Developing a microRNA body fluid identification test for use in forensic casework

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Abstract

Body fluid identification (BFID) forms a crucial aspect within forensic investigations. BFID is particularly important where the origin of a DNA profile is also needed e.g. sexual assaults. The aim of this work was to develop a RNA-based BFID for use in forensic casework. The test was developed in three stages: exploring co-isolation and single isolation strategies, screening and selecting RNA markers and different casework conditions e.g. low-level, non-human, degraded and mixed samples. Blood, saliva, skin, semen and vaginal material samples were collected. Samples underwent single or co-isolation (DNA, total RNA or messenger RNA), DNA quantification, cDNA synthesis and qPCR using a number of different candidate markers and reference genes for microRNA (miRNA) and messenger RNA. During the development of the test the following novel findings were observed: utilisation of complex co-isolation methods was not need for co-analysis of RNA and DNA; six highly discriminative miRNA markers were identified for miRNA analysis: blood (miR-451 and miR-194); saliva (miR-205); vaginal material (miR-224 and miR-335) and semen (miR-891a). In terms of casework applications, microRNA analysis showed potential for greater sensitivity than current enzymatic methods with the use of appropriate reference gene; species specificity was observed for reference gene RNU44; sample stability was observed in 1-yr bloodstains with miR-451 and RNU44 and successful resolution of major and minor components was achieved.

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List of abbreviations

BFID…………..………………………………..Body fluid identification

cDNA…………………………………………..Complementary DNA

DNA……………………………….…………Deoxyribonucleic acid

mRNA……………………………….…………Messenger RNA

miRNA, miR…..………………………….….....MicroRNA

Pri-miRNA…………………………….....…...Primary miRNA

Pre-miRNA………………………….....….….Precursor miRNA

RNA…………………………………….........Ribonucleic acid

PCR……………………………….….…Polymerase chain reaction

qPCR. ………………………….….…...Quantitative polymerase chain reaction

RT……………………………….………...Reverse transcription

sl………………………….…….…......Stem-loop

STR.……………………….…..………….…Short tandem repeats

dNTPs…..……………………….….…Deoxyribonucleotide triphosphates

MMLV……………………….…......Moloney Murine Leukaemia Virus

AQ…………………….…………….Absolute quantification

Cq……………………………….….……Quantification cycle

LOD…………………………..………..Limit of detection

LOQ……………………......…......Limit of quantification

RQ………………………………….Relative quantification

bp…………………………….……...Base pair

nm………………………………….Nanometres

SAP…..……………………..…………………........Semen acid phosphatase

VAP…..……………………..…………………….....Vaginal acid phosphatase

UV…………………………………………………….Ultra violet light

RSID…………………………………………………Rapid stain identification

Fe^{2+}…..…………………………..………………..Iron in an oxidation state of $^{2+}$

H_2O_2…………………………………………..……Hydrogen peroxide

DTT…………………………………………….....Dithiothreitol

EDTA………………………………………………Ethylenediamine tetraacetic acid

SDS…………………………………………..…Sodium dodecyl sulphate

UNG...Uracil N-glycosylase

CCD…………………………………………...……Charge coupled device

NIRS………………………………………..…….....Near infrared Raman spectroscopy

FT…………………………………….…...…..Fourier transform

SERS……………………………………….………Surface-enhanced Raman spectroscopy

WL…………………………………..……..Woods lamp

C. elegans…………………………..……….....*Caenorhabditis elegans*, (roundworm)

B. primigenious…………………………………Bos primigenious (cattle)

C. herengus……………………….……...……*Caspius herengus* (herring)

Cervidae………………………….…………........Deer

G. gallus……………………….…………...…*Gallus gallus* (chicken)

Phasianinae……………….………………..…....Pheasant

O. aries………………….………..…….………...*Ovis* aries (sheep)

S. scrofa. ……………………….…………….....*Sus scrofa* (pig)

Chapter 1
Introduction

1.1 Body fluid identification

Body fluid identification (BFID) plays a crucial role in forensic casework. It provides valuable information regarding the body fluid origin and can provide evidentiary strength to a DNA profile. DNA profiling has become a powerful tool for forensic investigations due to the tremendous efforts that have gone behind developing this technology.

DNA profiling is one of the most common methods used for identifying an individual. Current DNA technologies utilise short tandem repeats (STRs), which are repeating DNA sequences between 100 to 400 bp in length [1]. STRs can be categorized by the repeat pattern: simple, compound and complex. Simple repeats are identical in sequence and length, compound are a combination of simple repeats while complex repeats contain a range of different repeating sequences [2]. DNA profiles utilise a combination of all three types STR markers [2]. For instance, the Life Technologies Next Generation Multiplex Select (NGM SE) kit for PCR utilises the SE33 locus, which is a complex marker [2]. The use of multiple STR markers increases discriminatory value. The NGM SE kit utilises a total of 17 loci to identify individuals. DNA profiling kits have also become very sensitive over the years. For instance the total input required for a full DNA profile using the NGM SE kits is 1.0 ng [3]. One of the major limitations of DNA profiling is that it cannot give information regarding the body fluid origin.

1.1.1 Forensic applications

Despite the evidentiary strength that DNA profiling provides within courtrooms, there are many cases where BFID would be a powerful addition to a DNA profile e.g. non-consensual intercourse, withdrawn consent, digital penetration, bestiality, cold cases and trace DNA.

1.1.1.1 Non-consensual intercourse

BFID would be valuable where multiple body fluids are present [4]. For example in a case of non-consensual intercourse, there may be vaginal material and semen heavily present within an item of evidence collected. Generally the identification through DNA profiling is sufficient for identifying the suspect and victim. However identification of additional body fluids could provide valuable insight into a case. For instance, the presence of blood could indicate forced entry or non-consensual vaginal intercourse [5-10]. The lack of lubrication may cause lacerations and abrasions in the vagina and labia majora and minora (skin protecting the vulva, urethra and vagina) and result in the presence of circulatory blood in the sample. Additionally, forced entry may cause tears along the frenulum (a string connecting the prepuce to the vernal mucosa) and cause blood to be released from the penis. The presence of menstrual blood also may be an indicator of forced entry or non-consensual vaginal intercourse as it is different to circulatory blood. In all three scenarios, BFID may provide important circumstantial evidential evidence to the DNA profile.

1.1.1.2 Withdrawn consent

Another example where BFID would provide valuable information is in cases of withdrawn consent. Consent is defined by Section 74 of the Sexual Offences Act 2003 as an agreement made by choice [11]. Withdrawn consent is therefore the removal of that initial agreement. The issue of consent can occur at any point before or during a sexual act. For example two people may initially agree to oral stimulation or intercourse e.g. fellatio, cunnilingus but then withdraw that consent due to feelings of discomfort [12, 13]. A victim may state that non-

consensual stimulation or intercourse took place where as the suspect may state that only the exchange of kisses on the mouth occurred. DNA profiling would not be able to resolve the context of this case. However BFID may give an indication of both the presence and the type of body fluids present e.g. saliva, semen or vaginal material, which could then be used to establish if fellatio or cunnilingus took place.

1.1.1.3 Digital penetration

BFID would also be very useful for cases of sexual assault through penetration [14-18]. Assault through penetration is defined in Section 2 of the Sexual Offences Act 2003 as non-consensual penetration of the vagina or anus through digital penetration or with a foreign object [19, 20]. Digital penetration can be with the fingers, tongue or toes [20]. An example of digital penetration is in a case of Regina vs. Z (2009) [20]. A suspect digitally penetrated the victim staying at her boyfriend's home. The victim's anus was penetrated three times. The suspect was convicted in this case. However it was not determined whether penetration occurred through direct contact in the anus or through clothing. BFID of anal swabs and clothing could help establish the method of contact in addition to the DNA profiles obtained.

Another example of sexual assault is through digital penetration with a foreign object [21]. Sturgiss *et al* (2010) reviewed 20 cases of sexual assault involving foreign objects. The majority of the objects reported were cylindrical, with the most common object of insertion being drink bottles in the vaginal cavity. Other objects reported in this study included items such as pasta jars, handles and pencils. Additionally, any household item may be used to digitally penetrate a person e.g. deodorant can. BFID from foreign objects such as these may indicate the circumstances of the assault. For instance the presence of circulatory blood and

vaginal material may reflect the level of violence behind the assault. Thus providing valuable circumstantial evidence to the DNA profiles.

1.1.1.4 Bestiality

In forensic casework it is also common to encounter body fluids from different species [22]. For instance, crimes that occur in the household may contain animal body fluids. Body fluids from animals may be prevalent in homes where domesticated pets or livestock are cared for. For instance in sexual assaults involving animals, semen from a human male may be present in an animals anal or vaginal cavities. Identification of the individual through DNA profiling is powerful evidence. However the identification of the body fluids (e.g. semen, vaginal or anal swabs) would provide crucial information to the circumstances in a case. In 2009, Imbschweiler *et al* (2009) described such case between a human male and an ewe. Successful conviction was achieved using a combination of DNA profiling and BFID through visualisation of semen and epithelial cells [23]. In addition, body fluids from animals may be present in the home from preparing food. The presence of animal blood would need to be put into context of a case. For instance it is unlikely that the presence of *Bos primigenious* (cow) blood will be of significance unless the crime had taken place at a farm.

1.1.1.5 Cold cases

BFID may also be useful in cases where samples are heavily degraded such as in cold cases. Cold cases are crimes that have undergone investigation but have not been resolved. The exact point in which a case becomes cold is somewhat ambiguous. However it can generally be described by points of inactivity or lack of leads in a case (e.g. 1 year) [24]. Cold case samples

can be particularly challenging to forensic practitioners. The standard operating procedures (SOP's) used to recover, collect, transfer, test, record and preserve samples may have varied. Practitioners may also have little or no sample remaining from the tests performed while the case was active. Therefore the use of tests such as DNA profiling and BFID is very important. BFID can provide valuable insight into the circumstances of a case, which can be very useful where a full, partial or no DNA profile has been obtained from the samples tested. BFID may also be useful in cases where samples have been heavily degraded by environmental factors such as ultra-violet (UV) light, temperature and humidity. DNA profiles may or may not be obtained from these types of samples. In these instances BFID can provide additional circumstantial information where DNA profiles cannot.

1.1.1.6 Trace DNA

Another example where BFID is valuable is in trace DNA samples [18, 25-28]. Trace DNA can be broadly described as DNA that cannot be associated to body fluids. Trace DNA can be transferred onto people and surfaces through direct or indirect contact. DNA profiles may be full, partial or absent depending on factors such as type of contact, surface, place, time and lifestyle of the individuals [28]. BFID can provide insight to such factors in a case. Touch DNA has been important such as in Regina v Reed (2006) [29, 30]. In this case, two brothers were convicted of murder. Partial profiles were obtained from the suspects and victim from plastic from two knife handles. DNA profiling was essential to identifying and convicting the two suspects. The body fluid origin of these profiles was not determined e.g. skin cells or saliva. However it was evident that BFID could have played a crucial role in providing insight into the circumstances of this case.

Additionally there may be cases where body fluids may not be present. However this does not necessarily eliminate the possibility that a crime took place. For instance, semen will generally remain longer in the vagina than in the oral or anal cavities of individuals [31]. A victim may report non-consensual intercourse long after a crime taken place e.g. weeks, years. In these types of circumstances, samples such as clothing may not be available, making DNA profiling or BFID methods unusable. Instead, courtrooms may have to turn to other forms of evidence such as eyewitnesses [32, 33].

1.1.2 Current BFID tests

There are a number of different BFID tests currently used in forensic casework. They can be divided into two sections: presumptive and confirmatory. Presumptive BFID testing plays an important role in forensic investigations. They allow for both forensic practitioners and police to decide the most effective approach and tests to use in casework, which is often limited by both time and budgetary constraints. Presumptive tests are generally rapid, accurate, safe, easy to use, inexpensive and portable. However they also generally lack in specificity (e.g. body fluids, animal species, plants, household products) and sensitivity (e.g. limited or mixed samples) resulting in false positive and false negative results. False positives can suggest the presence of a particular body fluid where there is none. Conversely, false negatives can suggest the absence of a particular body fluid when it is present.

Confirmatory BFID tests form the other half of testing in forensic investigations. They are considered to be confirmative because they can identify components that are characteristic or unique to the particular body fluid type. For instance sperm cells are only found in semen. Confirmative BFID tests are generally more accurate, sensitive and specific than presumptive

tests. They also tend to require lower sample input, which is very beneficial for in forensic casework where sample is often limited. Furthermore these qualities generally lower the chances of false positive and negative results. The most casework relevant presumptive and confirmatory BFID tests have been described in this section.

1.1.2.1 Blood

Blood is a commonly encountered body fluid in forensic casework. Blood consists of three major components: plasma (54%), erythrocytes (45%), leukocytes and thrombocytes (1%). [34]. Plasma consists primarily of water (92%) and protein (8%). The water is rich in electrolytes, vitamins, acids and hormones (e.g. magnesium, potassium, amino acids, insulin), which is essential for maintaining homeostasis in the body. The proteins (e.g. serum albumin) in plasma also help maintain homeostasis in the body. Osmotic pressure is maintained by serum albumin. Erythrocytes are cells that do not contain a nucleus. They have a round slightly concave exterior that is comprised mainly of lipids while the interior contains a globule of iron-rich protein. The protein is called haemoglobin and is comprised of two α- and two ß-subunits [34]. The four subunits of haemoglobin can be further divided into haem, which is comprised of iron (Fe^{2+}) and protoporphyrin IX (organic compound) [34]. The characteristic red colour often observed in blood is from the iron present in erythrocytes [35]. Erythrocytes are produced in the bone marrow. Their main role in the body is to transport oxygenated and deoxygenated blood through the body. Leukocytes are cells that contain a nucleus. They can be classed into three major categories: granulocytes, lymphocytes and monocytes [36]. Their main role in the body is to protect it from foreign material and disease. Thrombocytes are cells that do not contain a nucleus. Their main role is to help with the healing process from injury by

aggregating around the injury and releasing histamine and serotonin that will eventually form a blood clot [37]. They are produced by megakaryocytes in the bone marrow.

1.1.2.1.1 Oxidation-reduction tests

There are several different presumptive techniques used to indicate the presence of blood [38-43]. Two of the most commonly used tests in forensic casework are the Kastle-Meyer (KM) and Leucomalachite Green (LMG) tests [44, 45]. The principle of both tests is very similar. The KM and LMG reagents in the presence of haem will undergo a standard oxidation-reduction reaction. Both KM and LMG are tested indirectly onto samples using filter paper or swabs. Both reagents are in a reduced state (colourless) before performing the test. KM or LMG reagent is first added to the samples. After a few seconds hydrogen peroxide (H_2O_2) is then added. In the presence of haem, the KM dye phenolphthalin will oxidise into phenolphthalein causing the sample to turn a bright pink colour. Similarly, with the LMG test, the base leuco will oxidise in the presence of haem turning the sample a vibrant green colour [46]. However it is important to mention that it is the peroxidase-like activity that causes this colour change in both of the tests. False positives can be indicated by a colour change between the addition of KM or LMG and addition of H_2O_2. Another common enzymatic method used in forensic casework is the luminol test. The principle behind the luminol test is very similar to the KM and LMG tests. Luminol (3-aminophthalhdrazide) will oxidise in the presence of haem turning the sample luminescent [46].

There have been a number of reports exploring the sensitivity and specificity of the KM, LMG and luminol tests in blood. Webb *et al* (2006) performed a comprehensive study comparing the sensitivity of presumptive tests for blood including the KM, LMG and luminol tests. In their findings, they found the luminol test to be the more sensitive than the KM and LMG tests. The sensitivity range of luminol was similar to other studies in this area, having sensitivity range of 1 in 5,000,000 dilutions [41]. The sensitivity range reported for the KM and LMG tests varied when compared to other research such as Cox *et al* (1991). For instance with the KM test, Webb *et al* (2006) detected blood-stained cloth down to 1 in 10,000 dilutions where as Cox *et al* (1991) detected it down to 1 in 100,000 dilutions. This may be down to a number of variables including reagent preparation. For instance, both Webb *et al* (2006) and Cox *et al* (1991) prepared their reagents from scratch. The wide range of sensitivity observed may also be a reflection of the target molecule, the more specific the target the more accurate the result.

Tobe *et al* (2007) performed a study comparing the specificity of presumptive blood tests including the KM, LMG and luminol tests. They found the least amount of cross reactivity in the luminol test. Luminol reacted only with metals (e.g. nickel chloride and cupric and ferric sulphate) [42]. The KM tests showed cross reactivity with other vegetables (e.g. horseradish, red onion), common household products (e.g. bleach) and body fluids (e.g. semen). The LMG test showed less cross reactivity than the KM test, reacting only with potato and red onion. Their findings agreed with previous literature where as the KM and LMG tests did not [38, 39]. The difference may be due to the type of sample used. For instance, Cox *et al* (1991) compared sweet potato and Irish potato, neither of which showed cross reactivity in the KM and LMG testing where as the type of potatoes tested in Tobe *et al* (2007) was not mentioned in their results.

Both the KM and LMG tests are sensitive methods. However their sensitivity is limited by their lack of specificity towards vegetables, household products and body fluids. The luminol test is more sensitive and specific than the KM and LMG tests and can be used to indicate the presence of blood that may have been cleaned up. However it is limited by the need to visualise the stains in the dark.

1.1.2.1.2 Crystal tests

Another common test used for blood in forensic casework are the crystal tests. Crystal tests are confirmatory methods. The Takayama and Teichmanns tests are two common methods used in forensic casework. The principles of both tests are very similar, utilising a chemical reaction to identify haem. This reaction will produce specific derivatives that can later be visualised using microscopy. The Takayama test uses an alkaline solution (contains sugar and pyridine) to form pyridine ferroprotophorphyrin [46]. The Teichmanns test uses a combination of heat and glacial acetic acid to produce ferriporphyrin chloride [46]. Both techniques are limited by their low sensitivity and inability to distinguish between different species [47].

1.1.2.1.3 Immunochromatographic tests

The use of species-specific blood tests can be useful in forensic cases where animal blood is present [43]. One common approach utilises immunochromatographic tests, which target the ß–units of haemoglobin (Hb) in primates to indicate the presence of blood. Immunochromatographic tests are presumptive techniques. One common technique used in forensic casework is the Hexagon OBTI test[®] [48]. The test is performed on a lateral test strip, which contains a sample and control region. These regions will contain monoclonal anti-

human Hb antibodies, which have a blue dye attached for testing. During analysis, suspected blood samples are placed onto the sample area. A buffer containing Tris is then added to the sample. In the presence of Hb, an antibody-antigen-antibody complex will form along the sample and control regions of the strip resulting in the appearance of two separate blue lines. Samples not containing Hb will only show a blue line in the control region [43]. Hochmeister *et al* (1999) performed an extensive validation study on the Hexagon OBTI test[®] exploring factors such as sensitivity, specificity, casework and the hook effect. Their findings showed that the Hexagon OBTI test[®] was primate specific and sensitive down to 1 in 100,000 dilutions in water. The test was also found to be robust, showing positive reactions in all casework samples e.g. blood-stained cloth 2-15 years old [48]. The Hexagon OBTI test[®] also exhibited the high dose hook affect where high levels of erythrocytes were present, highlighting one of the main limitations of this test. The other main limitation of this test is that it is not human-specific and has tested positive in other primates including *Pongo pygmaeus* (Orangutan).

Summary

The identification of blood can be very important in forensic casework. There are a handful presumptive and confirmatory tests that have been used to indicate the presence of blood. These tests target different components in blood. One of the main presumptive methods described was the oxidation-reduction tests e.g. KM, LMG and luminol. These tests are rapid, accurate, sensitive and easy to use. Both the KM and LMG tests however lack in specificity towards other body fluids, plants and common household products. Luminol has additional advantages over the KM and LMG tests including greater sensitivity and specificity. It is however limited by the need to visualise stains in the dark. Another presumptive technique described was immunochromatographic blood tests. They are rapid and sensitive techniques.

Their main drawback is the lack specificity towards human blood. Two confirmative tests were also described; Takayama and Teichmann. These tests are accurate however they have lower sensitivities than in other presumptive blood tests. Despite the range of different presumptive and confirmatory blood tests there are currently no confirmative tests that can differentiate between trauma and menstrual blood.

1.1.2.2 Saliva

Saliva is another commonly encountered body fluid in forensic casework (e.g. biting, licking, oral sex) [49-51]. Saliva is generally comprised of three major components: water (99.5%), organic (0.3%) and inorganic compounds (0.2%) [52-55]. The organic components of saliva consist mainly of proteins (e.g. α-amylase, albumin, histatins, mucins), acids (e.g. uric acid) and fats (e.g. cholesterol and lipids) [54]. The inorganic components of saliva comprise mainly of cations (e.g. calcium, magnesium, potassium, sodium) [54]. Three major glands in the mouth produce saliva: paratoid, sublingual and submandibular [56]. The major roles of saliva include deglutition, lubrication of deglutition (e.g. food) and protection of the enamel and from foreign viruses, bacteria and fungi of the mouth [52, 54]. Humans produce on average 1.3 L of saliva per day [46].

1.1.2.2.1 Amylase tests

There are a handful of presumptive techniques that are used in forensic casework to indicate the presence of saliva. One of the most commonly used presumptive tests in forensic casework is with the presence of enzyme α-amylase. Humans contain two different types of α-amylase: pancreatic α-amylase (HPA) and salivary α-amylase (HSA). Both have an important role in

digestion and aiding in the breakdown of complex carbohydrates (e.g. polysaccharides) into simple sugars (e.g. mono-saccharides). Humans normally have high levels of amylase in the body. However this can vary between individuals due to natural variation, disease and time of day [57, 58].

One of the main kits used to indicate the presence of saliva in forensic casework is the Phadebas® tests [57, 59-62]. The Phadebas® tests utilises the α-amylase activity to indicate the presence of saliva. In the presence of α-amylase, a water-insoluble polymer attached to a soluble blue dye will hydrolyse releasing the dye and will result in the appearance of a dark blue colour. There are liquid and paper versions of this test. The liquid version will test for α-amylase activity using aliquots of suspected samples before undergoing quantification with a spectrophotometer (620 nm). The paper Phadebas® press test is generally applied onto a piece of filter paper, moistened with water and placed onto the suspected sample regions (e.g. clothing) [58, 59, 63, 64]. The presence of α-amylase is then determined through visualisation of the stain.

Another common test used to indicate the presence of saliva is the starch-iodine test. It utilises similar principles to the Phadebas® test, relying on the activity of α-amylase activity to produce the appearance of a dark blue colour. However starch is used to identify α-amylase activity. The test is performed in a petri dish. An agarose gel will be set with starch, which will result in a blue colour. Samples are added to wells in the gel and immersed in iodine solution. A white colour in the well (due to diffusion) indicates amylase activity. The level of amylase present can be quantified by the size of the white area and standard curve (e.g. large white area are indicative of high levels of amylase) [46].

Summary

Amylase tests form an important part of forensic investigations involving saliva. The presence of amylase can give forensic practitioners insight on the circumstances of a case and how to proceed. In addition they are also relatively inexpensive and easy to use [62]. Amylase tests are limited by a number of factors. The tests are not specific to HSA and can be mistaken for HPA found in other areas of the body (e.g. blood, semen, vaginal material) [46]. Animals (e.g. primates, some rodents), plants (e.g. potatoes) and common household products (e.g. lotions, detergent) also contain high levels of amylase and as a result can generate false positives [51, 57, 65].

1.1.2.2.2 Microscopy

Alternatively, buccal cells can be visualised using microscopy. It is relatively quick and easy to use. Epithelial cells can be stained prior to visualisation. The haematoxylin and eosin (H&E) stain can be used in forensic casework to enhance the appearance of epithelial cells. The nucleus will be dyed blue while the surrounding region of the cell will be dyed red. Microscopy can be useful for identifying epithelial cells. However its inability to differentiate the morphology of epithelial cells found in vaginal material and skin, means that it cannot definitively confirm the presence of saliva.

1.1.2.2.3 Immunochromatographic tests

A more accurate, sensitive and specific approach for indicating the presence of saliva is using immunochromatographic tests, which target α-amylase. Immunochromatographic methods are presumptive techniques. One common immunochromatographic test used in forensic casework

is the rapid stain identification (RSID™) for human saliva test [50, 51]. They utilise lateral flow strips similar to pregnancy tests, which contain both sample and control regions. Both the sample and control region contain two monoclonal antibodies. The presence of the specific antigens will result in binding to the antibodies and a red line in both sample and control regions will appear. Samples not containing these antigens will only show a red line in the control region [50, 66].

Old *et al* (2009) performed a study exploring different casework applications (e.g. sensitivity, specificity, stability, and mixed body fluids) using the RSID™ human saliva test. In their findings, the RSID™ tests showed greater sensitivity than with the generic amylase tests. In their specificity studies, the RSID™ saliva test showed slight cross reactivity towards milk and post-coital samples containing vaginal material but no cross reactivity in blood and semen. The RSID™ test for saliva showed α-amylase stability in samples stored at high temperatures (e.g. 37 ° C) as wells as in samples stored over long periods of time (e.g. 30 days) [50, 67]. Saliva samples showed specificity in mixed samples containing blood or semen. The studies performed by Old *et al* (2009) reiterate the strengths of using an immunochromatographic test in forensic investigations. However their study also highlights the need for a confirmatory BFID test for identifying saliva.

1.1.2.2.4 Alternative light sources

Other less commonly used tests include alternative light sources (ALS). ALS is a presumptive technique. The general principle of ALS involves the absorption of light from shorter wavelengths followed by the emission of light at longer wavelengths and fluorescence of a sample. Saliva can fluoresce between 200-550 nm depending on the light source [68]. For

instance, Polilight[®], which is a common ALS source can indicate the presence of saliva when using orange goggles and a wavelength of 450 nm [26]. A study by Vandenberg and van Oorschot (2006) explored Polilight[®] and its applications on forensically relevant body fluids, which included saliva. In the majority of their studies (e.g. appearance on different fabrics, cleaning products, diluted and mixed samples), the presence of saliva could be indicated. However it is clear through their work that body fluid differentiation with ALS is limited by factors such as the composition of body fluids, mixed body fluids and the presence of household cleaning products [26].

Summary

The identification of saliva can be very important in forensic casework. There are a handful presumptive tests that have been used to indicate the presence of saliva. These tests target different components of saliva. One of the main presumptive methods described was α-amylase test and immunochromatographic tests. Both tests are relatively accurate, inexpensive and easy to use. However the immunochromatographic test has greater specificity than the amylase tests, which gives false positives in other body fluids, animals, plants and chemicals. Microscopy with staining (via H&E) is another technique that can be used to identify epithelial cells. The test is relatively quick and easy to use. However its main drawbacks include the inability to differentiate the morphology of saliva from vaginal material and skin as well as its destructive nature. ALS is another presumptive method that was described. Its main advantage over the other techniques is the ability to visualise the area of the stain. However it lacks in specificity towards other body fluids and common household products. The main drawback to these presumptive BFID methods in forensic casework are they cannot distinguish saliva from vaginal material [69].

1.1.2.3 Semen

Semen is another commonly encountered body fluid in forensic casework (e.g. sexual assaults) [65, 70-73]. It normally contains two components: 5% spermatozoa (sperm) and 95% fluid [74]. Spermatozoa have a head, which contains DNA and RNA, a tail, which is used for mobility and a complex mid-piece, which connects the two parts. The fluid consists of a number of components necessary for the survival of sperm during reproduction. The main energy sources for sperm include fructose and glycerophosphorylcholine. They are derived from storage areas such as the ampulla and epididymis, as well as the prostate glands and seminal vesicles. The environment of the sperm (e.g. pH 7-7.5) is regulated mainly by these glands and vesicles [74]. They will release a number of different enzymes, hormones, acids and elements including acid phosphatase, diamine oxidase, y-glutamyl transpeptidase, prostaglandins, semenogelins, citric and lactic acid and calcium, sodium, potassium and zinc [65, 74, 75]. In addition, the urethral glands will release mucus. The secretions produced by these glands and vesicles will also aid in the motility of sperm during reproduction. A healthy male on average releases about 3 ml of semen, which can contain up to 10 million sperm [74].

1.1.2.3.1 Acid phosphatase tests

Over the years a wide range of presumptive and confirmatory tests that have been developed to identify semen [46]. One common presumptive method used to indicate the presence of semen is the prostate acid phosphatase (PAP) test [76-81]. The PAP tests are used by forensic investigators to identify the samples that contain semen. This test is accurate and relatively

easy to use. Acid phosphatase is an enzyme produced by the prostate gland. Its main function is to increase acidity of semen [82]. PAP is normally found at high levels in semen.

There are two common AP tests used in forensic casework: the α-naphthyl phosphate and the 4-methylumbelliferone phosphate test [46, 65]. Suspected casework stains are treated with moistened filter paper or swabs to preserve the original sample [81]. The filter paper is subsequently treated with these substrates and additional reagents or light sources to indicate the presence of semen.

The chemistry behind both tests is relatively similar. Acid phosphatase in the presence of α-naphthyl phosphate or 4-methylumbelliferone phosphate will cause removal of these phosphate groups through hydrolysis [46, 65]. Semen is then indicated through precipitation using Brentamine Fast Blue B, which gives a purple colour or through the examination with ultra-violet respectively.

The acid phosphatase test is an effective test for indicating the presence of fresh semen [80, 83]. The test is also quick and portable making it useful for forensic casework. However there are a several limitations with this test. Acid phosphatase is also found in other body fluids, plants, chemicals (e.g. vaginal phosphatase, tea, toilet cleaner) and can yield false positives [78, 79, 81]. It is worth noting that indirect testing of the sample area has been shown to be a contributing factor to false positives [81, 84]. Acid phosphatase also degrades when exposed to different conditions such as putrefaction and heat [65, 75]. There are tests available that are more suited for degraded semen described later.

1.1.2.3.2 Microscopy

Samples containing acid phosphatase then undergo confirmatory testing using microscopy [65]. Spermatozoa is visualised through the microscope directly or through cytological staining. There are three popular methods used by the forensic community; the nuclear fast red and picroindigocarmine, alkaline fuchsin and haematoxylin and eosin tests [65, 72, 85]. Spermatozoa are prepared onto slides by drying (e.g. air drying or heat drying) and treatment with chemicals such as ether or alcohol [85]. Slides are then stained with the dyes and visualised under a microscope. The current standard for conviction within forensic casework is the presence of a single sperm head in addition to a DNA profile [85, 86].

The staining process for these dyes is relatively similar. The nuclear fast red and picroindigocarmine dye will target two different regions of the spermatozoa. Nuclear fast red is basophilic and will bind to the sperm head dying it red while the picroindigocarmine is acidophilic and will bind to the sperm tail dying it green [65, 85, 87]. The haematoxylin and eosin (H&E) stain performs in a similar manner except the sperm head is dyed blue while the sperm tail is dyed red. The alkaline fuchsin stain will dye the sperm red [87].

It is with no doubt that microscopy is a powerful confirmatory tool in forensic casework. It has been used to convict thousands of individuals [7, 15, 16, 88]. However identification of sperm is limited by its presence in a sample. Medical conditions and lifestyle choices may affect the presence of sperm. Individuals may have medical conditions such as aspermia where they do not produce semen, azoospermia where they have no sperm in the seminal fluid or oligospermia where they have a low sperm count. Other individuals may have no sperm present because of a vasectomy, a medical procedure that does not allow sperm to leave the

body. Alternative tests have been developed for establishing the presence of semen in a sample.

1.1.2.3.3 Immunochromatographic tests

Another common confirmatory method used to indicate the presence of semen are immunochromatographic tests [71]. They are generally rapid, accurate and easy to use. A common immunochromatographic test using in forensic casework is the prostate-specific antigen (PSA, P30, Kallikrein 3). PSA is a protein produced by the prostate gland. Its main role is to add volume to semen to encourage motility of sperm during reproduction. Semen normally contains high levels of PSA (e.g. 5.0 mg/ml) [89]. Its high concentration and relative stability make it particularly useful for identifying heavily degraded semen samples (e.g. 55 years) [48, 89-91].

The seminal vesicle-specific antigen test is another common immunochromatographic test used for semen samples. Semenogelins are comprised of two main proteins (semenogelin I and semenogelin II) and are produced by the seminal vesicles. Their main role is to add viscosity to semen during ejaculation. They are found at higher levels than PSA making them particularly useful for heavily degraded samples [91].

Two commonly used immunochromatographic tests used in forensic casework are the ABAcard® p30 test and RSID™ of human semen test [66]. These tests utilise the presence of the PSA or semenogelins to identify semen respectively. As mentioned previously, they utilise strips, which contain both sample and control regions. Both the sample and control region contain specific antibodies. The presence of specific antigens will result in binding to the

antibodies and a pink line in both sample and control regions will appear. Samples not containing these antigens will show a pink line in only the control region [66].

Boward and Wilson (2013) performed a comprehensive study exploring the sensitivity, specificity and cost-effectiveness of both ABAcard® p30 and RSID™ semen test using fresh, frozen, post-coital, vasectomised and mixed samples. The overall findings from their study indicated similar levels of specificity. In terms of cost-per-analysis, ABAcard® p30 was slightly cheaper than the RSID™ semen test. Also interestingly, the ABAcard® p30 showed varying levels of sensitivity when compared to the RSID™ semen test [66]. For instance in the mixed samples the RSID™ semen test showed greater sensitivity than with the ABAcard® p30, which was expected as semenogelins are generally found at higher levels than PSA. However the ABAcard® p30 showed greater sensitivity in post-coital samples. This was surprising as high levels of semenogelins are released prior to ejaculation. The difference in sensitivity may be down to variation in PSA and semenogelins levels between individuals. It may also be due to the use of different commercial kits.

Immunochromatographic tests are important in forensic casework. They provide an essential link to the presence of semen when spermatozoa are absent. In addition they are also rapid in throughput, accurate and easy to use. However there are couple of limitations to immunochromatographic tests. They can produce false negatives in the presence of too much sample (e.g. PSA levels of 50,000 ng/ml and above), a behaviour known as the high dose hook effect [48]. They can also produce false positives when sample amounts are limited. Prostate specific antigens and semenogelins are can also be found present in other body fluids (e.g. blood, milk, urine) and tissues (e.g. colon, kidneys, trachea) respectively [46, 91]. Furthermore, low levels of these antigens may also be mistaken for the presence of other body fluids.

1.1.2.3.4 Alternative light sources

Other less commonly used methods to identify semen in forensic casework include ALS. Alternative light sources are presumptive methods. They are rapid, non-destructive and easy to use. ALS can be useful for visualising biological stains on individuals and crime scenes (e.g. sexual assaults) [92, 93]. As mentioned previously, ALS will absorb light from shorter wavelengths to emit light at longer wavelengths; resulting in fluorescence of a sample [26]. One common ALS used in forensic casework to identify semen is the Woods lamp (WL). The WL will emit a wavelength at 360 nm, which falls in the ultra-violet (UV) light region of light; 200-400 nm [94, 95]. Semen with the WL will generally fluoresce between 300-500 nm [26, 68, 96]. Another common ALS used in forensic casework is the Polilight®. Polilight® emits light in the range 310-610 nm, which is also in the UV light region [26].

Wawryk and Odell (2005) performed a study on ALS. They explored the fluorescence of semen and other substances on skin using the Woods lamp. They reported that the appearance of semen stains was generally more faint on skin than on items such as sheets and clothing. They also performed a study comparing the fluorescence of azoospermic, vasectomized and normal semen. However they were not able to distinguish between these sample types. ALS relies heavily on visual interpretation of a sample.

ALS can be a useful tool for forensic investigations e.g. visualizing biological stains on bed sheets. Alternative light sources are generally easy to use, non-destructive and cost-effective. However ALS can give false positives from the presence of common household products e.g.

hand creams, soaps, detergents and ointments and must be interpreted with caution [26, 94, 96]. In addition, ALS is not a confirmatory method for identifying semen.

Summary

The identification of semen can be crucial in forensic investigations, especially in cases of sexual assault. There are a number of different presumptive and confirmatory techniques that were described for targeting different components of semen. Microscopy with staining was one of the main confirmatory methods described. Its use within forensic casework is powerful as the presence of one sperm head in addition to a DNA profile is enough to convict an individual. The main limitation of this technique is that it can only be used in the presence of sperm. The AP and immunochromatographic methods (e.g. PSA) were two other presumptive methods described. Both tests are generally used in cases where sperm is absent, with the latter method being of particular use for aged semen stains. In addition, these techniques are relatively accurate and easy to use. The main drawback of both tests is their lack of specificity towards other body fluids, plants and chemicals. Another limitation is that it can produce false negatives if the hook effect occurs. ALS is another presumptive technique that was described. Its main advantage over the other techniques is the ability to visualise the area of the stain. However it lacks in specificity towards other body fluids and common household products.

