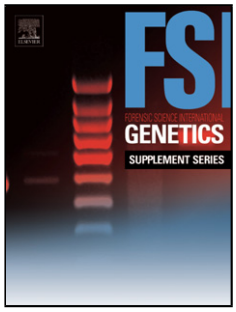

Title: Characterising the fluctuation of microRNA expression throughout a full menstrual cycle

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Characterising the fluctuation of microRNA expression throughout a full menstrual cycle.

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Abstract
When processing a crime scene, obtaining a DNA profile that can identify an individual is extremely important. However, the identification of the body fluid that the sample was obtained from could provide key information of the events that occurred. microRNA (miRNA) expression analysis is a technique that has the potential to differentiate body fluids. The presence and expression of body fluid specific miRNA would provide a fast and effective tool for progressing crime scene investigation, especially alleged sexual assault cases. Forensic case work lacks methods for identifying vaginal material, venous blood, menstrual blood and aspermatozoic seminal fluid within samples. A large screening study followed by a 31 day study on five female volunteers was performed utilising RT-qPCR on a large panel of body fluid markers. Screening showed a selection of markers were suitable to differentiate each body fluid, in some cases however, expression fluctuated when analysed over a 31 day period. The data shows that hsa-miR-412 may be suitable for identifying menstrual blood, expression from markers hsa-miR-124 and hsa-miR-205 varied significantly over the 31 day period and between individuals and therefore were less suitable for body fluid identification. The data supports the use of miRNA markers for the identification of certain body fluids such as menstrual and venous blood; however markers for the identification of body fluids such as vaginal material and saliva require further investigation.

Keywords:
microRNA, Body fluid identification, qPCR, Vaginal material, Menstrual cycle

Introduction
The identification of body fluids is extremely important for progressing forensic case work. In cases of sexual assault, the identification of a body fluid can impact case level and reveal information that can support the prosecution or the defense. The capability of body fluid identification however, is limited. This is due to the non-existence of presumptive identification tests for vaginal material and aspermatozoic seminal fluid. In cases of alleged sexual assault where DNA from a victim is found on a penile swab, the identification of the body fluid present will alter case level; if a technique can differentiate between saliva and vaginal material it may support either the defense or prosecution version of events. In cases of assault where a male cannot produce semen, a DNA profile would be unlikely. If body fluid identification techniques can identify seminal fluid regardless of spermatozoa being present it may give a better understanding of the events of the case. Equally, although presumptive tests for blood are available, there are no currently used methods for differentiating between menstrual and venous blood. In cases of sexual assault, trauma blood is likely to be present, if this can be determined to be venous and not menstrual blood the context of the case would become clearer.
The characterization of miRNA: short, stable, non-coding RNA's which modulate gene expression post transcription present a solution to body fluid identification problems [1, 2]. Their precise role in gene regulation suggests the presence of body fluid specific miRNAs is likely, thus making them a robust tool for body fluid identification. The menstrual cycle consists of physiological changes such as ovum release and the building and detachment of the endometrial lining as well as the fluctuating hormonal changes of oestrogen, progesterone and FSH. Since these dynamic changes are likely to be regulated by miRNA, the expression level of specific markers is likely to vary throughout the cycle.

Material studied, methods, techniques
Samples of venous blood, menstrual blood, semen, seminal fluid, vaginal material and saliva were collected from volunteers for initial screening (n = 10-25). Sample collection for the menstrual cycle study involved low vaginal swabs (LVS) being collected from 5 individuals on a daily basis for a period of 31 days. The LVS were extracted within two hours of collection and stored in a -4°C freezer. Extraction followed the DNA purification from buccal swabs protocol from the QIAamp DNA Mini kit from Qiagen. Each sample was then quantified following the Quantifiler™ Human DNA Quantification protocol from Applied Biosystems and diluted to ensure all samples had a concentration of 5ng/µl. Stem-loop reverse transcription (RT) was then performed per marker, per sample, adhering to the TaqMan® Small RNA Assays protocol. Samples were held at 16 °C for 30 minutes, 42 °C for 30 minutes followed by a 5 minute hold at 85 °C. Each RT reaction was then prepared for qPCR using PrecisionPLUS 2x FAST qPCR MasterMix and custom made 10X assays. Once prepared, samples were placed in triplicate within 72 wells of the Rotor-Gene® Q. The run consisted of 50 cycles of 95°C for 3 seconds, 60°C for 20 seconds after an initial hold of 95°C for 20 seconds.

Results and Discussion
Results from initial screening of over 50 markers indicated a selection of miRNA that suggested body fluid specificity (data not shown). Three of these markers; specific to menstrual blood (hsa-miR-412), vaginal material (hsa-miR-124a) and saliva (hsa-miR-205) were carried forward for the present study. The 31 day investigation showed that hsa-miR-412 was specific to menstrual blood, where distinct expression (>15ΔCq) was observed during volunteer menstruation (figure 1A). Expression was not seen in volunteer C due to a cessation in menstruation due to contraception. Initial screening results showed that hsa-miR-412 was highly expressed in menstrual blood but not venous blood, indicating it is a suitable candidate for identification and differentiation of blood.

Previous studies have reported hsa-miR-124a to be specific to vaginal material [1, 3-5]. Although initial screening results supported hsa-miR-124a as being specific for vaginal material, the results between individuals throughout the menstrual cycle varied significantly (figure 1B). Volunteers A and C showed strong expression however ΔCq values fluctuated significantly throughout the cycle. Expression from samples from volunteers D and E was negligible suggesting hsa-miR-124 expression varies between individuals. Where expression occurs, the level of expression is likely to be affected by other factors throughout the cycle.

hsa-miR-205 has been recognised as a marker for saliva by previous authors[5-7] and this was supported in the majority of initial screening samples within this investigation. Within the 31 day study expression throughout the menstrual cycle fluctuated over the course of sampling and in some individuals was not expressed at all (figure 1C). Some volunteers showed very low levels of expression,

suggesting it could be useful for differentiating between vaginal material and saliva. Limitations may exist where excessive expression occurs; volunteer A showed expression that was higher than expression levels from saliva samples (red dotted line, figure 1C). This high expression did however fluctuate significantly over the course of the menstrual cycle.

A selection of suggested endogenous controls has-miR-93, has-miR-1260b, has-miR-203a, has-mi-508 and SNORD-47 were analysed to ensure validation and comparison between body fluids. Screening revealed multiple markers that showed consistent expression throughout all body fluids, this continued throughout all volunteers over the menstrual cycle (data not shown)

Conclusion

The expression of miRNA within the samples fluctuated continuously throughout the menstrual cycle and significant variation was observed between individuals. The expression levels for hsa-miR-205 in volunteer A were above the expression levels seen in saliva samples, indicating that it may not be a suitable biomarker for differentiating between vaginal material and saliva within casework. Expression of hsa-miR-124 was inconsistent not only over the 31 day period, but also between individuals and is therefore not suitable for casework. These initial findings were supported by lifestyle information provided by the volunteers, providing underpinning evidence that expression is likely to vary not only through hormonal changes, but also through other factors such as contraception, smoking habits, diet and pregnancy history. hsa-miR-412 was highly specific for menstrual blood across all volunteers capable of menstruation, and therefore the data implies that the further identification of suitable miRNA markers is worthy of further study for incorporation into casework.

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Conflict of interest statement

The authors declare that they have no conflict of interest

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Figure 1: Figure 1 Delta Cq values of expression of miRNA across 5 volunteers over a 31 day cycle employing marker specific to menstrual blood (hsa-miR-412,A), vaginal material (hsa-miR-124,B) and saliva (hsa-miR-205,C).