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Title: Quantitative PCR analysis of blood and saliva specific microRNA markers following DNA extraction

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Keywords: Forensic genetics; body fluid identification; microRNA; DNA extraction; blood; saliva

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Abstract: The use of mRNA for the identification of body fluids is of particular interest within forensic science, and increasing support has been demonstrated for the use of microRNA analysis. miRNA is more stable than mRNA, and has been shown to be differentially expressed in body fluids. No studies have yet to be shown involving miRNA analysis from previously extracted DNA samples. The aim of this experiment was to determine if it was possible to conduct miRNA analysis on samples that have been previously extracted using standard DNA extraction. Blood and saliva samples were extracted using DNA and RNA kits, followed by cDNA synthesis, and then undergoing quantitative PCR analysis.

A direct comparison of ΔC_t values shows a larger abundance of miRNA following DNA extraction as opposed to total RNA extraction for both blood and saliva specific markers. By carrying out the comparison between the amounts of said markers, it could be seen that the expression of a blood specific marker is higher in blood than in saliva, and vice versa for the saliva specific marker. The results obtained could have a profound impact within cases where the sample has already undergone DNA extraction, such as in cold cases.



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Dear Editor,

Please find a re-submission to the journal 'Analytical Biochemistry' entitled '**Quantitative PCR analysis of blood and saliva specific microRNA markers following solid phase DNA extraction**'.

The original submission has been given the number ABIO-12-931.

Although you advised that it could be reduced in size for the 'Hints and Tips' section; the basis of that decision seemed to be based on the assumption that only two samples were used; which is incorrect. Therefore, I have resubmitted this as a full article; with the revisions emphasising the sample number.

If you still feel that this is insufficient; then I shall endeavour to reduce it in size for inclusion in to the 'Hints and tips' section.

I hope that the revisions are to your satisfaction.

Yours sincerely

Dr Graham Williams MFSSoc
Senior Lecturer in Forensic Science & Consultant Forensic Biologist

Response to Reviewer's comments

Thank you for your time and efforts in reviewing my article.

You appear to be under the impression that there were only two volunteers. This is not correct, there were six volunteers, each of whom provided two blood and two saliva samples each. I apologise the lack of clarity and I have rectified this in the revised manuscript.

I agree that that one old stain is insufficient, which I why I have not presented the data, but refer to it as a point of interest. I have currently kept this in the revised manuscript, but if you wish me to remove this paragraph, then I can do so.

With respect to controls, both reverse transcription and PCR negative controls were included in the study. I have amended the manuscript to reflect this.

With respect to the generation of Figure 1 and 2 - I feel that it is clear in that the dCt values were derived from the qPCR stage and simply plotted in a bar chart. As stated in the figure legend, the error bars are one standard deviation.

With respect to Figure 3 and 4 - You are correct, this is simply a representation of Figure 1 and 2, which I felt was useful for clarity. However, in light of your comments, I have removed Figure 3 and 4 and merged the narrative discussing these.

With respect to the title, I have adjusted the title away from "body fluid" to "blood and saliva specific"

RNase (and DNase) free water and reagents were used throughout the study - I have adjusted the text to reflect this.

Quantitative PCR analysis of blood and saliva specific microRNA markers following solid phase DNA extraction

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West Yorkshire, HD1 3DH, United Kingdom

* = Corresponding author – g.a.williams@hud.ac.uk (Tel: +44 (0)1484 472158)

Short Title: MicroRNA analysis from DNA extracts

Quantitative PCR analysis of blood and saliva specific microRNA markers following solid phase DNA extraction

Abstract

The use of mRNA for the identification of body fluids is of particular interest within forensic science, and increasing support has been demonstrated for the use of microRNA analysis. miRNA is more stable than mRNA, and has been shown to be differentially expressed in body fluids. No studies have yet to be shown involving miRNA analysis from previously extracted DNA samples.

The aim of this experiment was to determine if it was possible to conduct miRNA analysis on samples that have been previously extracted using standard DNA extraction. Blood and saliva samples were extracted using DNA and RNA kits, followed by cDNA synthesis, and then undergoing quantitative PCR analysis.

A direct comparison of ΔC_t values shows a larger abundance of miRNA following DNA extraction as opposed to total RNA extraction for both blood and saliva specific markers. By carrying out the comparison between the amounts of said markers, it could be seen that the expression of a blood specific marker is higher in blood than in saliva, and vice versa for the saliva specific marker. The results obtained could have a profound impact within cases where the sample has already undergone DNA extraction, such as in cold cases.

Keywords: Forensic genetics; body fluid identification; microRNA; DNA extraction; blood; saliva

The use of RNA for the identification of forensically relevant body fluids and their respective stains has been prominent over the last few years, with methods being developed using mRNA [1; 2; 3; 4]. This area of interest is particularly important within forensic investigations. However, in more recent reports, it is becoming increasingly apparent that the small RNAs known as microRNAs (miRNAs) could become more predominant in this area of research, as it has been well established that miRNAs are specifically expressed within forensically relevant body fluids, such as blood and saliva [5; 6; 7; 8].

MicroRNAs are a class of small, non-coding ribonucleic acids within the human genome. These molecules are post-transcriptional regulators that bind to complimentary sequences on target messenger RNA within the cell [9]. Their small size of between 18-22 bp gives them greater stability [10]. It is this stability that puts miRNA at a distinct advantage within forensic case work as opposed to mRNA, which is considered less stable and more susceptible to degradation [10].

