Evaluation of Stem-Loop Reverse Transcription and Poly-A Tail Extension in MicroRNA Analysis of Body Fluids

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Abstract: MicroRNA has been demonstrated to be a viable tool for body fluid identification purposes in forensic casework. Stem-loop reverse transcription (slRT) is regularly used for cDNA synthesis from mature miRNA, along with poly-A tail extension. Both have been used in a forensic context, but no direct comparison has been carried out. It has also not been shown whether poly-A tail extension can be used upon DNA extracts, as previously shown with slRT. Blood and saliva samples were collected and underwent DNA extraction with or without on-column DNA digestion. All samples were then aliquoted and underwent slRT and poly-A tail extension separately. qPCR was then conducted targeting microRNA markers hsa-miR-451 and hsa-miR-205. It was shown that the DNA digestion step did not affect the ability to differentiate between blood and saliva. It was also shown that this differentiation was possible using poly-A tail extension, and that poly-A tail extension exhibited more amplification than slRT. So whilst the choice of slRT and poly-A tail extension for the purpose of forensic body fluid identification is not critical, it may be best to use poly-A tail extension, particularly where there are low traces of sample.

Keywords: Blood, forensic genetics, MicroRNA, poly-A tail extension, qPCR, saliva, stem-loop reverse transcription.

INTRODUCTION

Forensic DNA profiling through the fragment analysis of short tandem repeats has revolutionised the criminal investigation. However, it is associated with limitations, one of which is the association of the DNA profile with the body fluid. For example, if it cannot be said that the DNA profile obtained from a particular body fluid, then it can be excluded as evidence in a court of law. Standard body fluid identification techniques have remained relatively unchanged for decades and there currently exists a major capability gap between DNA profiling and body fluid identification. There are currently a number of strategies to address this, including microRNA analysis.

MicroRNA (miRNA) analysis has been demonstrated to be a viable tool for body fluid identification in forensic case work [1-4], which provides a much more reliable, robust and sensitive assay than is currently available. A full range of body fluid markers has been identified [1] and their effectiveness is evaluated in the context of body fluid mixtures [4] and alternative end-point analysis, such as capillary electrophoresis (CE) [5-7].

One of the key aspects of miRNA analysis is the cDNA synthesis step. In messenger RNA (mRNA) analysis, this is carried out using reverse transcription. However, the mature miRNA marker can be ~20–26 bp in length. This presents a number of problems relating to reverse transcription;

namely, lack of binding sites for random primers and low melting temperature (due to short fragment size). Consequently, an elongation of the product during cDNA synthesis is required, thus giving more bindings sites for subsequent PCR and a larger, more thermo-stable, amplicon.

There are currently two widely used techniques available for the production of larger miRNA amplicons; stem-loop reverse transcription [8] and poly-A tail extension [9]. Both have been utilised for body fluid identification purposes.

Stem loop reverse transcription (slRT) was proposed by Chen *et al.* in 2005 [8], which partially reversed the biogenesis procedure in which the primary microRNA (pri-miRNA) is formed into a stem-loop. slRT requires a long primer sequence, which is partially self-complimentary; this creates a stem-loop structure and a 6 bp overhang. This 6 bp overhang can then be designed to hybridise to the 3' end 6 bp 'tab' on the mature miRNA sequence. A polymerase such as MMLV reverse transcriptase will then extend the 6 bp overhang to generate a complimentary copy of the mature miRNA sequence. Upon denaturation, the self-complimentary section of the stem-loop will unbind and the mature miRNA sequence will degrade, resulting in a single first strand consisting of the stem-loop primer sequence and a complimentary copy of the miRNA. This first strand then forms the basis of the template for cDNA synthesis, utilising a Taqman probe complimentary to the mature miRNA sequence and an unlabelled reverse primer complimentary to the stem-loop primer sequence. An alternative version to this was proposed by Li *et al.* in 2014 [6] which utilises an un-labelled forward primer, and a labelled reverse probe.

