ABSTRACT

Forensic RNA analysis is gathering pace with reports of messenger RNA analysis being used in case work, and with microRNA being increasingly researched. Such techniques address a fundamental issue in body fluid identification, namely increased specificity over existing chemical tests, and the incorporation of additional body fluids such as vaginal material. The use of RNA analysis will be of particular value to sex offences, where there can be a mixture of multiple body fluids from different people. The aim of this study was to determine whether microRNA based body fluid identification tests can be applied to mixed body fluid samples.
Blood and saliva were acquired from volunteers and underwent total RNA extraction. Mixed samples were prepared using a range of ratios from 1:1 to 10:1. Each mixed sample then underwent a blood-saliva differentiation test developed in-house, which includes stem-loop reverse transcription and real-time PCR analysis. Aliquots following mixture preparation also underwent standard STR analysis, utilising Quantiplex and Next Generation Multiplex kits.
Data relating to the development of an in-house blood-saliva differentiation test is presented, in which it has been demonstrated that such a test has a lower limit of detection than the enzymatic equivalent.
It has been shown that not only is it possible to determine the presence of more than one body fluid, it is also possible to determine the major body fluid contributor as well as the minor contributor.

Keywords: Forensic genetics; DNA; microRNA; stem loop reverse transcription, body fluid identification; mixtures; blood; saliva

Introduction

Forensic RNA analysis is increasingly being used for more definitive body fluid identification (BFID) with a focus on messenger RNA (mRNA) [1-5] and more increasingly on microRNA (miRNA) [6-11]. MicroRNA is, in theory, the preferred marker to target as it is more stable due to its small size (~22-26 bp) and relatively high copy numbers within the cells [11-17]. There are some concerns relating to its species specificity, but this may be resolved by using a single channel simultaneous extraction technique [18] and the associated DNA profile, which is human specific.

Such RNA based methodologies address a particular gap in body fluid identification, namely the use of vaginal material specific markers. This is of obvious benefit in addressing sexual offences; however, such offences are also heavily associated with mixed DNA profiles and mixed body fluids. The interpretation of mixed DNA profiles are a particularly complex area in case work [19-24], and it would currently be unreasonable to assume that one contributor of the DNA result came from one particular body fluid over the other contributor.

The aim of this study was to assess and evaluate the effect of mixed body fluids on a miRNA BFID test developed in-house. In addition to this, strategies for interpreting mixtures of body fluids were also explored. Although the issue being investigated predominantly relates to sexual offences, blood and saliva was used in order to obtain a relatively high number of samples, due to the difficulties in obtaining intimate samples.

Methods and materials

Marker selection

Following a review of the literature in which the body fluid specificity, or miRNA markers were explored, a series of markers were identified. For each marker, the mature miRNA sequence was identified using an online microRNA database (www.mirbase.org) [25-28], along with their corresponding stem-loop sequences to ensure that the mature sequences were sufficiently different, particularly with respect to the 6bp sequence on the 3' end. These markers were screened using expression data (from an in-house study) [18], and the markers which were deemed to be preferentially expressed in the relevant body fluid were selected for this study. RNU-44, a small nuclear RNA (snRNA), was used as the endogenous control [10]. The term 'preferentially expressed' is used where there was significantly more of one marker than the other as determined using a paired sample T-test.

TABLE 1

Marker Specificity

As stated, there appears to be issues relating to species specificity in that the markers targeted may also be expressed in body fluids from other species. Although it would be better if these were human specific, it is still an improvement over the current enzymatic tests, which are not only non-specific in terms of species, but can also react in the presence of non-biological material. Table 2 indicates those species which can also express the selected markers.

TABLE 2

Sample acquisition

Blood and saliva samples were acquired from a number of volunteers with informed consent and with ethical approval from the Applied Sciences Ethics Panel. Saliva samples were recovered using buccal swabs. Blood samples were acquired by using the finger prick method, in which Unistix lancets were used to draw blood and the blood was then spotted onto sterile filter paper.

Sample extraction

Total RNA were extracted from the blood and saliva samples using the buccal swab method of the RNeasy extraction kit (Qiagen, UK). For the blood samples, a $1cm^2$ section was cut out and treated as if it was a swab. Identical extraction methods were used for verisimilitude, as in case work samples the identity of the body fluid would not necessarily be known.