1.1.2.4 Vaginal material

Vaginal material is another commonly encountered body fluid in forensic casework. Vaginal material contains a number of different components including glycogenated epithelial cells, enzymes, proteins, acids, carbohydrates, organic compounds and microflora [97-101]. The

vagina is comprised of glycogenated epithelial cells. They will secrete vaginal transudate (e.g. glycogen, mucin, potassium, sodium) that will keep the vaginal walls moist during or without stimulation as well as during different points during the menstrual cycle [102-104]. Glycogen will also serve as one of the main energy sources for microflora (e.g. lactobacilli), which are naturally present in the body. They will produce organic acids (e.g. lactic acid) that will help act as a natural barrier against antigens based on the low pH (<4.5) [98, 105]. The vaginal walls will also renew itself on a regular basis through shedding. In this way it can keep the vaginal clean from bacteria build up. The average female will produce 3 ml of vaginal transudate per day [105]. This will be combined with other lubricating fluids from the upper and lower reproductive tracts. The endometrial and tubal glands found in the cervix will produce cervical mucus [106]. This mucus is thick and rich in glycoprotein and carbohydrates, serving as a protective barrier to the uterus [101]. The sebaceous glands surrounding the labia minora will produce sebum that will help protect and lubricate the entrance to the vagina during stimulation or no stimulation [106]. All of the components found in vaginal material are responsible for reproduction and health of the vagina.

1.1.2.4.1 Microscopy

There are a few presumptive tests used for indicating the presence of vaginal material [107]. One of the main presumptive methods utilises microscopy. Glycogenated epithelial cells are stained and visualised under a microscope. There are two main staining techniques used in forensic casework: the Lugol iodine and periodic acid-Schiff (PAS) test. The principles of both techniques are very similar. Samples are isolated in water then placed on a slide to dry (via air dry, methanol) and stained with either the Lugol or PAS reagent [108-110]. Both the Lugol

iodine and PAS reagent are acidophilic and will turn the cytoplasm of the glycogenated epithelial cells dark brown and red respectively [65, 87, 111].

Jones and Leon (2004) performed a study exploring the glycogen content from different body fluid types (e.g. saliva, vaginal material) using both Lugol iodine and PAS tests. In both methods, they found a low glycogen content in saliva (1-8%) when compared to vaginal material (>10%) [110]. The use of these techniques could be particularly useful for woman who are menstruating as they have been reported to have high levels of glycogenated epithelial cells in the vagina [109]. The level of glycogen in saliva was also interesting, as both tests have shown higher glycogen levels than in saliva and semen [108, 109, 112, 113]. Both the Lugol iodine and PAS tests can be useful tools for indicating the presence of vaginal material. However the use of these tests is limited by the ability to confirm the body fluid origin of glycogenated epithelial cells (e.g. saliva, vaginal material, semen), particularly where little sample is available [114]. In addition, microscopy using Lugol and PAS staining can give false negatives as non-menstruating women do not always have glycogen epithelial cells present [65]. It is also destructive to the sample. Other methods have been developed to indicate the presence of vaginal material.

1.1.2.4.2 Immunoelectrophoretic tests

Another presumptive technique used to indicate the presence of vaginal material is immunoelectrophoretic tests [79, 97, 115, 116]. Vaginal material contains low levels of acid phosphatase. One immunoelectrophoretic method that has been used to separate vaginal acid phosphatase in forensic casework is the Laurell immunoelectrophoretic test [116]. The principle involves separation of acid phosphatase on a gel (e.g. polyacrylamide) using

isoelectric focusing (e.g. pH 4) [115, 116]. The gel is then stained with a dye e.g. Brentamine fast black. The bands are then visualised under a light source such as a UV light. In a gel, smaller molecules tend to migrate faster than larger molecules.

Ablett (1983) performed a study comparing the mobilities of acid phosphatase from vaginal fluid and semen using twelve isoforms of acid phosphatase. Characteristic bands were observed for vaginal acid phosphatase (VAP) and semen acid phosphatase (SAP) suggesting that Laurell immunoelectrophoretic separation could be used to differentiate the two [116].

Adams and Wraxall (1974) also performed a study on range of different acid phosphatases e.g. VAP, SAP and other body fluids and plants. Their findings showed that immunoelectrophoretic separation could be used to indicate the presence of VAP or SAP. Their work also highlighted the limitations of this test (e.g. false positives). For instance, AP was also detected in yeast and plants [79].

1.1.2.4.3 Alternative light sources

Other methods that have been used to indicate the presence of vaginal material are ALS. ALS is a presumptive technique that is rapid, non-destructive and easy to use. The principle of ALS is it will absorb light from shorter wavelengths to emit light at longer wavelengths; resulting in fluorescence of a sample. Vandenberg and van Oorschot (2006) explored Polilight® and its applications on forensically relevant body fluids, including vaginal material. Their findings showed that vaginal material gave similar fluorescence to saliva (450 nm wavelength) with orange goggles [26, 97].

Summary

The identification of vaginal material is crucial to forensic investigations, especially in cases of sexual assault. There are a few presumptive tests that have been used to indicate the presence of vaginal material. These tests target different components of vaginal material. One presumptive method described was microscopy with staining. Glycogenated cells are stained using the Lugol or PAS reagent. Its use in forensic casework is limited due to its lack of specificity towards other body fluids (e.g. saliva, vaginal material and semen) and women at different developmental stages (e.g. menstruating, non-menstruating). It is also destructive to samples [97].

Another presumptive method described was immunoelectrophoretic testing, which uses isoelectric focusing to indicate the presence of VAP. The technique can distinguish between VAP and SAP but not other body fluids (e.g. saliva) and plants. It is also destructive to the sample [97]. The last presumptive method described was ALS. This method utilised light to indicate the presence of vaginal material. ALS is rapid, easy to use and non-destructive. However its main limitation was that it lacked specificity towards other body fluids and household products. The main drawback of all of these methods is that there are currently no presumptive BFID tests that can distinguish vaginal material cells from skin cells.

1.1.2.5 Skin cells

Skin cells are also commonly encountered in forensic casework (e.g. trace DNA, digital penetration). Skin consists of three main layers: the hypodermis, dermis and epidermis [117]. The hypodermis is the innermost layer, comprising mainly of tissue and glands (e.g. sweat). Its main function is to help insulate the body. The dermis is the middle layer, consisting mainly of muscle, nerves, hair follicles, glands (e.g. oil and sweat), vessels (e.g. lymph, blood) and tissue

[117]. It is responsible for thermoregulation, sensation and protection of the skin. The epidermis is the outermost layer, consisting of keratinised skin cells (contains no nuclei). Its main role is to provide a protective barrier from the environment (e.g. bacteria, chemicals).

1.1.2.5.1 Microscopy

There are a few tests that have been used to indicate the presence of skin in forensic casework. One presumptive method that has been used is microscopy through staining (e.g. H&E). French *et al* (2008) performed a study developing a staining technique that could differentiate epithelial cells collected from the ear or elbow from other areas of the body e.g. oral and vaginal cavities [104]. They explored a number of different dyes (e.g. Dane's, Ayoub-Shklar) and fixtures (e.g. 100% methanol, 95% ethanol) to observe the morphology and colour of the cells. Dane's technique utilises one dye, which stains the nuclei and cytoplasm a red-orange colour. It has been used to target protein (e.g. keratin) and carbohydrates (e.g. mucin). Ayoub-Shklar method utilises two dyes, which stain nuclei blue and cytoplasm red. In their study they found that a combination of Dane's method and methanol could distinguish epithelial cells from all cell types. Epithelial cells from the ear and elbow gave an orange colour while the buccal cells gave red-orange colour and vaginal cells gave bright orange colour and blue hue [104].

French *et al* (2008) performed a further study using blind samples containing epithelial cells either from the ear, elbow, oral or vaginal cavities. They were able to successfully distinguish 95% of the samples tested based on red-stained keratin in the skin cells [104]. The studies by French *et al* (2008) have demonstrated the potential for using histological staining to identify and distinguish epithelial cells from different areas of the body. However their studies did not

explore skin collected from the mid-layer e.g. dermis, which could be of relevance where a suspect has produced a deep scratch on a victim during a sexual assault. This could affect the ability to distinguish different epithelial cells because of the skin from the dermis layer contains nuclei.

Summary

Skin cell identification can be very important in forensic investigations. It has been suggested by French *et al* (2008) that microscopy combined with histological staining could be powerful tool for indicating the presence epithelial cells from different cell types [104]. However their method may be limited by identification of epithelial cells collected from the epidermis. Furthermore there are currently no presumptive or confirmative methods available in forensic casework that can distinguish skin cells from cells collected from the oral and vaginal cavity.

Current BFID tests summary

There is a wide range of presumptive and confirmatory BFID tests that are currently be used in forensic casework. For blood these include the KM, LMG, luminol, Takayama, Teichmanns, Hb immunochromatographic tests. For saliva these include amylase, α-amylase tests microscopy with staining (e.g. H&E) and ALS tests. For semen these include microscopy with staining (e.g. nuclear fast red and picroindigocarmine, alkaline fuchsin or H&E dyes, AP, PSA and ALS tests. For vaginal material these include microscopy with staining (e.g. Lugol, PAS) and ALS tests. Skin tests include mainly microscopy with staining (e.g. Dane test).

Despite the large number of body fluid identification tests available there are still no tests that can differentiate trauma blood from menstrual blood. Similarly there are no tests that can currently differentiate epithelial cells collected from skin, saliva or vaginal material. The ability to be able to identify the origin of a body fluid can be particularly important to understanding the context of a case.

1.1.3 New BFID tests

There are a number of new techniques that are currently being developed to overcome these challenges within forensic casework. These techniques can be divided by their application: forensic or non-forensic (e.g. biology, chemistry, material science). Non-forensic methods include Raman, fluorescence, hyper spectral imaging and nanotechnology. Forensic methods include DNA methylation, messenger RNA and microRNA analysis.

1.1.3.1 Raman spectroscopy

One qualitative method that is currently being explored for forensic purposes is Raman spectroscopy. It was discovered and invented by Chandrashekhara Raman in the early 1920s [118]. The basic principle of Raman spectroscopy relies on inelastic scattering of molecules to obtain characteristic vibrational signatures of a sample in a solid, liquid or gas state [71]. There are three different mechanisms of scattering: Stokes Raman, anti-Stokes, and Rayleigh [118]. Stokes Raman scattering occurs when the frequency is higher than the energy exchanged between a molecules and photons. Conversely anti-Stokes occurs when the frequency is lower than the energy exchanged between a molecules and photons. Rayleigh scattering occurs when the frequency is the same as the energy exchanged between molecules and photons [118].

There is another form of scattering known as elastic scattering. This occurs when there is no change in frequency or energy between molecules and photons. The frequency of molecules are generally higher in samples containing functional groups e.g. alkenes, alkynes. Molecules are generally excited using a laser such as an argon ion (488.0 and 514.5 nm) [118].

Raman spectroscopy has been used in a wide range of fields including biology, chemistry, material science and pharmaceuticals [118]. Its use within forensic casework has only recently been explored. Virkler and Lednev (2008) performed a handful of studies exploring the use of Raman spectroscopy for identifying blood, saliva, vaginal material and semen. In their studies they utilised near infrared (NIR) Raman spectroscopy using a confocal Raman spectrophotometer [118]. The instrument as the name suggests, uses a combination of microscopy and Raman scattering to produce a characteristic chemical spectrum that is read through a charge coupled device (CCD) camera.

Virkler and Lednev (2008) performed a series of BFID studies using NIR Raman spectroscopy at a wavelength of 785 nm. In their work they characterised dried blood, saliva, vaginal material and semen from multiple sample donors. Blood gave a characteristic spectrum containing two peaks; one of haemoglobin and other of fibrin. Saliva also gave a unique spectrum containing three peaks similar to protein, acetate and saccharide and amino acid. Semen gave a characteristic spectrum with three peaks: choline, tyrosine and spermine. Vaginal material also showed a spectrum containing three peaks: protein, urea and lactic acid [65, 71, 97, 119-121]. Their findings highlighted the potential of using Raman spectroscopy as a BFID method in forensic casework. However their work also highlighted spectral variations observed due to the complex nature of body fluids.

Raman microscopy such as NIR Raman spectroscopy has a number of advantages over other presumptive and confirmatory BFID tests [65, 119]. One of its main advantages is its ability to penetrate and produce a three-dimensional image without any destruction to the sample. Casper *et al* (2003) performed a study exploring the composition of skin using confocal Raman spectroscopy. In their findings they were able to identify both the cellular (e.g. blood cells, epithelial cells from hypodermis, dermis and epidermis) and molecular structures of skin (e.g. amino acid levels and moisture in skin) [122]. Applications explored in Casper *et al* (2003) study may be very useful in resolving the issue of indicating the presence of epithelial cells from various cell types in forensic casework. For instance it could be used to indicate the presence vaginal material, skin or saliva based on the chemical composition of each body fluid type. Raman spectroscopy has also shown potential for indicating the presence of mixed, trace and animal body fluids, which could be very useful for casework [65, 118]. Other advantages include ease of use and portability [123, 124].

However despite these advantages, Raman spectroscopy is limited by the variations that can occur in the spectrum (e.g. body fluid heterogeneity and sample variation between individuals), which can cause false positive results [65, 118, 120]. Also unless Raman spectroscopy is coupled with another technique (e.g. NIR) it can be subject to fluorescence interference from other molecules [120].

1.1.3.2 Hyper spectral imaging

Another qualitative method that is currently being explored for forensic purposes is hyper spectral imaging (HSI). HSI combines spectroscopy and imaging to obtain the chemical composition and distribution of samples [125]. The principle of this technique is based on a

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cubic (hypercubic) structure, which consists of three planes: x, y and λ (one wavelength). Samples undergo temporal scanning, which involves stacking of narrow spectral bands from x, y and λ planes to form three-dimensional images. Samples can be scanned according to points, lines or area. The point scanning imaging system (whiskbroom), targets two points along the spatial planes (x, y) to produce a hypercube. The line scanning system (pushbroom), targets one line along the spatial (x,y) axes and one line along the spectral (λ) axes to produce the cube. The area scanning system (staredown), targets a sequence of images along the spatial planes (x, y) to produce the cube [125]. Samples are visualised along the electromagnetic spectrum (e.g. NIR, IR, UV).

HSI has been used for a wide range of applications including satellite imaging, medicine and pharmaceuticals [125]. Its applications within forensic casework such as aged samples or trace samples are recent [126, 127]. Bo *et al* (2013) performed an interesting study exploring the spectral changes of dried equine blood over a 30-day period using pushbroom HSI. In their work they were able to establish the average age of bloodstains (±1.2 days) through spectroscopic changes observed when blood ages. Both α- and ß– units of haemoglobin will oxidise (HbO_2) into meta-haemoglobin (met-Hb) then hemichrome (HC) [127]. Their findings showed that the first oxidation stage, met-Hb occurred in ≤ 3 days while second oxidation stage, HC occurred in ≥ 30 days [127]

Studies such as Bo *et al* (2013) are very useful for forensic casework as they require no contact with the sample, are non-destructive and provide both spectral and spatial information of a sample. However they did not compare spectral signatures with other commonly encountered body fluids, which is also important for BFID in forensic casework. The use of HSI for BFID may be limited by the complex nature of body fluids. The interpretation of casework samples

may add an additional challenge as samples exposed to different environmental changes (e.g. temperature, humidity) can affect the final spectra.

1.1.3.3 Nanoparticles

Another emerging technique within the forensic community is nanotechnology [128-130]. Nanotechnology can be defined as a technique that can manipulate samples at cellular or molecular levels. It uses nanoparticles to target the sample of interest (e.g. biological, chemical, mechanical, optical, physical). Nanoparticles are generally 1-100 nm in size and are also often coated with metals (e.g. Au, Ag) or polymers (e.g. chitosan, silica) to further target properties [128]. Nanoparticles have been used for a number of different applications including biology, chemistry and biomedical science [128]. Its use within forensic casework has been very limited e.g. toxicology, fingerprints.

A study by Kestell and Gabriel (2010) explored the use of gold nanoparticles to identify drugs and their metabolites in urine using surface-enhanced Raman spectroscopy (SERS). In their work they spiked benzodiazepine and metabolites (e.g. 1,4-benzodiazepam) and tested them against different agents to enhance the interaction and sensitivity of the spectra [130]. They found that magnesium chloride ($MgCl_2$) gave the highest sensitivity amongst the spiked urine samples tested. Their findings could be especially useful in cases of drug-facilitated sexual assaults. Further studies could also be performed with other body fluids such as saliva and blood. SERS is a useful technique as it is more sensitive than Raman spectroscopy alone. However the performance of SERS is limited by selecting the appropriate aggregating agent.

1.1.3.4 DNA methylation

Another technique that is currently of forensic interest is DNA methylation [131-134]. The principle of this technique utilises the addition of a methyl group (-CH$_3$) onto the 5' position of cytosine using DNA methyltransferases [135, 136]. DNA methylation generally occurs where cytosine-phosphate-guanine (CpGs) are present. CpGs are where a cytosine is directly preceded by guanine. CpGs are often found clustered along the DNA sequence in areas known as CpG islands. CpG islands can be completely methylated or unmethylated. They can also contain a combination of the two. DNA methylation plays a fundamental role human development. It will regulate gene expression through methylated and unmethylated CpG sites. The majority of CpGs are methylated in humans (e.g. 60-90%) [136].

DNA methylation patterns can be determined using several different methods [136-138]. One common technique involves chemical modification of cytosine. The principle utilises sodium bisulfite to convert unmethylated cytosine into uracil while keeping the methylated sites unaffected. Samples then undergo PCR during which, any converted regions containing uracil will be recognised as thymine. DNA methylation patterns can then be identified through a comparison of these CpG sites and the original target sequence [139]. One method that can be used to identify these regions is methylation-specific PCR (MSP). It uses a set of methylated or unmethylated primers to produce PCR amplicons. Bisulfite treatment is a sensitive, flexible and versatile technique [140]. It can be used for either qualitative or quantitative assessment and can be applied to a wide range of applications. Its main drawback is the effectiveness of the bisulfite treatment. Incomplete conversion of unmethylated cytosines can result in false positives [141]. The chemical alteration of DNA sequences may result in inefficient priming resulting in non-specific product or low PCR yields [136].

Another common method utilises methylation-sensitive restriction enzymes [136, 140]. The principle of this technique involves digestion of methylated CpG sites along a target DNA sequence. Samples then undergo PCR where primers will bind to the target regions. The forward primer will bind left of the enzyme recognition site while the reverse primer will bind right of this site. Fully digested samples will be fragmented and will show no amplification during PCR while samples that have not digested will show amplification. A commonly used methylation-sensitive restriction enzyme is the HhaI enzyme [142]. It targets the sequence 5'…GCGC…3', which contains a methylated cytosine directly after the first guanine in the 5' direction [143]. Methylation-sensitive restriction enzymes are an inexpensive, sensitive and method for determining methylation patterns. They also can be multiplexed which can be useful in BFID. However the enzymes ability to successfully recognise and cleave the target DNA sequence and CpG sites limits this technique.

Methylation patterns have been used in a wide range of fields including chemistry, biology and medicine [140]. Its use in the forensic genetics is relatively new [131, 137, 142, 144, 145]. Frumkin *et al* (2011) performed the first study exploring the use of DNA methylation for BFID purposes. They analysed a number of forensically relevant body fluids such as blood, saliva, semen, skin and vaginal secretions. They utilised DNA isolation, methylation-sensitive restriction enzyme HhaI and PCR to determine methylation patterns. Their BFID panel included fifteen loci and three internal controls. Frumkin *et al* were able to successfully determine unique methylation patterns for each body fluid type through a comparison of peak height ratios [142]. Their findings showed the potential for using methylation patterns to identify body fluids within forensic casework. However their technique is limited by the need

to perform DNA profiling and methylation separately, which results in higher sample consumption.

Wasserstrom *et al* (2012) performed a study expanding on Frumkin *et al* (2011) work. They explored the applications of DNA methylation in forensic casework using a kit they developed called the Nucleix DNA source identifier (DSI)-semen kit [131, 146]. It uses the same principles and methods as in their earlier BFID study [142]. They tested the sensitivity and specificity of this kit using casework samples containing body fluids such as semen, blood, saliva and vaginal material. In their work they were able to successfully identify and differentiate semen from these body fluids. They findings were comparable to microscopy of sperm cells. This technique is limited in its use towards mixed body fluids, as it cannot distinguish semen when it is a minor component.

DNA methylation patterns have also shown potential use in trace body fluids. Xu *et al* (2012) performed a study on trace bloodstains using a modified bisulfite sequencing method. They utilised a combination of Qiagen's micro kit and EpiTect bisulfite kit to isolate and treat samples respectively [147]. Samples then underwent methylation-specific PCR (MSP) and direct sequencing. Their findings showed that their modified method gave a high conversion rate in the differentially methylated region of the SNRPN gene [148, 149]. Thus demonstrating its potential application for trace body fluids. However their technique is limited to trace blood samples. Further work needs to be performed to determine whether this method can be used for forensic casework.

DNA methylation patterns have also been used for a number of other forensic applications. For instance it has been used to differentiate monozygotic twins [137, 150, 151]. The DNA

methylation pattern in monozygotic twins is identical at birth. However differences in lifestyle factors, growth, diet and health can affect their methylation patterns. Li *et al* (2013) performed an interesting study exploring the difference in methylation profiles of peripheral blood between monozygotic twins. They collected blood samples from a total of 22 female and male monozygotic twins aged from 17 to 74 years old [152]. Blood underwent DNA isolation and bisulfite treatment. They used a Beadchip, consisting of 27,578 CpG sites to establish methylation sites in the blood samples [152]. In their study, they identified 92 CpG sites that were significantly different between each pair monozygotic twins. Their findings demonstrated the potential of using DNA methylation patterns for BFID in forensic casework. However the applications of their technique are limited by the need to explore methylation patterns within other body fluid types such as semen and vaginal material.

The use of epigenetic markers to identify body fluids has shown potential within forensic casework via characteristic methylation patterns. The techniques used to determine DNA methylation patterns also provide additional advantages to forensic investigators. Both the bisulfite treatment and methylation n-restriction sensitive enzyme are relatively sensitive, flexible and versatile. However both techniques are limited in their reliability. The bisulfite treatment is dependent on complete conversion of unmethylated cytosines to uracil. Similarly, the methylation-restriction sensitive enzymes are dependent on their ability to correctly recognise and cleave the target site. It has been demonstrated that a combination of techniques can enhance the reliability of a result. However it is not ideal. DNA methylation is perhaps not the best application for forensic casework as samples are often very limited in quality and quantity.

1.1.3.5 Messenger RNA analysis

Another confirmative method used for BFID is messenger RNA (mRNA) profiling [153-158]. The principle of this method utilises gene specific expressions to identify body fluids. Initially, mRNA is transcribed from DNA after which it is processed. There are several different mechanisms for processing mRNA. One of the main mechanisms is through splicing. Splicing is the removal of introns (non-coding sequences). Messenger RNA can code for single or multiple proteins depending on the method of splicing. Messenger RNA makes up approximately 4% of total RNA [154]. Mature mRNA from the 5' to 3' direction contain a 5 guanidine methyl cap, a 5' untranslated region, a coding region, a 3' UTR region and a poly(A) tail.

Messenger RNA gene expression levels can be determined using a number of different methods. Some of the most popular mRNA analysis methods include Northern blot analysis, in situ hybridization, microarrays, nuclease-protection assays and RT-PCR. A common mRNA analysis method for BFID utilises RT-PCR. In order to analyse mRNA it must first be isolated. Two common methods used to isolate RNA: solid-phase and liquid-liquid extractions [4]. Solid-phase extractions utilise a solid platform and a combination of high and low ionic buffers to isolate and purify RNA. Liquid-liquid phase methods (organic extractions) use a liquid platform and a combination of different buffers and alcohols to isolate and purify RNA. There are a number of methods and kits available for isolating RNA in body fluids. A common solid-phase RNA isolation kit is Qiagen's RNeasy mini kit, which uses a silica-gel membrane and high and low ionic buffers. A commonly used organic method is the guanidine isothiocyanate phenol/chloroform extraction with isopropanol precipitation [159]. RNA extracts then undergo reverse transcription (RT). Reverse transcription is process where mRNA is replicated to form

a single stranded molecule called complementary DNA (cDNA). RT is performed using a combination of buffers, primers, deoxynucleotide triphosphates (dNTP's), reverse transcriptase, RNase inhibitor and PCR grade water. An example of a RT kit is Ambion's RETROscript® kit which utilises components such as Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT).

Samples then undergo PCR. There are a number of different PCR technologies that can be used to amplify RT products. The two main PCR technologies used in mRNA analysis are end-point and real-time PCR [160]. Both techniques have their advantages and disadvantages. End-point PCR is an effective method for detecting the presence or absence of particular transcripts. However it cannot measure the gene expression levels during a reaction. Real-time PCR is a more accurate and sensitive method of analysis. It measures gene expression levels with reference genes. Reference genes are ubiquitous and are responsible for the basic functioning of cells. A common reference gene used for mRNA BFID is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [160, 161]. PCR products are then separated using electrophoresis. In mRNA analysis samples are generally separated in gels or capillaries. The general principle involves the separation of molecules based on size and charge through the application of an electrical field. Commonly used capillary electrophoresis instruments include Life Technologies 310, 3130 and 3500 genetic analysers. Messenger RNA analysis has been used in a broad range of field including chemistry, biology and biomedical science. Over the past decade there have been an increasing number of forensic research groups that have explored mRNA profiling for BFID [4, 18, 27, 155, 156, 160-184]. The main reasoning behind this includes their sensitive and specific expression within different body fluids and tissues.

Bauer and Patzelt (2002) performed the first study exploring mRNA analysis as a potential body fluid identification method in forensic casework. They focused primarily on developing a co-isolation technique for DNA and RNA. They used blood (venous and menstrual) and epithelial cells for their study. Samples underwent mRNA isolation, RT, PCR and gel electrophoresis. In their work they developed a suitable co-isolation method for mRNA analysis. However their study was limited to dried bloodstains and epithelial cells. Further work addressing the stability of mRNA would be useful.

Juusola and Ballantyne (2003) also performed a study exploring the potential of mRNA BFID using dried blood, saliva and semen swabs. Samples underwent total RNA isolation, cDNA synthesis, PCR and gel electrophoresis. In their study they identified suitable reference genes (e.g. GAPDH) for all of the tested body fluids. They also identified tissue-specific genes for saliva [155]. Juusola and Ballantyne (2005) expanded their mRNA panel to include blood, saliva, semen and vaginal secretions. Samples underwent similar processing steps before except they separated samples using capillary electrophoresis. In their work they developed a multiplex RT-PCR system for indicating the presence of blood, saliva, semen and vaginal secretions in both single and mixed samples [156]. In a further study, Juusola and Ballantyne (2007) utilised their multiplex RT-PCR system containing body fluid specific markers and reference genes. They were able to successfully indicate the presence of different body fluids [161]. Their findings from the three studies demonstrate the potential of using mRNA as BFID tool in forensic investigations. The technique was shown to be sensitive, specific and robust. The main drawback is the stability of mRNA when exposed to environmental factors. Additionally this technique has the potential to produce false positives if sample input is limited.

Fleming and Harbison (2010) also developed an mRNA multiplex system for single and mixed dried blood (peripheral, menstrual), saliva and semen stains. They also explored the sensitivity and specificity of this test. Samples underwent co-isolation, RT, PCR and capillary electrophoresis. In the majority of their studies they were able to obtain both DNA and mRNA profiles from both single and mixed samples [4]. Their findings also showed the test to be both sensitive and specific. Their overall findings demonstrated the potential of using mRNA BFID in forensic casework. However their studies did not test the stability of mRNA, which is one of the main issues associated with this technique.

Lindenbergh *et al* (2012) conducted a study to develop an mRNA BFID test for dried blood, saliva, skin and semen stains. Messenger RNA profiling was performed using a series of co-isolation, reverse transcription, PCR and capillary electrophoresis steps. Their main study explored the use of candidate body fluid specific markers and reference genes. Additional studies included stability of aged body fluids. In their studies they were able to successfully develop a 19-plex mRNA multiplex system for indicating the presence of body fluids including blood, saliva and semen. In addition they were also able to obtain full DNA profiles from these samples. They were also able to demonstrate the use of this mRNA-multiplex on aged body fluids [182]. Lindenbergh *et al* (2013) performed a further study exploring the use of their mRNA-multiplex system in forensic casework. They utilised a similar protocol to the previous study. However they included a step-by-step procedure for potential interpretational guidelines of an mRNA profile e.g. research perform analysis with clear interpretational guidelines with no knowledge in the context of the case followed by interpretation and collation of profiles with reporting officer and researchers [168]. In their study they were able to successfully establish a preliminary approach to the interpretation of RNA profiles within forensic casework. The authors notes that additional factors will need to be taken into account during

interpretation e.g. chain of custody [168]. Their findings from both studies highlight the potential of using an mRNA BFID method in forensic casework. Their work showed relatively high sensitivity and specificity. However one of the main limitations of this work, apart from developing this test is the issue of stability of mRNA. In Lindenbergh *et al* (2012) study, they were able to obtain both DNA and mRNA profiles from stains. However this work utilised dried stains in controlled humid-free conditions.

Messenger RNA BFID has also been explored in collaborative exercises performed by the European DNA profiling (EDNAP) group. A total of 24 different forensic genetic laboratories participated during these studies. They covered a number of forensically relevant body fluids including blood, saliva, semen, skin and most recently, vaginal material and menstrual blood [173-177]. They performed these studies using a wide range of commercially available isolation, reverse transcription, amplification kits of their choice. During their studies they developed multiplex systems for each body fluid. In their first and second collaborative exercise they identified a highly sensitive duplex and moderately sensitive pentaplex of mRNA markers for bloodstains [175, 176]. In their third exercise they identified sensitive mRNA triplex for saliva and pentaplex semen [173]. In their fourth and fifth exercise they identified potential mRNA markers for vaginal material and two triplexes for menstrual blood [174]. In all of their studies they were able to indicate the presence of dried blood, saliva, semen, vaginal material and menstrual blood using different multiplex of body fluid identification markers. They also explored the stability of mRNA in many of these body fluids stored at room temperature or freezing. The majority of the laboratories were also able to obtain full DNA profiles or partial profile with one or two allelic dropouts from these samples. They were also able to obtain RNA and DNA profiles from aged body fluids. Their studies using a wide range of methodologies and demonstrated the versatility and potential ease of utilising mRNA BFID

into current forensic genetic laboratories. However their studies were limited by the use of multiple technologies. The findings from these groups could not always be directly compared to one another. Furthermore their stability studies were limited to the use of dried stains and did not include factors such as humidity.

Roeder and Haas (2013) also performed a smaller study on mRNA BFID using dried blood, saliva, skin, semen and vaginal material swabs. Samples were analysed through single or co-isolation, RT, PCR and capillary electrophoresis. Their initial approach in developing a mRNA BFID test was to use one or two mRNA markers to indicate the presence of a particular body fluid [183]. However they found that this could give lead to false positives. Therefore they incorporated a minimum of five body fluid specific mRNA markers to identify each body fluid. They also included reference genes in their study. Their findings showed that the use of additional mRNA markers gave greater accuracy in BFID and lowered the number of false positives. However their findings also show that mRNA BFID could still produce false positives, which can be problematic where sample amount is limited. Furthermore their study is also limited by the stability of mRNA when exposed to environmental factors.

Xu *et al* (2014) recently performed an mRNA BFID study on a wide range of body fluids including blood (circulatory, menstrual), saliva, semen vaginal secretions, sweat, urine and nasal secretions [169]. Samples underwent co-isolation, RT, PCR and capillary electrophoresis. Their initial panel of candidate BFID markers were collated from many of the other mRNA research groups including Juusola *et al* 2007, Fleming and Harbison 2010, Lindenbergh *et al* 2012. From their studies they were able to develop a sensitive and specific mRNA 12-plex system containing body fluid specific markers and reference genes. They were also able to obtain full DNA profiles from these body fluids. Their findings highlight one of

the main advantages of this technique, which is the ability to multiplex markers. However their work is still limited by the stability of mRNA.

Messenger RNA analysis has shown potential as a BFID method in forensic casework. The technique is both sensitive and specific and can be applied to mixtures. It can also be multiplexed, which can reduce overall cost and time. It also offers a quality that many of the current BFID do not have, which is compatibility with current DNA profiling techniques. There are also drawbacks to this technique including the potential to generate false positive results. The main drawback is the instability of mRNA when exposed to external factors such as UV-light, temperature, dust and humidity. Despite these challenges mRNA analysis is still being studied extensively as a BFID method in forensic casework. In fact, a number of strategies have been used to implement mRNA profiling into current DNA profiling methods. Many research groups have utilised co-analysis to obtain both DNA and mRNA profiles [157, 159, 164, 173-175, 182, 185]. In forensic casework, samples are often limited in both quality and quantity. Thus a number of research groups have also explored co-isolation of DNA and mRNA as a means of maximising sample.

Bauer and Patzelt (2003) performed a study on co-isolation of DNA and mRNA using dried venous blood, menstrual blood and semen stains. Co-isolation of DNA and mRNA was performed using an organic extraction method with precipitation [164-166]. They utilised phenol/chloroform to separate both aqueous and organic phases. RNA was removed from the aqueous phase. They also compared their study with a standard DNA isolation method used in routine forensic laboratories, the Chelex-100® extraction [186]. The principle of this method utilises the alkalinity of chelating resin and boiling to isolate and purify DNA. In the majority of their work they were able to obtain DNA and mRNA profiles using the co-isolation protocol

[166]. Their findings also showed that their co-isolation strategy was not as sensitive as the standard DNA isolation protocol tested. However it was still sensitive enough to use on casework samples.

Alvarez *et al* (2004) performed a co-isolation study of DNA and mRNA using dried blood, saliva, semen and vaginal secretion swabs and cloth [157]. They utilised an organic isolation method with precipitation to co-isolate DNA and RNA. Samples were treated with an extraction solution containing Tris-HCl, EDTA, SDS and DTT. Phenol:chloroform (5:1 pH 4.5) was then added to separate both organic and aqueous phases. DNA and total RNA were collected from the aqueous phase. DNA fractions were separated through overnight precipitation using 100% ethanol. Similarly RNA fractions were separated using isopropanol. Samples then underwent RT, PCR and gel electrophoresis. DNA extracts underwent PCR and gel electrophoresis. RNA extracts underwent RT, PCR and gel electrophoresis. In their study they also compared a standard DNA isolation using an organic solvent method and total RNA isolation method using guanidine isothiocyanate-phenol:chloroform method [155, 159, 187]. Their study showed efficient separation of DNA and RNA using the co-isolation method. They also able to obtain both DNA and mRNA profiles from the samples tested. The co-isolation results were also comparable to the standard DNA and total RNA isolation methods. Their study demonstrates one of the main advantages of utilising a co-isolation technique in forensic casework, the use of minimal sample consumption. Often if samples are limited, they will only undergo DNA profiling [164]. However their study also utilises an overnight precipitation step, which can be costly in terms of overall analysis time. Furthermore there technique requires separation of DNA and RNA fractions post extraction, which can lower the evidential strength of associating a particular body fluid with a DNA profile.

Fleming and Harbison (2010) performed a side study on co-isolation of DNA and mRNA during an mRNA BFID study using blood (peripheral, menstrual), saliva and semen stains. They modified a total RNA method used by Juusola and Ballantyne (2005) to co-isolate DNA and RNA. The original protocol can be divided into two stages: organic extraction using guanidine isothiocyanate phenol:chloroform and precipitation using isopropanol [4, 159]. Co-isolation of DNA and RNA was achieved by initially replacing one of the components in the guanidine isothiocyanate denaturing solution (e.g. 2-mercaptoethanol) with DTT. DNA was precipitated from both the organic and interphase using 100% ethanol. DNA fractions underwent PCR and capillary electrophoresis. Their findings from their study showed that the majority of DNA was detected in the aqueous phase and did not agree with Bauer and Patzelt (2004) who recovered DNA in the organic phase. Fleming and Harbison (2010) modified their protocol further by precipitating DNA and total RNA from the aqueous phase using 2-propanol. DNA and total RNA were then separated using enzymatic digestion before performing DNA and mRNA analysis. From their study they were able to successfully obtain both DNA and mRNA profiles from the tested body fluids. Their study demonstrated one of the main advantages of using co-isolation in forensic casework, which includes reduced sample consumption. However their technique also required an overnight treatment of DNA and total RNA, which can be costly in terms of time in forensic casework. Perhaps most important, their method requires separation of DNA and mRNA post-extraction, which can reduce the ability to associate a DNA profile with a particular body fluid.