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Poly A tail extension was proposed by Fu *et al.* in 2006 [9], where mature miRNA markers undergo polyadenylation by a poly(A) polymerase and then reverse transcribed using oligo dT primers. The polyadenylation constructs a 'poly-A tail'. This poly-A tail serves the same function as the stem-loop primer in slRT.

Currently, no studies have explored yet whether slRT or poly-A tail extension provides a 'better' technique for forensic body fluid identification; consequently, this study carries out both slRT and poly-A tail extension upon the same samples in order to determine whether or not the selection has an impact on the end result. In addition, this study will build upon the study by Omelia *et al.* [10], in which it was demonstrated that miRNA could be characterised following solid phase DNA extraction, by carrying out a DNA digestion step, in order to assess whether or not this has an effect on the results.

MATERIALS AND METHODS

Marker Selection

For the purpose of this study hsa-miR-451 and hsa-miR-205 has been selected as a blood specific miRNA marker and a saliva specific miRNA marker, respectively. These markers have been repeatedly shown to be stable body fluid specific markers by a number of research groups around the world [5-7, 11]. Whilst other body fluid specific markers are available, these two have been the subjects of most researches in relation to body fluid identification.

Sample Preparation

Body fluids were collected from six healthy volunteers with informed consent and ethical approval. Blood was recovered via the finger-prick method and spotted on to a sterile swab. Saliva samples were collected using buccal swabs. The tips of the swabs were cut and placed into a sterile microcentrifuge tube for immediate extraction. The volunteers provided multiple samples at different times to reflect any individual variations.

MicroRNA Extraction with On-Column DNA Digestion

Extraction was carried out on all samples using the QIAamp DNA mini solid-phase extraction kit (Qiagen, UK), as per the manufacturer's instructions. Omelia *et al.* had previously shown that miRNA is retained within the extracts [10]. On-column DNA digestion was performed between the binding phase and the washing phase of the extraction protocol using the TURBO DNA-free Kit (Life Technologies, UK). The column was washed with 350 µl AW1 buffer, after which 100 µl DNA digestion mix containing 10 µl 10X TURBO DNase Buffer and 10 µl TURBO™ DNase was applied to the column and incubated at 37 ºC for 30 minutes. Afterwards, the column was washed with 500 µl AW1 and the remainder of the manufacturer's instructions were followed. On-column DNA digestion was omitted from a separate set of samples.

cDNA Synthesis Via Stem-Loop Reverse Transcription

Stem-loop reverse transcription [8] was carried out using the stem-loop primer from the Taqman MicroRNA assays (Life Technologies, UK) and the Taqman MicroRNA Reverse Transcription kit, (Life Technologies, UK) using the Veriti Thermocycler (Life Technologies, UK) as per the manufacturer's instructions.

cDNA Synthesis Via Poly-A Tail Extension

Poly-A tail extension [9] was carried out using the miScript II Reverse Transcription kit, (Qiagen, UK) following the manufacturer's instructions, along with the HiSpec Buffer using a Veriti Thermocycler (Life Technologies, UK). Following cDNA synthesis, the product was diluted 1 in 10.

Quantitative PCR

The slRT product then underwent qPCR using the forward and reverse primers, and the Taqman probe from the Taqman MicroRNA assay along with TaqMan Universal PCR Master Mix II, no UNG (Life Technologies, UK) using the Qiagen Rotor-Gene Q, following the manufacturer's recommended settings.

The poly-A tail extension product underwent qPCR using 10X miScript Primer Assays and the miScript SYBR Green PCR kit (Qiagen, UK) on the Qiagen Rotor-Gene Q with a 72-well rotor with 0.1-ml strip tubes and caps, following the manufacturer's recommended settings.

All samples were run in triplicate and included the full range of negative controls. 40 PCR cycles were used for all qPCR studies.

Data Analysis

Data was analysed using ΔCq values (40-Cq value). Significance was assessed using paired sample T-tests (p < 0.05).