Mixture preparation

Samples were mixed post-extraction so that a more accurate idea of mixing ratios prior to analysis could be obtained. A range of mixing ratios was prepared, including 1:1, 2:1, 5:1 and 10:1. These ratios were derived from sample volume post-extraction. Single source controls were also included in this study.

cDNA synthesis

Stem loop reverse transcription [32] was used to synthesise cDNA from the mature microRNA sequences. Commercially available MicroRNA Reverse transcription kits (Life Technologies, UK) were used on the Veriti Thermocycler (Life Technologies, UK) as per manufacturer's instructions. The stem-loop reverse transcription primers were also available from Life Technologies. The mature miRNA sequences provided with the kits were checked against the miRBase database [25-28].

Real-time PCR

Quantification by real-time PCR was carried out on the ABI 7500 Fast Real-Time PCR Machine (Life Technologies, UK). Taqman labelled probes were used as per manufacturer's recommendations, with a minor modification in that the number of PCR cycles was increased from 40 to 50 (Ct_{MAX}), thus allowing all reactions to achieve the plateau phase. The threshold was automatically defined by the SDS Software and used for determining the cycle threshold (Ct) values.

Data analysis

ΔCt values were derived either using $Ct_{RNU44} - Ct_{marker}$ or $Ct_{MAX} - Ct_{marker}$. The ΔCt values were used for subsequent data analysis. The Ct_{MAX} value was applied to single source

samples and Ct_{RNA44} value was applied to mixtures. For data analysis of mixtures, a normalising formula was derived from the 1:1 mixing ratio sample (m) to compensate for variance between markers and samples. This formula (below) was then applied to all samples, where 205 refers to marker hsa-miR-205, and 451 refers to marker hsa-miR-451.

$$\mathbf{\Delta Ct_{205} - (\Delta Ct_{205m} - \Delta Ct_{451m})}$$

Results

Single Source Results

FIGURE 1

The first set of results in Figure 1 relates to single source samples, which indicates a high expression of hsa-miR-451 in blood and a low expression of hsa-miR-205. Conversely, this set of results also shows a high expression of hsa-miR-205 in saliva and a low expression of hsa-miR-451. There is a significant difference in expression (p=0.00), and consequently, along with an n-value of 50, these results indicate that these two markers are more than sufficient to distinguish between blood and saliva. For the purpose of this study, a significant difference of <0.05 is deemed the threshold at which a sample can be called blood or saliva.

FIGURE 2

A blind study was carried out in which a series of blood and saliva samples were prepared and anonymously labelled. These samples were prepared after extraction, so that they could not be visually distinguished. Four such samples are shown in Figure 2. All samples were correctly identified as being blood or saliva (all with a p-value of 0.00).

FIGURE 3

Following the development of the in-house test, a sensitivity study was conducted in which 10µl of blood were serially diluted. The blood was regularly tested using the leucomalachite green (LMG) and the Kastle-Meyer (KM) test at each stage of dilution. At a dilution of 1:50,000, both the KM and LMG tests were negative. The miRNA BFID test was then carried out on the blood samples. As indicated in Figure 3, although the expression of hsa-miR-451 and hsa-miR-205 was somewhat less, there is still a significant difference (p=0.00) between the two.

Body fluid mixture results

FIGURE 4

It can be seen in Figure 4 that, whichever body fluid is the major contributor, the respective marker is significantly more expressed than the opposing marker. The single source body fluids show a relative large difference in marker expression. When the minor contributor is compared with the single source body fluid it can be seen that there is higher expression in the mixture, suggesting that the minor body fluid is actually present. Although the mixing ratios are not maintained post-PCR, the correct major contributor can be identified every time, and likewise for the minor contributor. Negative controls were included in all studies.

Discussion

The aim of this study was to apply microRNA analysis to stains containing a mixture of body fluids. This was achieved by carrying out stem-loop reverse transcription and real-time PCR on samples containing both blood and saliva, utilising body fluid specific miRNA markers.

A test was developed in-house in which hsa-miR-451 and hsa-miR-205 were capable of differentiating between blood and saliva with a 100% success rate. This test underwent a blind study in which the body fluid was correctly identified every time. Serial dilutions of

blood and saliva samples were carried out and the test was carried out on samples diluted 1:50,000 (beyond the limit of detection of the enzymatic tests) with a 100% success rate.