Bowden *et al* (2011) took an interesting approach on co-isolation using dried blood, saliva, semen and vaginal fluid swabs. They used a combination of Promega's DNA IQ system and Zymo's Research Mini Isolation™ II kit to co-isolate DNA and mRNA from the samples [185]. DNA isolation was performed using Promega's silica coated magnetic beads. Total

RNA was then isolated from the DNA lysis buffer discard using Zymo's isolation kit. Two separate extracts containing either DNA or total RNA were then used to perform STR or mRNA analysis respectively. They also compared this method with Fleming and Harbison (2010) organic co-extraction method for DNA and mRNA. Bowden *et al* (2011) was able to obtain both DNA and mRNA profiles using their modified approach. They also obtained comparable results to Fleming and Harbison (2010) study. Their work in this study provides a few advantages over the organic co-isolation method [4]. Their use of the discarded lysis and binding buffers to isolate total RNA reduces overall sample consumption. However their need for an additional kit to isolate total RNA adds to the total sample processing time. Their use of magnetic bead technology can be advantageous to casework samples as the beads can offer 360º binding. However this modified approach does require separation of both DNA and total RNA extracts (e.g. lysis/binding step), which can lower the evidential value in court. Furthermore the authors describe the potential for automation of their technique. However given the nature of casework samples this may not be necessary.

A number of other groups have incorporated co-isolation method during the development of an mRNA BFID test for forensic casework. For instance in the BFID collaborative exercises performed by the European DNA profiling (EDNAP) group. They utilised samples from blood, saliva, semen, skin and most recently, vaginal material and menstrual blood [173-175]. The majority of the laboratories performed co-isolation using Qiagen's AllPrep RNA/DNA Mini kit [188]. This kit utilised a silica-gel membrane to isolate DNA. Total RNA was then separated using the DNA wash. Thus resulting in two different extracts then underwent DNA or mRNA profiling separately. In their studies they were able to obtain full DNA and mRNA profiles from their body fluids tested. The advantages of using this technique include reduced

sample consumption. However their method requires separation of the sample, which can lower the ability to associate a DNA profile with its body fluid origin.

Many of the co-isolation methods described both separate DNA and total RNA fractions post-extraction or during the extraction procedure [173-175, 189]. One research group even explored different mediums to co-isolate mRNA and DNA from the same stain e.g. magnetic beads [185]. Also the majority of co-isolation efforts using magnetic beads have been more of non-forensic interest. The development of a co-isolation method whether neither fractions (DNA and RNA) are separated could offer a number of advantages over the co-isolation techniques utilised by other groups [4, 173-175, 185]. The main advantage would be the ability to closely associate a DNA profile with a body fluid. In addition, the use of a single co-isolation method would reduce the consumption of sample and time, which are often crucial factors in forensic casework.

Despite the recent collaborative efforts that have gone into developing mRNA profiling for current forensic casework the question remains of whether mRNA profiling is the best-suited method for BFID [4, 168, 170, 172-177, 184]. As described previously one of the main drawbacks of mRNA is its stability. Messenger RNA degradation plays an important role in normal living cells. It helps regulate the amount of mRNA translated and thus the amount of gene produced in a cell. The lifetime of an mRNA depends on its role in the body. Some mRNA will be present for minutes while other may be present for hours or even days. For instance the half-life of c-*fos* message is 15 min while the half-life of ß-globin mRNA is 24 hours [190]. There are two main regulatory mechanisms for mRNA [154, 190]. One mechanism utilises AU rich elements (ARE) in the 3'UTR of mRNA. ARE's contain motifs such as AUUUA and range between 50-150 nt in length [191]. ARE-binding proteins will bind

to the 3' UTR regions and will either promote translation or degradation of the mRNA. The other mechanism involves removal of the methylguanosine (m^7G) cap in the 5' end of mRNA [190]. The removal of the m^7G cap will allow for degradation of the mRNA. Messenger RNA degradation in biological stains is more variable and is strongly dictated by external factors from the environment such as bacteria, ribonucleases (RNases), temperature, light and humidity [154]. Therefore stability in a forensic context can be defined as a sample that survives from time of deposition and to time of collection. A number of groups have explored the stability of mRNA in body fluids [27, 154, 164, 173-175].

Setzer *et al* (2008) performed a comprehensive study exploring the stability of mRNA in blood, saliva, semen and vaginal secretions under different environmental conditions. Blood, saliva and semen were aliquot and dried onto cotton cloth. Vaginal secretions were collected onto polyester swabs [192]. Samples were stored indoors at room temperature and exposed to light, dark, humid or non-humid environments. They were also stored outdoors and exposed to humidity, light and heat with or without rain. All samples were exposed to these conditions over a period of 0 to 547 days [158]. Samples underwent total RNA isolation, RT, PCR and gel electrophoresis. In their indoor studies blood was stable in all room temperature conditions up to 365 days. Saliva was also stable in all room temperature conditions for 365 days except in UV-light, where it was detected for 180 days. Vaginal secretions were more variable in the room temperature conditions and were detected from 90 to 547 days. In their outdoor studies protected from rain, vaginal secretions were detected for 180 days, blood for 30 days and saliva and semen for seven days. In their outdoor studies exposed to rain, samples showed a significant decrease in mRNA stability e.g. semen was detected between one to seven days, blood and vaginal secretions for three days and saliva for one day [158]. The findings from their outdoor studies may be a result of intra- and extracellular activities e.g. osmosis, bacteria

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and RNases. Thus indicating that mRNA BFID may not be the most appropriate method for forensic casework.

Zubakov *et al* (2008 and 2009) performed two studies on the stability of mRNA in blood and saliva stains. Blood and saliva were aliquoted onto cotton swabs and dried at room temperature. In Zubakov *et al* (2008) study samples were stored over a period of 0 to 180 days in a dust and humid-free environment. Samples were stored under the same conditions in Zubakov *et al* (2009) but over a period of 2-6 years and 13-16 years. All samples were exposed to natural daylight in the laboratory. Samples were isolated for total RNA using Qiagen's RNeasy kit. Blood and saliva specific mRNA markers were then selected using microarray and gene expression data analysis. Samples then underwent RT followed by qPCR utilising SYBR® green chemistry. They identified 14 mRNA markers that were stable in blood stored over a 180-day and 16-year period and saliva over the 180-day and 6-year period [193, 194]. Both studies provide useful insight into the stability of mRNA in blood and saliva when samples are dried and tested under relatively controlled conditions e.g. light. However none of their work addresses the stability of mRNA under more adverse conditions such as prolonged exposure to temperature and humidity.

Kohlmeier and Schneider (2011) performed a study on the stability of mRNA in blood when dried onto different substrates (e.g. carpet, fabric, jeans, wallpaper, leather and wood). Bloodstains were stored for 23 years in a dark, dust and humid-free environment. Samples were co-extracted for total RNA and DNA using Qiagen's AllPrep DNA/RNA Mini kit. RNA fractions underwent cDNA synthesis using SuperScript III RT kit and qPCR using a panel of body fluid specific markers adapted from Juusola *et al* (2005) and Haas *et al* (2009). DNA fractions underwent DNA quantification using Life Technologies Quantifiler kit and amplified

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using Life Technologies AmpFlSTR® SEfiler Plus™ kit. Their findings showed stability on all the substrates tested up to 23 years. Also full DNA profiles were obtained in all blood samples. Haemoglobin ß (HBB) is currently being explored as a marker for forensic casework [175]. Haas *et al* (2011) findings provide additional insight to HBB. However this study does not address the stability of HBB on these substrates when exposed to prolonged periods of humidity or UV-light, which can be relevant to casework samples [195].

Additionally there have been several other groups that have explored the stability of mRNA in their mRNA profiling development work. Haas *et al* (2011) performed a side study exploring mRNA stability in blood under different environmental conditions. Their study was performed over a shorter period of time (1 day to 1 year) and with a single type of body fluid where as Setzer *et al* (2008) stored blood, saliva, semen and vaginal secretions over 547 days. However Haas *et al* (2011) work was more comprehensive as they recorded details such as the amount of rain, humidity level and temperature range into their findings [153]. Additionally they explored high heat (37 ℃) which none of the other groups exploring stability in blood performed. Samples underwent co-isolation. RNA fractions underwent RT, PCR while DNA fractions underwent PCR and capillary electrophoresis. Interestingly samples that had been covered were detected in a shorter time frame (3 days) than the uncovered samples (1 month), which was the reverse of what Setzer *et al* (2008) found. DNA profiles were obtained from the majority of covered samples however none were obtained in the uncovered samples. The authors emphasize the potential of mRNA BFID. However this study also showcases the instability of mRNA. Messenger RNA profiling may not be best method for BFID.

Jakubowska *et al* (2013) performed a side study exploring the stability of mRNA in vaginal fluid and menstrual blood. Vaginal fluid was collected onto cotton swabs or sanitary towels

and stored over a period of 0 to 12 years at room temperature or 0 to 18 years in the freezer. Menstrual blood was collected onto different substrates (jeans, cotton or leather) and stored for two years at room temperature. Samples underwent total RNA isolation, reverse transcription, PCR and capillary electrophoresis. In their work they detected mRNA in menstrual blood stored for one year at room temperature. They also detected mRNA in vaginal fluid stored for two years at room temperature and 18 years when frozen [178]. Their findings on mRNA stability agreed with Setzer *et al* (2008) who also interestingly detected mRNA in vaginal stains aged over a period of 547 days. Jawbowski *et al* (2013) provides additional insight on the stability of mRNA in vaginal stains after long-term storage in the freezer, which none of the previous studies had explored. This information could be useful for casework samples collected during colder months. However their findings were limited to controlled parameters e.g. temperature. It would be interesting to see the effect of mRNA stability over time during which samples are exposed to different temperatures for set time intervals.

Messenger RNA has been shown to be stable in a range of forensically relevant body fluids such as blood, saliva, semen and vaginal material. High stability was observed in samples that had been stored indoors and exposed to different temperature, lighting and humidity. Rapid degradation was observed in samples that had been stored outdoors, particularly when exposed to rain. Messenger RNA profiling may not be the best tool for identifying forensic casework samples as they are often exposed to a variety of different environmental conditions that can degrade mRNA. A potentially more suited and stable candidate for BFID is microRNA.

1.2 MicroRNA

MicroRNAs (miRNA, miR) are short sequences of RNA between 18-25 nucleotides in length. They are found in the non-coding regions of DNA. They are often clustered and conserved along the intergenic regions of the human genome [196]. Although not as common, they can also be found within the intronic regions of the human genome [197, 198]. MicroRNAs found within the same clusters or "families" often exhibit similar functional roles within the human body [199]. From an evolutionary standpoint it is thought that miRNAs are clustered along the genome as a way of preserving information from generation to generation [200-202].

There are two main types of small non-coding RNAs: miRNA and short interfering RNA (siRNA) [203]. Both siRNAs and miRNAs play a key role in negative-regulation of genes in the human body. They play essential roles in biological processes including cell growth, proliferation, differentiation, glucose homeostasis, fat metabolism and immune regulation [204]. They also play important roles in various cancers, neurodegenerative disorders and disease (e.g. infections, autoimmune, cardiovascular) [204-206].

1.2.1 Biogenesis
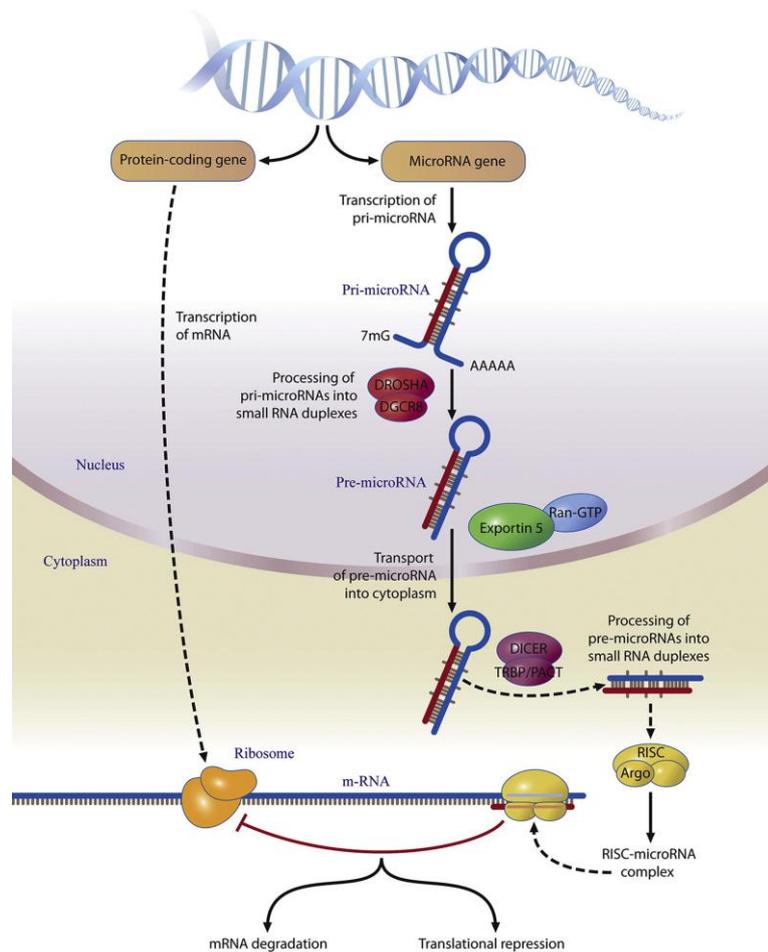
Figure 1. Showing the biogenesis of microRNA in eukaryotic cells. Image taken from Sand et al (2010).

The biogenesis of microRNA is shown in Figure 1 [207-209]. Mature miRNA can form by first cleaving the primary miRNA (pri-miRNA) transcript with enzyme Drosha and co-factor DGCR8. The precursor miRNA (pre-miRNA) will then be transported out of the nucleus and into the cytoplasm using enzyme Exportin 5 and co-factor Ran-GTP. There, pre-miR transcript will be cleaved a second time to remove the stem-loop using enzyme Dicer and co-factor TRBP/PACT [210, 211]. One of the miRNA strands will then degenerate. The remaining strand will bind with the Argonaut protein and form an RNA inducing silence complex (RISC).

1.2.2 Discovery

MicroRNAs were first discovered about a decade ago [208]. Gene inhibition was first explored by Van der Krol *et al* (1990) during a study with flavonoid genes in petunias. They used anthocyanins (pigmented) and flavonols (non-pigmented) petunias for their study. They evaluated two flavonoid genes: dihydroflavonol-4-reductase (DFR) and chalcone synthase (CHS) to observe the effects of flower pigmentation [212]. In the majority of their work, the introduction of DFR and antisense CHS mRNA into the *Petunia hybrida gene* did not give a noticeable change in flower pigmentation. However in 25% of these samples, they observed a dramatic reduction in gene expression and flower pigmentation. Their study inadvertently stumbled upon a potential mechanism of gene silencing.

Fire *et al* (1998) explored this gene inhibition mechanism further in a study using *Caenorhabditis elegans* (*C. elegans*). They injected *C. elegans* with single stranded RNA (ssRNA) and double stranded RNA (dsRNA). They utilised genes with well-characterised phenotypes for their study including the unc-22 gene. The unc-22 gene codes for myofilament protein found in striated muscle cells [213]. Partial inhibition of this gene will cause muscle twitches where as full inhibition will result in impaired mobility and structural defects in the muscle [213]. Injection with ssRNA (antisense or sense), showed reduced activity in *C. elegans* while injection with dsRNA resulted in complete loss of muscle activity. Fire *et al* (1998) work was crucial to understanding the gene inhibition mechanism and won them the Nobel Prize for Physiology and Medicine in 2006.

Montgomery *et al* (1998) expanded on Fire *et al* (1998) work with *C. elegans.* They injected *C. elegans* with dsRNA derived from the unc-22 gene. They performed a series of studies to

determine whether or not gene inhibition was occurring during the pre-transcriptional, transcriptional or post-transcriptional stages of mRNA. They compared the effect of dsRNA during the initial stage of biosynthesis and observed no changes in the gene or primary transcripts [214]. They also explored the transcriptional of effects of dsRNA when utilising genes clustered in an operon. They observed no changes in activity upstream or downstream regions of the gene during transcription. In their final study they utilised *in situ* hybridization to determine the effects of dsRNA. They identified minimal changes in the nucleus and significant changes in the cytoplasm. Their findings from their study suggested that gene inhibition occurred at the post-transcriptional stage of mRNA.

Parish *et al* (2000) performed a study identifying the structural features of dsRNA for gene inhibition in *C. elegans*. They explored a number of different factors including RNA bases, length, sequence, homology and helical structure. Their findings showed a wide range of dsRNA could promote gene inhibition. Interestingly they found dsRNA as short as 25 nt in length could promote RNA interference [215]. Lee *et al* (1993) identified the first miRNA during a study comparing the heterochronic patterns of lin-4 and lin-14 genes in *C. elegans*. Both lin-4 and lin-14 are responsible for the development of larvae [216, 217]. In their studies they identified two lin-4 transcripts, 22 and 61 nt in length that were complementary to the sequence the 3'UTR of lin-14 [218]. They found these transcripts would indirectly or directly inhibit the translation of lin-14 mRNA. Thus lin-4 was first miRNA to be discovered. Reinhart *et al* (2000) discovered the second miRNA in *C. elegans* during a study comparing the heterochronic patterns of let-7 with lin-14, lin-28, lin-41, lin-42 and daf-2 genes in *C. elegans*. They identified let-7 directly inhibited the 3'UTR of lin-14, lin-28 and lin-41. The inhibition of lin-41 was detrimental resulting in complete loss of muscle function [219]. Thus let-7 was the second miRNA to be identified. Since then thousands of miRNAs have been identified across

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different species and been recorded on a database called miRBase [198, 220-226]. The latest version of miRBase (v21) currently holds information for over 2,500 human miRNAs [227].

1.2.3 MicroRNA analysis

A recent technique that has sparked interest amongst the forensic community is microRNA (miRNA) analysis [204, 228]. MicroRNA has a number of advantages over mRNA analysis. Their short sequence length of approximately 18-25 nt provides greater inherent stability compared to mRNA and as consequence has become of great forensic interest, particularly in BFID in forensic casework [229-236]. Their expression patterns in different species types are also of interest. Also their relatively high abundance within cells make them excellent candidates for BFID in forensic casework samples especially where low level samples and mixed body fluids are involved. MicroRNA analysis has been mainly used for clinical and medical fields. Its use within a forensic context is recent. There have been a handful of studies that have been performed using miR analysis in forensic casework [229-233, 235, 237]. The microRNA analysis techniques performed by these groups are similar to the messenger RNA method described for RT-PCR, except it utilises special primers to accommodate for microRNA shorter size.

Hanson *et al* (2009) performed the first study exploring miRNA analysis as a potential body fluid identification method in forensic casework. Five different body fluids were used in their study: blood (venous, menstrual), saliva, semen and vaginal secretions. Samples underwent total RNA isolation using an organic method [159]. Samples then underwent reverse transcription using poly-T tails incorporated after polyadenylation and qPCR using SYBR® Green chemistry. From their findings they identified nine potential miRNA markers that could

be used to indicate the presence of blood (miR-16 and miR-451), saliva (miR-205 and miR-658), semen (miR-10b and miR-135), vaginal secretions (miR-124a and miR-372) and menstrual blood (miR-412 and miR-451) [230]. They also selected two potential reference genes for blood (RNU6B and RNU44) and one reference gene for semen (RNU6B) during sample normalization (shown in Table 1). Their findings demonstrate the potential of using miRNA analysis as a BFID in forensic casework. However there studies also demonstrate the need to develop this BFID test, as this study was limited. Further work is needed in the identification of body fluid specific miRNA markers. Also extensive work exploring low-level, non-human, degraded and mixed samples is needed to determine the suitability of this test on forensic samples.

Zubakov *et al* (2010) performed a more comprehensive study exploring miRNA analysis as a potential BFID technique by performing a larger screen on the miRNAs. Five different body fluids were used in this study: blood (venous, menstrual), saliva, semen and vaginal secretions. Samples underwent total RNA isolation followed by microarray analysis or RT-qPCR. In their microarray studies they utilised LNA™-modified oligo-nucleotide capture probes to screen the microRNA repository (version 10.1), which contained 718 human miRNAs [231]. From their findings they identified 294 potential miRNAs. They selected 14 of the most promising candidate markers to undergo RT using stem-loop primers and qPCR using TaqMan® chemistry. From their work they identified four potential miRNA markers that could be used to indicate the presence of venous blood (miR-144 and miR-185) and semen (miR-135a and miR-891a). However they were not able to identify miRNA markers for menstrual blood, saliva, semen or vaginal secretions. They also identified three potential references genes (RNU24, RNU44 and RNU48) (shown in Table 1). Their findings show the potential of using miRNA as

a BFID method. However their studies again highlight the need for more BFID studies in this area as different markers were identified in Hanson *et al* (2009) work.

Zubakov *et al* (2010) also performed brief work exploring the stability, sensitivity and specificity of the blood and semen markers. In their stability study, they stored dried blood and semen for 1 year in a dust and UV- free environment with controlled humidity and temperatures. Their findings showed stability in all of the blood and semen markers tested. The authors do highlight that their study was limited. Further research on the stability of miRNA is needed with regards to environmental factors including prolonged exposure to humidity and UV-light. In their sensitivity study they serially dilute blood and semen samples 20 to 0.002 ng [231]. In their study they were able to differentiate all of the body fluid markers down to 0.002 ng, suggesting that miRNA analysis is more sensitive than mRNA BFID. Their study on specificity amongst species was very limited. They performed alignment studies on these markers and found they were also expressed on other animals. It is clear that much more work is needed in this area to establish the sensitivity, specificity and stability of miRNA.

Courts and Madea (2011) also conducted a study on miRNA analysis using dried blood and saliva stains. In their studies samples underwent total RNA isolation followed by microarray analysis or RT-qPCR. Microarray studies were performed using Geniom® Biochips, which utilise the reverse complements of mature miRNAs to screen the miRNA repository (v14.0) containing 800 miRNAs [235]. From their study they selected a total of six candidate miRNA markers to undergo RT-qPCR analysis using SYBR® Green chemistry. They identified three potential miRNA markers for blood (miR-451, miR-150 and miR-126) and three markers for saliva (miR-205, miR-203 and miR-200c) [235]. They also identified a potential reference gene for blood and saliva (RNU6b) during sample normalization (shown in Table 1). Their

study again highlights the potential of using miRNA BFID in forensic casework. However their findings also demonstrate that further investigation is needed to identify the most suitable miRNA markers for BFID as both Hanson (2009) and Zubakov (2010) have also identified a range of miRNA markers.

In the same study Courts and Madea (2011) also explored the stability of these markers and their application in mixed samples. In their stability study they stored a bloodstain in the dark for one year. They found that both markers showed stability in the bloodstains. Although this study was useful, it was very limited. Further work exploring the stability of miRNA is needed across a wide range of body fluids. In their mixed body fluid study they combined both blood and saliva by volume. They detected the presence of blood and saliva. However the authors did not describe whether they were able to determine the mixing ratios in this test. It is clear that further studies need to be conducted to explore the effect of mixing ratios and whether this miRNA BFID test is suitable for distinguishing a single source or mixed sample.

Wang *et al* (2013) performed an interesting study comparing the work of Hanson *et al* (2009), Zubakov *et al* (2010) and Courts and Madea (2011). Blood (venous, menstrual), saliva, semen and vaginal material were used in their study. Samples in their work underwent total RNA isolation followed by microarray analysis of RT-qPCR. Microarray studies were performed using TaqMan Array Human MicroRNA cards, which contain 754 known human miRNAs. From their study they selected five miRNA markers for RT using stem-loop primers and qPCR using TaqMan® chemistry. In their work they identified three new miRNA markers for indicating the presence of blood (miR486), semen (miR-888) and menstrual blood (miR-214) [233]. They also identified the same marker for blood (miR-16) and vaginal material (miR-124a) as in the study by Hanson *et al* (2009) and semen (miR-891a) as in the study by Zubakov *et al* (2010). They also identified selected a potential reference gene for BFID (RNU6) (shown

in Table 1). Their studies again show the potential of using miRNA analysis as a BFID method in forensic casework. However further work needs to be done for identifying the most suited miRNA BFID markers.

Wang *et al* (2013) also performed a study briefly exploring the stability and sensitivity on several of the body fluids. In their study they tested the stability of blood (venous and menstrual) and semen when stored for 1-month in the dark. Their study showed stability of miRNA in all of the body fluids tested, which can be useful information for samples stored over a shorter periods. There study however was very limited. It did not explore longer storage times or environmental conditions. In their sensitivity study they explored miRNA in all of the body fluids (10-0.001 ng). They found that miRNA could be detected in all of the body fluids.

Bai *et al* (2013) also recently conducted a study comparing Hanson *et al* (2009), Zubakov *et al* (2010) and Courts and Madea (2011) work. Four different body fluid types were used in this study: blood, saliva, semen and vaginal secretions. Samples underwent total RNA isolation, RT and qPCR using SYBR® Green chemistry. In their work they identified new miRNA markers for semen (miR-10b, miR-135b). They also identified the same markers for blood (miR-16 and miR-451), saliva (miR-205 and miR-658), and vaginal material (miR-124a and miR-372) as in the study by Hanson *et al* (2009) (shown in Table 1). They also identified similar markers for blood (miR-451) and saliva (miR-205) as in Courts and Madea (2011) work. None of the markers identified were the same as Zubakov *et al* (2010). The chemistry may have an impact of the identification of the markers. Bai *et al* (2013) utilises the same chemistry, SYBR® Green as Hanson *et al* (2009) and Courts and Madea (2011) [236]. This study demonstrates the need for further work in this area.

	Hanson (2009)	Zubakov (2010)	Courts (2011)	Wang (2013)	Bai (2013)
	SYBR Green	TaqMan	SYBR Green	TaqMan	SYBR Green
Blood	miR-16 miR-451	miR-144 miR-185	miR-126 miR-150 miR-451	miR-16 miR-486	miR-16 miR-451
Saliva	miR-205 miR-658		miR-200c miR-203 miR-205		miR-205 miR-658
Semen	miR-10b miR-135	miR-135a miR-891a		miR-888 miR-891a	miR-10b miR-135b
Vaginal material	miR-124a miR-372			miR-124a	miR-124a miR-372
Reference gene	RNU6b RNU44	RNU24 RNU44 RNU48	RNU6b	RNU6	

Table 1. Summary of the body fluid specific miRNA markers and reference genes identified by Hanson et al (2009), Zubakov et al (2010), Court and Madea (2011), Wang et al (2013) and Bai et al (2013).

1.3 Project aims

The aim of this project was to develop an RNA-based body fluid identification test for use in forensic casework. Three main areas were covered during the development of this test. The first topic explored both single and co-isolation strategies for DNA, microRNA and mRNA. The overall aim was to develop a single or co-isolation method that did not separate DNA and RNA fractions. Thus allowing the DNA profile to be more closely associated to a body fluid. Once a sample isolation method was determined the next project was to screen a panel of body fluid specific RNA markers as selection of markers can vary depending on the technique used. Finally the applications of the RNA-based BFID test were examined including sensitivity, specificity, stability and mixtures. This was to determine whether this test was suitable for forensic casework.

Chapter 2

Methods and Materials

2.1 Ethical approval

One of the fundamental factors to consider when developing a new body fluid identification test is to ensure that the test is ethical. This is essential, as both the development and nature of the test relies on the use of donors. The physical and mental safety, comfort and privacy of the donors must be maintained if the method is to be implemented by the forensic community. A new BFID test was developed and approved by the University of Huddersfield School of Applied Sciences Ethics Committee.

2.2 Sample collection

When developing a new BFID test it is important that the body fluid is collected as efficiently and effectively as possible. In this way, maximum use of the sample can be achieved and contamination from surrounding areas can be minimised. Poor sample collection methods can be a host for a number of unnecessary problems. It can lead to limited and low quality yields during sample isolation and can affect downstream processes including cDNA synthesis and qPCR. It can ultimately lead to difficult to interpret results or in worst, loss of a case sample.

Both the UK and US police forces have standard operating procedures (SOPs) for collecting body fluids. One common technique used for body fluid collection is with the use of cotton swabs. Cotton swabs are a relatively non-invasive method for obtaining DNA profiles from victims and suspects. Cotton swabs are also used for collecting more intimate body fluids in cases of sexual assaults. For instance after a rape, both the victim and suspect will generally taken to a hospital, where a trained specialist will carefully collect vaginal and

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penile swabs. If the suspect is not apprehended but a condom is present, then the cotton swab may be used to collect semen from the inside of the condom.

In other cases, the entire item is collected for body fluid identification. Items of clothing can be a popular source of body fluids. For instance, a T-shirt may be collected for trace amounts of blood and saliva from the victims and suspects. The collection methods prepared in this work have been developed to simulate such case scenarios. It has also been developed to require minimal modification if it is incorporated into routine casework.

2.2.1 Blood samples

Blood was collected onto sterile filter paper (Fisher Scientific, UK). Disposable Unistik 3 comfort lancets (Barrier Healthcare, UK), which are commonly used to help measure diabetic blood levels, were used to create a pinprick on sanitised fingertips (Brosch Direct, UK). Blood was then stained onto filter paper and immediately packaged into sterile RD polyethylene bags to minimise contamination (Fisher Scientific, UK). The purpose of using this method of collection was to simulate dried bloodstains found on surfaces at a crime scene.

2.2.2 Saliva samples

Two different methods were used. One collection method used buccal swabs. This involved swabbing each cheek for 30 s using sterile buccal swabs (Sarstedt, UK). This particular length of time was chosen to ensure that a sufficient amount of epithelial cells would be collected. Also, all samples were collected with at least 1 hr. between food or drink consumption to minimise experimental variables such as bacterial contamination.

This collection method was used to simulate samples collected from sexual assaults e.g. linking a victims saliva on a penile swab during non-consensual oral sex.

The second collection method used was with collection tubes. Donors followed the same guidelines as before e.g. a minimum 1 hr. between food and the drink consumption. Donors then deposited saliva directly into sterile 50 ml BD Falcon™ tubes (VWR, UK). This collection method was also selected to simulate samples collected from an assault e.g. linking a suspect to a victim to a bar fight through the presence of saliva deposits on the victim shirt.

2.2.3 Skin samples

Skin was collected using a combination of sterilised rulers and cotton swabs. Skin from the back of the hand was selected, as it was easy to access and sanitise for donors. Gentle abrasion was applied with a sterile ruler to help lift epithelial cells. A cotton swab was then used to collect these cells from the hand and ruler. This collection method was developed to simulate samples that may be collected during an assault e.g. linking suspects to a victim through the presence of the suspects skin on a victims fingernail clippings.

2.2.4 Vaginal material samples

Vaginal material was also used for the development of the BFID test. Donors signed consent forms and were provided with overnight sample collection kits by a designated female staff member. Additional information such as time since last period or time since last intercourse were not requested as only a handful of donors came forward during this study.

Sample kits for collecting vaginal material contained additional information about the study, collection items (e.g. 2 sterile cotton swabs, a biohazard bag) and instructions on collecting, packaging and storing samples. Samples were then returned to the designated female staff member and placed into the laboratory. This collection method was developed to broadly simulate samples that may be collected from sexual assaults.

2.2.5 Semen samples

Semen was also used for the development of the BFID test. Donors signed consent forms and were provided with overnight sample collection kits by a designated male staff member. Additional information such as whether donors were vasectomised were not requested due to the potential sensitivity of donors.

Each sample kit contained information about the study, the collection items: 2 sterile cotton swabs and a biohazard bag and instructions on collecting, packaging and storing samples. Samples were then returned to the designated male staff member and placed into the laboratory. This collection method was developed to broadly simulate samples that may be collected from sexual assaults.

2.3 Sample isolation

Body fluids are complex by nature. They contain a range of components including enzymes, proteins and cellular debris that can interfere with BFID analysis. Therefore it is important, that an appropriate isolation method is selected. This need is exemplified by the

fact that many body fluids collected at a crime scene are limited and or of poor quality. To further complicate matters the source of the body fluid may be unknown, especially if found in trace amounts at a crime scene.

Therefore part of this research was focused on finding a universal isolation method for body fluids. Four different isolation methods were identified. All isolations were performed without specialist treatment towards specific body fluids (e.g. DTT treatment for semen).

2.3.1 Dynabeads® magnetic separation technology

One method explored was the Dynabeads® magnetic beads technology by Life Technologies. The technology is different from other commercial bead based kits (e.g. by Promega and Qiagen) as the uniform spherical nature of the sample allows for 360° binding of samples. Dynabeads are also paramagnetic or in other words are magnetised when a magnetic field is present. This means that the separation of genetic material is far gentler than with methods that use centrifugation. This is advantageous from the police and forensic practitioners perspective as there is a heavy backlog for processing casework samples.

Two different isolation kits were selected: the Dynabeads® mRNA DIRECT kit, which uses an oligo-dT tail for isolating polyadenylated mRNA and the Dynabeads® DNA DIRECT™ Universal kit, which uses a silica-like chemistry (Life Technologies, UK).

2.3.1.1 Messenger RNA isolation using oligo-dT beads

The standard mRNA isolation protocol was used on all body fluids [238]. The oligo® $(dT)_{25}$ beads were first prepared in this protocol. The beads are heavier than the solution and tend to gather at the bottom of the tube. Therefore homogenization is required before pipetting the solution into sterile 1.5 ml collection tubes (Fisher Scientific, UK). Note the beads come immersed in a pre-lysis/binding solution, 1 X PBS (pH 7.4).

The tube is then placed onto a DynaMag™-2 magnet (Life Technologies, UK) for 30 s to allow sufficient time for the beads and solution to separate. In most cases, separation of the beads occurs within the first 10 s of applying a magnet. A gelatinous looking streak then forms along the side of the tube wall allowing for the surrounding solution to be carefully removed, usually with smaller filtered pipette tips. The tube is then removed from the magnet to allow for the beads to separate. An additional lysis/binding buffer containing strong chaotropic agents is then added to the beads.

Preparation of the sample involved modification to the original protocol since it is designed for tissues and cultured cells. Samples collected using filter paper were prepared by cutting 1 cm (diameter) hole punches into collection tubes. Samples collected using cotton swabs were prepared by removing the entire swab head with sterile disposable scalpels (Swann Morton, UK). Samples were then treated with the same lysis/binding buffer and vortexed to break down the cell walls.

Samples then underwent mRNA extraction. All samples were transferred into the 1.5 ml tubes containing Dynabeads. Tubes were then rotated for 15 min. using an orbital shaker (VWR, UK) at room temperature to allow for hybridization between mRNA and oligo-dT beads to occur. Sample tubes were then placed on the magnet for two min to allow for the mRNA/Dynabeads complex to form. The supernatant was then carefully removed using a pipette. Samples were then washed four times with two different wash buffers, during which the mRNA/Dynabeads complex became more compact due to removal of impurities. Samples were then eluted using Tris-HCl (10 mM, pH 7.5).

2.3.1.2 DNA isolation using silica beads

The standard Direct DNA isolation protocol was used to isolate DNA [239]. The protocol was very similar to the mRNA protocol, except the Dynabeads came readily prepared in the lysis/binding buffer solution. The sample preparation protocol was similar to the sample preparation method developed for the mRNA protocol with exception of adding 1 X PBS to lyse and bind the cells.

Samples then underwent extraction by adding the Dynabeads to the solution in a single rapid pipetting action and incubated to allow for lysis and binding of the cells to occur. Samples were then separated with the Dynal magnet and allow for the DNA/Dynabeads complex to form. The lysis/binding solution was then removed through pipetting and then taken from the magnet. The sample was then washed twice with a single wash buffer to remove impurities. Samples were then eluted with a low buffer to reverse the binding conditions.

2.3.2 Qiagen silica gel membrane technology

The Qiagen silica gel membrane technology was also explored as it is commonly used technique amongst forensic science providers. Two isolation kits were selected: RNeasy mini kit and QIAamp DNA blood mini kit (Qiagen, UK).

2.3.2.1 Total RNA isolation using silica columns

The "Purification of total RNA from animal cells using spin technology" protocol was used to isolate total RNA from body fluids [240]. This protocol can be divided into two steps: sample preparation and sample isolation. Minimal modifications were required for this protocol.

Samples collected using cotton swabs and filter paper were prepared in a similar manner as before, except a combination of high salt lysis/binding buffer and 70% ethanol were used to breakdown the cell walls.

Samples were then transferred to RNeasy® spin columns and centrifuged using a Hettich MIKRO temperature controlled centrifuge (VWR, UK). Flow through from the spin columns was discarded. Samples were then washed three times with two different wash buffers to remove impurities. Samples were then eluted in RNase-free water to reverse binding conditions in the silica bed.

2.3.2.2 DNA isolation using silica columns

The "DNA purification from cotton swabs (spin protocol)" was used to isolate DNA [241]. This protocol has been divided into two steps: sample preparation and sample isolation. Minimal modification was required for this protocol.

Samples collected using cotton swabs and filter paper was prepared in a similar manner as before, except a combination of a high salt lysis/binding buffer, PBS (pH 7.5) and proteinase K were used to breakdown cell walls. Samples were then vortexed and incubated. Ethanol was then added to further promote binding.

Samples were then transferred into QIAamp mini spin columns and treated in the same manner as the RNeasy isolation method. They were then washed twice with two different wash buffers to remove contaminants and then eluted with a low salt elution buffer to remove the purified DNA sample from the silica bed.

