A Capillary Electrophoresis Method for Identifying Forensically Relevant Body Fluids Using miRNAs

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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. MicroRNAs (miRNAs) have shown tissue and cell-type specific expression and the stability necessary for a robust body fluid identification (BFID) system.

A common strategy for miRNA profiling systems is to compare relative expression values (ΔCT) of various miRNAs 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 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. While the linear primer system addresses the technical challenges of the stem-loop primers, only one universal primer for each dye channel was designed and tested by Li et al. [1].

Therefore, as novel markers are continually being discovered for BFID and normalization, it would be advantageous to expand the linear primer system to more markers and additional dye channels. In this study we designed a miRNA multiplex with seven BFID markers, and one reference marker.

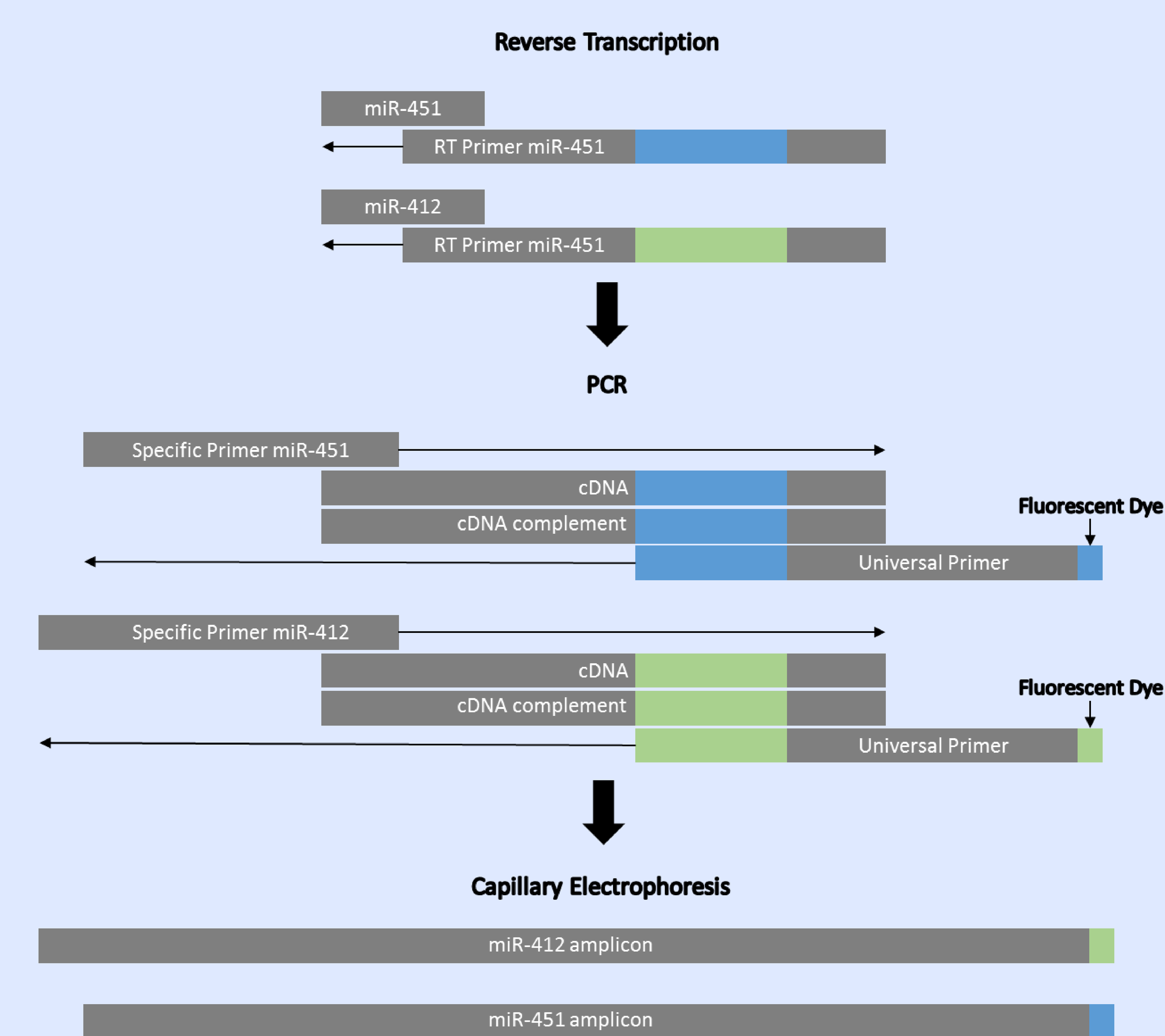

Figure 1. Schematic of linear primer system.