Consequently, an effective miRNA based body fluid identification test has been developed in-house, which is more sensitive than enzymatic based blood tests. Although it is not obvious why such a test is needed to differentiate between blood and saliva, this demonstrates the principle with a high sample number.

There are also circumstances where it is not possible to definitively identify a stain as being blood, such as when an area that is LMG/KM +ve is not actually visible (for example a small drop on a black shirt or a diluted stain on a surface). In such cases, a blood vs. saliva test would be useful if it was necessary to determine the presence of blood, as opposed to a false positive or an underlying body fluid such as saliva. The sensitivity test has demonstrated that if an LMG/KM reaction has been observed, it is possible to get a more definitive answer using this test. Furthermore if an area is suspected to be blood, but no LMG/KM is detected (for example on a black shirt that has been laundered), then it may still be possible to detect the presence of blood using this miRNA BFID test.

This test was then applied to the issue of mixtures. This was initially carried out in order to determine what the effect of mixed body fluids had on the results, mainly to determine whether or not it could be distinguished as a mixture. Prior to this point, the ΔCt value was derived from Ct_{MAX}-Ct_{value}. The need for an endogenous control was very quickly realised and this was implemented using RNU-44 (following an in-house screen). As RNU-44 is a small nuclear RNA (snRNA) rather than a true miRNA endogenous control, the 1:1 mixture sample was used as a control, and a formula was derived and applied to all samples.

The formula derived from the 1:1 mixture allowed for the correction of the relative amount of genetic material available in blood and saliva. For example, if the ΔCt value for blood in the 1:1 mixture was 5 and the ΔCt value for saliva in the same sample was 10, then the value for saliva would be corrected to be equal to blood, in this case by subtracting 5 from the saliva ΔCt value, thus giving a new ΔCt value for saliva as 5. This same value would then be subtracted from all saliva samples within that run.

Following this step, the 2:1 mixture sample was not sufficient to significantly discern a major contributor, which is comparable with DNA mixtures in which a 2:1 mixture would be classed as a complex mixture rather than a simple mixture (or major/minor). At a 5:1 mixing ratio, the difference between the major body fluid and minor body fluid is sufficient to classify as a simple mixture, again, comparable to that of DNA mixtures. This was even more pronounced in the 10:1 mixing sample, as would be expected.

Consequently, this demonstrates that the miRNA based BFID test can be used on mixed body fluid samples with reliable results. Not only can the mixture be identified, it is possible to determine the major/minor contributors. Currently, the association of a major DNA profile with a particular body fluid should not be carried out, given the uncertainties of the relative amounts of body fluids in the stain. This miRNA BFID test clearly demonstrates that this capability can be embedded within the forensic laboratory.

At this stage, the reporting language should be fairly limited. For example, in a sample where the blood is the major body fluid and the saliva is the minor contributor, it should be reported

as ‘In my opinion, the major DNA profile was more likely to be obtained from the blood rather than the saliva’.

If a single stream strategy, such as that suggested by van der Meer et al. [18], is applied to this test and incorporates a full miRNA marker panel, then the language could be strengthened to ‘In my opinion, the major DNA profile was obtained from the blood.’ However, such a marker panel would need to include skin cell specific markers, and said markers need to be sufficient to differentiate between salivary and vaginal epithelia.

Summary

A effective in-house miRNA based body fluid identification test was developed that is capable of distinguishing between blood and saliva. This test, which was demonstrated to be more sensitive than the LMG/KM tests, was also successfully applied to mixed body fluids.

Future work will involve exploring this phenomenon on the more relevant body fluids, such as vaginal material, seminal fluid and in particular, skin cells. Such work includes exploring potential case work issues and development of interpretational guidelines in partner with a forensic science provider and end user organisations such as the police. Finally, a future study will incorporate DNA profiling using the single stream strategy proposed by van der Meer et al. [18].

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TABLE 1

Marker name	Body Fluid	Mature Sequence (5'-3')
Hsa-mir-451[7, 30]	Blood	aaaccguuaccauuacugaguu
Hsa-mir-205[7]	Saliva	uccuucauuccaccggagucug

Table 1 – Selected markers along with their body fluid specificity and mature miRNA sequence. The underlined section indicates the 6bp tab required for stem-loop reverse transcription [25-29].