2.4 Sample quantification

Sample quantification is an important step when developing a new BFID test. This is because different BFID tests require different quantities and qualities of starting material. For instance, chemical BFID tests generally require higher volumes to work where as RNA-based BFID tests should require lower volumes.

If a new BFID test is to be developed and incorporated into existing DNA profiling methods, it is important that the amount of RNA required is comparable. Current DNA

profiling kits require as little as 1.0 ng of DNA to achieve full profiles (e.g. NGM SE) [3]. One of the challenges with developing a new RNA-based BFID test is that there is currently no reliable absolute quantification test available for RNA. However there are several methods available for indicating the quantity and quality of RNA including the use of bioanalysers and UV-Vis spectrophotometry. Nano volume UV-Vis spectrophotometry was selected for assessing the quantity and quality RNA while qPCR was used to quantify the amount of human DNA present in samples.

2.4.1 Nano volume UV-Vis spectrophotometry

Samples were quantified using a NanoVue Plus™ spectrophotometer (VWR, UK). The path length of this instrument was 0.5 mm path length and the absorbance was set to 260 nm. Reference measurements (e.g. elution buffer) were taken at the start of sample measurements to establish a baseline. Both 260 nm/ 280nm and 260 nm / 230 nm ratios from each measurement were used to asses the quality of nucleic acid.

2.4.2 Quantitative PCR

Samples were also quantified for human DNA using the Investigator Quantiplex Kit (Qiagen, UK). The "Quantification of DNA using the Rotor-Gene Q" protocol was followed [242, 243].

Since the kit is an absolute quantification method, a series of known standards were prepared. A four-fold serial dilution was performed using control DNA Z1 (20 ng/µl) and QuantiTect nucleic acid dilution buffer. A total of seven standards were prepared. A master

mix containing qPCR reaction mix and primer mix was then prepared and added to the standards, samples and no template negative control.

2.5 Complementary DNA synthesis

Complementary (cDNA) synthesis is an essential step in RNA-based BFID analysis. RNA must become cDNA in order to undergo PCR. In this protocol two different primers types were used. A standard reverse transcription method was used for large RNA transcripts e.g. mRNA (1500 to 2000 nt). In this method a combination of RT reagents including primers were used to synthesize a cDNA strand.

A stem-loop reverse transcription method was used for shorter RNA transcripts e.g. mature miRNA (18-25 nt). The shorter length of mature miRNA makes it difficult for primers to bind and accurately synthesize a cDNA strand. Either poly-T primers utilised after polyadenylation (SYBR® Green chemistry) or stem-loop primers (TaqMan® chemistry) can be used for this. In this work, stem-loop primers were selected due to their high specificity (e.g. of as little as one nucleotide difference) and use in forensic RNA research [244].

During RT, the 3' of the stem-loop primer will bind to the last 6 bp of the mature miRNA sequence while the 5' end, which is artificial (in nature) will fold on itself (shown in Figure 3). MMLV-RT will then extend this sequence. The stem-loop will unfold during PCR.
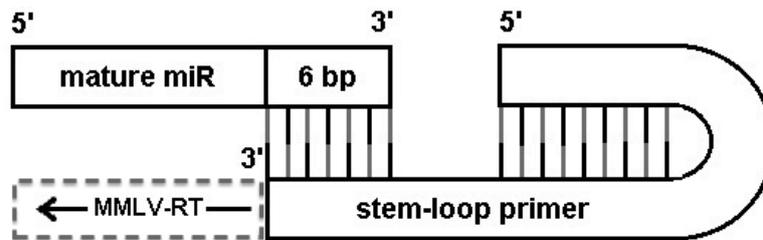
Figure 2. A diagram showing cDNA synthesis using stem-loop primers. During reverse transcription, the 5' end of the stem-loop primer will fold onto itself while the 3' end of the stem-loop primer will bind to the last 6 bp in the mature miRNA sequence. The sequence is then extended using an enzyme e.g. MMLV-RT.

2.5.1 Standard reverse transcription

The RETROscript® first strand synthesis kit (Life Technologies, UK) was used for mRNA transcripts. Samples underwent standard reverse transcription following the RETROscript® protocol [245]. Samples were initially heat denatured with 50µM random decamers and nuclease free water on a Veriti thermocycler (Fisher Scientific, UK) for 3 min at 75 ℃ to remove any secondary structure that may inhibit reverse transcription. A master mix containing the essential components for reverse transcription (2.5 mM dNTP mix, 10X RT buffer, 10 units/µl RNase inhibitor and 100 units/µl MMLV-RT) were then added to the reaction. Samples then underwent standard RT using the following conditions: 60 min at 42 ℃, 10 min at 92 ℃.

In all studies a negative control containing all components for reverse transcription except the sample template was prepared. A negative control containing all components except Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT) was also prepared.

2.5.2 Stem-loop reverse transcription

The TaqMan® microRNA reverse transcription kit and TaqMan® microRNA assays (Life Technologies, UK) were used for miRNA transcripts. The protocol used was the "TaqMan® small RNA protocol" [246].

A RT master mix containing nuclease-free water, 10X reverse transcription buffer, 50 U/µl MMLV-RT, 20 U/µl RNase inhibitor and 100 mM dNTPs was prepared. The mastermix, 5X primer and extract was then combined into a single tube. Samples then underwent stem-loop RT on a Veriti thermocycler with the following cycling conditions: 16 °C, 30 min at 42 °C, 5 min at 85 °C and held at 4 °C. In all studies the same negative controls prepared as those in the standard reverse transcription protocol.

2.6 Quantitative PCR

As mentioned previously, there is currently no reliable method for quantifying RNA. An alternative method of quantification is through the comparison of gene expression levels via qPCR e.g. mRNA and miRNA. There are two different types of chemistries that can be used for qPCR analysis including Life Technologies TaqMan® chemistry and SYBR® Green chemistries. Many of the forensic research groups utilise these chemistry for BFID due to its high specificity and sensitivity and as such, has been selected for the development of this BFID test. Previous research by the University of Huddersfield research team explored SYBR Green chemistry for RNA-based BFID analysis. Findings from their

studies showed lack of specificity to mRNA targets and as such, TaqMan chemistry was explored in this work.

2.6.1 qPCR of stem-loop RT products

The "TaqMan® small RNA protocol" used in the cDNA synthesis section 2.5.2 was also used for the qPCR section. A single master mix containing 2X TaqMan® universal PCR master mix II no UNG (Life Technologies, UK) and nuclease free water was prepared in triplicate for each experiment in sterile 1.5 ml tubes (shown in Table 2). A 20X primer solution containing a TaqMan probe, a specific forward primer and universal reverse primer for miRNA (Ambion, UK) and RT product were then prepared in separate 0.2 ml PCR tube. A negative control containing all components of the PCR reaction apart from RT product was included in all studies.

Samples were then transferred to MicroAmp® optical 96-well reaction plate (Life Technologies, UK) and sealed with a MicroAmp® clear adhesive film and applicator (Life Technologies, UK). Quantitative PCR was performed on a 7500 fast real-time PCR system (Life Technologies, UK) using the following PCR cycling conditions: enzyme activation for 10 min at 95 °C and 40 cycles of denaturation for 15 s at 95 °C, annealing for 60 s at 60 °C and an extension during the temperature ramp of annealing and denaturation steps.

QPCR MASTER MIX COMPONENTS	1 REACTION IN TRIPLICATE (μl)	10 REACTIONS IN TRIPLICATE (μl)
TAQMAN® UNIVERSAL PCR MASTER MIX II (2 X) NO UNG	40.00	400.0
NUCLEASE-FREE WATER	30.68	306.8
TOTAL VOLUME	70.68	706.8

Table 2. Showing an example of a calculated qPCR master mix when using a total of 10 reactions.

2.6.2 qPCR of standard RT products

The "TaqMan® universal master mix II" protocol (Life Technologies, UK) was used to amplify RT product. A qPCR reaction mix containing 2X TaqMan® universal PCR master mix II no UNG, RT product or nuclease-free water and 20X TaqMan® primer assay was prepared in triplicate. A PCR negative control was also included in each study. Quantitative PCR reaction mixes were then transferred onto a 96-well plate and run on the fast 7500 qPCR machine with the following the same PCR cycling conditions as in the previous protocol.

2.7 Data analysis

In qPCR, quantification cycles (Cq) are used to assess gene expression levels in a sample. Cq is the point at which the expression of a sample is reaches above the background signal. It is used to indicate the amount of gene present in a sample. The ΔCq values were

presented following the minimum information for publication of real-time PCR data (MIQE) guidelines described by Bustin (2009) [247].

There are two ways that ΔCq was calculated. In the presence of a reference gene, ΔCq was calculated by subtracting the Cq of a reference gene by the Cq of the marker. Alternatively in the absence of a reference gene, the ΔCq is calculated by subtracting the maximum number of cycles used by the Cq of the marker (shown below).

$$\Delta Cq = Cq_{\text{max or reference gene}} - Cq_{\text{marker}}$$

Establishing the significance of a result or test is also important during data analysis. There are number of different statistical tests that can be used in qPCR analysis (e.g. ANOVA, paired sample T-tests). The statistical approach using in this work was paired sample T-tests, with a 95% confidence interval using Minitab® statistical software v17.

2.8 Enzymatic tests

Enzymatic tests are often used as initial body fluid indicators at a crime scene. Some of the commonly used tests include the Kastle-Meyer (KM) test for blood. These tests are often labelled presumptive due their lack in discrimination towards certain plants, cleaning products and clothes. Despite their limitations, enzymatic tests are regularly used at crime scenes and as such, have been explored in this work to compare against the new BFID test. Three enzymatic tests have been selected for comparison, the Kastle-Meyer (KM) and Leucomalachite Green (LMG) test for blood and the Phadebas® test for saliva.

2.8.1 Kastle-Meyer and Leucomalachite Green tests

The KM and LMG tests were used to indicate the presence of blood during limit of detection studies. Blood samples undergoing KM or LMG tests were dried onto filter paper before use. In each experiment a blank filter paper and filter paper with water was included as negative controls. KM and LMG testing was then performed by adding one drop KM or LMG reagent onto the stain. A maximum of 30 s was given before adding a drop of H_2O_2. This was to monitor oxidative false positives. The presence of blood was indicated by an immediate colour change; bright pink for KM and aquamarine for LMG.

2.8.2 Phadebas® test

The Phadebas® amylase kit (Maggle Life Sciences, UK) was used to indicate the presence of saliva. The Phadebas amylase test protocol was followed with minimum modification to the manufactures instructions [248]. Saliva deposits were collected and diluted into sterile 15 ml BD Falcon™ tubes (VWR, UK). The tubes were then incubated in a 37 °C water bath (VWR, UK) before adding a Phadebas tablet. The tube was then vortexed and returned to the bath for 15 min to activate the tablet. 0.5 M NaOH (Sigma Aldrich, UK) was pipette into each tube to stop the reaction. Samples were then centrifuged for 5 min with a Thermo Scientific Sorvall ST centrifuge (Thermo Scientific, UK) before measuring the samples on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, UK) [249]. A standard plastic cuvette with a 1 cm light path was used to measure the absorbance of samples and negative controls. An absorbance range of 400-800 nm was selected for saliva.

Chapter 3

Evaluating four different isolation kits for RNA analysis

When developing a new BFID protocol for forensic casework it is crucial to use an efficient and effective isolation method. This is very important to establish, as casework samples are often limited in both quantity and quality. For the purposes of developing a robust test for forensic casework, abundant samples were used.

3.1 Co-isolation using magnetic bead technology

One of the daily changes faced by forensic practitioners is working with casework samples. Body fluids that are generally collected from a crime scene will often be limited in quality and quantity. Furthermore body fluids may be the only source available for obtaining a DNA profile from a suspect. The development of a BFID test that could be used alongside current DNA profiling techniques would be very useful. The ability to use the same body fluid stain for BFID and DNA analysis would allow the forensic practitioner to more closely associate the DNA profile and body fluid origin when in court.

Co-analysis may be useful where it is important to know both the DNA profile and body fluid origin e.g. sexual assault. It may be important to know if the blood present on a suspect is a result of trauma blood or menses blood due to a rape. The ability to associate a DNA profile to the specific blood type may strengthen the evidentiary value within a case.

The challenges that casework samples bring (e.g. limited sample quality and quantity) have caused forensic groups to look at alternative methods of analysis. Co-isolation is an area that a number of mRNA research groups have considered as a way to overcome this problem [4, 156, 163, 165, 168, 173-177, 182, 185, 189, 250-260]. There are two main approaches. One approach involves samples isolation by splitting DNA and RNA into two difference fractions. The other approach involves isolation of one nucleic acid (e.g. DNA) followed by isolation of a second nucleic acid (e.g. RNA) using wash buffer that has been retained. Both methods utilise either silica gel columns or organic isolations. However one of the main drawbacks is the decrease in association between DNA profile and body fluid.

Aim

Thus aim of this work was to develop a co-isolation method where neither the DNA or mRNA fraction is separated. In this way, a DNA profile can truly be considered to originate from a particular body fluid and analysis time is reduced.

A number of different technologies have been used to co-isolate samples. The majority of research groups looking into co-isolation use spin column or organic based isolation methods as described previously. Very few forensic research groups have looked into magnetic bead based technology to co-isolate DNA and RNA e.g. Bowden *et al* (2011) and none of these groups have explored mixing beads. Furthermore the majority of research focusing on magnetic bead based technology has been non-forensic related [261-271]. Thus the aim of this work was to use different ratios of beads, oligo-dT and silica to co-isolate mRNA and DNA respectively.

Experimental design

In this study, two commercially available magnetic bead isolation kits were used for DNA (Life Technologies DNA Dynabeads Universal kit) and mRNA (Life Technologies mRNA Dynabeads Direct kit). A total of 6 different co-isolation methods were used. The standard protocol for both kits served as 2 of the co-isolation methods. Four modified protocols were prepared: a DNA kit using oligo-dT beads, a mRNA kit using silica beads, a DNA kit using a ratio of 50 oligo-dT beads:50 silica beads and a mRNA kit using a ratio of 50 oligo-dT beads:50 silica beads (shown in Table 3). Saliva swabs were the focus of the study as these were the easiest to obtain. Samples then underwent the standard and modified isolation

protocols, quantified using UV-Vis spectrophotometry, cDNA synthesis and qPCR using

KRT13 (saliva-specific), MNDA (blood-specific) and GAPDH (reference gene).

Standard isolation methods		Modified isolation methods	
Isolation kit	Magnetic beads	Isolation kit	Magnetic beads
DNA	Silica	DNA	Oligo-dT
mRNA	Oligo-dT	mRNA	Silica
		DNA	50 Silica:50 Oligo-dT
		mRNA	50 Silica:50 Oligo-dT

Table 3. Comparing the standard magnetic bead isolation methods with the modified magnetic bead isolation methods. The standard DNA isolation method utilised silica beads. The standard mRNA isolation method utilised oligo-dT beads. The modified DNA isolation methods utilised either oligo-dT beads or a ratio of 50 silica and 50 oligo-dT beads. The modified mRNA isolation methods utilised either silica beads or a ratio of 50 silica and 50 oligo-dT beads.

3.1.1 DNA concentration in saliva swabs
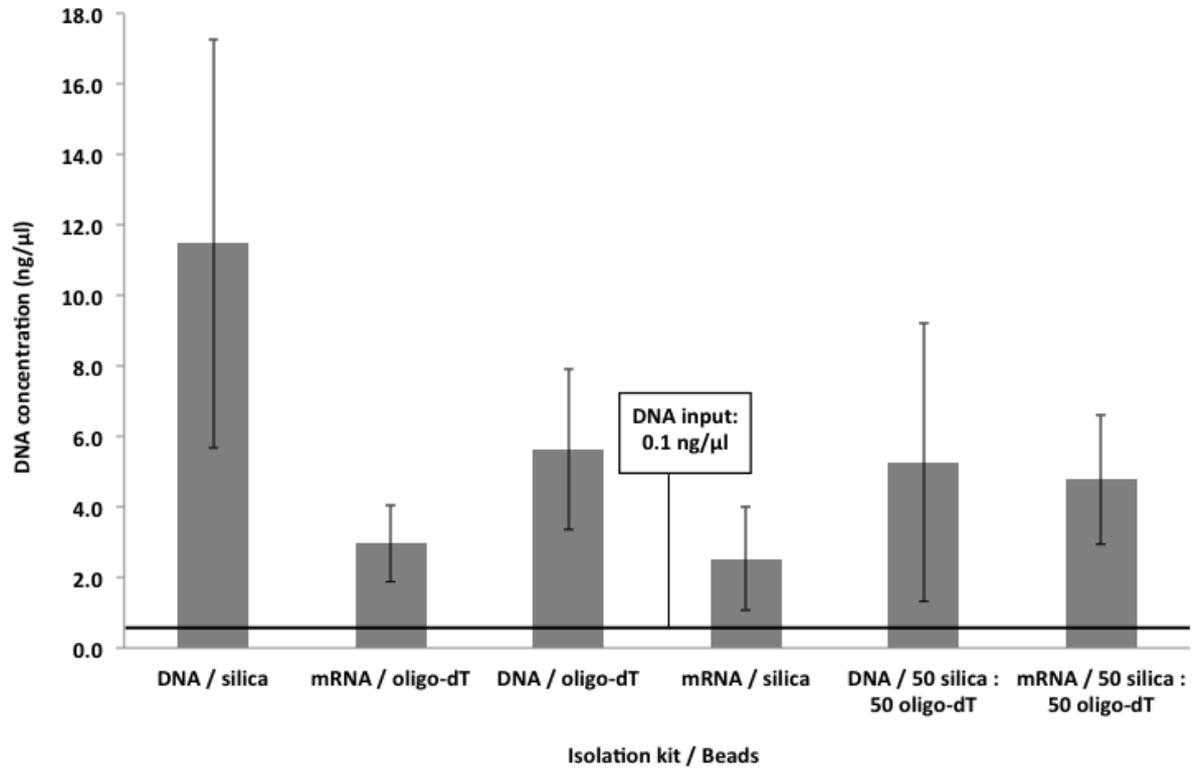
Figure 3. Showing the average DNA concentration obtained using the 6 different isolation methods. Error bars represent one standard deviation (n=15).

Figure 3 shows the average DNA concentrations obtained using the 6 different co-isolation methods. All 6 co-isolation methods gave sufficient concentrations of DNA (0.1 ng/µl), indicating that the 6 methods could be incorporated into current DNA profiling techniques.

3.1.2 mRNA expression in saliva swabs
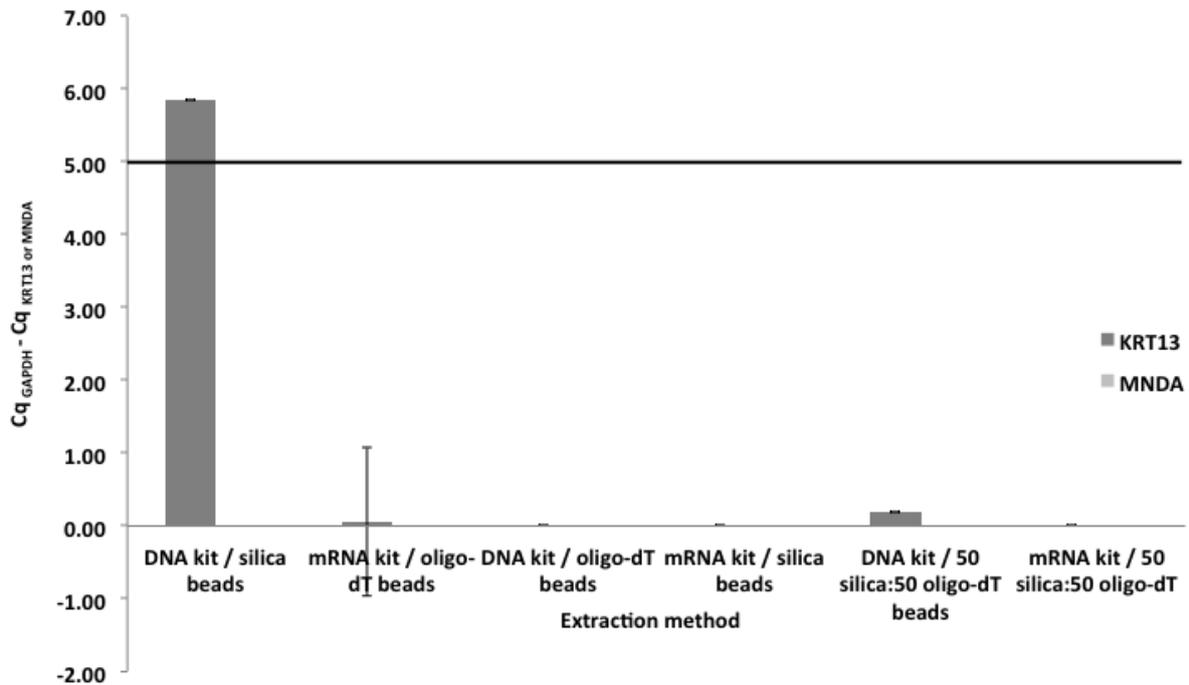
Figure 4. Showing the relative expression of KRT13 and MNDA in 6 different isolation methods. The black line is indicates a set threshold of ΔCq 5. Error bars represent one standard deviation (n=15)

Figure 4 shows the expression of KRT13 and MNDA when normalised with GAPDH in 6 different isolation methods. In this study KRT13 (saliva) showed the highest expression in using the DNA isolation method and minimal expression in the other isolation methods. This was surprising as it was thought that KRT13 would show the highest expression using the standard mRNA isolation kit. The third EDNAP collaborative exercise supported this finding. MNDA (blood) showed no expression in the 6 co-isolation methods [173]. No amplification was observed in the negative controls. The expression of the mRNA markers and reference gene were also presented separately to better understand their expression within saliva swabs.
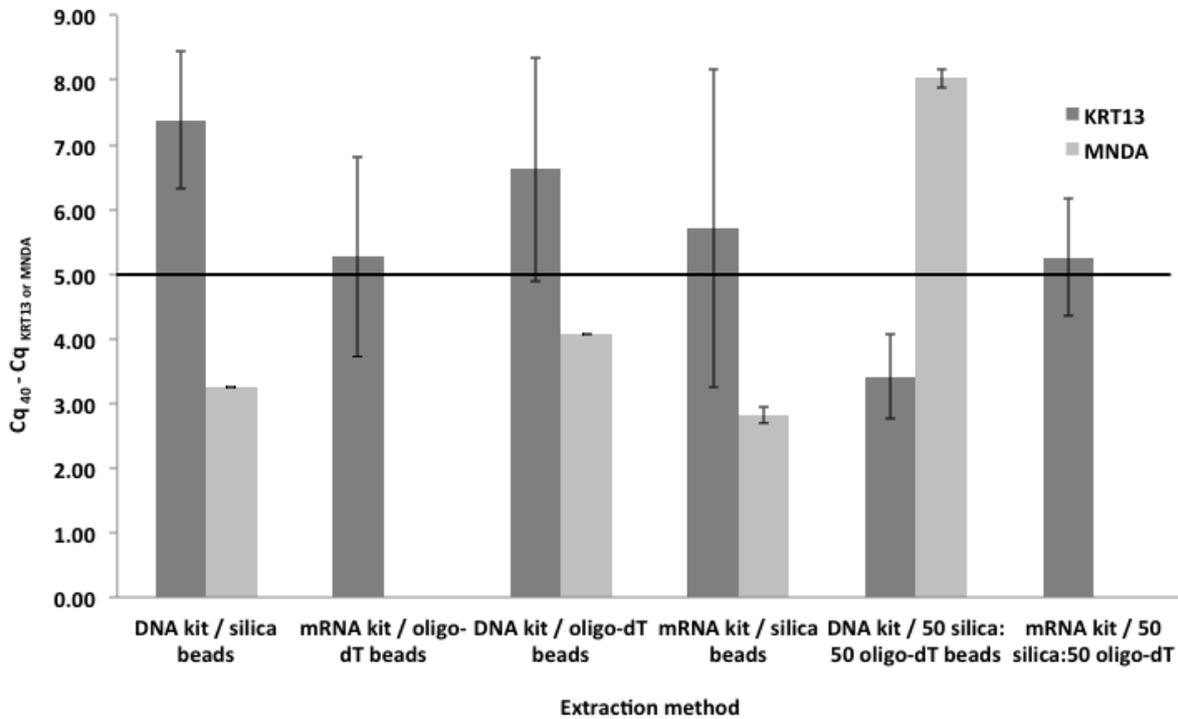
Figure 5. Showing the expression of saliva-specific KRT13 and blood-specific MNDA across 6 different isolation methods in saliva swabs. The black line is indicates a set threshold of ΔCq 5. Error bars represent one standard deviation (n=15).

KRT13 presented on its own showed moderate expression with standard DNA kit and lower expression using the five other methods (shown in Figure 5). MNDA showed minimal amplification in all 6 methods except in the modified protocol using the DNA kit with 50 silica : 50 oligo-dT beads, which KRT13 showed moderate expression. The third EDNAP collaborative exercise also supported the moderate levels of expression and discrimination within saliva deposits [173].
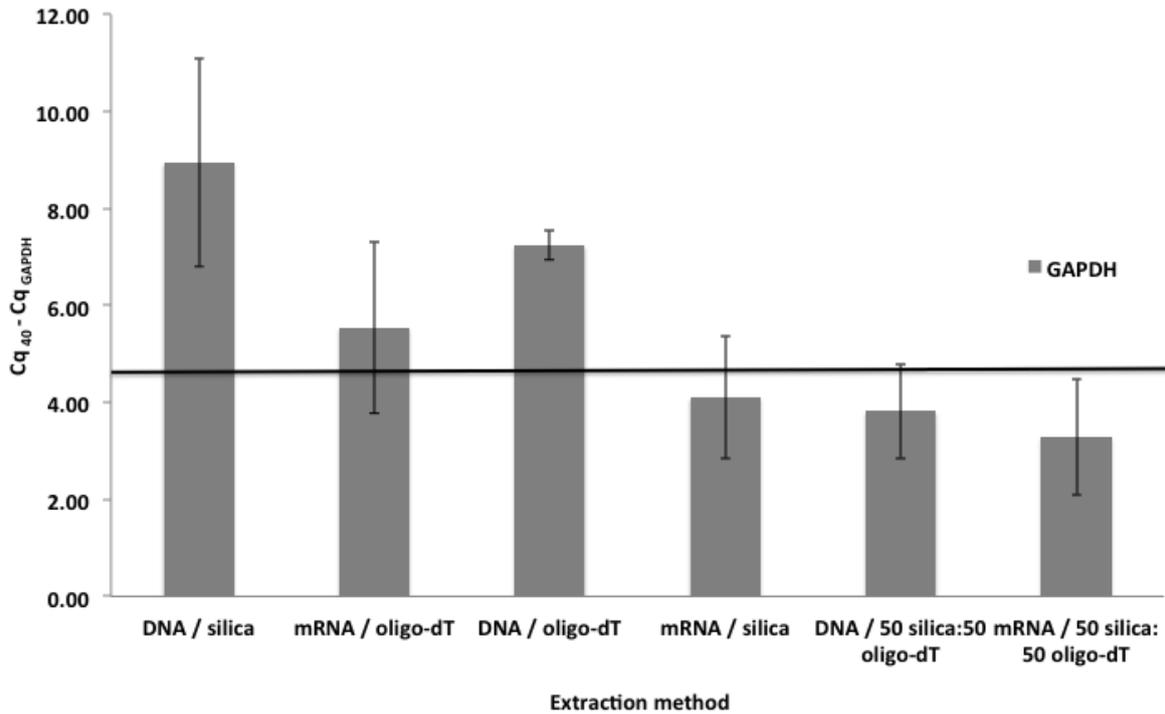
Figure 6. Showing the GAPDH expression across 6 different isolation methods. The black line is indicates a set threshold of ∆Cq 5. Error bars represent one standard deviation (n=15).

GAPDH when presented on its own showed moderate expression in one of the standard

isolation methods and in one of the modified methods (shown in Figure 6). GAPDH showed

low expression levels in the remaining isolation methods. Bowden *et al* (2011) agreed with

the GAPDH expression levels shown in the standard isolation protocols.

Discussion

The aim of this work was to develop a unique co-isolation method using two commercially available DNA and mRNA kits; Life Technologies Dynabeads Universal DNA direct kit and mRNA direct kit. Four modified methods were developed: DNA isolation kit with oligo-dT beads, mRNA isolation kit with silica beads, DNA isolation kit with 50 silica : 50 oligo-dT beads, mRNA isolation kit with 50 silica: 50 oligo-dT beads.

Of the six isolation methods, the standard isolation methods performed the best. KRT13 showed moderate levels of specificity towards saliva. The third EDNAP collaborative exercise supported the level of specificity of KRT13. MNDA (blood specific) showed minimal amplification across six isolation methods [173]. The studies from this result demonstrated that co-isolation method was not necessary.

3.2 Isolation using silica gel membrane technology

The use of RNA-based BFID tests is currently being considered in forensic investigations. A number of forensic research groups have shown mRNA to be stable under a number of conditions e.g. aged, post-mortem, in-vitro for years at a time [158, 193-195, 272, 273]. As a result, many of these research groups have tailored their work to develop and implement mRNA BFID into forensic casework [155-157, 173-177].

Despite the tremendous efforts that have been put toward developing mRNA BFID and the work supporting the stability of mRNA, a handful of these research groups have begun to use microRNAs for BFID e.g. Hanson *et al* (2009), Zubakov *et al* (2010), Courts and Madea (2011) and Wang *et al* (2013). MicroRNAs possess a number of very attractive qualities from a casework perspective. They are generally highly abundant and their short sequence length (18-25 nt) makes them prime candidates for casework samples, which are often limited and poor in quality. They also play a key role in gene regulation and as a result have been rapidly sought out for BFID.

Aim

Consequently the aim of this work was to explore miRNA analysis as a potential BFID method for its potential use in forensic casework. Hanson *et al* (2009), Zubakov *et al* (2010), Courts and Madea (2011), Wang *et al* (2013) have utilised either total RNA or miRNA isolation kits for their miRNA BFID methodologies. Although this approach is logical the study here explores and compares DNA isolation and total RNA isolation. The ability to use a DNA isolation method over total RNA isolation would provide an advantage

that none of the other groups have. It would allow for the practitioners to integrate miRNA analysis with more ease as they would be able to continue to use their existing methodologies for DNA profiling without the additional sample handling steps.

Experimental design

Thus two commonly used DNA and total kits were used in this study (e.g. the QIAGEN silica-gel membrane chemistry). Bloodstains and saliva swabs were collected and isolated using the total RNA or DNA kits. Samples then underwent human DNA quantification, cDNA synthesis and qPCR using miR-451 (blood) and miR-205 (saliva). Negative controls were also included in this study.

3.2.1 MicroRNA expression in DNA and total RNA kits
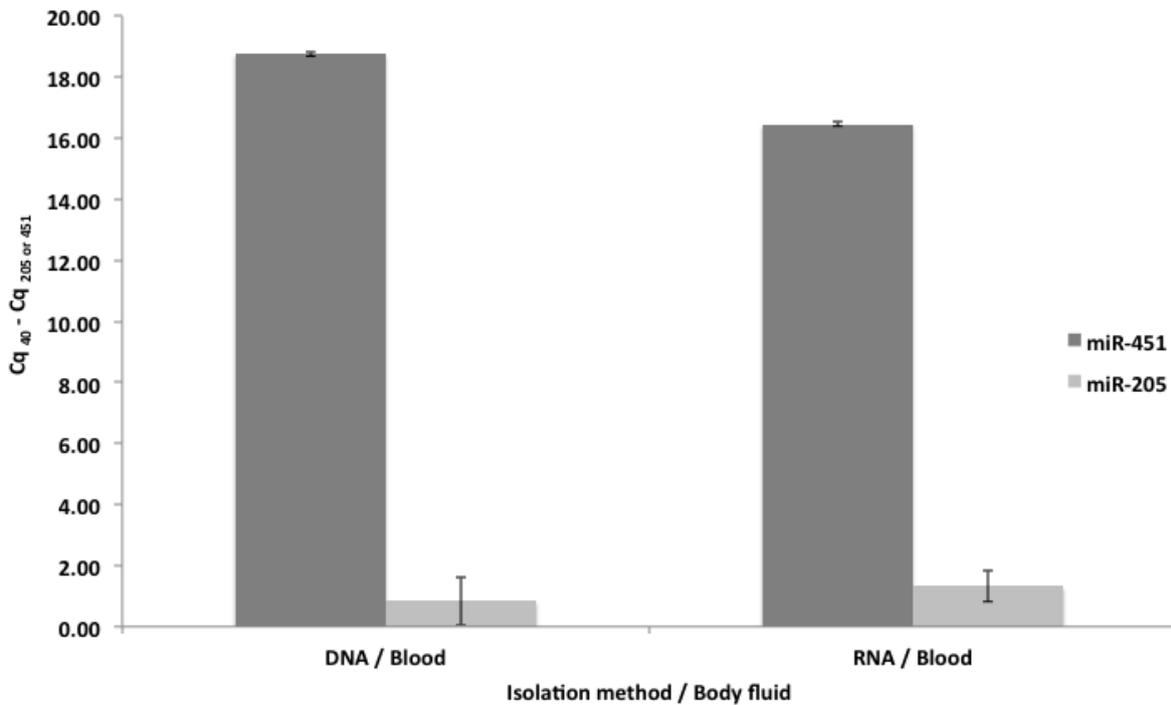
Figure 7. Showing the expression of miR-451 and miR-205 in bloodstains isolated using the DNA and total RNA isolation kits. Error bars represent one standard deviation (n=6).

Body fluid specificity was demonstrated using both the DNA and total RNA isolation kits (shown in Figure 7). In both kits miR-451 showed significantly more expression in blood than miR-205 ($P<0.001$). Hanson *et al* (2009) and Courts and Madea (2011) support this finding. No amplification was detected in the negative controls. A similar experiment was performed for saliva swabs.
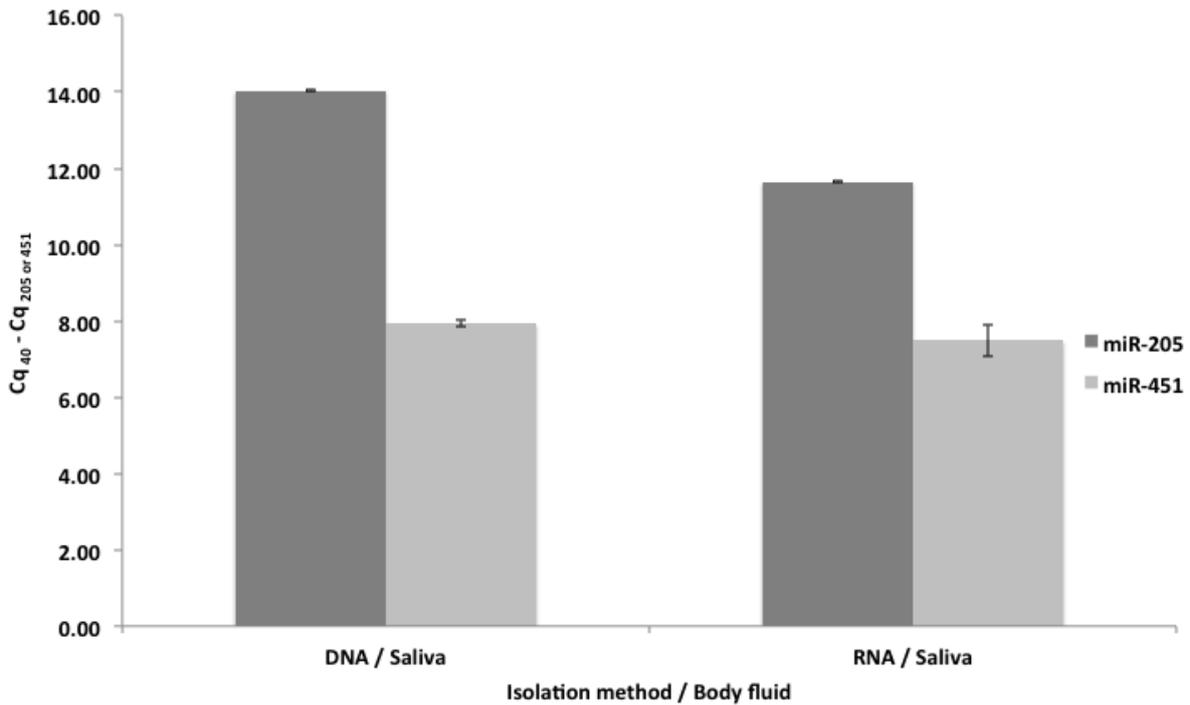
Figure 8. Showing the expression of miR-205 and miR-451 from saliva swabs isolated with the DNA and total RNA isolation kit. Error bars represent one standard deviation (n=6).

Body fluid specificity was demonstrated using both the DNA and total RNA isolation kits (shown in Figure 8). In both kits miR-205 showed significantly more expression than miR-451 ($P<0.001$). Surprisingly in both experiments (shown in Figures 7 and 8), the DNA isolation method gave significantly more expression of miRNA than with the total RNA isolation method ($P<0.05$).