Previous work has been based upon retrieval of miRNA through standard RNA extraction processes, or non-routine procedures [4; 6; 8; 11]. Co-isolation of DNA and RNA has also been developed in order to minimise the level of sample being used [6]. Currently, there appears to be no research carried out on whether such RNA based body fluid identification tests can be carried out on samples that have already undergone DNA extraction, i.e. cold cases.

In the UK, there is a requirement that all washes, supernatants, and extracts are retained under Crown Prosecution Service guidelines. Consequently, the aim of this study is to determine whether or not it was possible to carry out microRNA analysis upon DNA extracts. When standard DNA extraction is performed, such as the commercially available solid phase extraction based QIAamp DNA mini kit (Qiagen, UK), it is reasonable to expect that the DNA quality within the extract would be high. Therefore the three wash steps

involved in the extraction process were also analysed, hypothesising that the RNA content, and thus miRNA, would be present in at least one of the washes. Such an outcome would mean that the miRNA based BFID test could be carried out on samples previously extracted using standard DNA extraction methods.

Methods and Materials

Marker Selection

miRNA has relative stability and therefore, for this study, one marker was selected for each of the two body fluids used: blood and saliva. Markers miR-451a was chosen for blood and miR-205 for saliva. These markers were initially selected by considering the findings of Hanson 2009 [5], and Zubakov 2010 [12]: the first utilised real-time PCR while the latter used microarray screening to determine selectivity of each marker.

Sample Recovery

Blood and saliva samples were collected from six volunteers with their consent and ethical approval from the Applied Sciences Ethics Panel. Two buccal swabs were used to obtain saliva samples from each volunteer, whilst blood was obtained using the finger prick method. Unistix lancets were used to prick the finger and the blood was then spotted onto sterile filter paper and placed into a sealed plastic bag. Once dried, the head of the buccal swab was removed using sterile scalpels and placed into microcentrifuge tubes for each volunteer. Two small squares of blood-stained filter paper at 0.5cm^2 were cut out and placed into microcentrifuge tubes. A 10 month old blood stain, which had been stored at 4°C , was also recovered.

DNA and total RNA Extraction

One of each of the 6 pairs of blood samples and pairs of saliva samples then underwent DNA extraction with the other undergoing RNA extraction.

As previously demonstrated, the QIAamp DNA Mini Kit (Qiagen UK) provides high yields of DNA and microRNA [6], therefore making this a preferred technique. Using spin column technology, the manufacturer's protocol was followed using the buccal protocol for both blood and saliva, given that the identity of the body fluid at this stage would be unknown. Rather than discarding the collection tube and flow-through, these were transferred into a microcentrifuge tube for storage at -20°C.

The total RNA extraction using RNeasy Mini Kit (Qiagen UK) was also used. As with the DNA extraction, the manufacturer's guidelines were followed with the additional steps of retaining the three washes throughout the extraction process into microcentrifuge tubes, with each wash being retained separately.

cDNA Synthesis

Synthesis of cDNA from miRNA was carried out using stem loop reverse transcription [13]. MicroRNA Reverse transcription kits and stem loop primers were used (Life Technologies, UK) using a 2720 Thermal Cycler (Applied Biosystems, UK) as per manufacturer's instructions. Reverse transcription negative controls were included; by preparing the same reaction mix, but with the reverse transcriptase omitted.

Quantitative PCR (qPCR)

Real-time PCR was performed on the ABI 7500 Fast instrument (Applied Biosystems, UK) using the Taqman® Fast Universal PCR Master Mix (Life Technologies, UK) along with Taqman® labelled probes (Life Technologies, UK) as per manufacturer's instructions.

Reverse transcription negative controls were also included along with PCR negative controls; where the sample was replaced with sterile RNase-free H₂O.

The cycle threshold (Ct) was automatically detected using the SDS Software. ΔC_t values were obtained for analysis using the equation $Ct^{MAX} - Ct$. For this study the Ct^{MAX} was 40.

All reagents used in this study were sterile and DNase and RNase free.

Data analysis

The ΔC_t value was used for comparison purposes and for statistical analysis. Paired sample T-tests were used to determine significant difference (<0.05).

Results

FIGURE 1

FIGURE 2

The results shown in Figures 1 and 2 indicate a high expression of hsa-miR-451a in blood and low expression in saliva. They also indicate high expression of hsa-miR-205 in saliva but not blood, and therefore suggest specificity where the two body fluids are concerned. There is a significant difference in expression ($p=0.00$) and consequently, these results indicate that these two markers are more than sufficient to distinguish between blood and saliva, with an n-value of 90.

The figures also relate to blood and saliva samples that were extracted using both DNA and total RNA methods previously mentioned. They gave a clear result, in that the DNA extraction process generates a greater microRNA product than that of the RNA extraction process. There is a significant difference in the two extraction methods ($p=0.00$), indicating that the QIAamp DNA Mini Kit provides higher concentrations of miRNA than the total RNA RNeasy Mini Kit.

FIGURE 3

FIGURE 4

Throughout the extraction process, the washes and final extracts were collected for all samples and taken through the process to qPCR. The result clearly shows that when using the QIAamp DNA Mini Kit, the final extract holds the majority of miRNA ($p=0.0$).

RNA has long had a reputation for its instability; however, recent publications have shown that mRNA can survive in blood stains up to 23 years old [14]. As miRNA is theoretically more stable than mRNA, stability is not considered to be an issue. Nevertheless, the miRNA BFID test was carried out on a relatively old stain, which was a 10 month old blood stain stored at 4°C. This limited sample set (therefore data not shown) indicated no significant difference between the expression of hsa-miR-451a in fresh blood and in 10 month old blood. This test also indicated that there was significantly more hsa-miR-451a in the 10 month old blood stain than in fresh saliva ($p=0.0$).