TABLE 2

Marker name	Body Fluid	Expressed in...
Hsa-mir-451	Blood	Human, mouse, rat, dog, pig, Rhesus monkey, Zebra fish, Carolina anole, chimpanzee, Bornean orangutan, Western clawed frog
Hsa-mir-205	Saliva	Human
RNU-44	Endogenous control	Human

Table 2 - The species in which the selected markers are expressed. These are the species known to date and are not exhaustive. This information is obtained from the manufacturer's technical specifications [29].

FIGURE 1

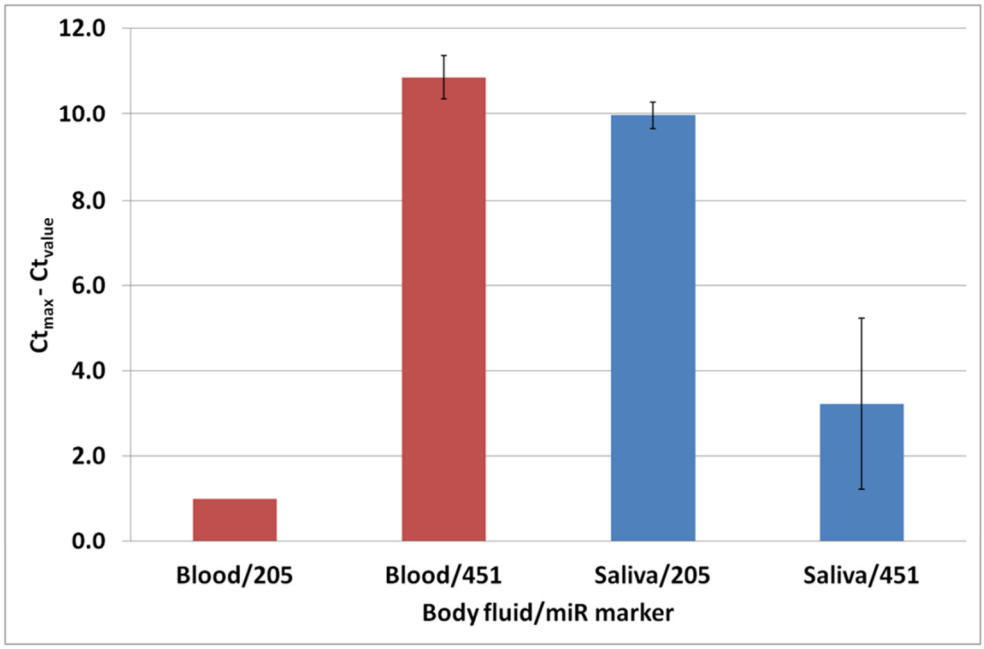

Figure 1 *- Bar chart indicating the expression of hsa-miR-451 and hsa-miR-205 in blood and saliva. The error bars represents 1 standard deviation. Paired sample T tests show a p-value of 0.00 showing significance difference in expression within each sample. The standard deviation for Blood/205 is virtually zero, thus error bars cannot be observed for this data set. Red bars indicate analysis of blood samples and blue bars indicate analysis of saliva samples.*