3.2.2 MicroRNA expression during different stages of sample isolation

To establish where the miRNA was being retained, the wash steps from each of the kits also underwent isolation. Blood and saliva swab samples underwent the same process as described previously, with the exception that all of the wash steps were retained and underwent isolation as well. A total of 6 blood and saliva samples were used in this study. Negative controls were also included.
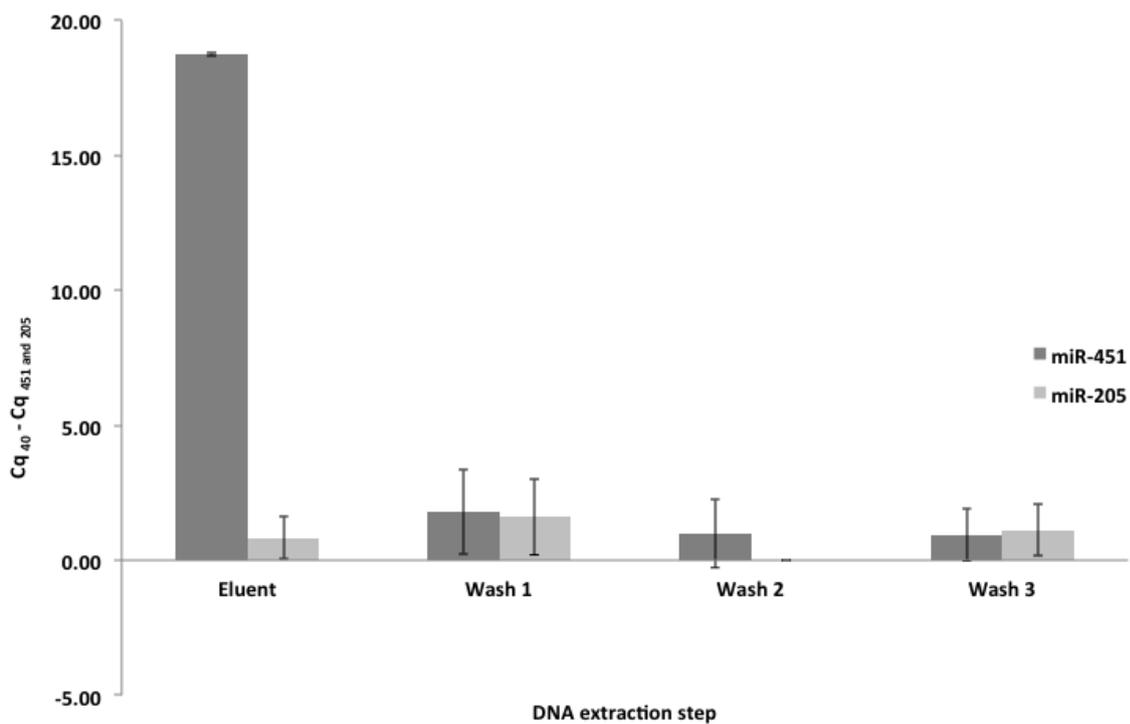
Figure 9. Showing the expression of miR-451 and miR-205 in bloodstains using the DNA isolation kit. Error bars represent one standard deviation (n=6).
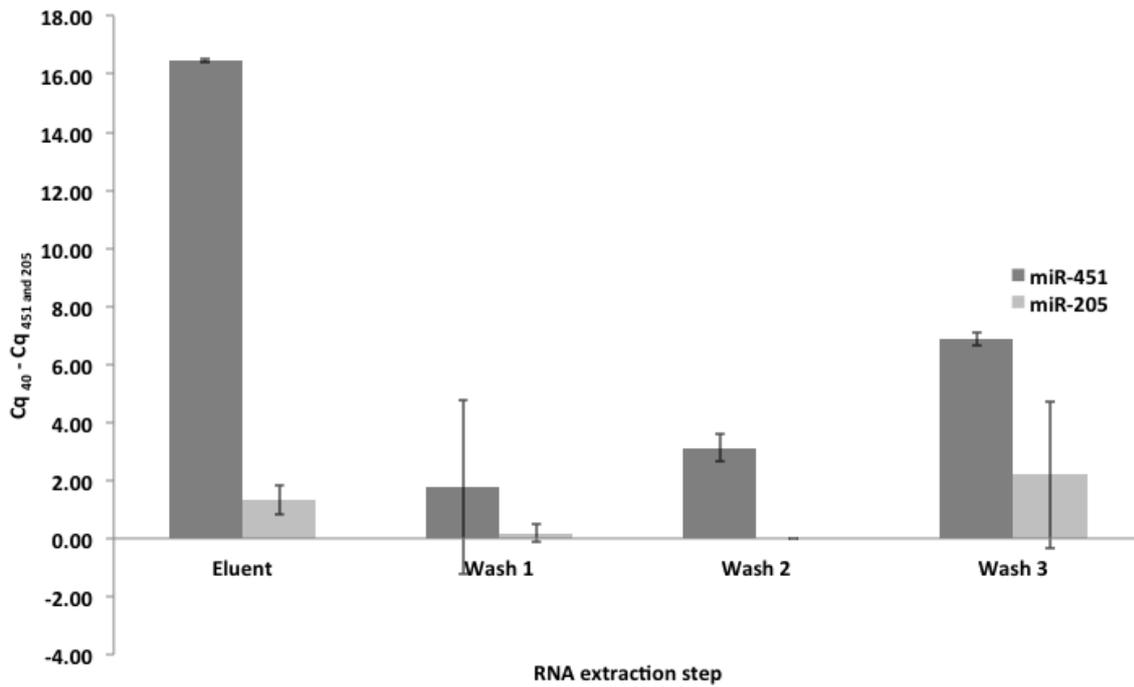
Figure 10. Showing the expression of miR-451 and miR-205 in bloodstains using the total RNA isolation kit. Error bars represent one standard deviation (n=6).

A similar experiment was performed for saliva swabs (shown in Supplementary section in the Appendix). A similar pattern was seen, with miR-205 giving the highest expression of miRNA in the eluent. No known research groups have studied the expression of miRNA during the wash and elution steps of sample isolation [185].

3.2.3 MicroRNA analysis of unknown samples

Since the aim in this study was to evaluate the use of miRNA analysis within a forensic context, a series of blood and saliva swabs underwent a blind process to simulate forensic samples. Blood and saliva samples underwent the same sample process as mentioned previously, with the exception that the sample origin was not known. Four samples were used in this study and labelled: A, B, C and D. Negative controls were included in this study.
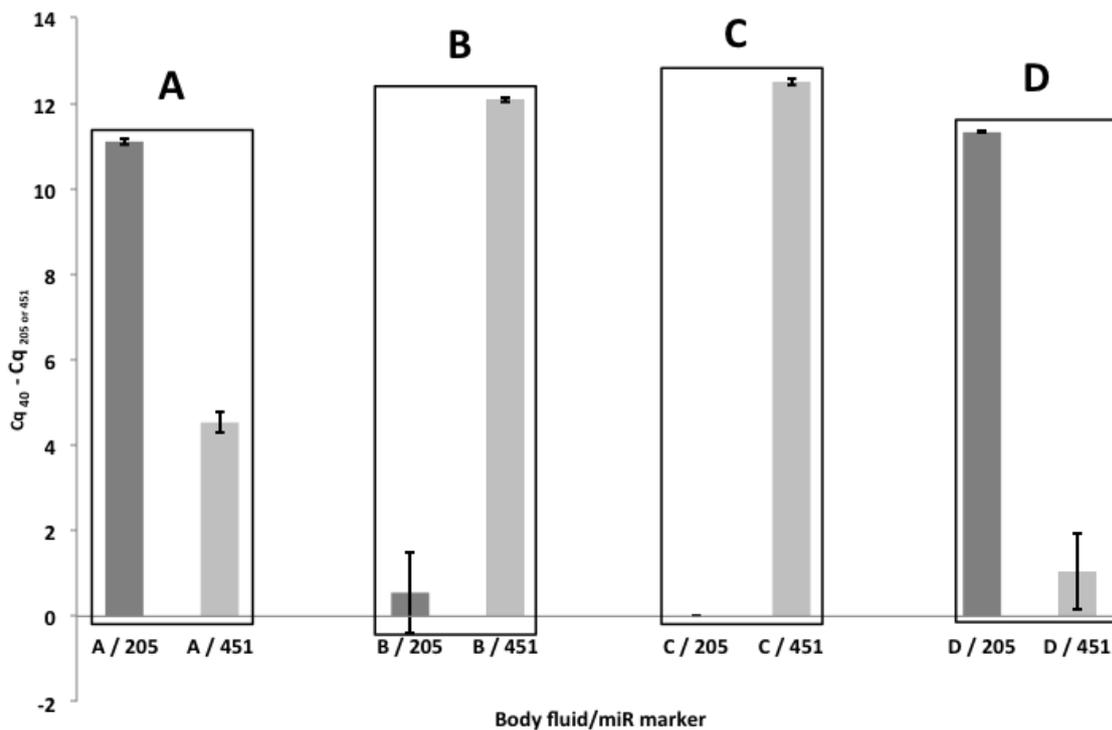
Figure 11. Showing the expression of miR-451 and miR-205 4 unknown body fluids (n=4). Error bars represent one standard deviation. Results indicate A = Saliva swab, B = Bloodstain, C = Bloodstain, D = Saliva swab (n=3).

Results

Body fluid specific expression was shown and correctly identified in all of the samples tested; A = saliva swab, B − bloodstain, C = bloodstain and D = saliva swab (shown in Figure 11). No amplification was observed in the negative controls. The findings from this study demonstrated the potential capability of using miRNA analysis for BFID in forensic casework.

3.2.4 MicroRNA analysis of saliva swabs

A final study was performed to increase sample numbers. Saliva swabs were the focus of this work due to ease of sample access. Samples underwent the same analysis process as before, except only with the DNA isolation kit. Negative controls were also included in this study.
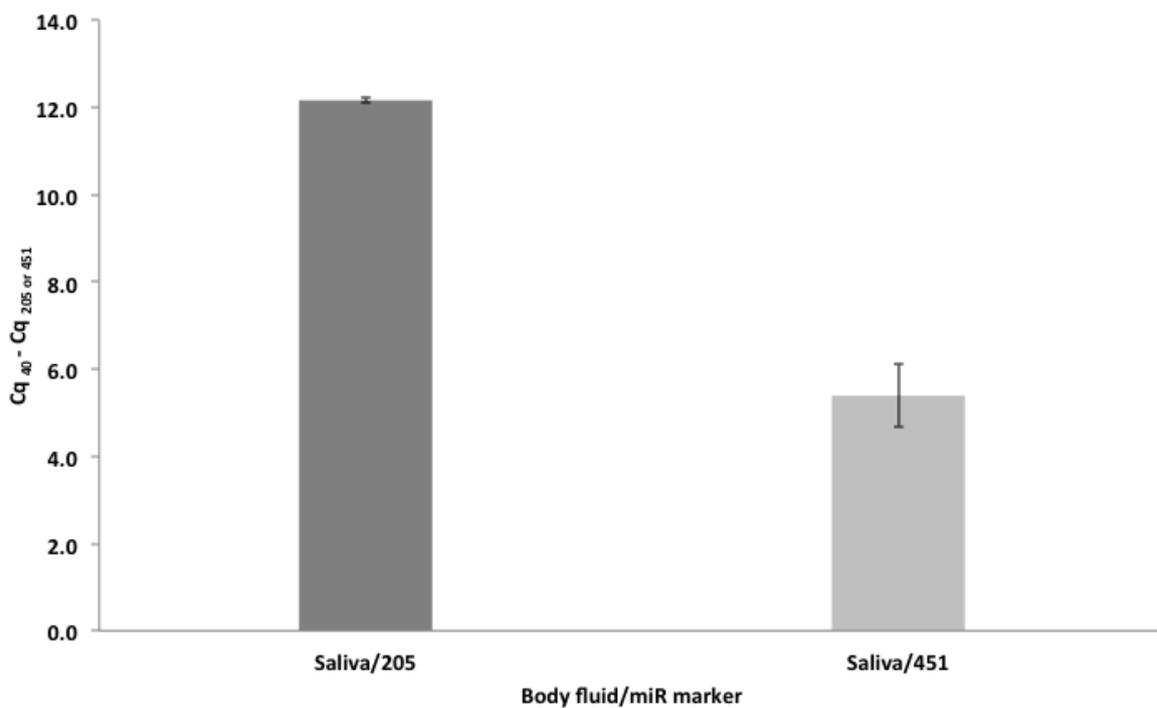
Figure 12. Showing the miR-205 and miR-451 expression in saliva swabs using the DNA isolation kit. Error bars represent one standard deviation (n=11).

Marker miR-205 showed significantly more expression in saliva than miR-451, as observed previously (shown in Figure 12). No amplification was observed in the negative controls. Hanson *et al* (2009), *Zubakov et al* (2010), and Courts and Madea (2011) support this finding.

Discussion

The aim of this study was to evaluate mRNA and miRNA analysis using different isolation methods. In the previous study using mRNA (Section 3.1.1), a co-isolation method was utilised. The findings showed that co-isolation of mRNA and DNA using a unique combination of magnetic beads; was not a requirement for BFID. Also since the aim of this chapter was to evaluate isolation methods in RNA analysis, it was important to explore miRNA as a body fluid indicator.

Thus the aim of this section of work was to explore miRNA analysis for BFID. Blood and saliva swabs were the focus of this study as it was the easiest to obtain out of the other forensically relevant body fluids (e.g. semen, vaginal material). Markers miR-451 for blood and miR-205 for saliva was selected as a starting point. The selection was based on both forensic and non-forensic related literature as well as through the main repository for microRNAs (miRBase).

In all of the studies performed relating to miRNA analysis e.g. DNA vs. total RNA isolations, expression of miRNA during wash steps of isolation, blind process and increasing sample numbers: miR-451 and miR-205 showed specificity to blood and saliva respectively.

There were a number of new findings shown in this section of the work. The major finding in this work was the expression of miRNA in DNA isolations. The major forensic research groups working on miRNA analysis e.g. Zubakov *et al* (2010), Hanson *et al* (2009), Courts and Madea (2011) utilised total RNA or miRNA isolation kits for miRNA. Although their

standpoint was logical, from a casework perspective it was thought that it might complicate current DNA profiling methods. Thus the first aim of this work was to compare the effectiveness of two common DNA and total RNA isolation kits (described in Chapter 2). It was hypothesised that since the conditions e.g. buffers for each kit were optimised to isolate either DNA or total RNA, that it would be likely that the total RNA isolation method would yield the highest amount of RNA. However a comparison of expression of miR-451 and miR-205 in blood and saliva revealed significantly more expression in the DNA isolation kit than the total RNA isolation kit ($P<0.05$). The level of miRNA expression shown using the DNA isolation kit was surprising. There also was a significant difference between the two kits (ΔCq 2-3). The potential impact of this find is substantial in the area of forensic casework. It gives the practitioner the choice to use either a DNA or total RNA isolation method without compromising their main aim, which is often to obtain a DNA profile.

It has been mentioned in the Qiagen protocols, and has been observed by forensic research groups e.g. Zubakov *et al* (2010), Wang *et al* (2013) and the EDNAP collaborative exercises 1-5, that total RNA isolation kits can also isolate DNA and conversely the same for DNA kits. However it appears that these groups have missed this finding, that higher recovery of miRNA can be achieved using DNA isolation kits.

One might argue whether the expression shown was microRNA. However given the specificity of the chemistry that was used, e.g. TaqMan, it is highly likely that the expression is microRNA. As mentioned earlier, the combination of the stem-loop primer and TaqMan chemistry is highly specific. It also comprises of a synthetic strand (not found in nature), which will bind upon itself via primer dimer formation. Thus the resulting product is truly that of the target of interest and is one of the major advantages of using

TaqMan over other chemistries such as SYBR Green. Furthermore no amplification was observed in the negative controls suggesting that the product is likely to be microRNA. An additional method of confirmation would be to send these products through sequencing.

The high expression of miRNA found in the DNA extracts lead this work to the next study; establishing where the miRNA was being most recovered. For comparative purposes, a similar experiment was prepared with the total RNA isolation kits. It was hypothesised in this experiment that the majority of non-target genetic material e.g. RNA would be lost during the wash steps as this is the stage where sample purification occurs. What was observed in the study was completely different. The majority of miRNA was released during the elution step.

The findings from this study could mean a number of things. It could mean that the miRNA because it is so small becomes entwined with DNA during lysis/binding step. Thus when samples are washed the miRNA remains intact. There is also a competing hypothesis. A significant amount of microRNA may be being lost during the wash steps. The wash steps contain powerful chaotropic agents e.g. Guanidine hydrochloride, which can cause PCR inhibition. However since microRNAs are generally high in abundance (e.g. 50,000 copies/cell), the loss of microRNAs may still mean that there is still enough eluted during the wash step.

To explore the potential of microRNA analysis within forensic casework, samples also underwent a blind process whereby the miRNA markers would be used to identify a body fluid. At this stage only single source samples were used, as the complexity that mixtures can add to interpretation was not needed. The blind process showed successful identification of blood and saliva samples showing the potential of using miRNA analysis

within forensic casework. It is important to note that because the aim is to use this within forensic casework, the use of additional body fluid specific markers and identification of reference genes are likely to be needed.

Finally to demonstrate the differentiating capabilities of miR-205 and miR-451 in saliva and blood, samples numbers were increased.   The results from this study showed successful identification of saliva using these markers. Since the aim of this work is to develop an RNA analysis method for use in forensic casework, a wider range of body fluids and body fluid specific markers needed. Thus the next chapter focuses on screening of such markers for BFID.

Conclusions

In this work, the identification of body fluid using miRNA analysis was explored. Two different body fluids and miRNA markers were explored to assess this test. Also two different isolation kits; DNA and total RNA isolation kit were explored. The results indicated successful use and application of miRNA analysis in forensic casework.

Chapter 4

Developing a miRNA panel for a miRNA analysis

4.1 MicroRNA marker screening

Body fluid specific screening forms a crucial step in developing a miRNA analysis method for use in forensic casework. There are two fundamental factors that need to be addressed when screening miRNA markers. First it is important to establish which miRNA markers are highly expressed in specific body fluid. Second is dependent on the specificity of that marker. If the miRNA marker is found to be body fluid specific or show no expression in other body fluids, then the test for that particular body fluid is ready. If the miRNA marker shows high expression in one body fluid but also some expression in other body fluids then it is important to determine their body fluid differentiation capability e.g. whether additional markers will be needed to identify a particular body fluid. Both factors are essential when establishing a new RNA-BFID test, as without specificity or differentiation capability of such markers it is not possible to develop a test. There are a handful of miRNA research groups working on miRNA BFID [230, 231, 234-236].

The majority of the research in screening miRNA markers has gone into the identification of blood and saliva markers e.g. Zubakov *et al* (2010), Courts and Madea (2011), Wang *et al* (2013) and Bai *et al* (2013). They utilised different chemistries e.g. TaqMan® or SYBR® green to identify body fluid specific miRNA markers (shown in Table 1). Thus for the purpose of developing a unique or original panel for this miRNA BFID method, miRNA markers for blood and saliva were explored.

One tissue type that has not been explored extensively in miRNA screening is skin. The majority of skin related miRNAs are related to non-forensic based research e.g. cancer [208]. It is worth noting that identification of skin markers has been made through mRNA

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analysis [18, 27, 184]. However since the aim in this project is to develop a miRNA analysis method this is not of much relevance.

Two other body fluids that are of major forensic interest are vaginal material and semen. Vaginal material has been difficult to identify and distinguish, as its cellular material e.g. epithelial cells are very similar to those found in saliva. Consequently in forensic casework it is difficult to differentiate vaginal material from saliva. Hanson *et al* (2009) and Zubakov *et al* (2010) identified potential miRNA markers for this. However again because different chemistries were used and the aim of this work is to create both a unique and highly discriminative panel of miRNA markers the identification of such miRNA marker was explored.

Semen poses a different set of challenges to the other body fluids. The majority of the genetic material is found encased in the sperm head, which is firmly held together by di-sulphite bonds. Generally, special treatment using strong agents such as DTT is needed to release this material. In cases where an individual has been vasectomised or is azoospermic this can give identification of semen additional challenges. Identification of semen will be reliant on the seminal fluid. Thus identification of a marker present in semen was also explored. Hanson *et al* (2009), Zubakov *et al* (2010) and Wang *et al* (2013) have identified potential markers but for the same reasons previously mentioned with the other body fluids, a semen or seminal fluid miRNA marker was explored.

Aim

The aim of this study was to build on the existing panel of body fluid specific miRNA markers from the previous chapter by identifying suitable miRNA markers for BFID.

Experimental design

A total of 13 different miRNA markers were screened with 6 different body fluids (shown in Table 4). Five different donors were used in this study based on sample availability. All body fluids underwent sample collection, DNA isolation, cDNA synthesis and qPCR. A random set of RT and PCR controls were included in each study.

Body fluid	miR marker	Mature miR sequence	miRBase accession ID	Reference(s)
Blood	miR-451a	AAACCGUUACCAUUACUGAGUU	MIMAT0001631	[196, 200, 230, 235, 274-279]
Blood	miR-16	UAGCAGCACGUAAAUAUUGGCG	MIMAT0000069	[230, 280-282]
Saliva	miR-205	UCCUUCAUUCCACCGGAGUCUG	MIMAT0000266	[200, 230, 235, 237, 283-292]
Saliva	miR-658	GGCGGAGGGAAGUAGGUCCGUUGGU	MIMAT0003336	[230]
Skin	miR-203	GUGAAAUGUUUAGGACCACUAG	MIMAT0000264	[200, 208, 235, 291, 293, 294]
Skin	miR-194	UGUAACAGCAACUCCAUGUGGA	MIMAT0000460	[208, 294]
Skin	miR-224	CAAGUCACUAGUGGUUCCGUU	MIMAT0000281	[208, 294]
Skin	miR-335	UCAAGAGCAAUAACGAAAAAUGU	MIMAT0000765	[208, 294]
Seminal fluid	miR-891a	UGCAACGAACCUGAGCCACUGA	MIMAT0004902	[231, 237]
Seminal fluid	miR-588	UUGGCCACAAUGGGUUAGAAC	MIMAT0003255	[232]
Vaginal material	miR-617	AGACUUCCCAUUUGAAGGUGGC	MIMAT0003286	[231]
Vaginal material	miR-372	AAAGUGCUGCGACAUUUGACGU	MIMAT0000724	[200, 230, 287, 295, 296]
Vaginal material	miR-124a	CGUGUUCACAGCGGACCUUGAU	MIMAT0004591	[230, 237, 297]

Table 4. Showing 13 candidate miRNA markers selected for BFID including their mature sequence, miRBase accession ID and referenc

4.1.1 Blood

Figure 13. Showing the expression of 13 different miRNA markers in bloodstain. The black line at ΔCq 5 represents a threshold. Values above this line were considered expression while values below this line were considered background amplification. Error bars represent one standard deviation (n=5).

Three potential miRNA markers were identified for blood: highly expressed miR-451 and miR-16 and moderately expressed miR-194 (shown in Figure 13). Two of the three markers were selected for the final body fluid panel: miR-451 and miR-194 (as described more later in this chapter). Marker miR-16 was not included as it showed similar levels of body fluid differentiation as miR-451. No amplification in the negative controls was observed in this study. Marker miR-194 was identified as a new marker for identifying blood. The inclusion of miR-451 in this panel was strongly supported by other research groups in this field e.g. Hanson *et al* (2009), Courts and Madea (2011), Zubakov *et al* (2010).

4.1.2 Saliva swabs

Figure 14. Showing the specificity of 13 different miRNA markers in saliva swabs. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

A combination of two or more markers is needed to identify saliva swabs (shown in Figure 14). One potential saliva swab marker that could be included in this panel is miR-205 (as described more later in this chapter). Marker miR-205 showed a high level of expression in saliva swabs when compared to the miRNA markers below the set threshold of ΔCq 5 (shown in Figure 14). The identification of one potential marker, miR-205 was strongly supported by the work of Hanson *et al* (2009), Courts and Madea (2011).

4.1.3 Saliva deposits
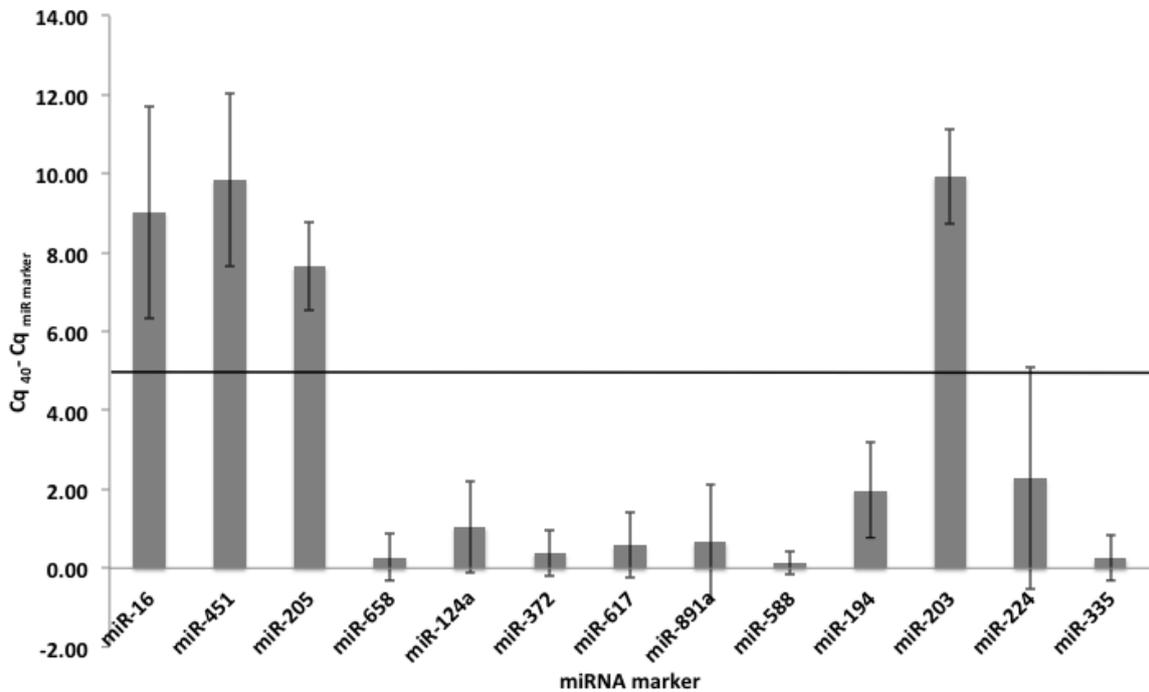
Figure 15. Showing the specificity of 13 different miRNA markers in saliva deposits. The black line at ∆Cq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

A combination of two or more markers is also needed to identify saliva deposits (shown in Figure 15). The same marker selected for the identification of saliva swabs, miR-205 could also be used to establish the presence of saliva (as described more later in this chapter). Additional markers could then be used to differentiate between other body fluids. The identification of miR-205 as one of the potential markers was supported by the work of Hanson *et al* (2009) and Courts and Madea (2011).

4.1.4 Skin
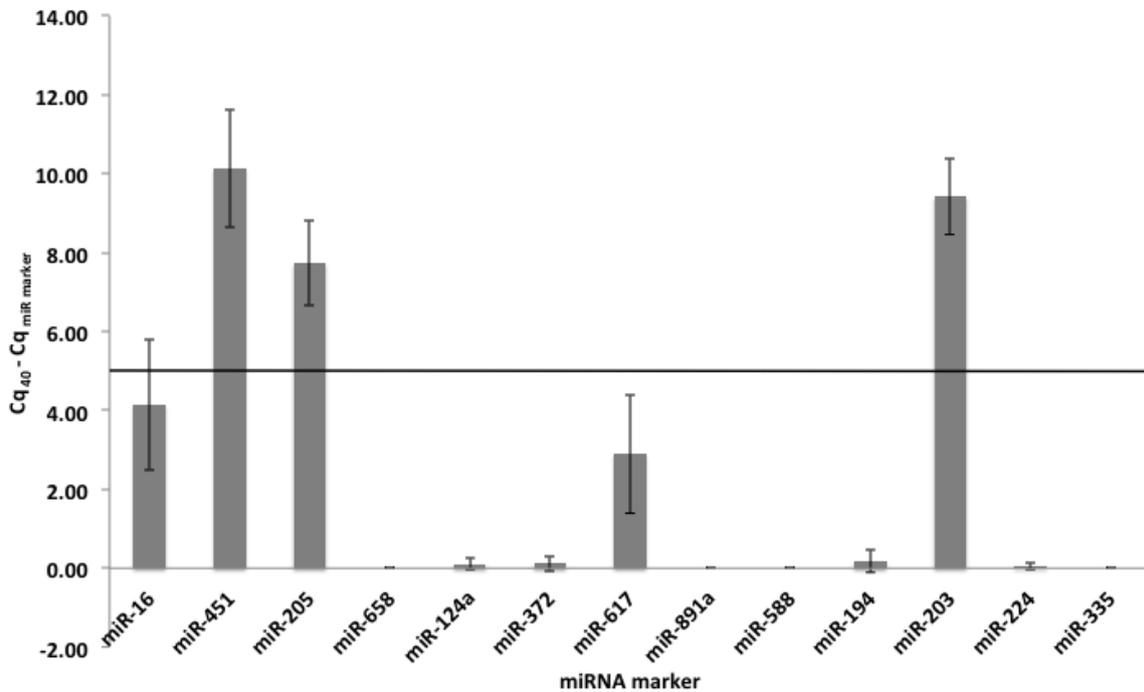
Figure 16. Showing the specificity of 13 different miRNA markers in skin. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

A miRNA marker was not identified for skin (shown in Figure 16). High expression was seen in one of the five of the potential skin markers tested; miR-203. However its high expression seen in saliva swabs, saliva deposits and blood did not make it suited identifying skin. Candidate skin markers from this study were eliminated (as described more later in this chapter). This research was the first efforts made to identify a skin miRNA marker for forensic BFID.

4.1.5 Semen
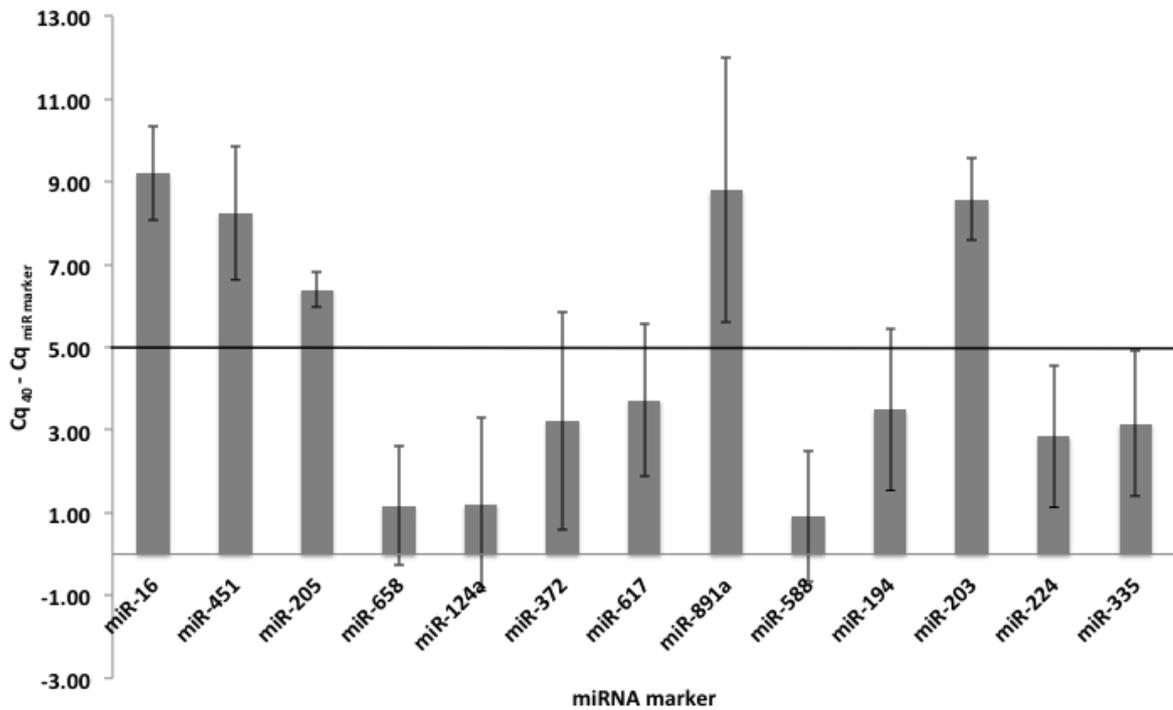
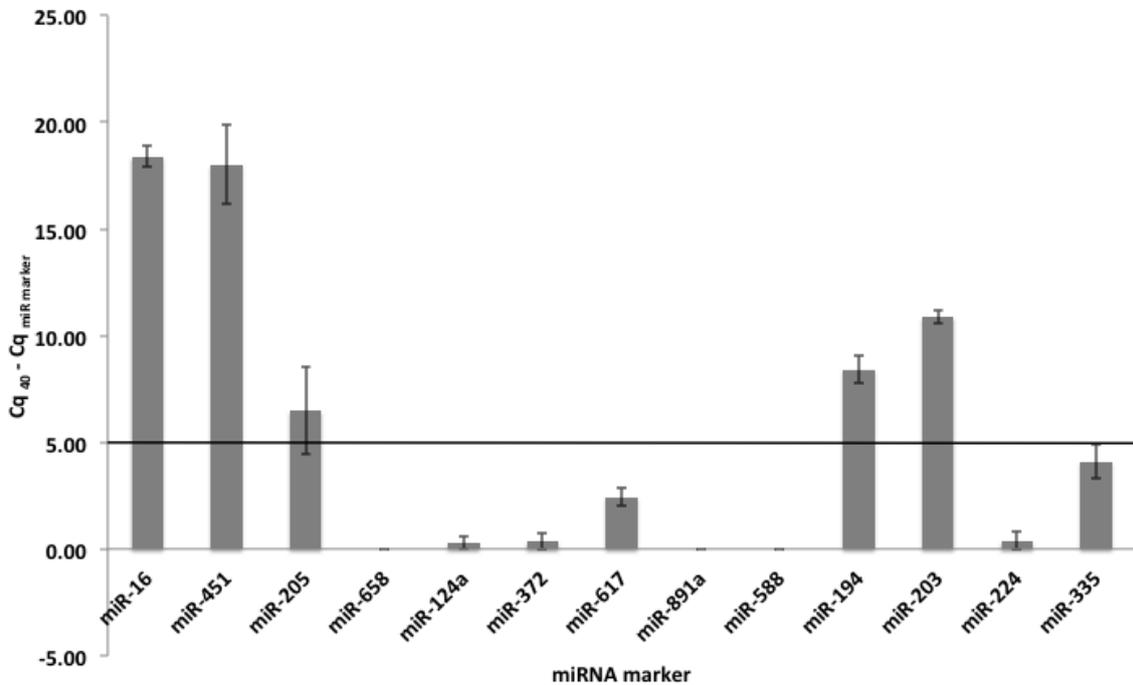*Figure 17. Showing the specificity of 13 different miRNA markers in semen. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).*

Marker miR-891a was identified as a potential marker for identifying semen (shown in Figure 17). Marker miR-891a showed significantly high levels of expression when compared to the markers below the set threshold of ΔCq 5. A single miRNA marker was identified for semen (as described more later in this chapter). The use of miR-891a as a potential semen marker was supported by Zubakov *et al* (2010) and Wang *et al* (2013).
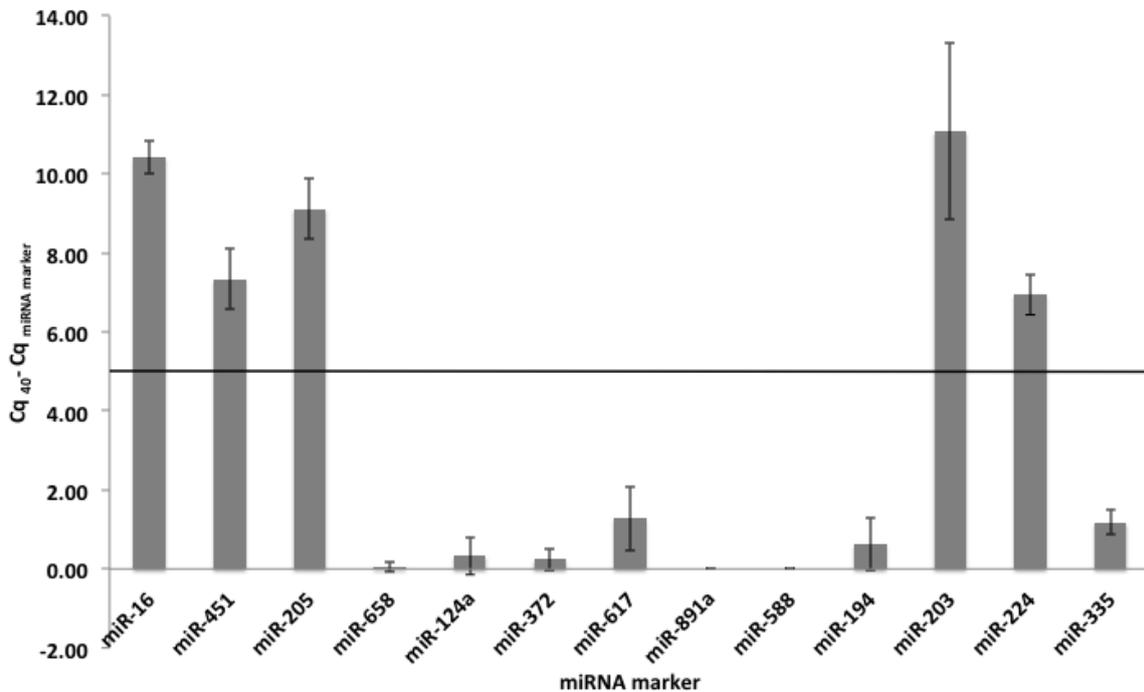
4.1.6 Vaginal material
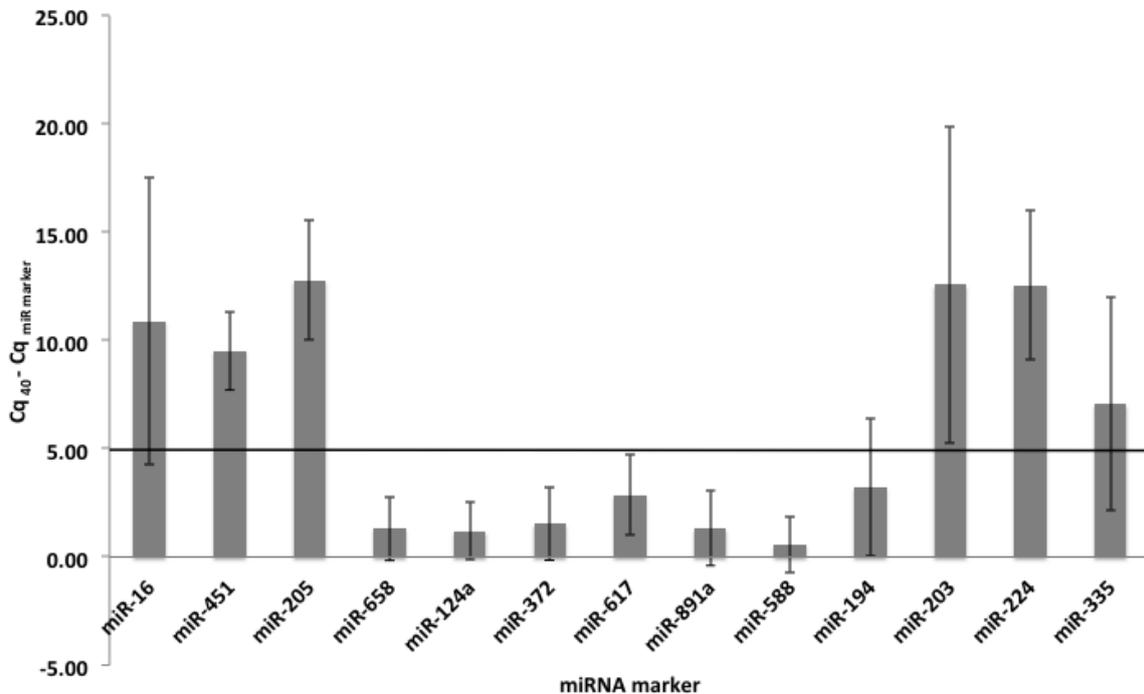
Figure 18. Showing the specificity of 13 different miRNA markers in vaginal material. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

A combination of two markers was identified for vaginal material (shown in Figure 18).