Discussion

The aim of this study was to establish whether or not a miRNA based body fluid identification analysis could be conducted on previously extracted DNA samples, using standard solid phase extraction methods. This was achieved by carrying out both DNA and total RNA extraction, followed by stem-loop reverse transcription and quantitative PCR analysis targeting the blood marker hsa-miR-451a, and saliva marker hsa-miR-205.

By carrying out the comparison between the amount of blood and saliva specific markers, it could be seen that the expression of hsa-miR-451a is higher in blood than in saliva, and vice versa for the saliva specific marker hsa-miR-205, thus demonstrating that the test developed in-house is capable of distinguishing between blood and saliva.

DNA and RNA extractions were carried out in order to provide a direct comparison of miRNA product retained following the application of the two commercially available kits. Prior to the experiment, it was expected that the RNeasy extraction kit would provide the higher amount of miRNA, as opposed to the DNA mini extraction kit. A direct comparison of the ΔC_t values obtained showed that significantly more amplification was observed following DNA extraction than there was following total RNA extraction for both blood and saliva specific markers. Although the relatively high copy number of miRNA accounts for why it may persist through the purification process, it does not explain why more miRNA material was present post DNA extraction than post RNA extraction.

Working on the hypothesis that, throughout the extraction process miRNA would be expelled within one of the three wash stages, the washes were retained for analysis. This was applied to all samples extracted, using both the DNA and RNA extraction kits. However, the results showed that, without exception, miRNA was predominantly present within the final extraction product rather than the retained washes.

There was a significant difference ($p < 0.05$) for every experiment carried out on samples following both DNA and RNA extraction, thus indicating that a miRNA body fluid identification test can be successfully applied to samples that have already undergone DNA extraction, thus demonstrating that such a miRNA based body fluid identification test can be applied retrospectively, for example, in cold case samples.

Summary

A miRNA BFID test was developed that is capable of distinguishing between blood and saliva, not only on samples that have undergone total RNA extraction, but also on samples that have undergone DNA extraction.

Preliminary data was also reported that suggested that the miRNA BFID test could also be carried out on relatively old samples that have undergone DNA extraction, supporting

the view that the miRNA BFID test can be applied to cold cases, thus opening up the number of cases that could benefit from forensic RNA analysis.

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FIGURE 1

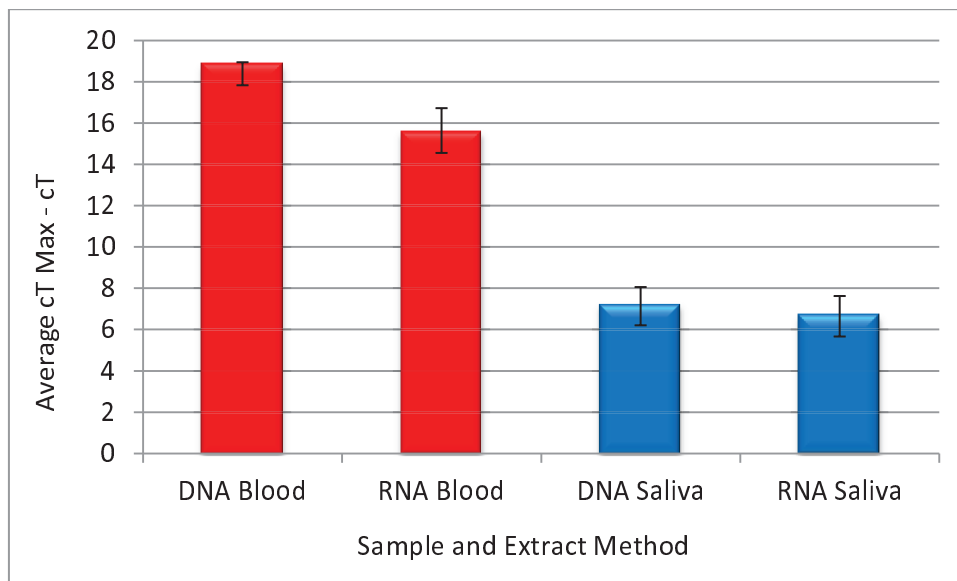


Figure 1 - A bar chart indicating the expression of hsa-miR-451 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits. All error bars represents 1 standard deviation.

FIGURE 2

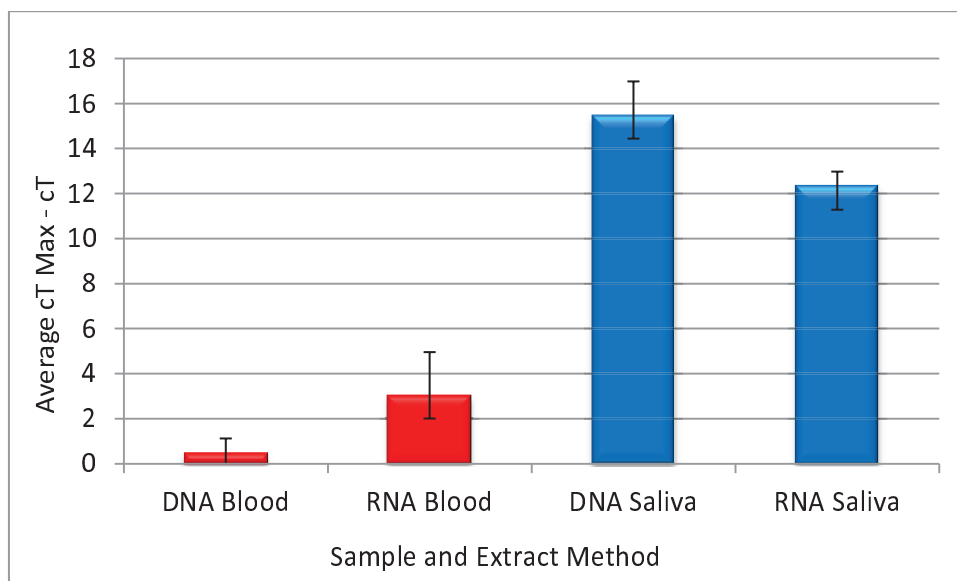


Figure 2 - A bar chart indicating the expression of hsa-miR-205 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits

FIGURE 3

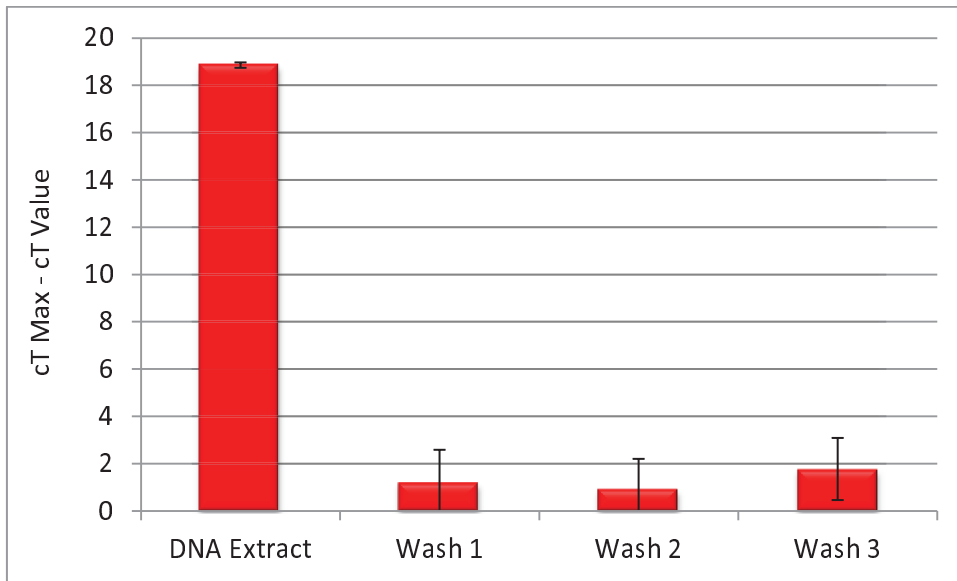


Figure 3 - A bar chart indicating the expression of hsa-miR-451 in blood when extracted with the QIAamp DNA Mini Kit collecting the wash steps and final extract.

FIGURE 4

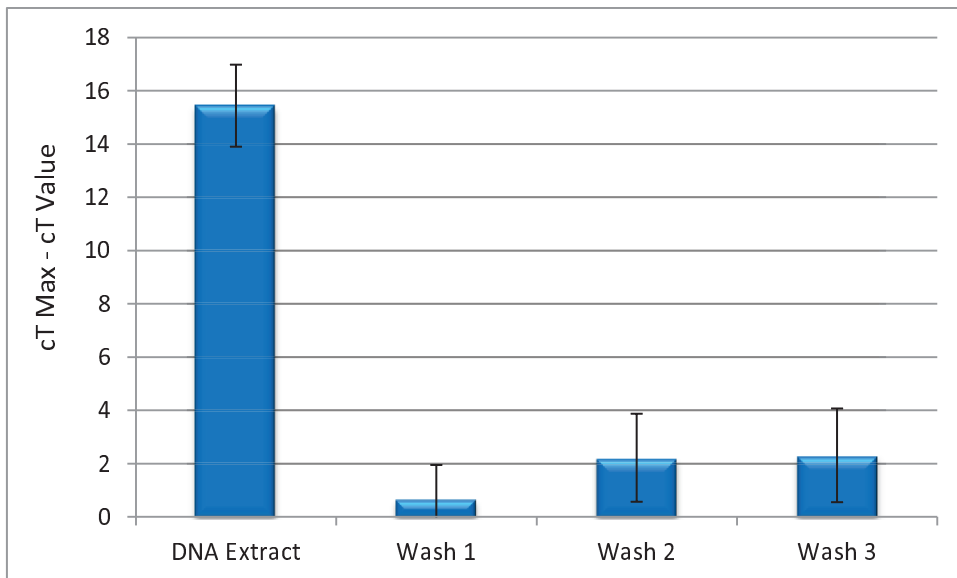


Figure 4 - A bar chart indicating the expression of hsa-miR-205 in saliva when extracted with the QIAamp DNA Mini Kit collecting the three wash steps and final extract.

FILE INVENTORY

Figure 1 - A bar chart indicating the expression of hsa-miR-451 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits. All error bars represents 1 standard deviation.

Figure 2 - A bar chart indicating the expression of hsa-miR-205 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits.

Figure 3 - A bar chart indicating the expression of hsa-miR-451 in blood when extracted with the QIAamp DNA Mini Kit collecting the wash steps and final extract.

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Quantitative PCR analysis of **blood and saliva specific microRNA markers
following solid phase DNA extraction**

Emma J. Omelia₁; Mari L. Uchimoto₁; Graham Williams₁*

₁ = Forensic and Analytical Research Centre, University of Huddersfield, Queensgate, Huddersfield,
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Abstract

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Keywords: Forensic genetics; body fluid identification; microRNA; DNA extraction; blood; saliva

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The ΔC_t value was used for comparison purposes and for statistical analysis. Paired sample T-tests were used to determine significant difference (<0.05).