FIGURE 2

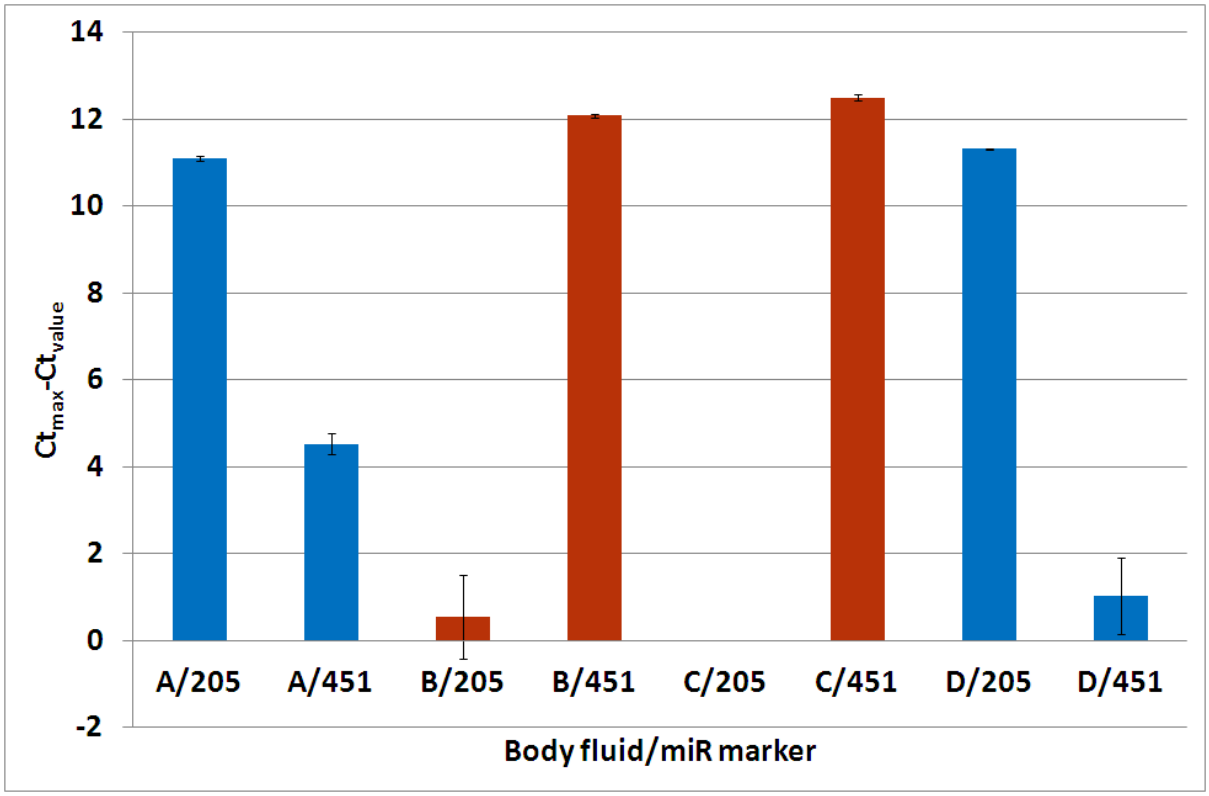

Figure 2 *– A bar chart showing the results of four samples following a blind study. A, B, C and D refers to the sample codes. 205 refers to hsa-miR-205 and 451 refers to hsa-miR-451. All error bars represent one standard deviation. Red bars indicate analysis of blood samples and blue bars indicate analysis of saliva samples.*

FIGURE 3

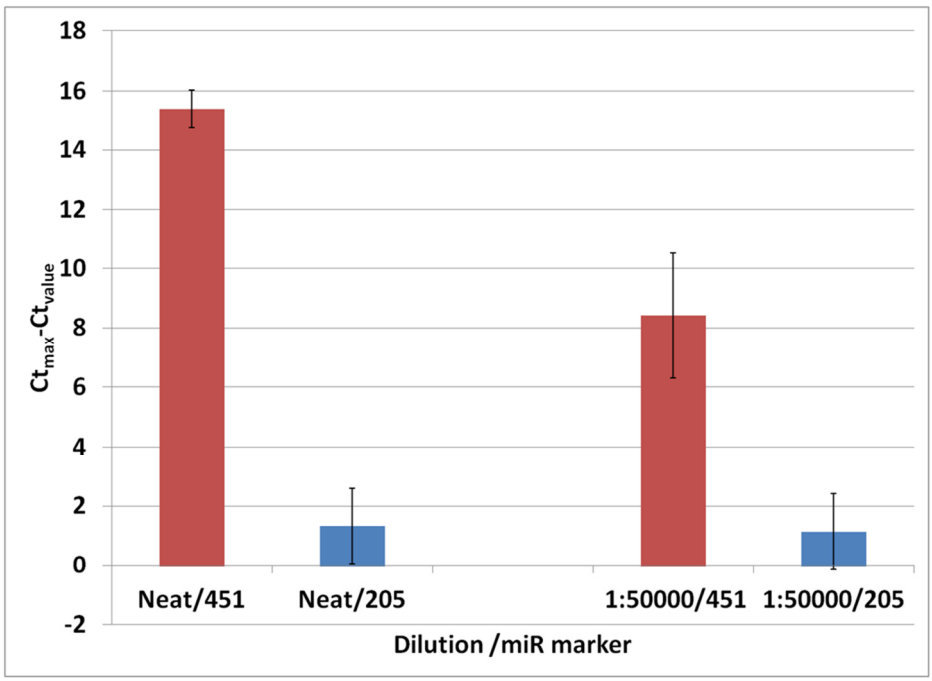

Figure 3 *- A bar chart indicating the expression of hsa-miR451 and hsa-miR-205 in undiluted blood samples and in blood samples diluted by a factor of 1:50,000. All error bars represent one standard*

deviation. Red bars indicate analysis of blood markers (451) and blue bars indicate analysis of saliva markers (205).