RESULTS AND DISCUSSION

Multiplex Reactions

Markers were chosen for menstrual blood, venous blood, semen, saliva, and an endogenous reference gene 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. Primer sequences were generated based on the model designed by Li et al. [1].

Table 1. MiRNA markers included in the multiplex and proportion of markers observed in the miRNA profiles generated in this study (N=5).

Body Fluid	Ref. Gene	Venous Blood		Menstrual Blood		Semen		Saliva
	let-7g	miR-451a	miR-142-3p	miR-141-3p	miR-412-3p	miR-891a	miR-10b	miR-205
Venous Blood	1	1	1	0	0	0	0	0
Menstrual Blood	1	1	0	1	0.6	0	0	1
Semen	1	0	1	0.8	0.8	1	1	1
Saliva	1	0	1	0.2	0.2	0	0	1

- The multiplex was able to distinguish between venous blood, menstrual blood, semen, and saliva.
- Endogenous reference gene was observed in all samples.
- An interpretation strategy was developed based on the presence/absence of markers (Fig. 2).
- Non-specific amplification was observed in the green channel in menstrual blood and saliva samples.
- Cross-reactivity of miR-141-3p and miR-412-3p complicates mixture interpretation.
  - A venous blood/semen mixture cannot be distinguished from a menstrual blood/semen mixture.
  - The presence of saliva would be masked by semen.

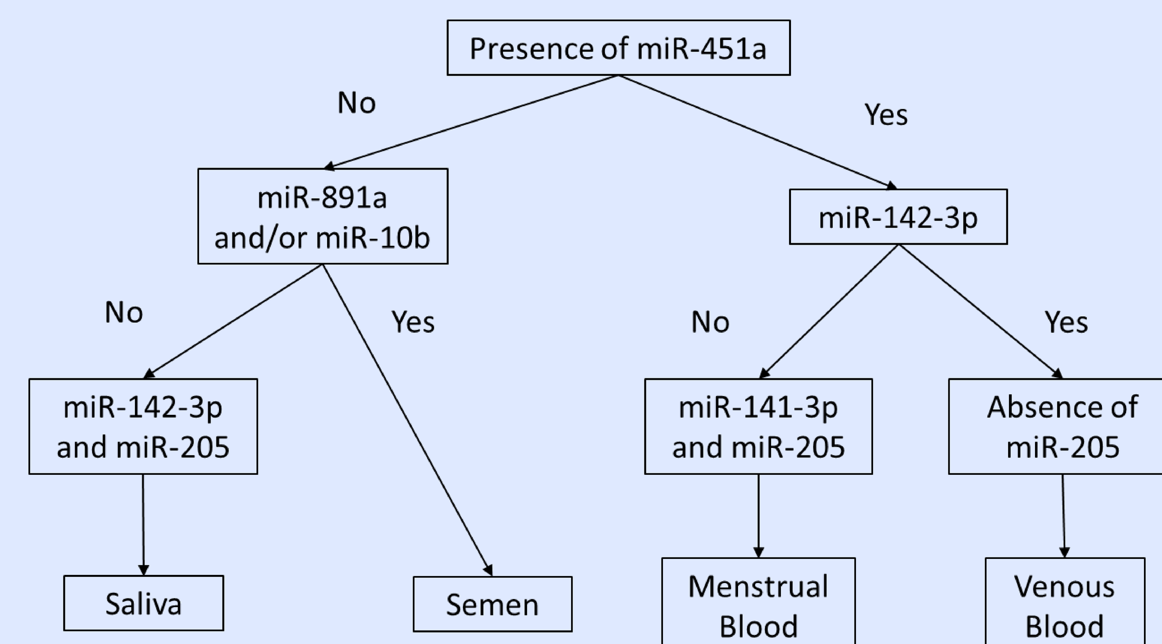

Figure 2. Decision tree for body fluid identification of single source samples.