Results

FIGURE 1

FIGURE 2

The results shown in Figures 1 and 2 indicate a high expression of hsa-miR-451a in blood and low expression in saliva. They also indicate high expression of hsa-miR-205 in saliva but not blood, and therefore suggest specificity where the two body fluids are concerned. There is a significant difference in expression ($p=0.00$) and consequently, these results indicate that these two markers are more than sufficient to distinguish between blood and saliva, with an n-value of 90.

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FIGURE 3

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Throughout the extraction process, the washes and final extracts were collected for all samples and taken through the process to qPCR. The result clearly shows that when using the QIAamp DNA Mini Kit, the final extract holds the majority of miRNA ($p=0.0$).

RNA has long had a reputation for its instability; however, recent publications have shown that mRNA can survive in blood stains up to 23 years old [14]. As miRNA is theoretically more stable than mRNA, stability is not considered to be an issue. Nevertheless, the miRNA BFID test was carried out on a relatively old stain, which was a 10 month old blood stain stored at 4°C. This limited sample set (therefore data not shown) indicated no significant difference between the expression of hsa-miR-451a in fresh blood and in 10 month old blood. This test also indicated that there was significantly more hsa-miR-451a in the 10 month old blood stain than in fresh saliva ($p=0.0$).

Discussion

The aim of this study was to establish whether or not a miRNA based body fluid identification analysis could be conducted on previously extracted DNA samples, using standard solid phase extraction methods. This was achieved by carrying out both DNA and total RNA extraction, followed by stem-loop reverse transcription and quantitative PCR analysis targeting the blood marker hsa-miR-451a, and saliva marker hsa-miR-205.

By carrying out the comparison between the amount of blood and saliva specific markers, it could be seen that the expression of hsa-miR-451a is higher in blood than in saliva, and vice versa for the saliva specific marker hsa-miR-205, thus demonstrating that the test developed in-house is capable of distinguishing between blood and saliva.

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Summary

A miRNA BFID test was developed that is capable of distinguishing between blood and saliva, not only on samples that have undergone total RNA extraction, but also on samples that have undergone DNA extraction.

Preliminary data was also reported that suggested that the miRNA BFID test could also be carried out on relatively old samples that have undergone DNA extraction, supporting

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References

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FIGURE 1

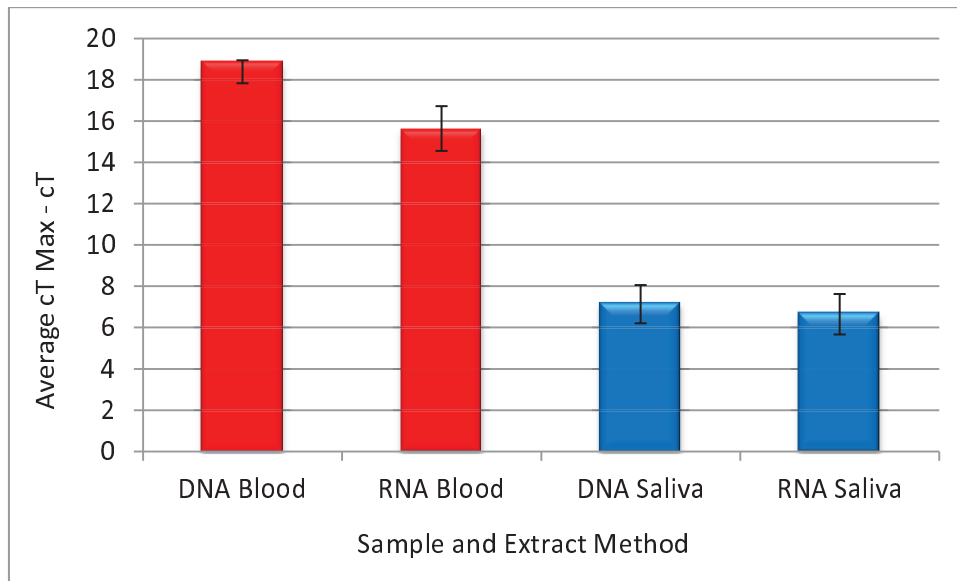


Figure 1 - A bar chart indicating the expression of hsa-miR-451 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits.. All error bars represents 1 standard deviation.

FIGURE 2

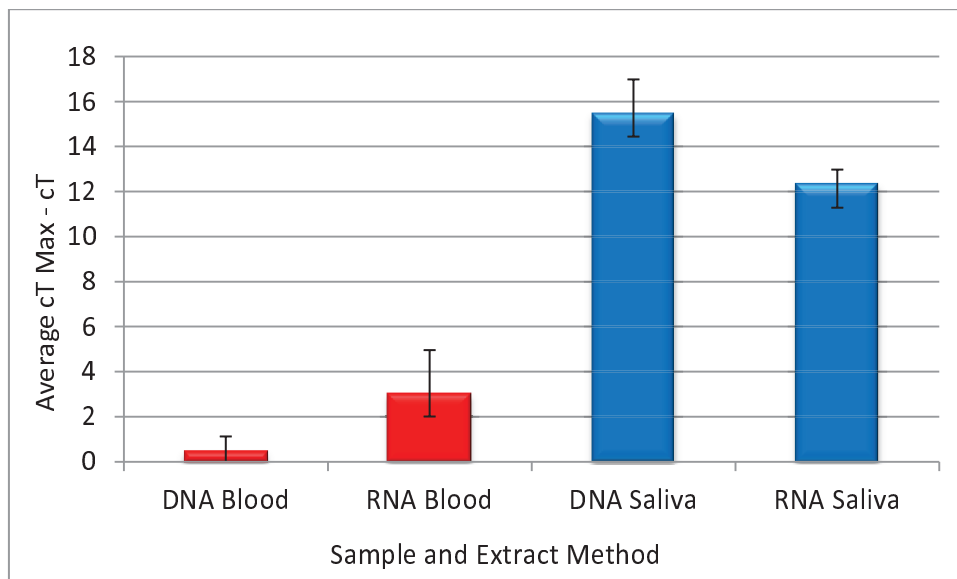


Figure 2 - A bar chart indicating the expression of hsa-miR-205 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits

FIGURE 3

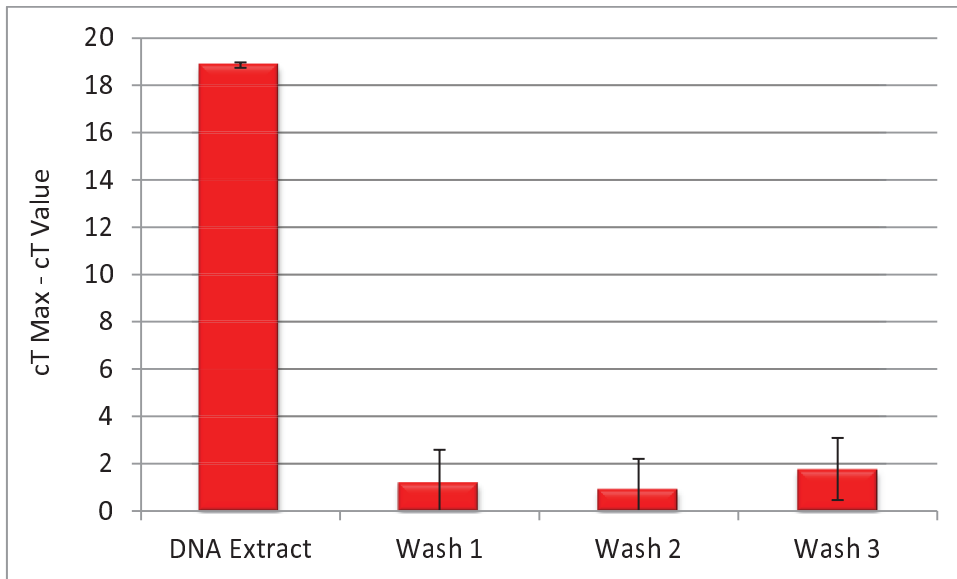


Figure 3 - A bar chart indicating the expression of hsa-miR-451 in blood when extracted with the QIAamp DNA Mini Kit collecting the wash steps and final extract.

FIGURE 4

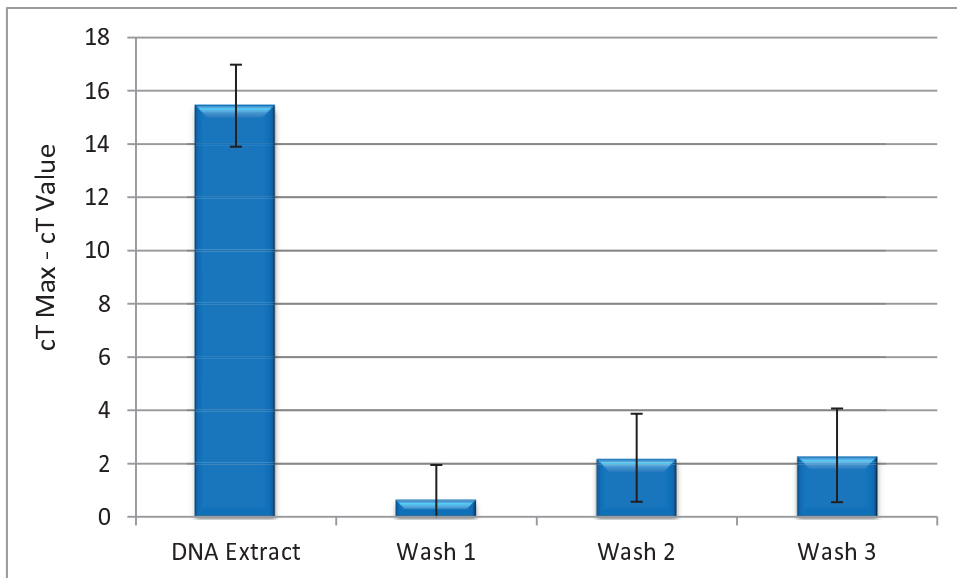


Figure 4 - A bar chart indicating the expression of hsa-miR-205 in saliva when extracted with the QIAamp DNA Mini Kit collecting the three wash steps and final extract.

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Figure 1 - A bar chart indicating the expression of hsa-miR-451 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits. All error bars represents 1 standard deviation.

Figure 2 - A bar chart indicating the expression of hsa-miR-205 in blood and saliva and when extracted with the QIAamp DNA Mini and RNeasy Mini Kits.

Figure 3 - A bar chart indicating the expression of hsa-miR-451 in blood when extracted with the QIAamp DNA Mini Kit collecting the wash steps and final extract.

Figure 4 - A bar chart indicating the expression of hsa-miR-205 in saliva when extracted with the QIAamp DNA Mini Kit collecting the three wash steps and final extract.

