Development and Initial Evaluation of a miRNA System for Forensically Relevant Body Fluids using Capillary Electrophoresis

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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 number of markers that can be amplified simultaneously. Additional reactions would be required to analyze multiple markers, which increase 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 in that study [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.

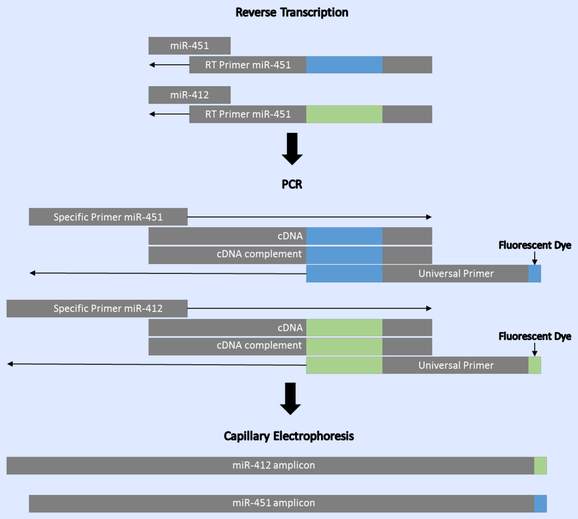

Figure 1. Schematic of linear primer system.

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.

Table 1. MiRNA markers included in the multiplex reaction.

Body Fluid	Venous Blood		Menstrual Blood		Semen		Saliva	
Marker	miR-451	miR-142-3	miR-141-3	miR-412	miR-891	miR-10	miR-205	miR-658

Multiplex Reactions

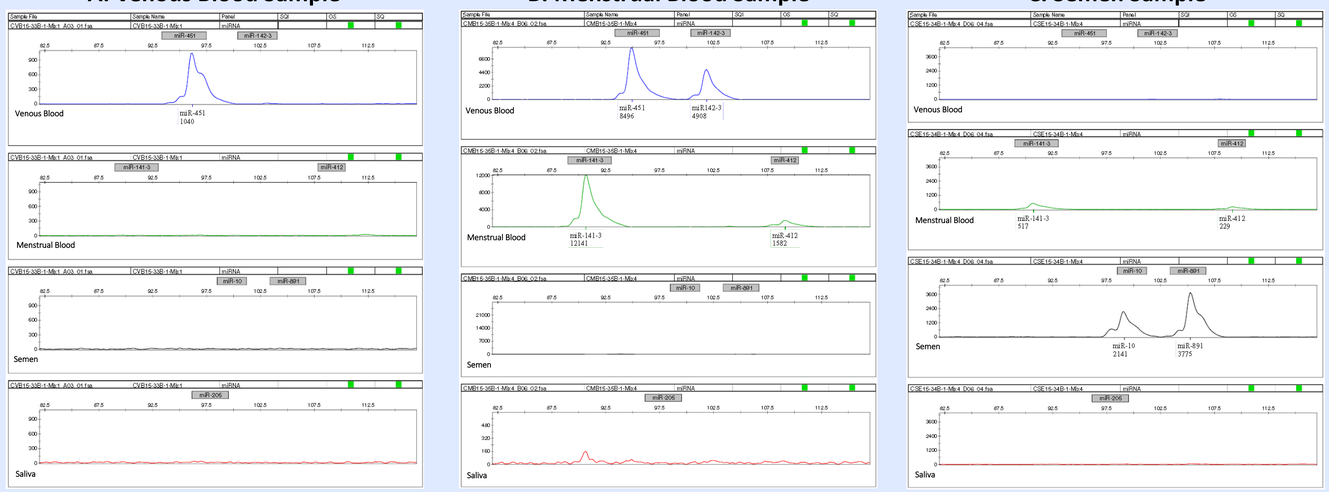

Figure 2. Electropherograms from miRNA multiplex reactions. A. Peaks miR-451 observed for venous blood. B. Peaks miR-451, miR-142-3, miR-412 and miR-141-3 observed for menstrual blood. C. Peaks miR-141-3, miR-10, miR-412, and miR-891 observed for semen.

- The multiplex is able to distinguish between venous blood, menstrual blood, and semen.
- Marker miR-451 appeared to be specific to venous blood and markers miR-891 and miR-10 were specific for semen.
- Saliva primers amplified genomic DNA and were removed from the multiplex system.
- The cross-reactivity observed for miR-141-3 (menstrual blood marker) with semen has not been previously reported.
- Marker balance of miR-142-3 between venous blood and menstrual blood has been difficult to achieve due to different expression levels between fluids.
- Low level transcription of miR-141-3 and miR-412 was observed in semen samples. Logistic regression analysis may be necessary.
- A standardized method of quantification in necessary to regulate the amount of input cDNA in the PCR reaction.

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. In ternational 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 Seri es. 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 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 STR 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

- The multiplex is functional but requires optimization.
- Co-extraction of DNA and RNA yielded high quality STR profiles.
- Saliva primers that amplified genomic DNA are currently being redesigned.
- Menstrual blood marker miR-141-3 showed 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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