Markers miR-224 and miR-335 showed high levels of expression when compared to the

markers below the set threshold of ΔCq 5. Markers miR-224 and miR-335 were selected

for the final miRNA panel (as described more later in this chapter). No other miRNA based

research groups have combined miR-224 and miR-335 as vaginal material markers (shown

in Table 1).

4.1.7 miR-451

Figure 19. Showing the specificity of miR-451 across 6 different body fluids The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

Marker miR-451 was the first of two markers selected from this study to identify blood (shown in Figure 19). Marker miR-451 showed higher expression in blood than in any of the body fluids tested. However its expression in all the other body fluids were above the set threshold, and as such a second blood marker was included.

4.1.8 miR-194
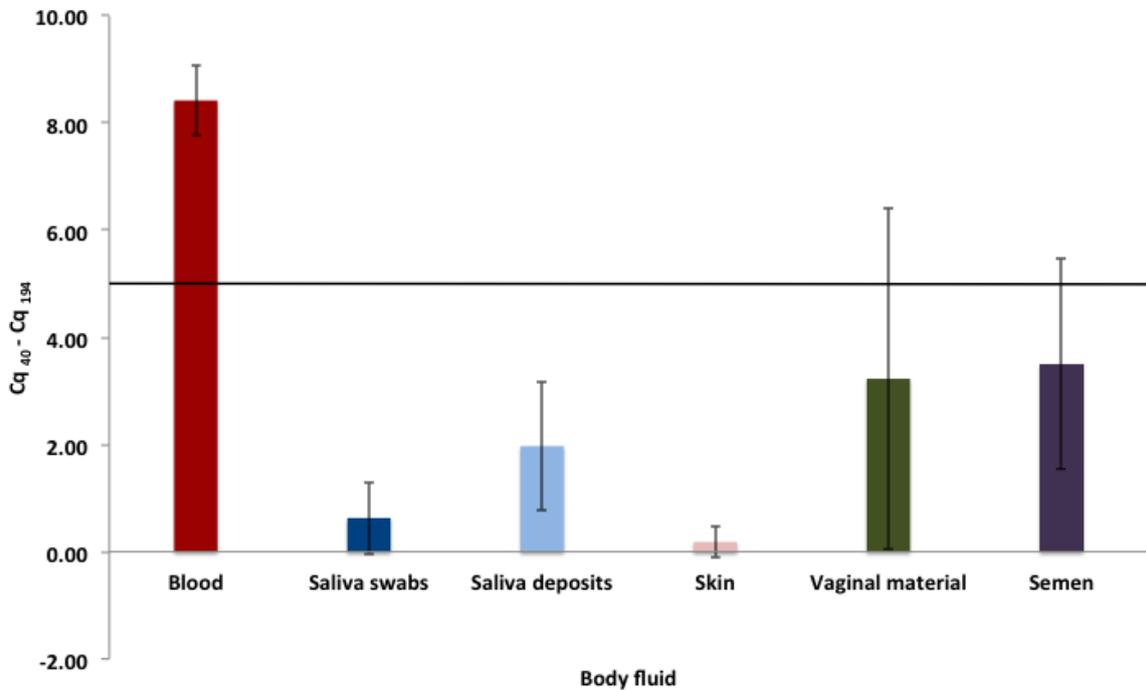
Figure 20. Showing the specificity of miR-194 across 6 different body fluids The black line at ∆Cq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

Marker miR-194 was the second of two markers selected to identify blood (shown in Figure 20). It showed moderate levels of expression in blood. Its expression in the majority of the other body fluids was below the set threshold. Marker miR-194 could differentiate blood from saliva swabs, deposits and skin. Also depending on sample variability, miR-194 could differentiate vaginal material and semen. A discriminative pair of blood markers: miR-451 and miR-194 was identified. A new blood marker was identified in this work, miR-194. Zubakov *et al* (2010), Courts and Madea (2011) and Wang *et al* (2013) support this work for using miR-451 as a blood marker.

4.1.9 miR-205
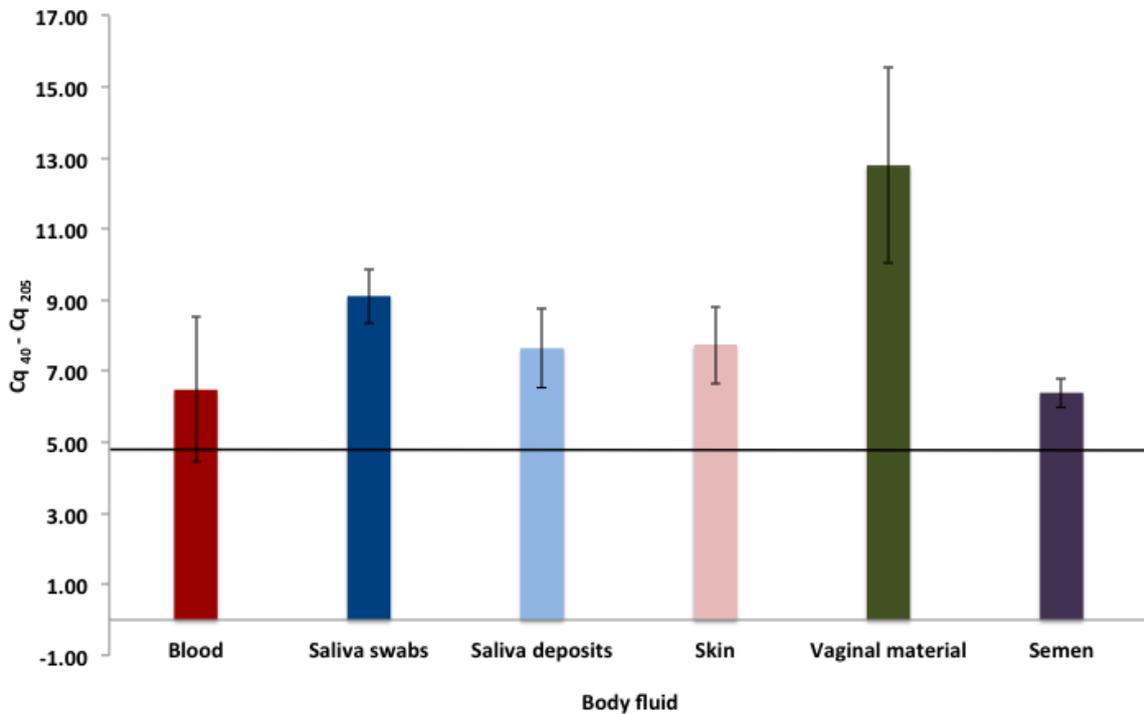
Figure 21. Showing the specificity of miR-205 across 6 different body fluids The black line at ∆Cq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

One potential marker was identified for saliva (shown in Figure 21). Marker miR-205 could be used to indicate the presence of saliva. It could also be used to potentially differentiate saliva swabs and saliva deposits from vaginal material. However additional markers will be required to differentiate saliva swabs from saliva deposits and skin. Marker miR-205 was identified as a potential marker for identifying saliva swabs and saliva deposits. Zubakov *et al* (2010), Courts and Madea (2011) and Wang (2013) support this work for using miR-205 as a saliva marker. Additional markers will need to be selected to differentiate saliva swabs from saliva deposits and skin.

4.1.10 miR-224
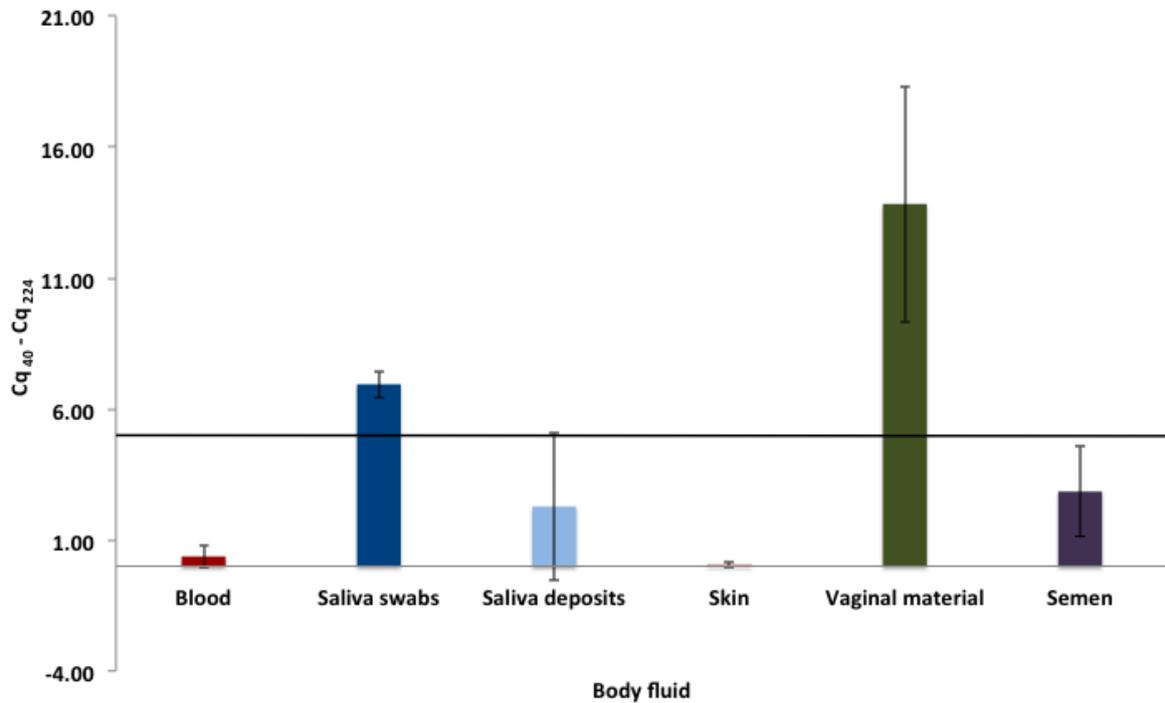
Figure 22. Showing the specificity of miR-224 across 6 different body fluids The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

Marker miR-224 was one of two markers selected to identify vaginal material (shown in Figure 22). It showed high levels of expression in vaginal material. Marker miR-224 could be used to differentiate vaginal material from blood, saliva deposits, skin and semen. Since miR-224 did show expression in saliva swabs, a second miRNA marker for vaginal material was selected.

4.1.11 miR-335
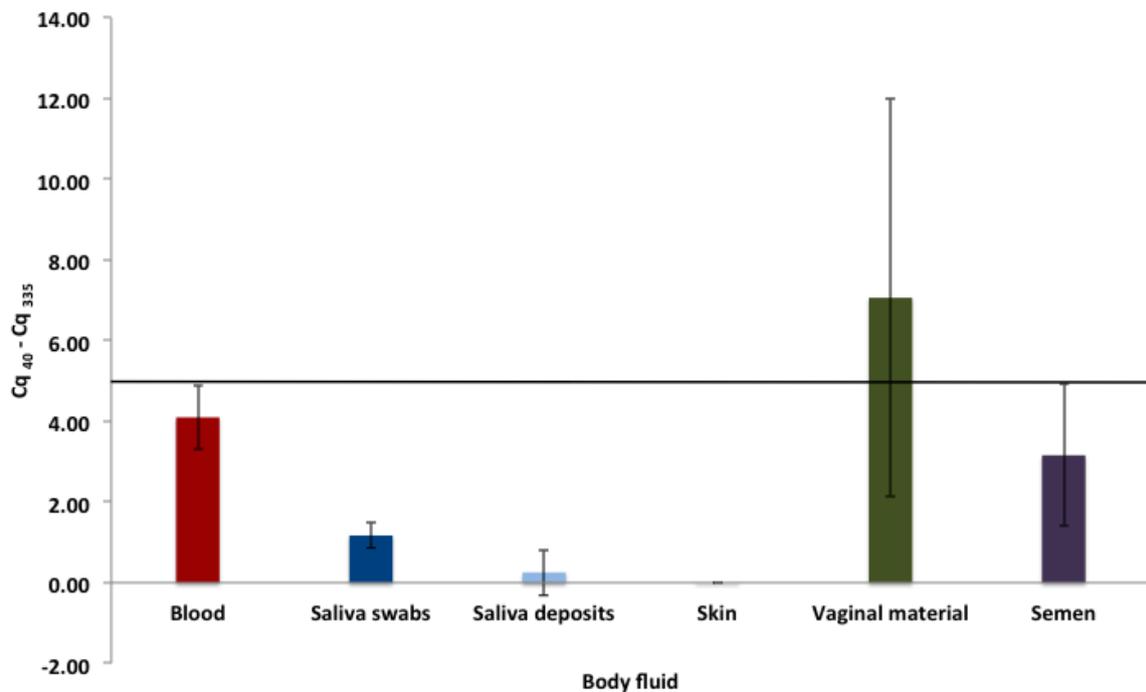
Figure 23. Showing the specificity of miR-335 across 6 different body fluids The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

Marker miR-335 was the second of two markers selected from this study to identify vaginal material (shown in Figure 23). It showed moderate levels of expression in vaginal material. It could be used to differentiate vaginal material from saliva swabs, saliva deposits and skin. Also depending on variability in samples, it could be used to potentially differentiate vaginal material from blood and semen. A discriminative pair of vaginal material markers: miR-335 with miR-224 was identified. Both miR-335 and miR-224 were identified as new markers for vaginal material.
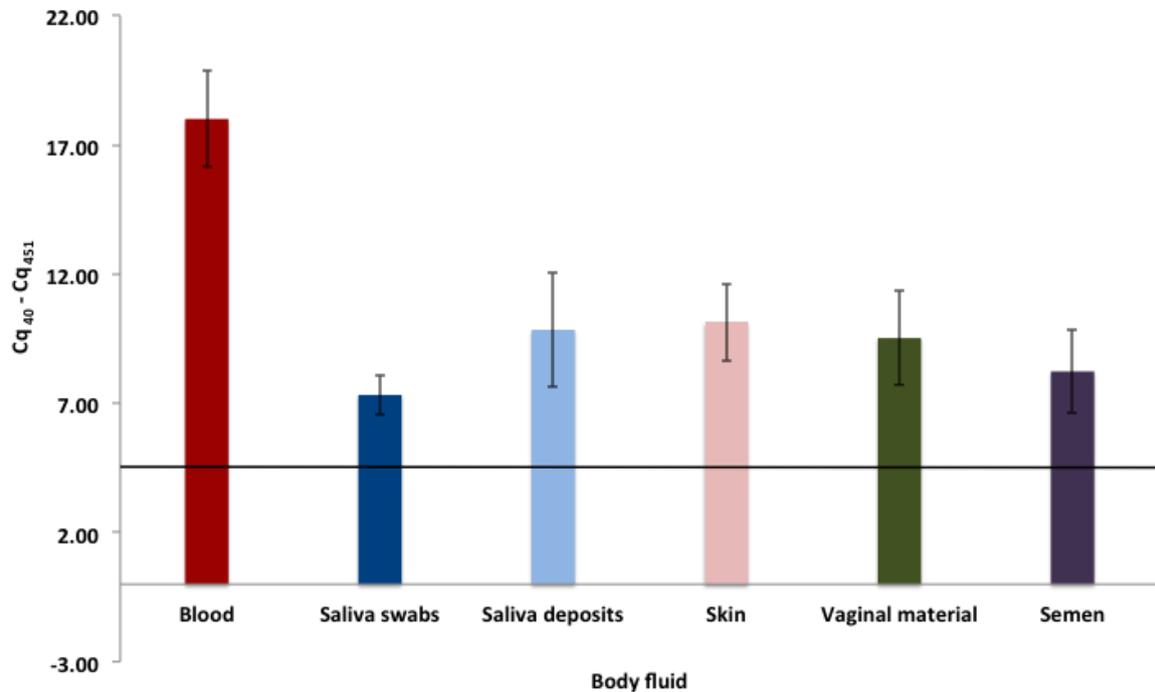
4.1.12 miR-891a
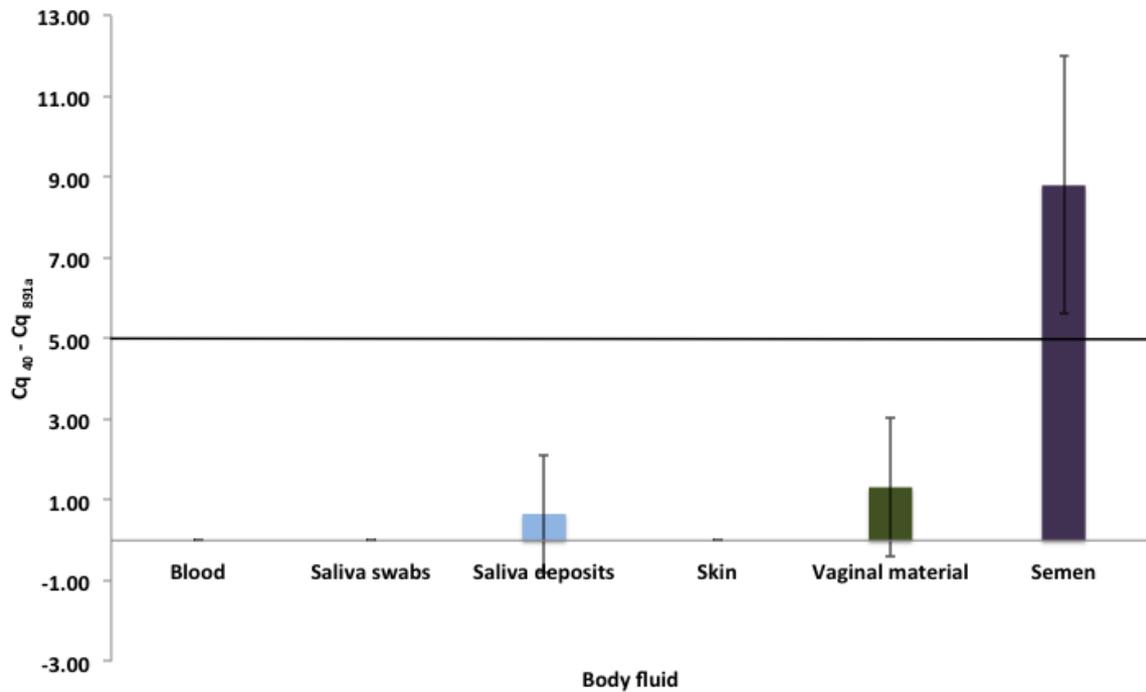
Figure 24. Showing the specificity of miR-891a across 6 different body fluids The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

MicroRNA marker, miR-891a was identified as a semen specific marker (shown in Figure 24). It showed moderate levels of expression in semen and could be used to differentiate semen from blood, saliva swabs, saliva deposits, skin and vaginal material. A semen specific marker was identified for miRNA analysis. Zubakov *et al* (2010) and Wang *et al* (2013) support this work for using miR-891a to differentiate from the body fluids tested.

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	Blood	Saliva swabs	Saliva deposits	Skin	Semen	Vaginal material
miR-451	18.01	7.33	9.85	10.14	8.24	9.53
miR-194	8.42	0.63	1.97	0.18	3.50	3.23
miR-205	6.48	9.11	7.64	7.74	6.39	12.79
miR-224	0.37	6.95	2.28	0.04	2.85	13.83
miR-335	4.10	1.17	0.26	0.00	3.16	7.06
miR-891a	0.00	0.00	0.66	0.00	8.80	1.32

Table 5. Showing the panel of miRNA markers. The average ΔCq values were shown in all of the boxes. Green boxes indicate identification of a body fluid. Yellow boxes indicate the average ΔCq values above the set threshold of ΔCq 5. Red boxes indicate ΔCq values below the set threshold of ΔCq 5.

Discussion

The aim of this work was to develop a highly discriminative panel of miRNA markers for miRNA analysis using six commonly encountered body fluids and tissues in forensic casework: blood, saliva swabs, saliva deposits, skin, semen and vaginal material.

A total of 13 different miRNA markers were selected for the screen: miR-451 and miR-16 (blood); miR-205 and miR-658 (saliva swabs and saliva deposits); miR-124a, miR-372 and miR-617 (vaginal material); miR-891a and miR-588 (semen); and miR-194, miR-203, miR-224 and miR-335 (skin).

Of the 13 miRNA markers tested, six were identified as body fluid specific, either singularly or using a combination of two markers: miR-451 and miR-194 (blood); miR-205 (saliva); miR-224 and miR-335 (vaginal material); and miR-891 (semen) (shown in Table 5). No markers were identified for skin.

The remaining seven miRNA markers were eliminated on the basis of: lack of specificity (e.g. miR-203), commonality in expression patterns (e.g. miR-16 exhibited similar levels of expression as miR-451) or low expression (e.g. miR-658, miR-124a, miR-372, miR-617 and miR-588).

The differentiation capabilities of each marker or marker set were as follows: a combination of markers, miR-451 and miR-194 were able to differentiate blood from the other five body fluids types tested. One potential marker was identified for differentiating saliva from vaginal material. However it was determined that additional markers would be needed to

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differentiate saliva swabs from saliva deposits, skin and seminal fluid. A combination of markers, miR-224 and miR-335 were able to differentiate vaginal material from the other five body fluid types tested. A single marker, miR-891a was identified for differentiating semen from the other five body fluid types tested (shown in Table 5).

The following miRNA markers were novel in this study: miR-194, originally selected for skin, was found to be blood-specific in this study. None of the other research groups in this area e.g. Hanson *et al* (2009), Zubakov *et al* (2010), Courts and Madea (2011), Wang *et al* (2013) and Bai *et al* (2013) have used miR-194 to identify blood. A combination of markers was used to identify and differentiate blood. Marker miR-451 showed high expression in blood, however it also showed expression in all of the other body fluids tested. Consequently if this test was to be applied to casework samples this would be problematic and the level of expression of miR-451 in blood may be proportional to the expression found in the other body fluids. As such, a second marker was included for the identification and differentiation of blood. Zubakov *et al* (2010) and Courts and Madea (2011) also support the use of a second marker. The selection of a second marker was based on the expression levels of all the markers in the other body fluids. It was initially thought that miR-16 might be a suitable second marker for blood. However upon closer study at the expression of miR-16 in all the other body fluids, it exhibited similar expression levels as miR-451.

Therefore it was felt that two markers showing the similar result would be redundant and not sufficient for further differentiation. Furthermore its relatively high expression in all of the other body fluids, except in skin made it potentially not suited for differentiation. Interestingly on a side note the low expression of miR-16 in skin in comparison to all of the

other body fluids could be useful for identifying skin. However additional markers for identifying skin would also be needed in case it was found to be exhibited in other body fluids such as sweat or urine.

The second marker selected for identification and differentiation of blood was miR-194. Initially this had been selected as a potential skin specific marker. However its low expression in skin made it unsuitable as a skin miRNA marker. Figure 20 shows the expression of miR-194 in all of the body fluids. It showed moderate levels of expression but lower levels of expression in all of the other body fluids. It is worth noting that a high variation was observed in miR-194 in both vaginal material and semen, possibly due to the lack of prior sample normalisation. So caution may need to be taken in interpretation. Nevertheless the combination of miR-451 and miR-194 made a discriminative pair for the differentiating blood from all of the other body fluids tested. When comparing the expression of miR-451 and miR-194 by body fluid is worth noting that although miR-451 exhibited relatively high expression in all samples, miR-194 showed expression above the set threshold in blood.

Identification of saliva swabs and saliva deposits and skin was particularly challenging and is currently still a work in progress. One potential marker was selected from this screen, miR-205. This was because it had been previously shown to be sufficient for differentiating saliva swabs from blood. Zubakov *et al* (2010), Courts and Madea (2011) and Wang *et al* (2013) support this finding of using miR-205 as one marker and support the use of additional markers for identifying saliva. In their work they had identified two other candidate markers, one of which was part of our panel e.g. miR-203. However since miR-203 exhibited similar levels of differentiation as miR-205 it was eliminated from the panel.

Thus it can be concluded that more studies need to be made to identify and distinguish between saliva swabs, saliva deposits and skin.

A combination of two different markers was identified for differentiating vaginal material from all other body fluids tested. Marker miR-224 showed high expression in vaginal material and low expression in all other body fluids types except saliva swabs. Saliva swabs showed lower expression than vaginal material however it crossed the set threshold, thus a second miRNA marker was included. The second marker selected, miR-335 showed moderate levels of expression (similar to miR-194), however it showed lower expression in all of the other body fluids e.g. values did not cross the set threshold. Again if the expression of miR-224 and miR-335 are observed by body fluid it was seen that miR-335 only crosses the threshold in vaginal material. Marker miR-224 showed high expression in vaginal material and moderate levels of expression in saliva swabs but in no other body fluids. However the differentiation of vaginal material and saliva swabs can be determined by averaging the two expression levels. In other words the average expression level of miR-224 and miR-335 will cross the set threshold in vaginal material but will not cross the threshold in saliva swabs (shown in Figure 14 and 18). Two new vaginal material markers originally selected for skin were identified in this study. Also briefly worth mentioning, miR-617, which was also a candidate marker for vaginal material, however it was eliminated on the basis that it did not cross the set threshold.

The final marker that formed part of the panel was miR-891a. It was the one marker that was found to be semen specific or in other words no expression was seen in any of the other markers. Both Zubakov *et al* (2010) and Wang *et al* (2013) supported this finding. An additional marker was explored for seminal fluid; miR-588. However minimal expression

was seen in semen and all of the other body fluids and was eliminated from this panel. Weber *et al* (2010) support this finding to a degree as it was identified as a highly expressed and specific seminal fluid marker. However it appeared that it was eliminated in the final panel of markers based on its ability to discriminate from other body fluids. The difference in expression observed by Weber *et al* (2010) and this study may be down to a couple of factors. One is the chemistry that was used, Weber *et al* (2010) used SYBR green based chemistry to identify their panel of body fluid specific miRNA markers. Another factor is sample; Weber *et al* (2010) used seminal fluid where as in this study semen was used. It may be that the ratio of miR-588 when compared to miR-891a is lower in semen than in seminal fluid.

An additional factor to consider in this study was sample variation. There was high degree of both inter and intra-variability seen in the miR-224 and miR-335 expression in vaginal material and miR-891a expression in semen. There are a number of possible explanations for the high variability in these results. Sample normalisation was performed according to volume rather than concentration. This approach was taken to more closely simulate casework samples. However it may have resulted in more variation between the samples. Environmental factors may also have contributed to the variation observed across the samples. For instance varying levels of bacterial contamination may have been present due to the sampling regions, resulting in different levels of miRNA expression in the body fluids. The high variation seen may have also been down to individual variation. In other words, the expression of miR-224, miR-335 and miR-891a may naturally vary between different individuals in vaginal material and semen respectively.

Furthermore it is important to add that although a panel of discriminative miRNA markers were identified for miRNA analysis, the criteria for the markers to be used in forensic casework will need to be adapted. For instance it is apparent that though the set threshold is suited for abundant samples it will most likely need to be adapted e.g. lowered for casework samples. Also with the high variability observed in casework samples (e.g. sample quality and quantity), reference gene will with no doubt be needed. It is however paramount to first establish a panel of miRNA markers within abundant samples before proceeding onto this next step.

Another approach that could be used to help identify miRNA markers may be to begin categorizing miRNA markers according to functional roles. For instance, highly expressed marker miR-451 for blood was also expressed in the other body fluids tested. This may suggest that miR-451 has a common functional role within these body fluids. Similarly could be said for miR-224 for vaginal material that was highly expressed in vaginal material but was expressed lower in the other body fluids (still above the set threshold).

Conclusions

A discriminative panel of miRNA markers was identified for miRNA analysis was identified for blood (miR-451 and miR-194), saliva swabs and saliva deposits (miR-205), vaginal material (miR-224 and miR-335) and semen (miR-891a) for use in forensic casework.

Chapter 5

The applications of miRNA analysis

Establishing the strengths and limitations of a miRNA BFID test is very important as with the development and improvement of any test. There are several key questions that need to be addressed. Firstly is this miR BFID test sensitive, specific and stable enough to correctly identify a body fluid? Secondly is this test comparable, stronger or weaker to current methodologies in use? Lastly is it possible to determine the components of a mixture especially as body fluids are often found in this form?

5.1 Sensitivity of miRNA analysis with enzymatic tests

Establishing the sensitivity of a test is very important. It gives an indication of the strengths and limitations of a test in context with existing methodologies. It also gives the forensic practitioner an indication of the best test to apply to a casework sample e.g. presumptive vs. confirmative tests.

It is also a requirement under quality procedures to establish the limit of detection. The technical and legal requirements (e.g. ISO17025 the international standard of lab accreditation) associated with introducing a new method must be met in order for it to be incorporated into a forensic laboratory [42].

Sensitivity is often described as the point which a target e.g. miRNA marker can no longer be detected or the limit of detection (LOD) [247]. Sensitivity is also described as the point at which a target (e.g. miRNA marker) is detected but falls below the range of size standards or the limit of quantification (LOQ). Since samples in this work were quantified by relative quantification (RQ) rather than absolute quantification (AQ), markers in this study were assessed by LOD.

In forensic casework, it is common practice to test samples with presumptive methods before proceeding with confirmatory techniques. Presumptive tests play an important role in forensic casework. They allow forensic practitioners and police to decide which confirmative tests to perform within a limited budget and time frame.

One of the main limitations of presumptive tests used in forensic casework is their sensitivity. A wide range of sensitivities have been reported for both presumptive blood tests: Kastle-Meyer (KM), Leucomalachite Green (LMG) and presumptive saliva tests: Phadebas [38, 39, 41, 42, 57, 59-62]. An equally or more sensitive BFID test would be advantageous in forensic casework.

Despite the rapid efforts shown by many mRNA (e.g. Juusola *et al* 2003, Alvarez *et al* 2004, Fleming *et al* (2010), EDNAP collaborative exercises 1-5 (2011-2014)) and miRNA (e.g. Hanson *et al* (2009), Zubakov *et al* (2010), Haas *et al* (2011), Wang *et al* (2013) and Li *et al* (2014)) research groups to incorporate RNA BFID analysis into current forensic casework, it appears only one study has compared the sensitivity of RNA analysis (e.g. mRNA analysis with presumptive tests) [168].

Furthermore none of these miRNA research groups have assessed the sensitivity of miR-451, miR-205 in miRNA analysis or assessed the sensitivity of reference genes. The lack of such studies is surprising as reference genes are one of the main methods used for assessing the relative expression levels of different miRNAs and mRNAs.

Aim

Thus there were two main aims in this work. The first aim was to assess the sensitivity of miRNA analysis with three presumptive tests: Kastle-Meyer (KM)), Leucomalachite Green (LMG) and Phadebas. Both the KM and LMG tested were selected on the basis that they are used internationally in by forensic practitioners and police forces [42, 298, 299]. The Phadebas

test was also selected for similar reasons. The second was to assess the sensitivity of miRNA analysis using markers miR-451, miR-205 and RNU44 (shown in Table 6).

Experimental design

There were two different experiments performed in this section. The first experiment assessed the sensitivity range of the KM, LMG and Phadebas tests. Dried bloodstains were used for the KM and LMG tests while saliva deposits were used for the Phadebas test. A 10-fold serial dilution was performed on both sample types. Samples then underwent KM, LMG and Phadebas testing.

The second experiment assessed the sensitivity range of miR-451 and miR-205 in blood and saliva deposits. The sensitivity range of RNU44 was also assessed in both body fluids as no previous research had been performed. A 10-fold serial dilution was performed on both sample types. Samples then underwent DNA isolation, cDNA synthesis and qPCR.

During the course of both studies a total of 5 different blood and saliva donors were used. Both positive controls (e.g. undiluted blood and saliva samples) and negative controls (e.g. blank filter paper, RT and PCR controls) were also included.

Reference gene	Sequence	NCBI Accession no.	References
RNU44	CCU GGA UGA UGA UAG CAA AUG CUG ACU GAA CAU GAA GGU CUU AAU UAG CUC UAA CUG ACU	NR_002750	[231, 233, 235]

Table 6. Showing the reference gene selected for BFID including its sequence, NCBI accession number and reference.

5.1.1 Kastle-Meyer, Leucomalachite Green and Phadebas tests

Both the Kastle-Meyer (KM), Leucomalachite Green (LMG) tests showed a sensitivity range between 1 in 100 – 1 in 10,000 dilutions in bloodstains (shown in Table 7). A similar range was also observed for the Phadebas® (PAT) test in saliva deposits. All positive and negative controls performed as expected. Cox *et al* (1991), Grodsky *et al* (1951), Webb *et al* (2006), Tobe *et al* (2007) and Johnston *et al* (2008) support this sensitivity range observed for blood. Willot (1980), Kipps and Whitehead (1975), Auvdel (1986), Keating and Higgs (1994), Hedman *et al* (2011) support this sensitivity range observed for saliva deposits.

	Neat	1 in 10	1 in 100	1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000
KM	+(5)	+(5)	+(5)	+(3)	-(0)	-(0)	-(0)
LMG	+(5)	+(5)	+(5)	+(3)	-(0)	-(0)	-(0)
PAT	+(5)	+(5)	+(5)	+(2)	-(0)	-(0)	-(0)

Table 7. Showing the sensitivity of KM, LMG and Phadebas® (PAT) when tested on neat, 1 in 10, 1 in 100, 1 in 10,000, 1 in 100,000 and 1 in 1,000,000 dried bloodstains or saliva deposits respectively. Positive reactions were indicated with a plus sign (+), while no reaction was indicated with a minus sign (-). Parentheses containing numeric values indicate the total number of positive reactions (n=5).