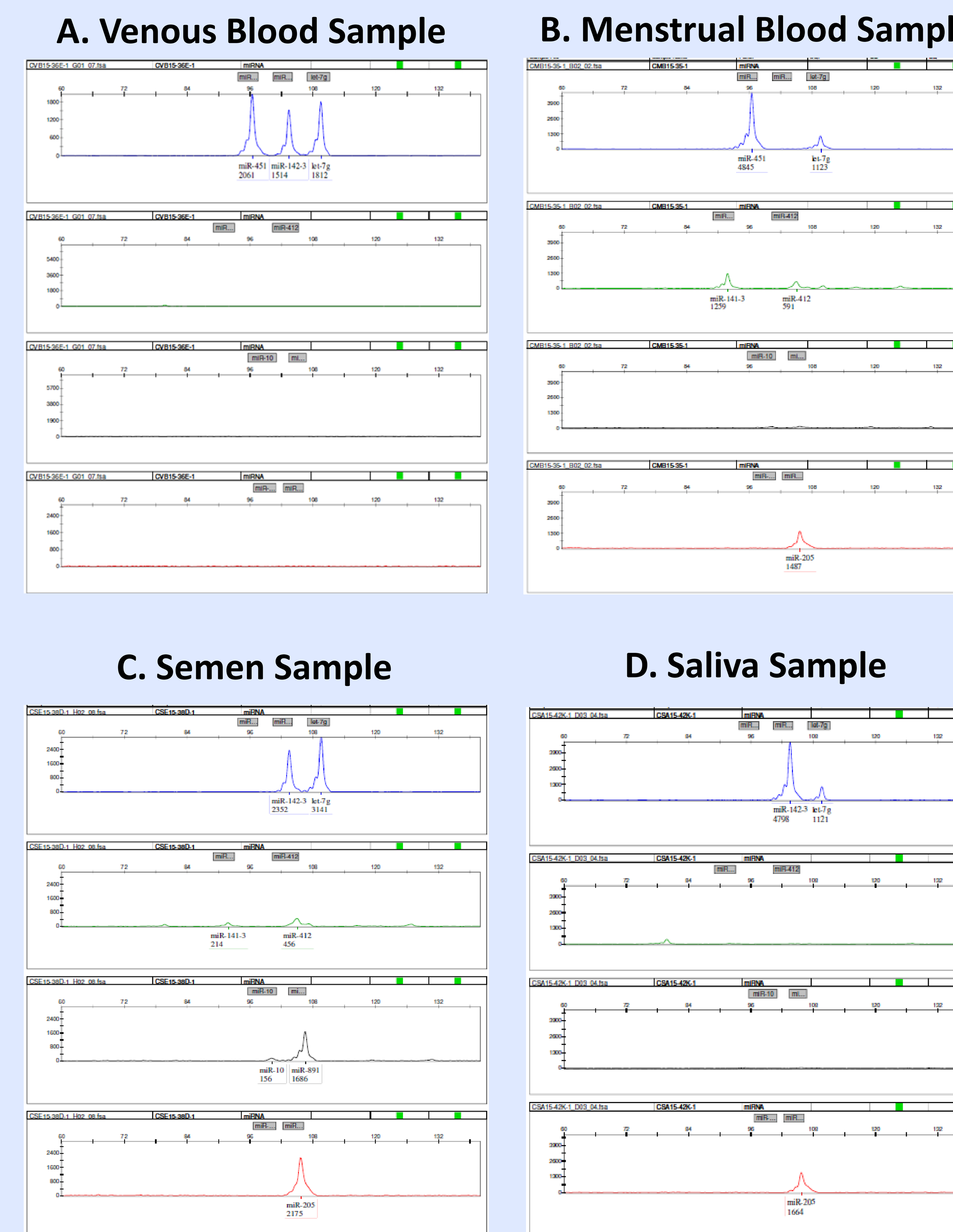

Figure 3. Representative electropherograms from miRNA multiplex reactions.

MATERIALS AND METHODS

Sample Collection Venous blood, menstrual blood, semen, and saliva (N=5) were collected according to 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 with 900 rpm shaking.

DNA Quantification and Amplification The Quantifiler™ Trio 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 Multiplex PCR Kit (Qiagen) in a reaction volume of 26 µL composed of 13 µL 2X PCR Mix, 3 µL cDNA, and 10 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 were analyzed using Genemapper® Software v. 5.3 (ThermoFisher Scientific). An analytical threshold of 150 RFU was applied.

CONCLUSIONS

- Co-extraction of DNA and RNA yielded high quality STR profiles.
- miRNA multiplex is functional but requires some improvements for mixture interpretation.
- Future work may include:
  - Refinement and/or replacement of select markers
  - Addition of markers for vaginal material
  - Stability study of biological stains (mock forensic casework samples)

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