RESULTS

As shown in Fig. (**1**), there are significantly lower amounts of amplification of miR-451 and miR-205 in blood and saliva samples that have undergone DNA digestion (p < 0.01) than those that have not. However, despite the lower amplification in digested samples, there is still sufficiently high amplification for it to be used. This decrease in amplification following DNA digestion may imply that some of the products are gDNA. However, lack of significance suggests that this is more likely due to the inclusion of the on-column DNA digestion step. It should be noted that the DNA digestion kit was not designed for on-column DNA digestion and as such is unlikely to be optimised.

The level of amplification following poly-A tail extension follows a similar pattern to slRT in that there is no significant difference between the amount of amplification between digested and undigested blood samples (p = 0.055). However there, is significantly less miR-205 in digested saliva than there is in undigested saliva (p ≤ 0.01). It is possible that this decrease in amplification is down to the removal of gDNA, or it could be due to the presence of the on-column DNA digestion step.

Stem-Loop Reverse Transcription vs Poly-A Tail Extension

Digested blood and saliva sample then underwent slRT and poly-A tail extension prior to qPCR targeting miR-451.
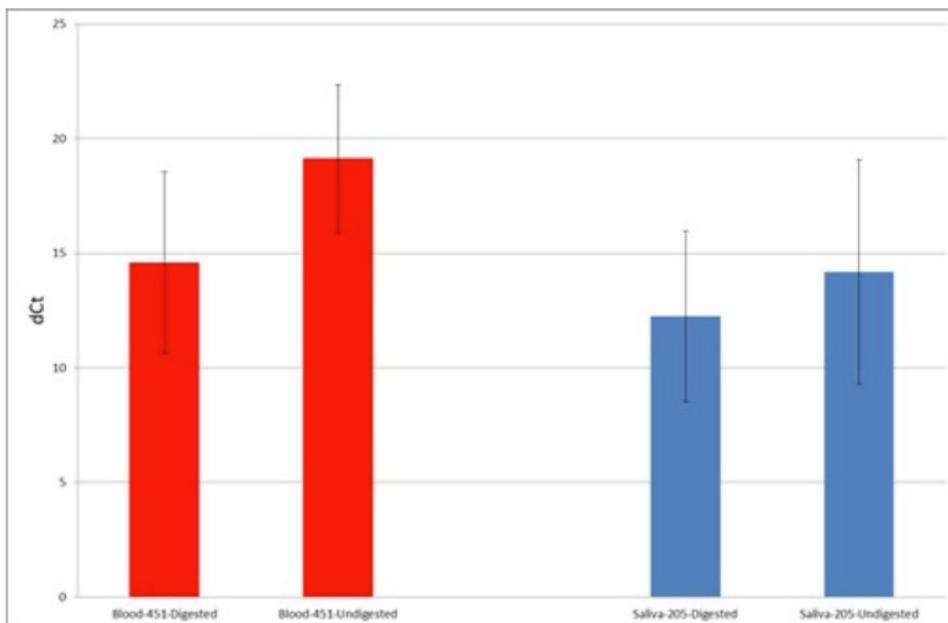
Fig. (1). Bar chart showing the detected amplification of miR-451 and miR-205 in blood and saliva samples that have not undergone DNA digestion and those that have. All samples in this data set underwent slRT. Error bars represent one standard deviation (n = 72).
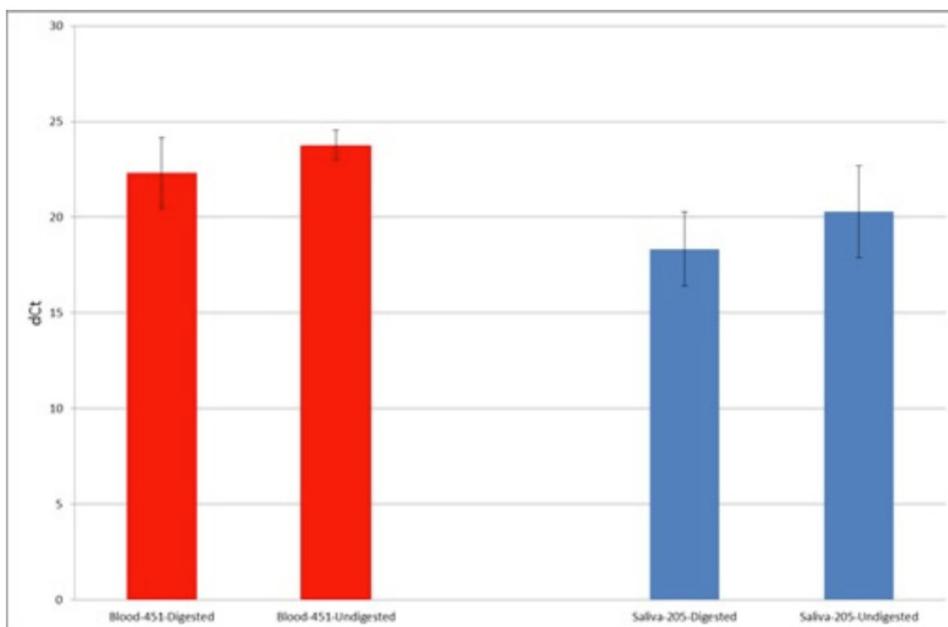