5.1.2 miRNA analysis on blood
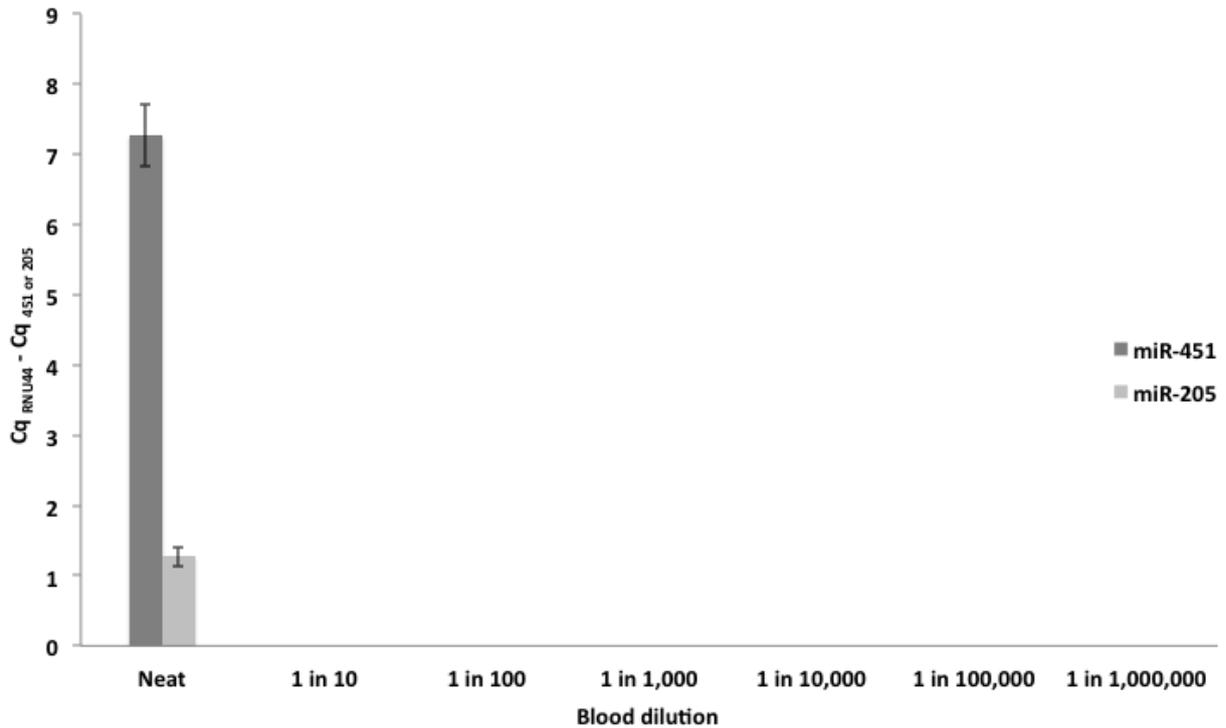
Figure 25. Showing the sensitivity of miR-451 in blood when normalised with reference gene RNU44. Blood was diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000 and 1 in 1,000,000. Marker miR-205 was included to assess performance of the test. Error bars represent one standard deviation (n=3).

The sensitivity range of miR-451 and miR-205 in blood when normalised with RNU44 was between neat and 1 in 10 dilutions (shown in Figure 25). The sensitivity range for miR-451 was surprising, as it had previously exhibited high levels of expression within blood. It was hypothesised that the expression in RNU44 was affecting the expression level in miR-451. No amplification was observed in the negative controls in the sensitivity studies with blood and saliva deposits.
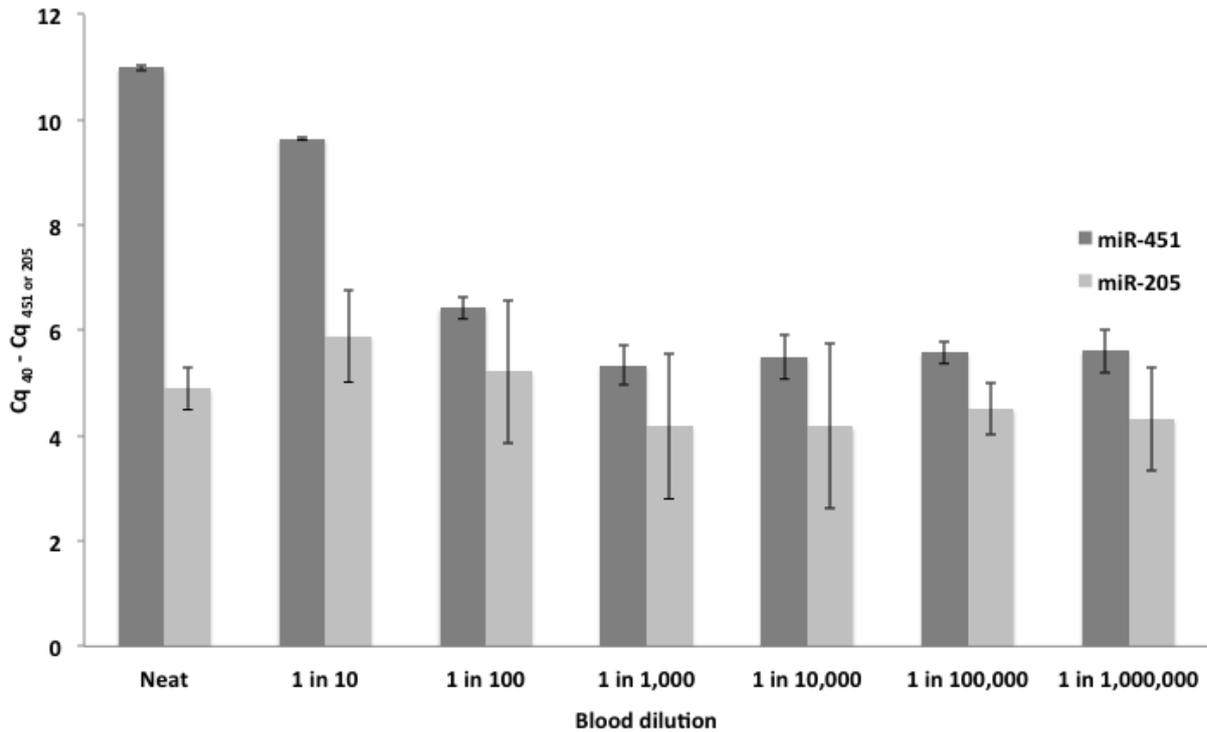
Figure 26. Showing the sensitivity of miR-451 in blood when presented on its own. Blood was diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000, 1 in 100,000 and 1 in 1,000,000. Marker miR-205 was included to assess the performance of the test. Error bars represent one standard deviation (n=3).

To test this hypothesis, miR-451 and miR-205 were presented without the reference gene (shown in Figure 26). Marker miR-451 showed moderate expression in neat blood. It also showed expression in all of the dilutions. Marker miR-451 showed a lower sensitivity range between 1 in 100 and 1 in 1,000 ($P<0.05$). Thus supporting the hypothesis that RNU44 had affected the results.

The sensitivity range of miR-451 was determined in this study to be between 1 in 100 and 1 in 1,000 dilutions. Thus indicating that a different blood marker may be needed for more diluted blood. No other research groups have explored the sensitivity of miR-451.
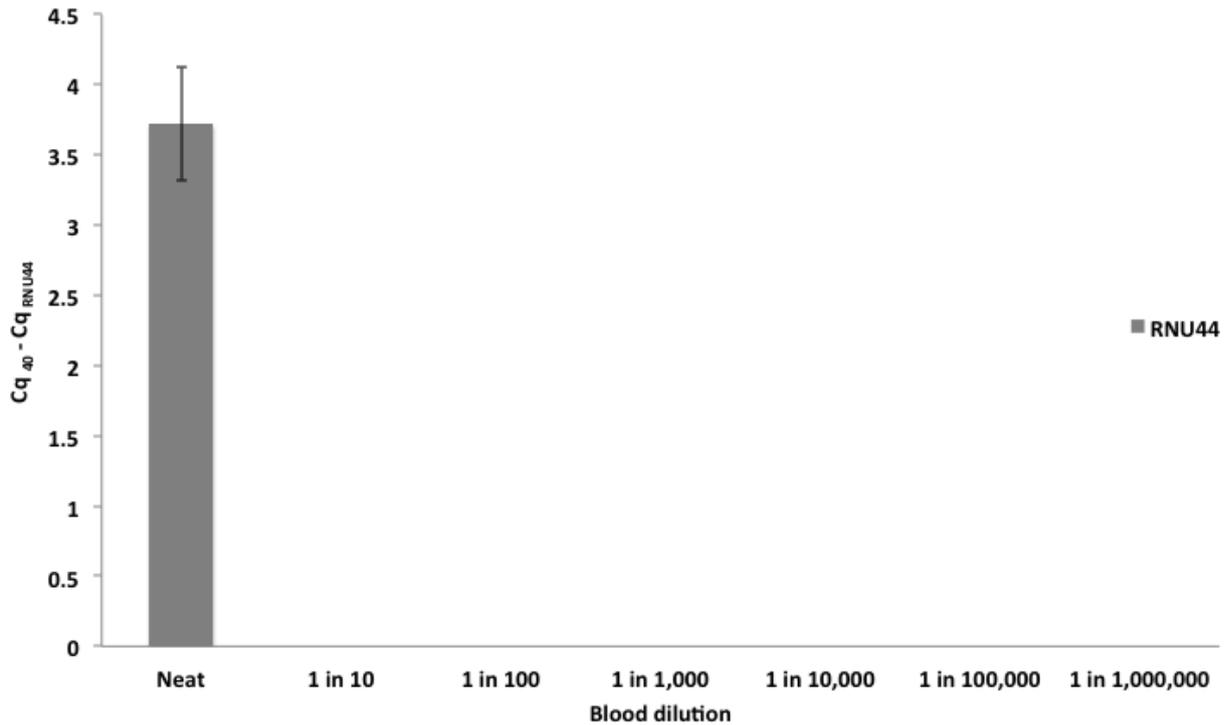
Figure 27. Showing the sensitivity of RNU44 in blood when presented on its own. Blood was diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000 and 1 in 1,000,000. Error bars in this study represent one standard deviation (n=3).

RNU44 was also present on its own (shown in Figure 27). RNU44 showed low expression in neat blood. RNU44 showed a higher sensitivity range between neat and 1 in 10 ($P<0.05$). Thus supporting the hypothesis that RNU44 had affected the sensitivity range of miR-451.

RNU44 was a suitable reference gene for neat blood samples and was supported by Zubakov *et al* (2010) and Wang *et al* (2012). However this study demonstrates the need for a different reference gene for diluted blood. No other research groups have explored the sensitivity of RNU44 in blood.

5.1.3 miRNA analysis on saliva deposits
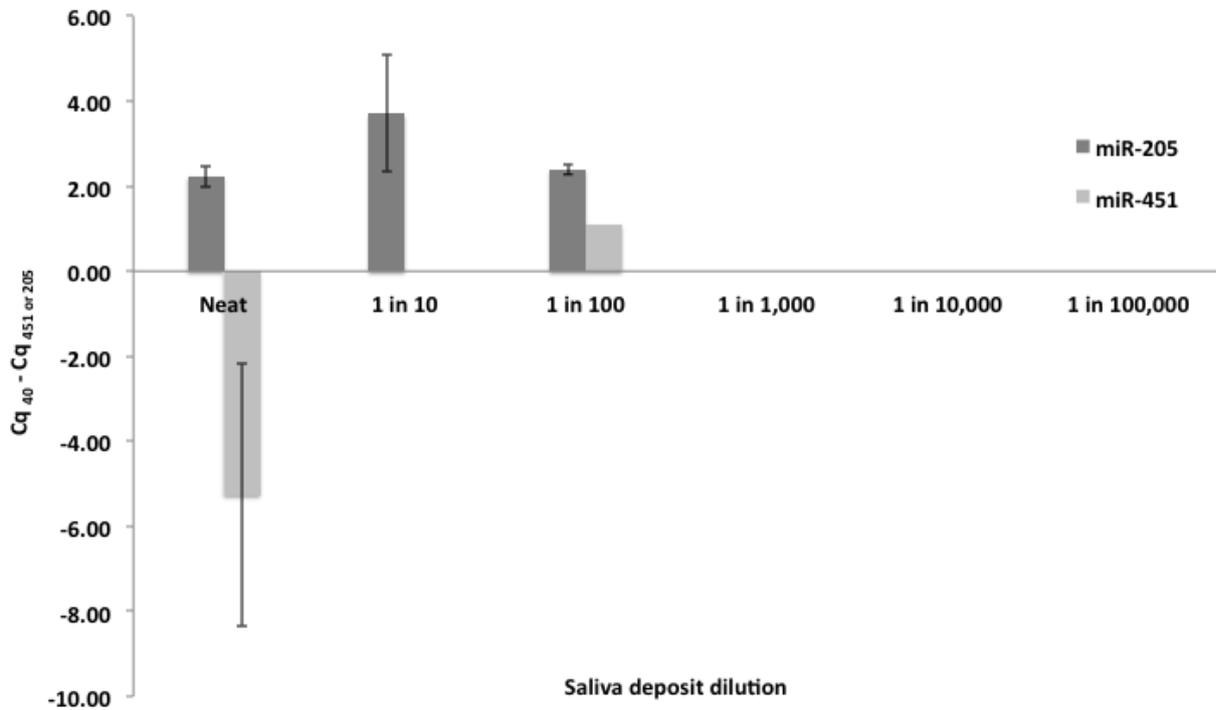
Figure 28. Showing the sensitivity of miR-205 in saliva deposits when normalised with reference gene RNU44. Saliva deposits were diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000. Marker miR-451 was included to assess the performance of the test. Error bars represent one standard deviation (n=3).

A similar sensitivity study was performed using saliva deposits (shown in Figure 28). The sensitivity range of miR-205 was between 1 in 100 and 1 in 1,000 ($P<0.05$). It was hypothesised that RNU44 had affected the sensitivity of miR-205 and miR-451 based on the expression levels seen between the neat and diluted saliva samples.
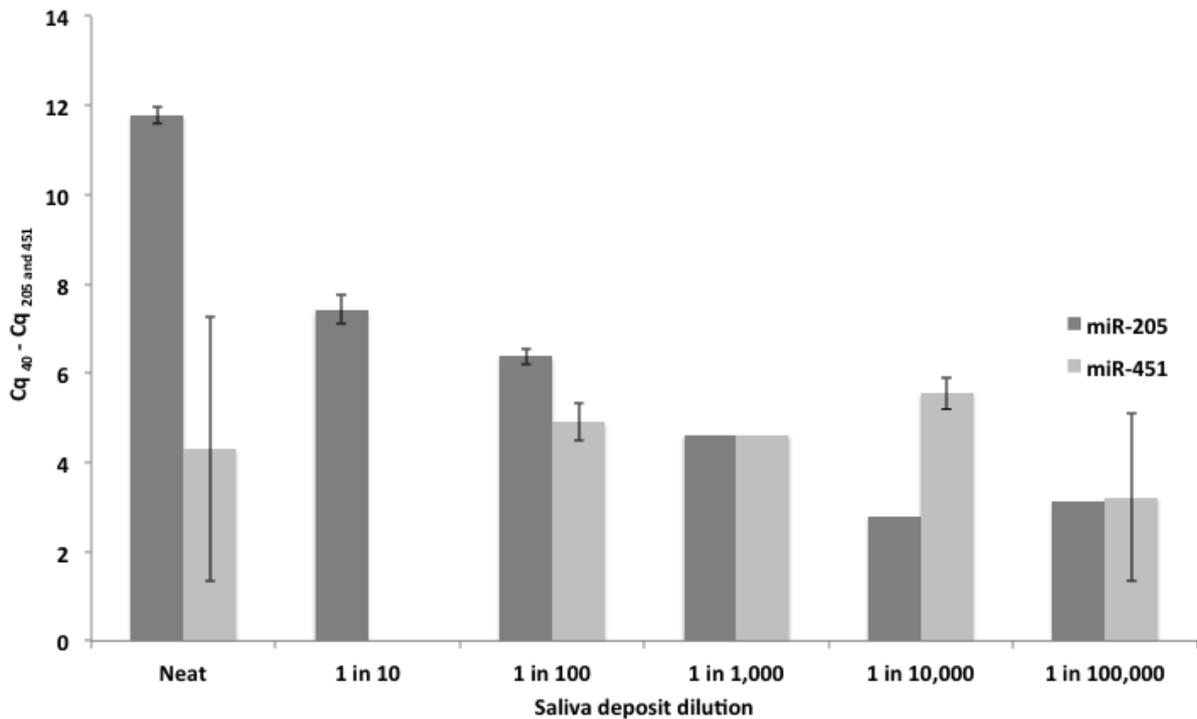
Figure 29. Showing the sensitivity of miR-205 in saliva deposits when presented on its own. Saliva deposits were diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000. Error bars represent one standard deviation (n=3).

To test this hypothesis, miR-205 and miR-451 was presented on its own (shown in Figure 29). Marker miR-205 showed high expression in neat saliva deposits. It also showed expression in all of the dilutions. The sensitivity range of miR-205 was the same as in the previous figure; and could no longer be distinguished after 1 in 1,000 dilutions (P>0.05).

The sensitivity range of miR-205 was determined in this study to be between 1 in 100 and 1 in 1,000 dilutions. Thus indicating that a different saliva deposit marker may be needed for more diluted saliva. No other research groups have explored the sensitivity of miR-205.

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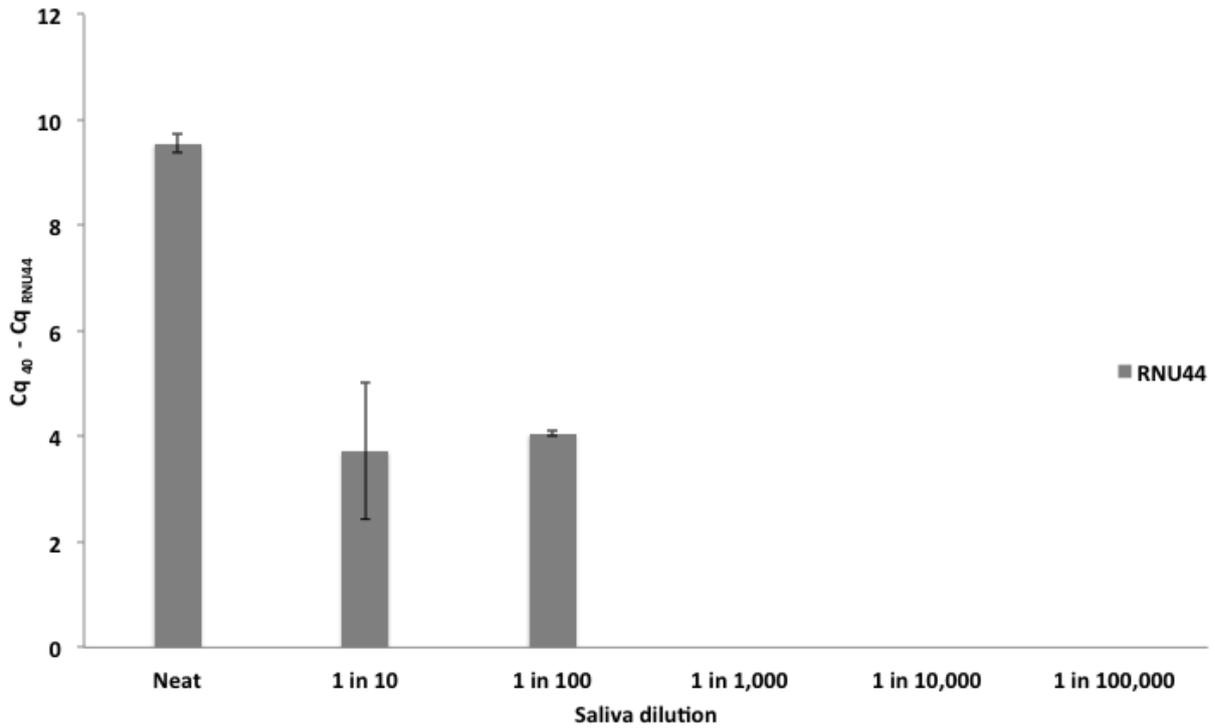
Figure 30. Showing the sensitivity of RNU44 in saliva deposits when presented on its own. Saliva deposits were diluted 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000. Error bars represent one standard deviation (n=3).

RNU44 was also presented on its own (shown in Figure 30). RNU44 showed high expression in the neat saliva deposits. The sensitivity range of RNU44 was between 1 in 10 and 1 in 100, which was 10-fold higher than miR-205.

RNU44 was a suitable reference gene for neat saliva deposits and was supported by Zubakov *et al* (2010) and Wang *et al* (2013). However this study demonstrates the need for a different reference gene for diluted saliva deposits. No other research groups have explored the sensitivity of RNU44 in saliva deposits.

Discussion

There were two aims in this section. The first was to assess the sensitivity of miRNA analysis with three presumptive tests: KM, LMG and Phadebas test. The second was to assess the sensitivity of individual markers miR-451, mir-205 and RNU44 in blood and saliva deposits.

In the presumptive studies the overall sensitivity range for the KM, LMG and Phadebas tests were similar to sensitivity ranges reported for blood by Cox *et al* (1991), Grodsky *et al* (1951), Webb *et al* (2006), Tobe *et al* (2007) and Johnston *et al* (2008) supported this sensitivity range for blood between 1 in 100 and 1 in 10,000. Similarly the sensitivity ranges reported for saliva by Willot (1980), Kipps and Whitehead (1975), Auvdel (1986), Keating and Higgs (1994), Hedman *et al* (2011); support the range in saliva. This range was expected in these tests as different preparation methods (e.g. dry vs. wet samples), protocols and tests (e.g. purchasing a test vs. making a test in the lab) were used.

In the miRNA analysis studies the sensitivity of the body fluid specific markers was more sensitive than the reference genes. The sensitivity range of miR-451 in blood and miR-205 in saliva deposits was between 1 in 100 and 1 in 1,000. The sensitivity range of RNU44 in blood was between neat and 1 in 10 while the sensitivity range of RNU44 in saliva deposits was between 1 in 10 and 1 in 100.

In this study miRNA analysis was less sensitive than presumptive tests: Kastle-Meyer, Leucomalachite Green and Phadebas. This was surprising as it was thought that a confirmatory method would be more sensitive than presumptive tests.

The sensitivity range for miR-451, miR-205 and RNU44 may be down to a few factors. It may be an indication of the sensitivity of the TaqMan chemistry. Markers miR-451, miR-205 and RNU44 were selected on the basis of high expression in abundant blood and saliva samples. A high expression level in this work was considered to be in the range of ΔCq 11 and above. The average expression levels in miR-451 in blood and miR-205 in saliva were very similar. Markers miR-451 and miR-205 with the TaqMan chemistry gave an average ΔCq of 11.5 in neat blood and neat saliva deposits respectively. The sensitivity range of miR-451 in blood and miR-205 in saliva deposits was between 1 in 100 and 1 in 1,000. It is not unreasonable to see that the detection limit of the TaqMan chemistry was within that range given the expression levels. Similarly, RNU44 with the TaqMan chemistry gave an average ΔCq 3.75 in neat blood and ΔCq 9.8 in neat saliva. Thus explaining why the sensitivity range of RNU44 in saliva deposits was lower (1 in 10 - 1 in 100) than in blood (neat - 1 in 10). These findings were supported by Zubakov *et al* (2010) who found similar levels of sensitivity using two different TaqMan blood markers.

Another factor that may have affected the sensitivity range was the samples. Bloodstains and saliva deposits were used, as they were similar to the sample types that may be encountered at crime scene. Samples were also not normalised before cDNA synthesis to also mimic the variation that may occur when receiving crime scene samples. However this lack of normalisation may have affected the overall sensitivity of this test. It is worth noting however that Zubakov *et al* (2010) found similar levels of sensitivity in their studies even with normalisation. So perhaps this may have been down more to the TaqMan chemistry.

It would be interesting to assess the sensitivity of miRNA analysis using SYBR® green chemistry to see whether the sensitivity range in miR-451 and miR-205 would be similar, lower

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or higher than the TaqMan® chemistry. Both Courts and Madea (2011) and Hanson *et al* (2009) have successfully identified a strong panel of miRNA markers using this chemistry. However as previously mentioned, neither of these groups has looked into the sensitivity of their chosen markers.

It is also clear that the use of miR-451, miR-205 and RNU44 is sufficient for comparing neat blood and saliva deposits. However the use of such markers and reference genes will need to be adapted if they are to be used in forensic casework. The use of perhaps additional markers or different markers may be needed where limited sample is available.

Conclusions

In this study, miRNA analysis was determined to be less sensitive than the KM, LMG and Phadebas tests. The use of miR-451, miR-205 and RNU44 was sufficient for differentiating neat blood and neat saliva deposits. However when applied in context of forensic casework, where samples are often low in quantity and quality, it was determined that additional or different blood and saliva markers would be needed.

Also found equally important was the expression of the reference gene. The expression of RNU44 was sufficient in neat saliva deposits where as it showed significantly lower sensitivity in neat blood, affecting the overall sensitivity of the results. The need for a good reference gene was further emphasized by the variability observed in casework samples. It is particularly important that the reference gene shows similar expression levels to the body fluid specific miRNA markers, if a body fluid is to be identified.

5.2 Specificity of miRNA and mRNA analysis

Species specificity is another important factor to establish. There are times in forensic casework where body fluids from human and animals are present. Many cases include but are not limited to animal cruelty and abuse e.g. bestiality, dog-fighting rings and neglect. In other cases, the presence of body fluids from animals may be subtle e.g. domestic animals or livestock. Body fluids from animals may also be present in the home if animal meat has been prepared. Thus it is important to establish whether or not the blood at a crime scene is human or not, particularly if the incident took place in a kitchen where animal meat has been prepared.

RNA analysis methods (e.g. miRNA or mRNA) may offer different levels of specificity. MicroRNA analysis uses short transcripts (18 to 25 nt) where as mRNA analysis uses longer transcripts (1500 to 2000 nt). The differences in sequence length alone can mean that total number of possibilities for a single miRNA targeting a single or multiple mRNAs is increased. Conversely the longer lengths of mRNA may mean that it is more specific. In addition a number of mRNA have exhibited high tissue specificity in humans where as miRNA have not.

Although a few studies on mRNA and miRNA specificity have been explored, none have compared miRNA analysis with mRNA analysis [153, 173, 175, 177, 231, 300, 301]. None of the research groups who have identified RNU44 as a suitable reference gene in humans have assessed its specificity amongst other species [231, 234, 235]. Also no miRNA research groups have explored the species specificity of miR-451 within a casework context e.g. expression of animal blood from animal meat.

Aim

Therefore there were three aims in this study: to compare the specificity of miRNA analysis with mRNA analysis, explore the specificity of miR-451 in bloodstains within a casework context and to explore the specificity of RNU44.

Experiment

Bloodstains were the focus of this work as it was the easiest to obtain. Bloodstains from: *Gallus gallus* (chicken), *Bos primigenious* (cow), *Cervidae* (deer), *Culpea pallassi* (herring), *Phasianinae* (pheasant) and *Sus scrofa* (pig) were purchased from a butcher. Blood samples were dried onto filter paper before undergoing DNA isolation, cDNA synthesis and qPCR. MicroRNA analysis was performed using miR-451 and RNU44 while mRNA analysis was performed using HBB and GAPDH. During the course of this study, a total of 14 different animal blood samples. Positive (e.g. human) and negative controls were also included.

5.2.1 Specificity of mRNA analysis
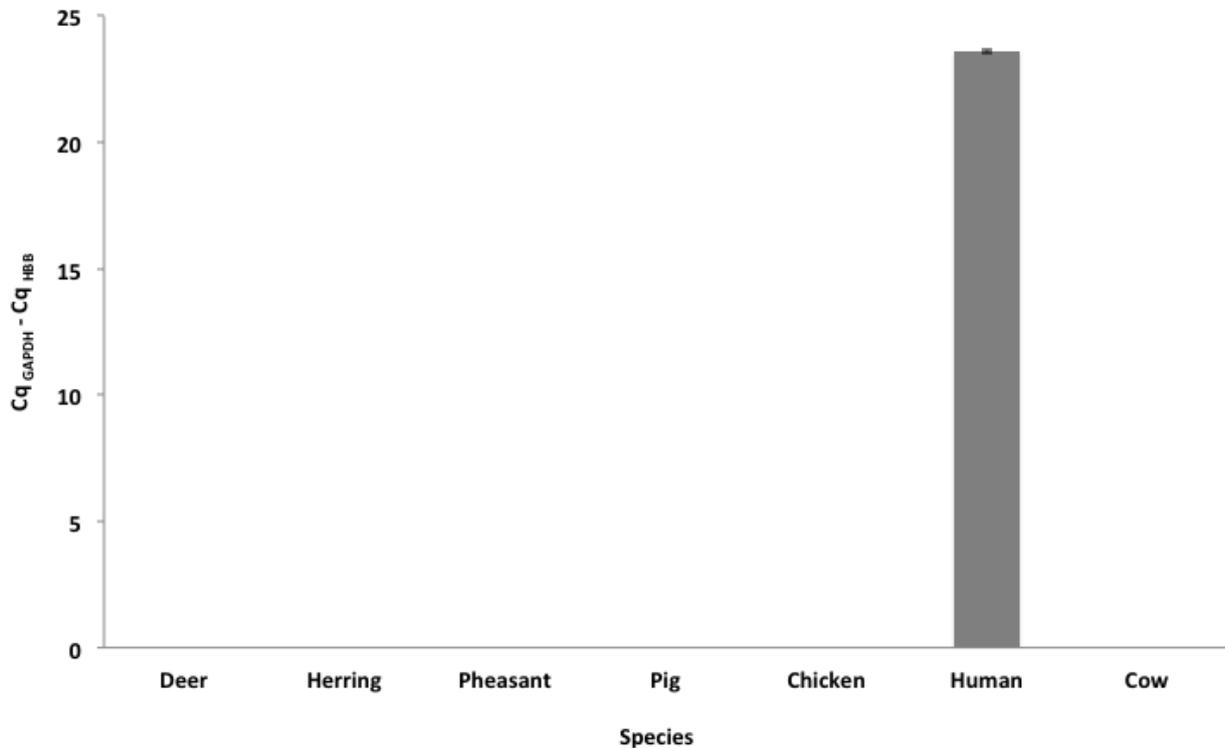
Figure 31. Showing the expression of HBB in chicken, cow, deer, herring, human, pheasant and pig when normalised with GAPDH. Error bars represent one standard deviation (n=3).

In the mRNA species specificity study, HBB showed high levels of expression when normalised with GAPDH (shown Figure 31). HBB showed no expression in the other body fluids. Haas *et al* (2011 and 2012) support the species specificity of HBB in blood. In this study, no amplification was seen in the negative controls. Also, the expression of GAPDH and HBB were presented individually to assess their specificity towards the 7 different species.
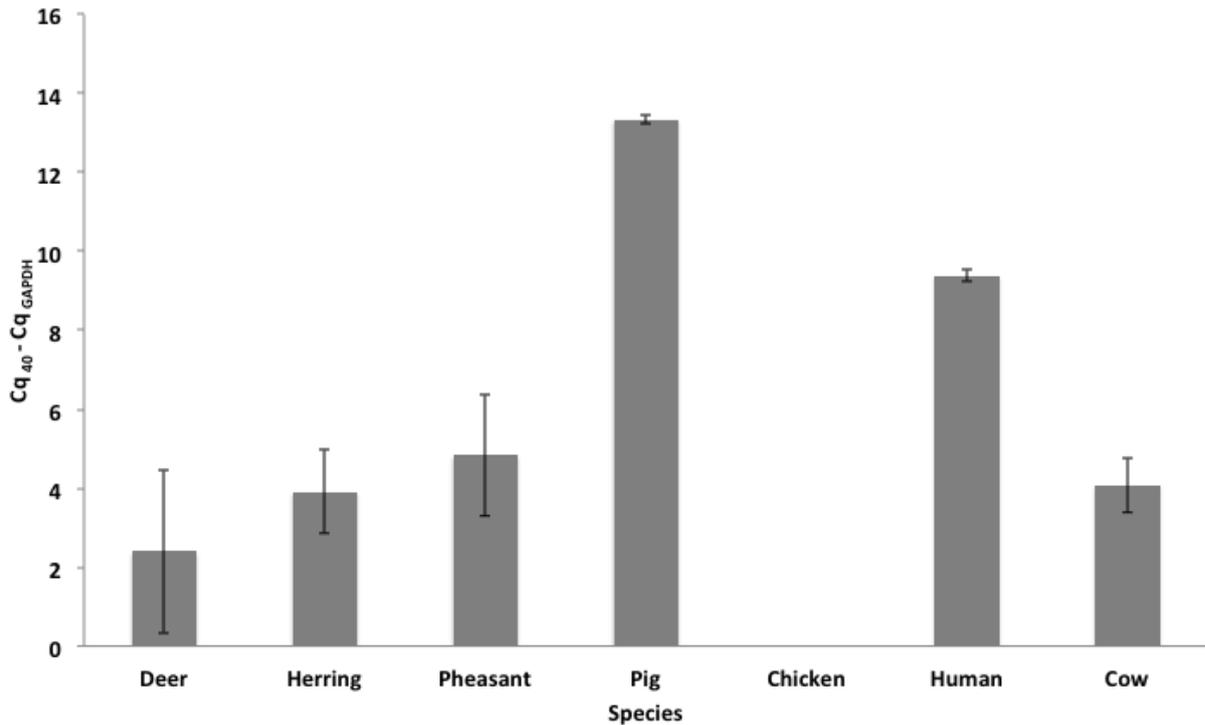
Figure 32. Showing the expression of GAPDH in in chicken, cow, deer, herring, human, pheasant and pig when presented on its own. Error bars represent one standard deviation (n=3).

GAPDH presented on its own showed expression in all animals except chicken (Figure 32). The highest levels of expression were seen in pig and human and gave shigher levels of expression in these animals than in the other animals tested (P<0.05).

In this work, GAPDH did not show species specificity in the 7 different animal blood tested. However GAPDH did exhibit high levels of expression in pig and human. Juusola *et al* (2003), Wang *et al* (2012), NCBI (2010) using GAPDH as a human reference gene supports this finding.
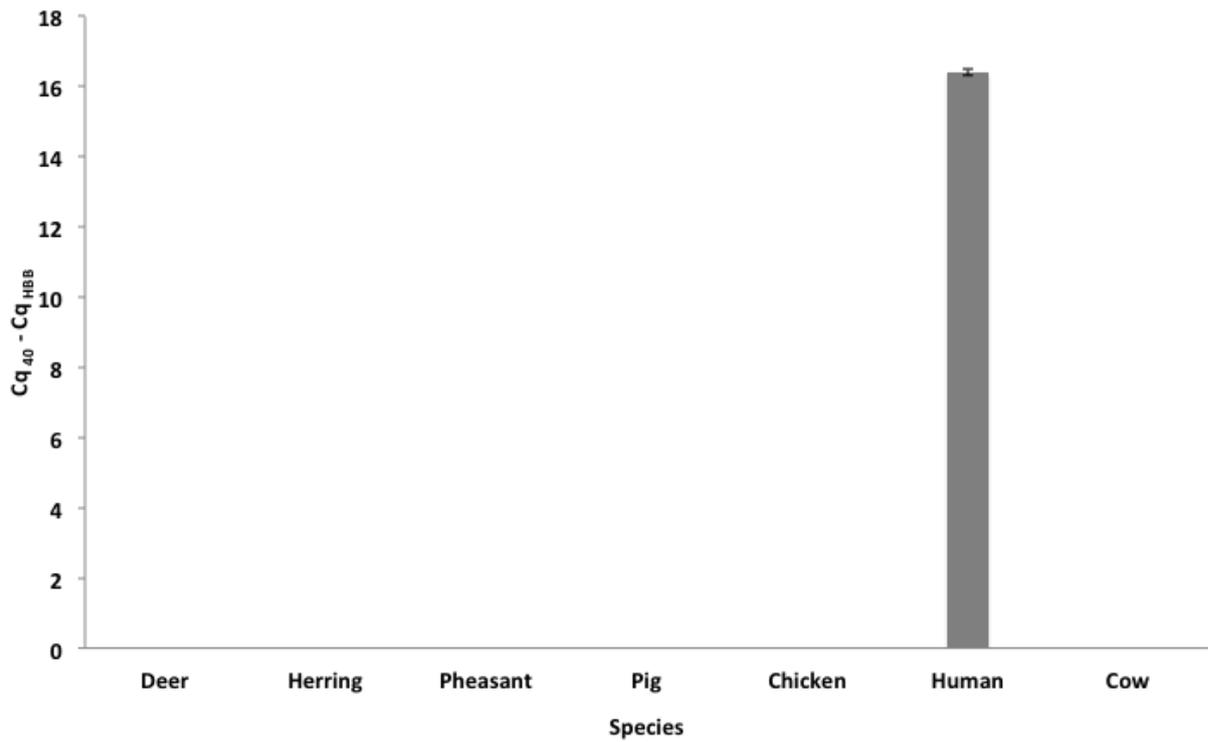
Figure 33. Showing the expression of HBB in chicken, cow, deer, herring, human, pheasant and pig when presented on its own. Error bars represent 1 standard deviation (n=3).