FIGURE 4

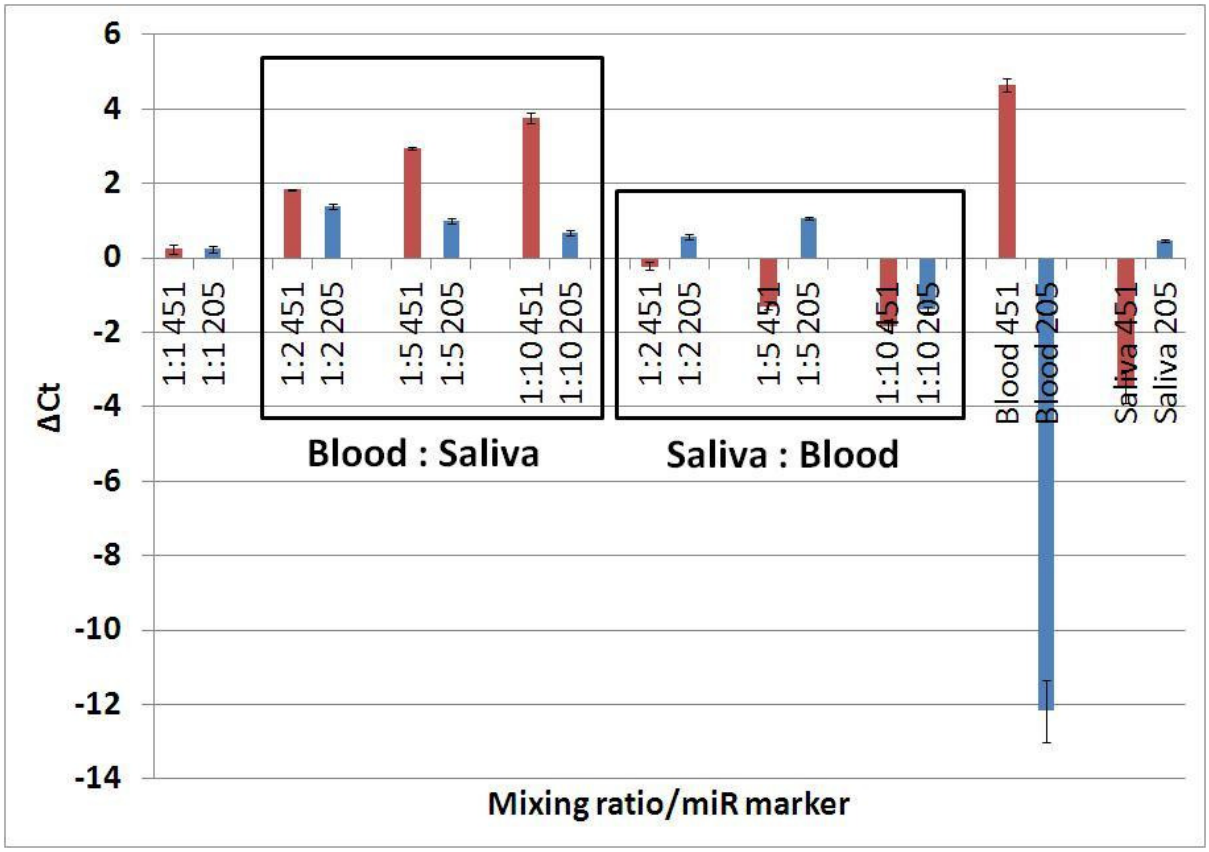

Figure 4 *- A bar chart indicating the expression of hsa-miR-451 and msa-miR-205 in body fluid stains containing a mixture of blood and saliva over a range of different mixing ratios. Single source blood and saliva samples are also included as controls. Red bars indicate analysis of blood markers (451) and blue bars indicate analysis of saliva markers (205).*