Samples were extracted with the AllPrep® DNA/RNA Micro Kit (Qiagen) according to manufacturer's Small RNA Purification protocol with the addition of 5 µL 14.3 M β-mercaptoethanol and an incubation of 2 hrs at 56°C.

DNA Quantification and Amplification The Quantifiler™ Human DNA Quantification Kit was used to quantify DNA extracts on a 7500 Real Time PCR System. DNA was amplified using the GlobalFiler™ PCR Amplification Kit (ThermoFisher Scientific).

Reverse Transcription Reverse transcription was performed using the SuperScript® III First Strand Synthesis System (ThermoFisher Scientific) with PAGE purified custom reverse transcription primers (IDT).

miRNA Amplification PCR amplification was performed with the Type-It® Microsatellite PCR Kit (Qiagen) in a reaction volume of 20 µL composed of 10 µL 2X PCR Mix, 1 µL cDNA, and 9 µL of specific primers (IDT) and fluorescently tagged universal primers (ThermoFisher Scientific).

Capillary Electrophoresis PCR products were separated and detected with an ABI 3500 Genetic Analyzer. Data was analyzed using Genemapper® Software v. 5.3 (ThermoFisher Scientific). An analytical threshold of 175 RFU was applied.

CONCLUSIONS

- Multiplex is functional but requires optimization
- Co-extraction of DNA and RNA yielded high quality STR profiles
- Saliva primers amplify genomic DNA and must be redesigned
- Menstrual blood marker miR-141-3 shows cross-reactivity with semen and saliva and may not be suitable for BFID
- Future work will include:
  - Addition of markers for vaginal material and an internal reference marker
  - Stability study of biological stains (mock forensic casework samples)

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