Fig. (2). Bar chart showing the detected amplification of miR-451 and miR-205 in blood and saliva samples that have not undergone DNA digestion and those that have. All samples in the data set underwent poly-A tail extension. Error bars represent one standard deviation (n = 72).

In all cases, there was significantly more miR-451 in blood samples than in saliva samples, as expected (p < 0.01). There was also significantly more product following poly-A tail extension in both blood and saliva (p < 0.01).

The same samples also underwent slRT and poly-A tail extension followed by qPCR targeting miR-205 in blood and saliva. In all cases, there was significantly more miR-205 in saliva than in blood, as expected (p < 0.01). It is also observed that there is significantly more product following poly-A tail extension than following slRT in both blood and saliva (p < 0.01).

DISCUSSION

The purpose of this study was three-fold. Firstly, it was based on the study by Omelia *et al.* [10] and verified that the miRNA persists following a standard solid phase DNA extraction procedure. Two sets of blood and saliva samples underwent a regularly used body fluid identification procedure as detailed in Omelia *et al.* [10], with one of the sets incorporating on-column DNA digestion. It was demonstrated that miRNA is still detected following DNA extraction with on-column DNA digestion. The incorporation of the DNA digestion step supports the view that miRNA is
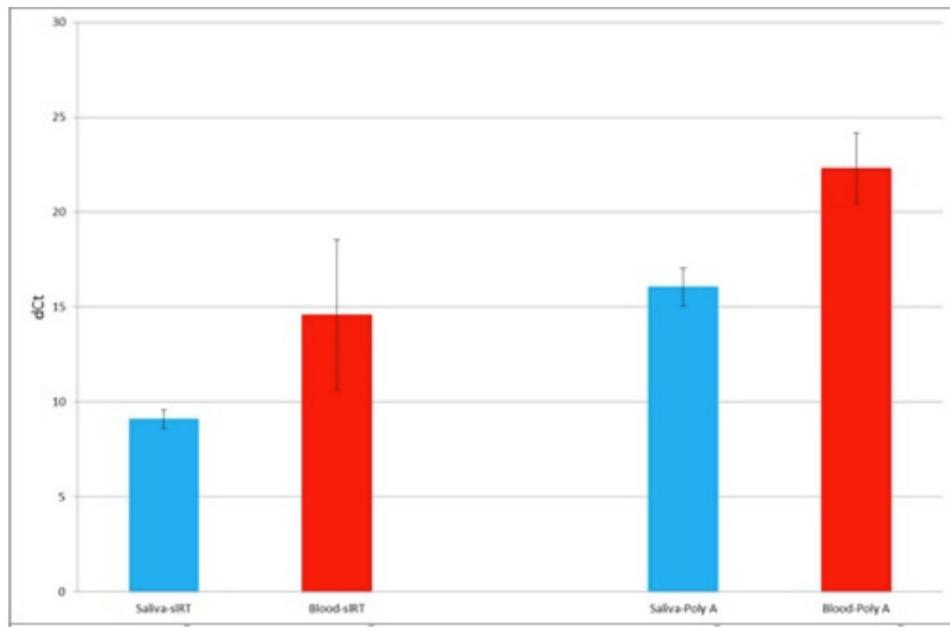
Fig. (3). Bar chart showing the detected amplification of miR-451 in digested blood and saliva samples following slRT and poly-A tail extension. Error bars represent one standard deviation (n = 72).
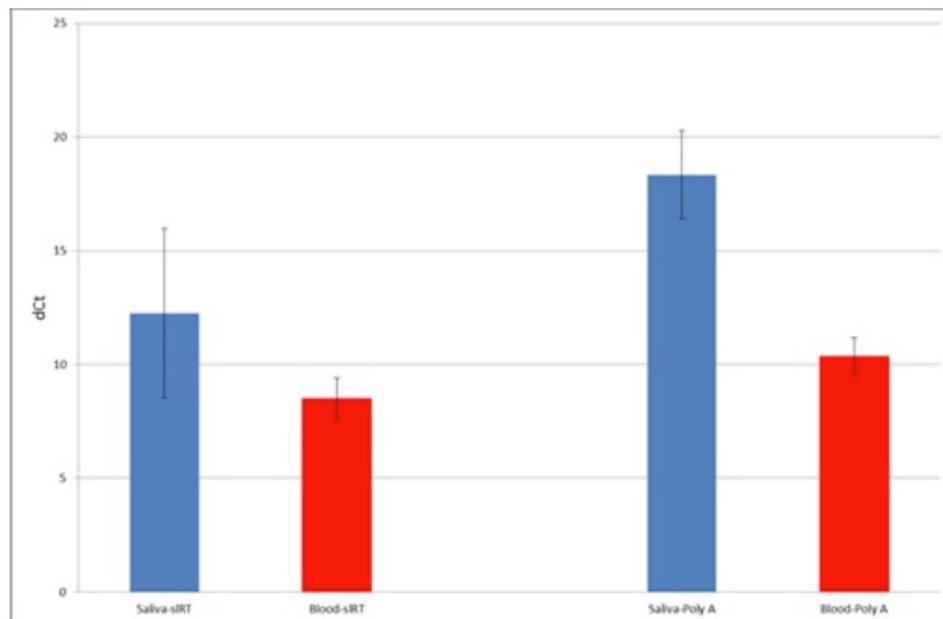

Fig. (4). Bar chart showing the detected amplification of miR-205 in blood and saliva following slRT and poly-A tail extension. Error bars represent one standard deviation (n = 72).