Figure 1
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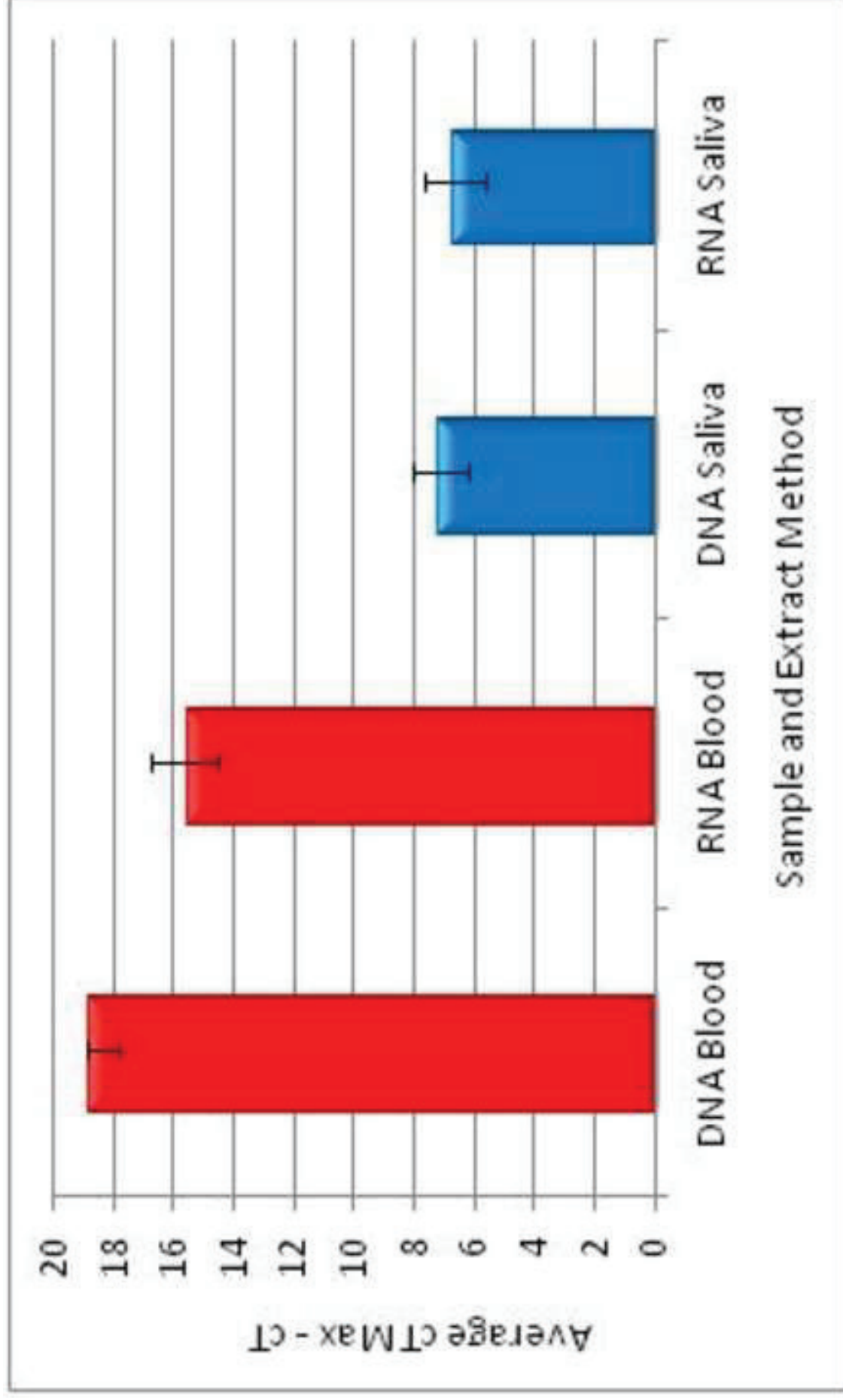


Figure 2
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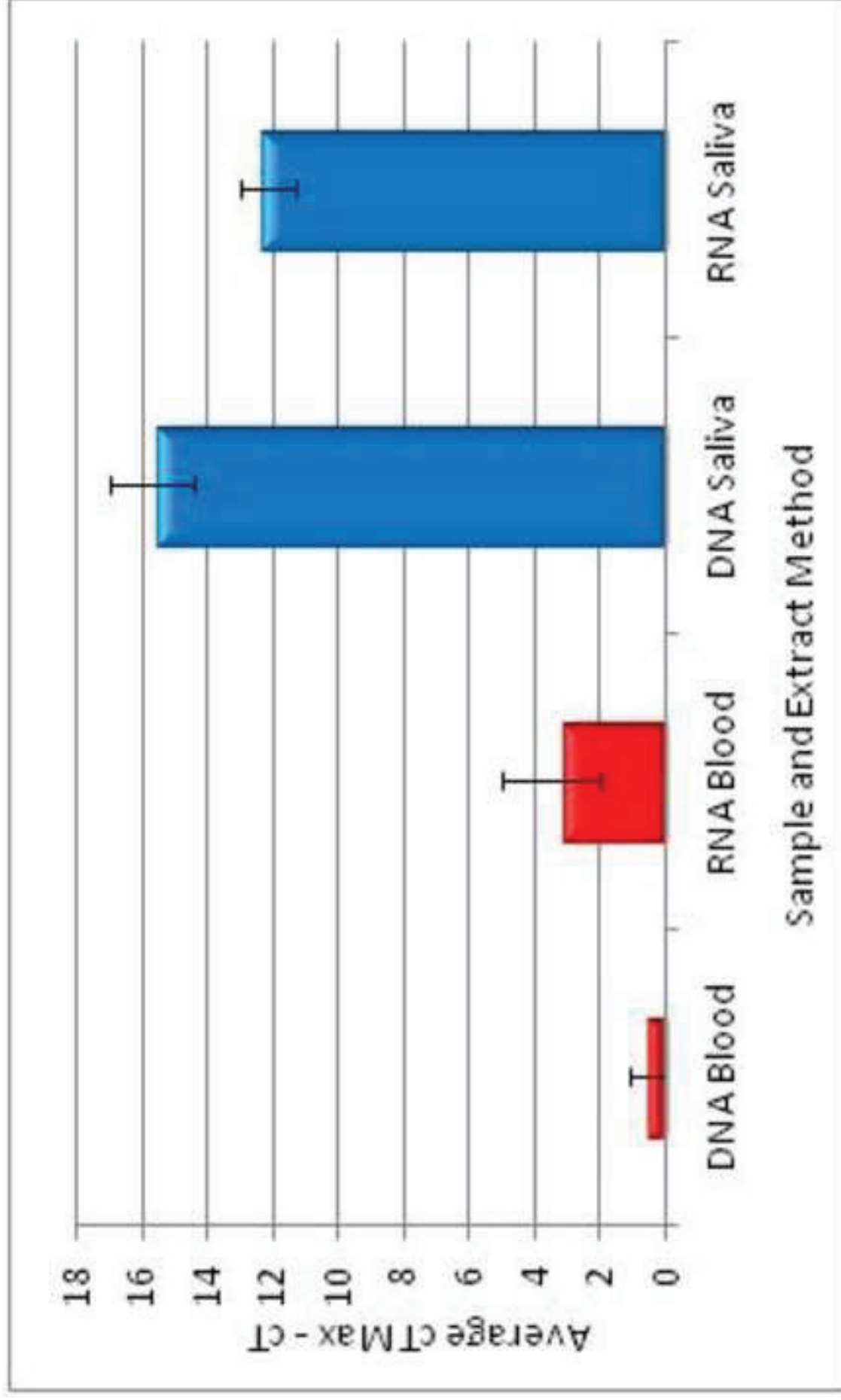


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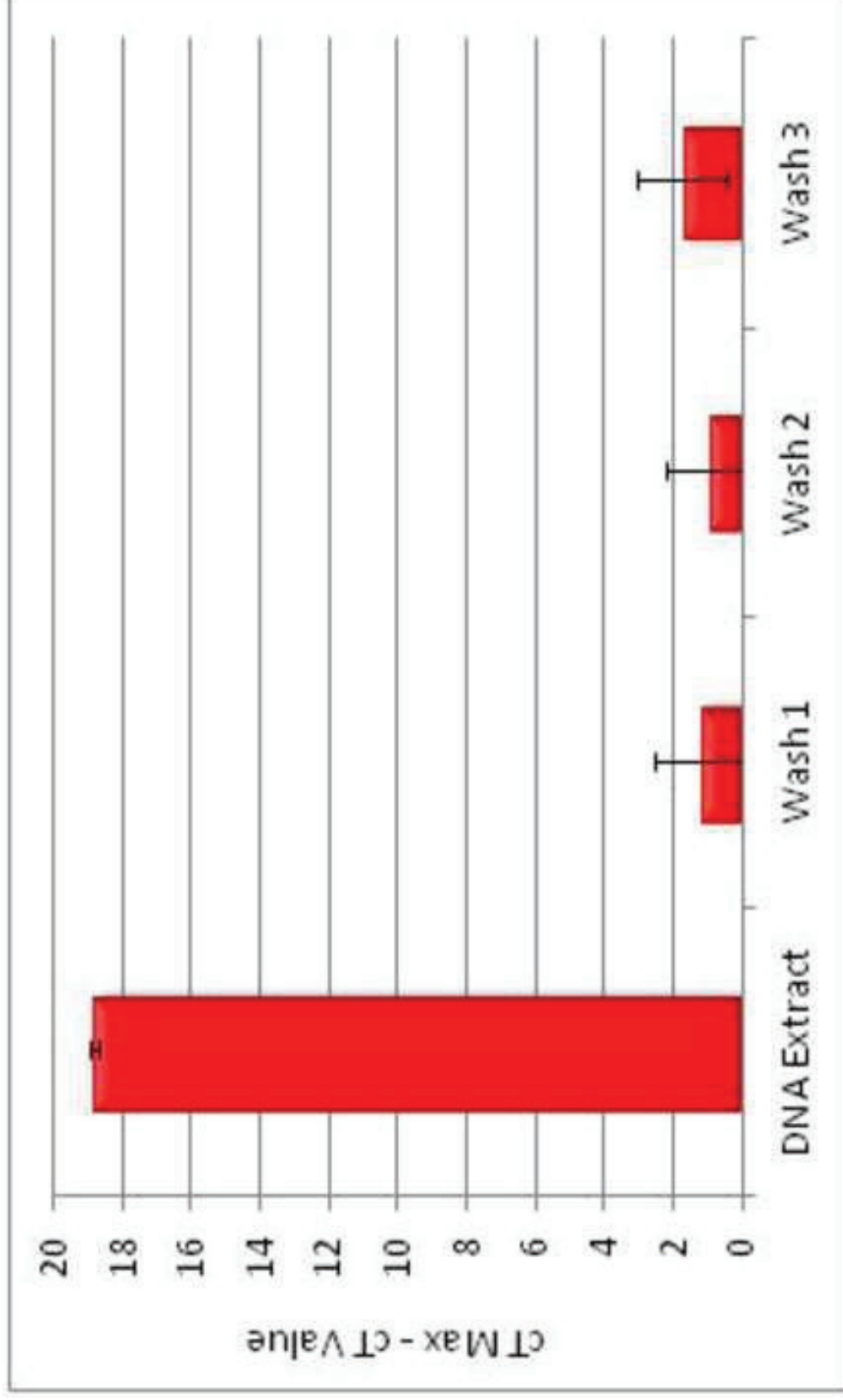


Figure 4
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