being detected rather than some variant of gDNA. This procedure was carried out using both slRT and poly-A tail extension. Both showed similar levels of performance.

It was observed that the digested samples had lower amplification than the un-digested samples. However, this was not significant ($p > 0.05$) and could be due to the incorporation of an additional step (on-column digestion).

The second purpose was to assess whether or not miRNA-based body fluid identification could be used following DNA extraction and poly-A tail extension. It was demonstrated that there was significantly more miR-451 in

blood than saliva and significantly more miR-205 in saliva than in blood. This indicates that poly-A tail extension is a valid reverse transcription step when carrying out miRNA analysis upon DNA extracts.

The third purpose of the study was to assess the performance of slRT and poly-A tail extension in the context of body fluid identification. This was conducted by carrying out both the slRT and poly-A tail extension method on the same samples. The results indicated that both methods were sufficient to differentiate between blood and saliva, using miR-451 and miR-205. Consequently, the choice of slRT and poly-A tail

extension is not a critical decision to make, at least in the context of pristine abundant samples. Although both techniques were sufficient for BFID purposes, it seems apparent that the use of poly-A tail extension detects significantly higher levels of both miR-451 and miR-205 in the samples than the use of slRT. However, it does not appear to significantly affect the relative difference in expression of the markers in their respective body fluids. Consequently, it may be more appropriate to use the poly-A tail extension method as this technique seems less likely to get 'drop-outs' than using slRT with low levels of body fluid samples, which can be commonly encountered in forensic casework.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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