HBB presented on its own showed human specificity in blood (shown in Figure 33). Haas *et al* (2011 and 2012) support human specificity of HBB in blood. This finding also supports the theory that mRNA is more specific than miRNA.

5.2.2 Specificity of miRNA analysis
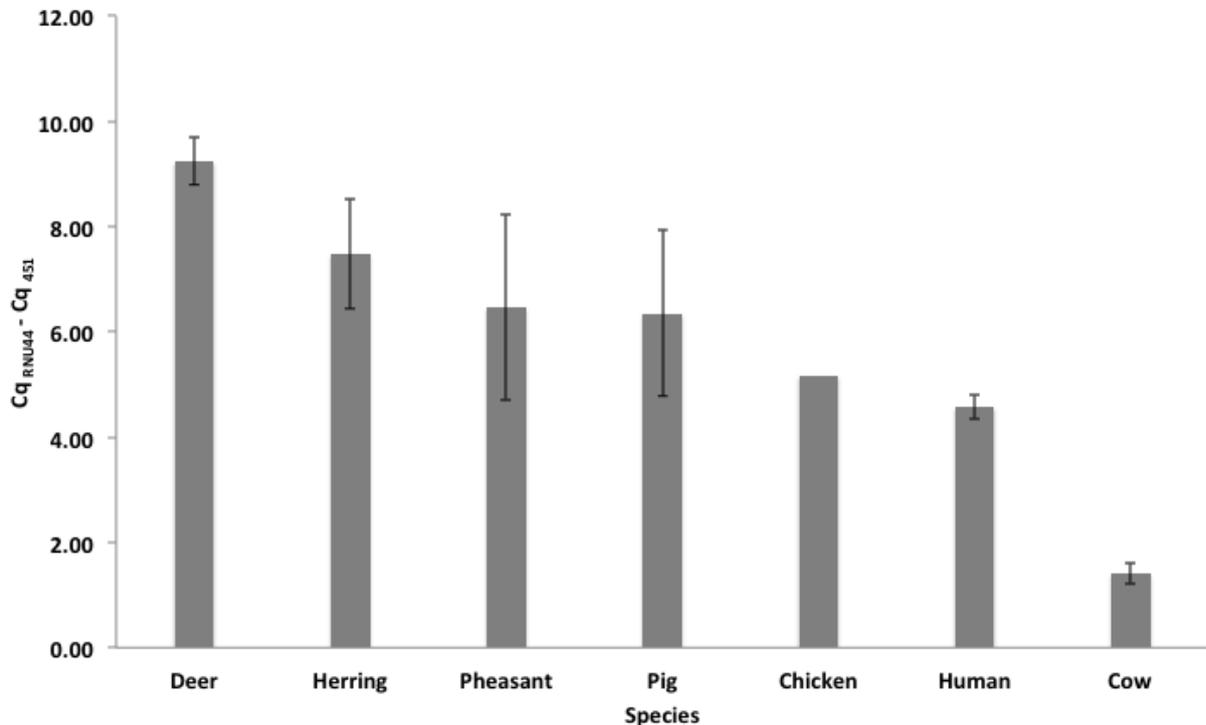
Figure 34. Showing the expression of miR-451 across in chicken, cow, deer, herring, human, pheasant and pig when normalised with RNU44. Error bars represent one standard deviation (n=3).

In the miRNA species specificity study, miR-451 showed the highest expression in deer and herring when normalised with RNU44 (shown in Figure 34). Moderate levels of miR-451 expression were seen in all other species. No amplification was observed in the negative controls.

Surprisingly, human gave one of the lowest levels of miR-451 expression in this study. To better understand the behaviour of the markers, the expression of miR-451 and RNU44 were presented separately.
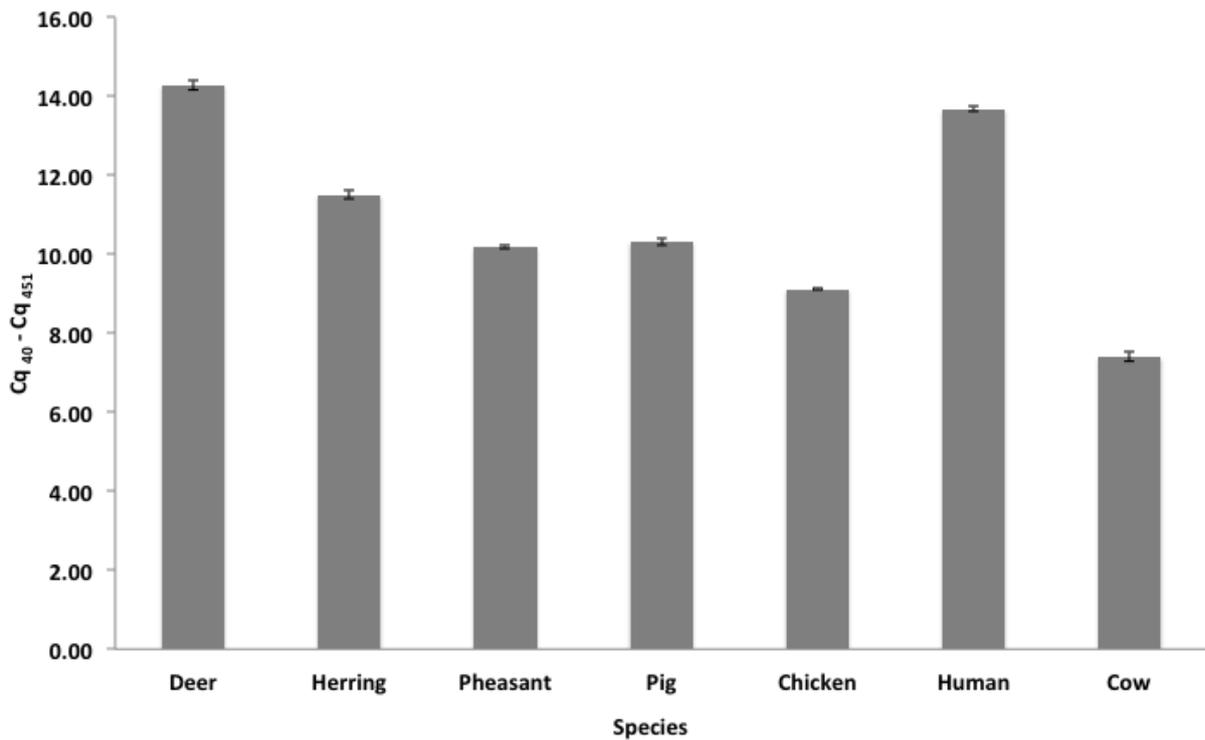
Figure 35. Showing the expression of miR-451 in in chicken, cow, deer, herring, human, pheasant and pig when presented on its own. Error bars represent one standard deviation (n=3).

When miR-451 was presented on its own the results appear much clearer (shown in Figure 35). The results were very similar to the normalised results shown previously. Deer gave the highest expression of miR-451. However human gave the second highest expression of miR-451. Moderate levels of miR-451 expression were seen in all other species tested.

In this study miR-451 was found expressed in all of the species tested suggesting it was not species specific. A study by Life Technologies support the finding that the miR-451 was not human-specific [302].
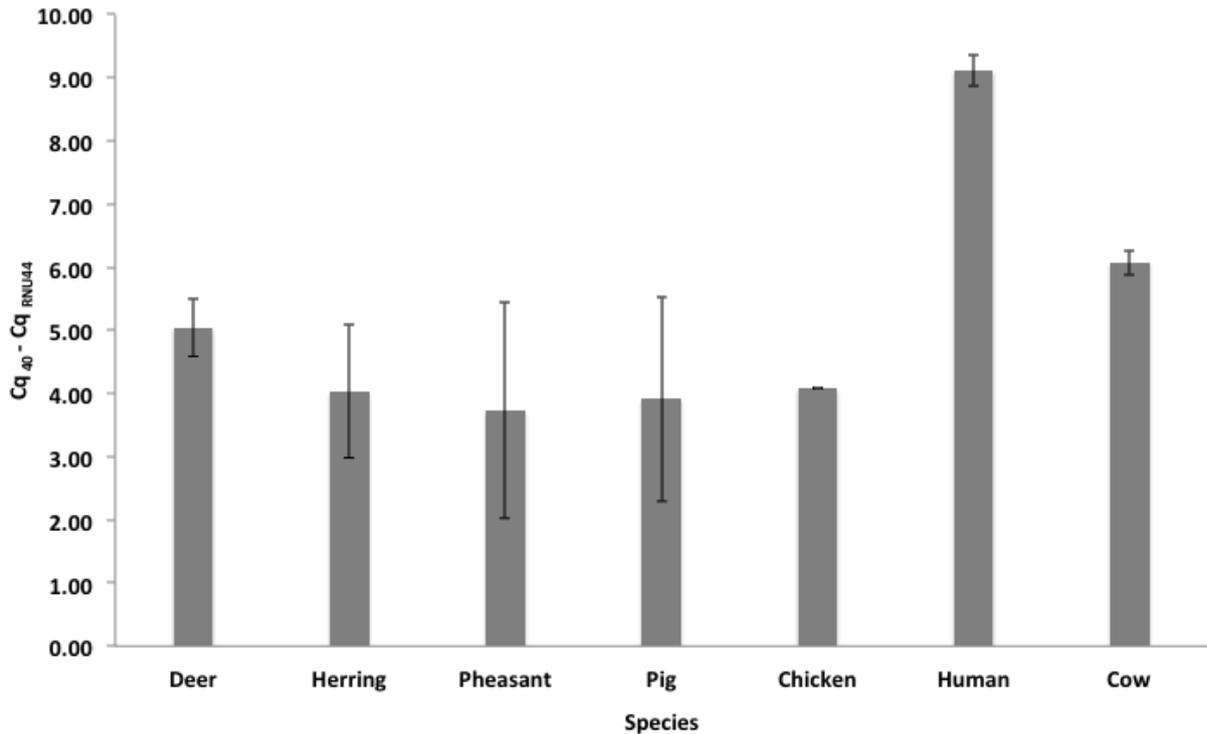
Figure 36. Showing the expression of RNU44 in in chicken, cow, deer, herring, human, pheasant and pig when presented on its own. Error bars represent one standard deviation (n=3).

The most striking aspect of RNU44 when presented on its own was its expression in human (shown in Figure 36). RNU44 in human showed significantly higher expression levels than in all of the other species ($P<0.05$). It was clear that the high expression seen in RNU44 and miR-451 in human and variation in expression of both markers in the other species was affecting the overall expression in the results.

In this study, RNU44 was identified as a potential human specific reference gene amongst the species tested.   It also highlighted the importance of finding a reference gene that is equally expressed amongst the species that are tested. No studies have been performed exploring the species specificity of RNU44 or reference genes for that matter.

Discussion

There were three aims in this work. The first was to compare the specificity of miRNA analysis with mRNA analysis. The second was to assess the species specificity of miR-451 as it has not been explored within a casework context e.g. its expression in blood derived from animal meat. Also, since no work has been published exploring the species specificity of RNU44, this was also assessed.

In the mRNA species specificity study HBB showed human specific expression in blood. This was expected as HBB, is the ß-subunit of haemoglobin in human and primates [230]. Hanson *et al* (2009) and Haas *et al* (2011) also support the finding that HBB was human specific in blood. The findings from this study also support the theory that mRNA is more specific than miRNA.

In the mRNA species specificity study GAPDH showed high levels of expression in pig and human. Studies have identified GAPDH as a human specific reference gene through human post mortem tissue studies [154, 303, 304]. Thus it was expected GAPDH showed high expression levels in human. Although these studies have shown that GAPDH is specific to human it was not surprising to see GAPDH highly expressed in pig [305].

In the miRNA species specificity study miR-451 was expressed in all of the species tested, suggesting that it was not specific in blood. Life Technologies support the finding that miR-451 was not species specific [302].

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In the miRNA species specificity study a potential human specific reference gene was identified; RNU44. This could be very useful for identification of human blood at a crime scene where blood from livestock is present. For instance in a murder where blood is present but it is not certain whether the blood is from meat cut on a kitchen counter top or whether the blood is human.

In this study, miRNA analysis was found to be less sensitive than mRNA analysis. These findings were supported by Life Technologies (2014) who found miR-451 to be non-species specific. This finding was further by a study by Li *et al* (2014) who also found that miRNA was not species specific (using a different blood marker miR-16). Zubakov *et al* (2010) (also found miRNA to be non-species specific (using a different blood marker miR-144). Zubakov *et al* (2010) non-species specificity was particularly interesting as miR-144 is clustered closely to miR-451 (<10kb) [306]. Generally miRNAs that are clustered near one another have similar co-regulation functions [307]. It could possibly be inferred that if miR-451 and miR-144 have similar functions, that it may also share other similar characteristics in species specificity. This possibility is supported by the fact the miR-451 and miR-144 originate from the same primary miRNA sequence.

A number of miRNA markers have been identified for BFID. However a limited number of tests have been performed on the species specificity of these markers. Thus it may be a case of identifying which of or if any of the miRNA markers identified are species specific. It may also be important to identify species-specific reference genes. Alternatively there may need to be a combination of both species specific miRNA markers and reference genes needed in order to resolve where the body fluid of human origin. It is likely that the latter is going to be needed

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as the expression levels of both miRNA markers and reference genes will be more difficult to interpret with casework samples which are often limited in both quantity and quality.

The potential for using miRNA analysis for casework related to species is still promising. It has been identified in this work that miR-451 was not specific towards the species tested, however a potential human specific reference gene was identified. Therefore it could be possible to more closely associate a DNA profile with a particular body fluid e.g. blood. It would be useful to be able to expand the capability of miRNA analysis with different species by identifying miRNA markers or reference genes that are specific to other species types.

It is evident that the use of miRNA analysis within species studies is still in its infancy. The studies that have performed for miRNA analysis have been limited by a combination of collecting high sample numbers, sample types and identify suitable markers. A collaborative exercise could be of use to help resolve this issue.

Conclusions

In this study, miRNA analysis was found less specific to mRNA analysis. However its use within casework remains positive. The identification of a potential human-specific reference gene in this study and lack of species specificity observed in the miRNA marker identified for blood have shown the potential of the use of miRNA analysis in cases relating to species specificity. The combination of both species-specific miRNA BFID markers and species-specific reference genes may provide a powerful test for body fluid discrimination.

5.3 MicroRNA stability

The issue of stability in RNA analysis is an important issue to investigate, not only because of the rigorous requirements in forensics, but also to address the general stigma of instability associated with RNA analysis. Stability can be described in a forensic context, as the minimum requirement is that the microRNA can survive within a stain for the time between deposition of the stain and extraction and subsequent analysis in the laboratory. Given, the uncertainties typically associated with crime scenes, such a length of time can be highly variable and in many cases, may be unknown. Ideally, it would be useful if microRNA could survive in a stain for years, thus including cold case capability within this technique.

It is important to establish the stability of miRNA markers so that the forensic practitioner can make a judgment as to whether or not to carry out a test given the case circumstances. For example, if it is demonstrated that miRNA may not persist after 6 months, and a case is considered where the stain is 1 year old, then the practitioner can decide not to carry out the test, thus minimising wastage. Conversely, if the stain in question is 4 months old, then it would be worth considering miRNA analysis on the sample.

Given the recent study in which mRNA was detected in a 23 year old blood stain, it is not unreasonable to expect the same for a miRNA marker, especially given that microRNA is considered to be more stable than mRNA [195]. Thus it is a reasonable hypothesis that miRNA could survive for even longer than 23 years. However, given the short duration of a typical PhD, this is not a hypothesis that could be explored as a part of this study. It is possible to obtain older sample from police forces or the Forensic Science Service Archives; however, it is

considered a priority to establish the test on fresher samples (e.g. 1 year old) first, before going for more challenging samples.

A handful of research groups have explored the stability of RNA within a forensic context e.g. Zubakov *et al* (2010), Courts and Madea (2011), Lindenbergh *et al* (2012), Wang *et al* (2013), EDNAP collaborative exercises 2-3.

Aim

The aim of this work was to establish the stability of miRNA when exposed to a variety of different environmental conditions such as UV-light exposure and temperature changes.

Experiment

In this study, bloodstains stored over a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year were used. A total of 3 blood donors were used for this study. Blood underwent DNA isolation, cDNA synthesis and qPCR analysis targeting miR-451, miR-205 and reference gene RNU44.

5.3.1 miRNA stability
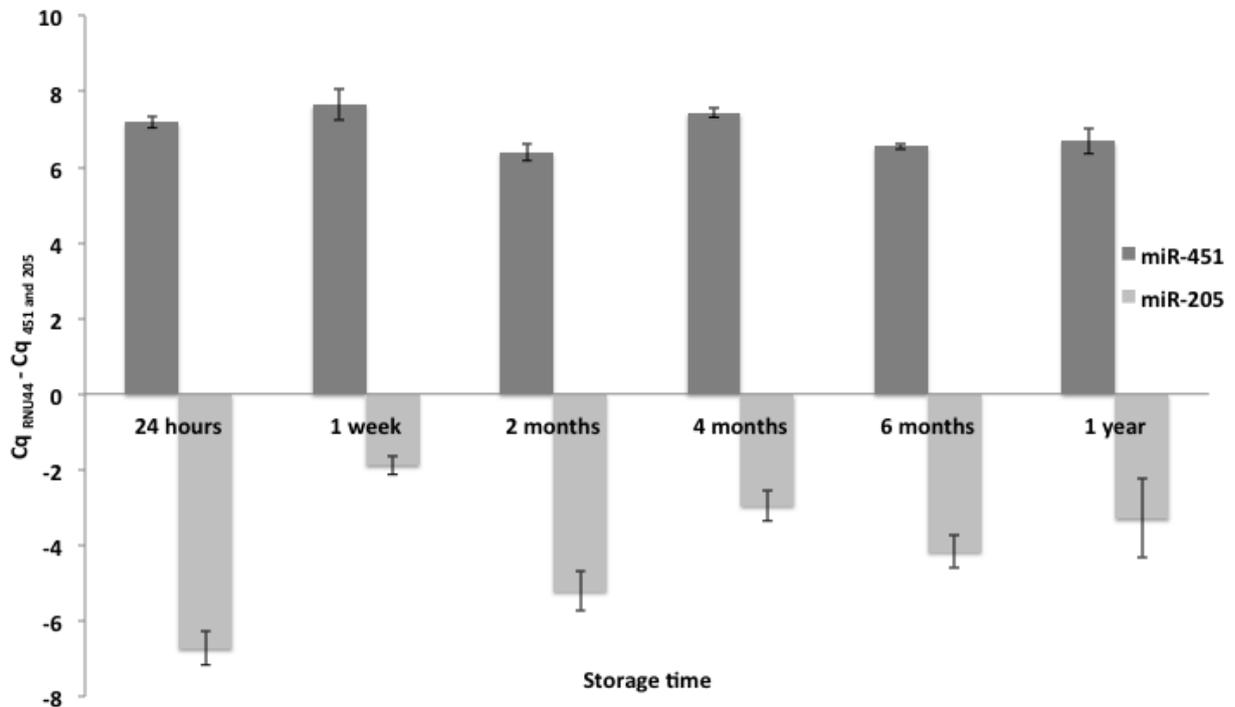
Figure 37. Showing the expression of miR-451 in bloodstains stored for a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year when normalised with RNU44. Error bars represent one standard deviation (n=3).

In this miRNA stability study there was no difference in miR-451 expression shown between the 24 hr and 1-year bloodstain (shown in Figure 37). To better understand the expression of miR-451 and RNU44 was presented on its own. Also, no amplification was observed in the negative controls.
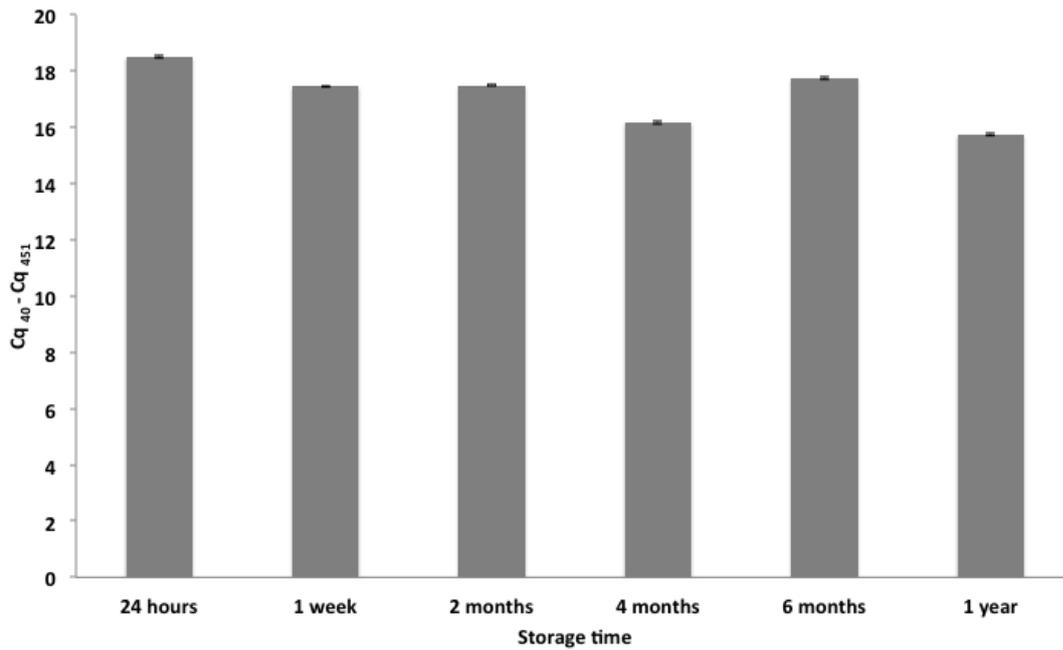
Figure 38. Showing the expression of miR-451 in bloodstains stored for a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year when presented on its own. Error bars represent one standard deviation (n=3).

The expression of miR-451 presented on its own showed similar levels of expression in all bloodstains (shown in Figure 38). Again no difference was observed between the 24-hour bloodstain and 1-year-old bloodstain.

In this study miR-451 was stable in 1-year old bloodstains. Both Zubakov (2009) and Courts and Madea (2011) support the stability of miR-451. Wang *et al* (2013) supported the variation in miR-451 expression in bloodstains.
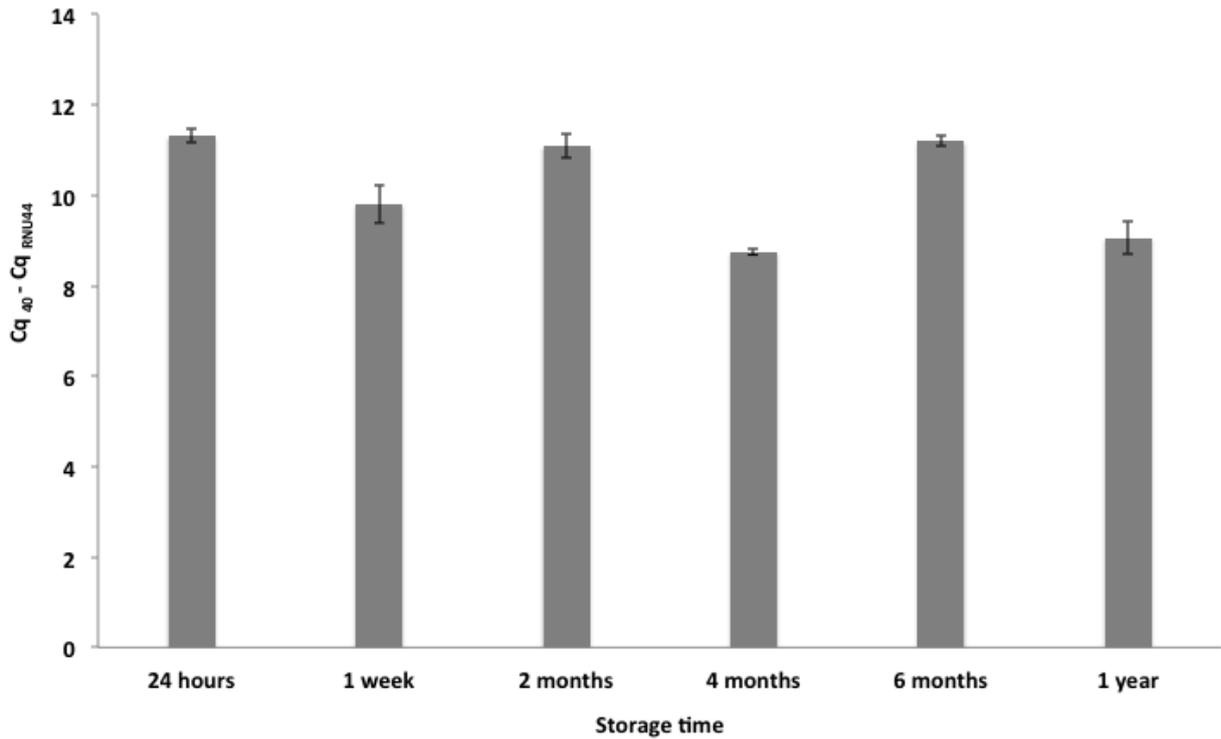
Figure 39. Showing the expression of RNU44 in bloodstains stored for a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year when presented on its own. Error bars represent one standard deviation (n=3).

The expression of RNU44 presented on its own showed varying levels of expression across the bloodstains (shown Figure 39). No other work has been published exploring the stability of RNU44 over a long period of time.

Discussion

The aim of this work to assess the stability of miR-451 in bloodstains stored over a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year. Zubakov *et al* (2010) supported the finding that miR-451 was stable in 1-year-old bloodstains. Courts and Madea (2011) also supported the stability of microRNA in 1-year-old bloodstains and used miR-16 as a blood-specific marker.

Bloodstains stored over a period of 24 hours, 1 week, 2 months, 4 months, 6 months and 1 year showed minor variation in miR-451 expression. Wang *et al* (2013) also supported these findings, observing minor fluctuation in expression levels in a 24 hour vs 1 month stain (ΔCq 2-3). There may be a number of reasons why the bloodstains from this study showed minor variation in miR-451 expression.

One possibility may be down to the samples. In this study, bloodstains were stored on the windowsill and as such were exposed to diurnal conditions (e.g. regular periods of light and dark) as well as changes in temperature. This may have caused variation in miRNA expression level e.g. seasonal changes. Additionally the sample number in this study was limited. The samples used were originally for a different stain-age study. A single bloodstain from different donors was used and as such the difference in miR-451 expression levels may have been a result of natural variation observed between individuals.

The minor variation in expression levels may be characteristic of the particular miRNA marker. For instance, miR-451 may exhibit significantly lower levels of expression if stored over a certain period of time e.g. 4-6 months. It may also be characteristic within the body fluid type

e.g. a specific expression pattern may be observed for a blood using a blood specific miRNA marker, where as the expression of a saliva specific miRNA marker may be different within that same sample.

RNU44 also showed moderate variation in expression across all bloodstains. Again the variability observed may be due variation in sample or a characteristic trait of RNU44 as previously discussed with miR-451.

This study has shown that miRNA analysis has the potential to be incorporated in to forensic casework. Markers miR-451 and RNU44 showed a high level of stability in 24 hour old to 1 year old bloodstains. The exact age of the bloodstain could not be determined in this study. However given the natural variation that may be observed in crime scene stains it may not be realistic to establish the exact age of the stain. It would however it helpful if a general range for particular miRNA markers and reference genes could be established. Then there could be a certain degree of confidence that could be included when reporting a result.

Also from a casework standpoint, it may be more logical or appropriate to use miRNA analysis as it is potentially more stable than mRNA analysis. This may become more evident if a wider range of aged stains is incorporated e.g. 5 years, 10 years and 15 years. The forensic community is slow to accept new methodologies and since BFID continues to play an important role within this framework, it is thought that miRNA analysis would be more suitable for aged body fluids.

Conclusions

In this study, miR-451 and RNU44 exhibited stability from bloodstains stored from 24 hour to 1 year. The results from this study demonstrate the potential for incorporating miRNA analysis into existing framework. The innate stability of miRNA may provide an advantage over mRNA, especially where samples are severely degraded or aged.

5.4 MicroRNA analysis of mixed body fluids

When developing a miRNA BFID test it is important to establish whether or not the test is suited for forensic casework samples. Mixed body fluids form a large portion of samples collected at crime scenes e.g. sexual assaults. Therefore it is important to ensure that miRNA analysis can be applied to mixtures.

Forensic practitioners often encounter a wide spectrum of mixtures at a crime scene. This range covers anything from 1:1 mixtures up to and above 20:1 mixtures. Mixtures also become increasingly difficult to interpret when there is multiple contributors involved e.g. two or more people.

The ability to identify the body fluid origin from a mixture is highly subjective. This is often due to the complex nature of body fluids and perhaps is the main reason why it has not been explored in research. There have been a handful of group who have looked into resolving mixtures using mRNA analysis [4, 156, 168, 169]. However there have been no extensive studies performed e.g. Courts and Madea (2011), which have attempted to resolve mixtures using miRNA analysis e.g. such as the resolution of mixing ratios.

Aim

The aim of this study was to assess whether or not miRNA analysis could be used to resolve mixtures.

Experimental design

In this study, blood and saliva swabs were collected. A series of 1:1, 2:1 and 5:1 mixtures was then prepared, where blood was major components and saliva was minor component and conversely where saliva was major and blood was minor. Samples then underwent cDNA synthesis and qPCR using miR-451, miR-205 and RNU44. A total of 4 different blood and saliva donors were used during the course of this study. Positive controls from single source body fluids (e.g. blood, saliva) and negative controls were included

5.4.1 miRNA analysis with mixtures
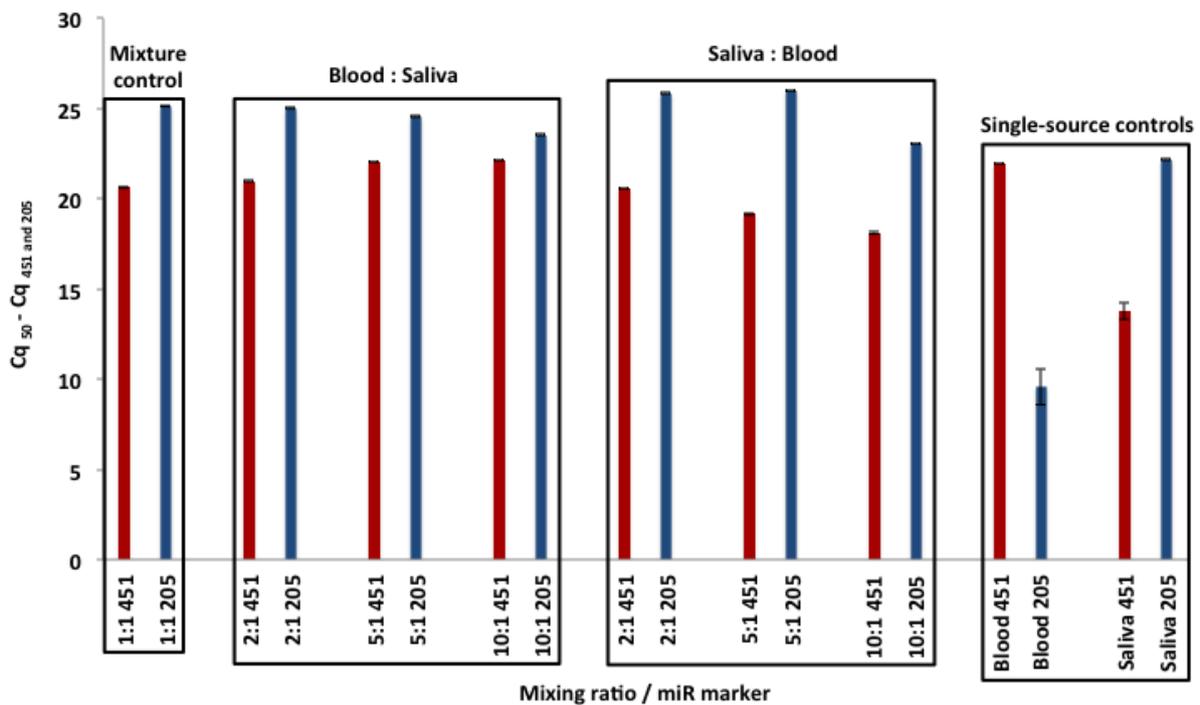
Figure 40. Showing the expression of miR-451 and miR-205 in blood and saliva (swab) mixtures. Mixing ratios of 1 blood:1 saliva, 2 blood:1 saliva, 5 blood:1 saliva, 10 blood: 1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva: 1 blood were used in this study. For clarity, blood is shown in red and saliva is shown in blue (n=3).

Figure 40 shows the results from the mixtures study. It was seen that the single-source controls for blood and saliva performed as expected e.g. the blood control miR-451 showed more expression in blood than miR-205. No amplification was observed in the negative controls. A different pattern of expression was shown in the mixtures. In the mixtures where saliva was the major component and blood was the minor component, miR-205 showed more expression than miR-451. In the mixed samples where blood was the major component and saliva was the minor component and where there were equal ratios of blood to saliva, miR-205 showed more expression than miR-451. Also none of the mixing ratios were maintained.
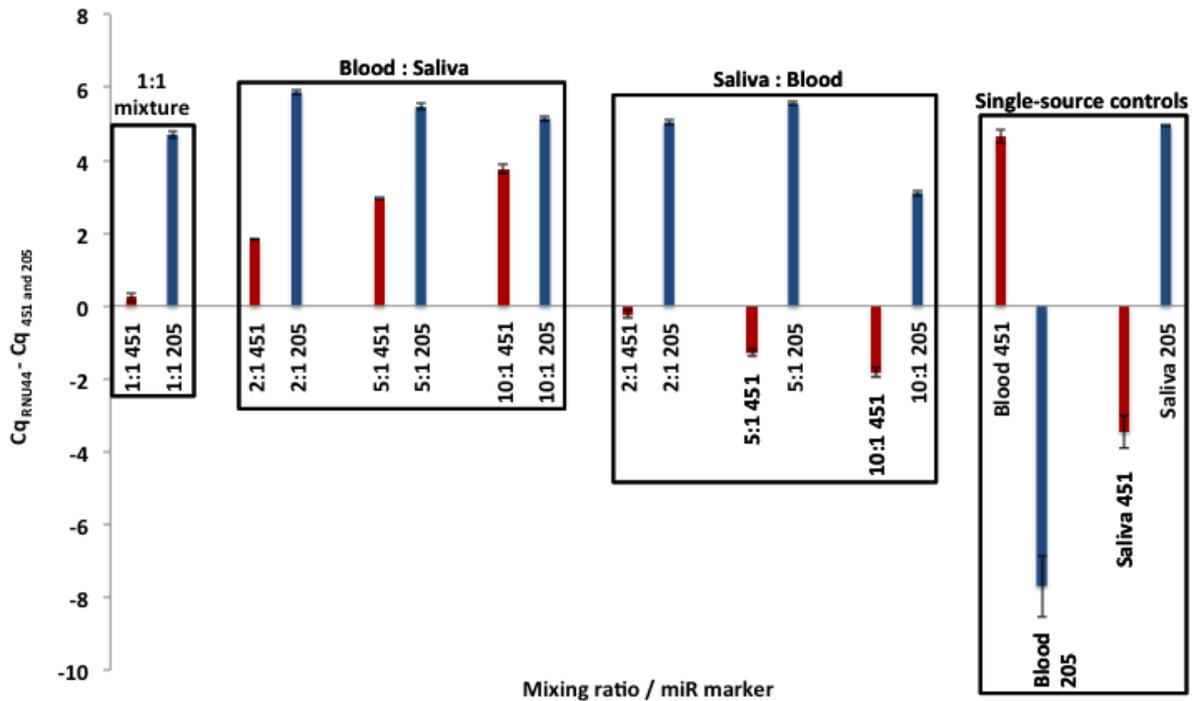
Figure 41. Showing the expression of miR-451 and miR-205 when normalised with reference gene RNU44. Mixing ratios of 1 blood:1 saliva, 2 blood:1 saliva, 5 blood:1 saliva, 10 blood: 1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva: 1 blood were used in this study. For clarity, blood is shown in red and saliva is shown in blue (n=3).

To resolve this issue RNU44 was incorporated into this study (shown in Figure 41). Single source controls performed as expected. An improvement was observed in the mixing ratios where saliva was the major component and blood was minor component. However miR-205 still gave more expression in the blood major and saliva minor mixtures and in the 1:1 mixture.
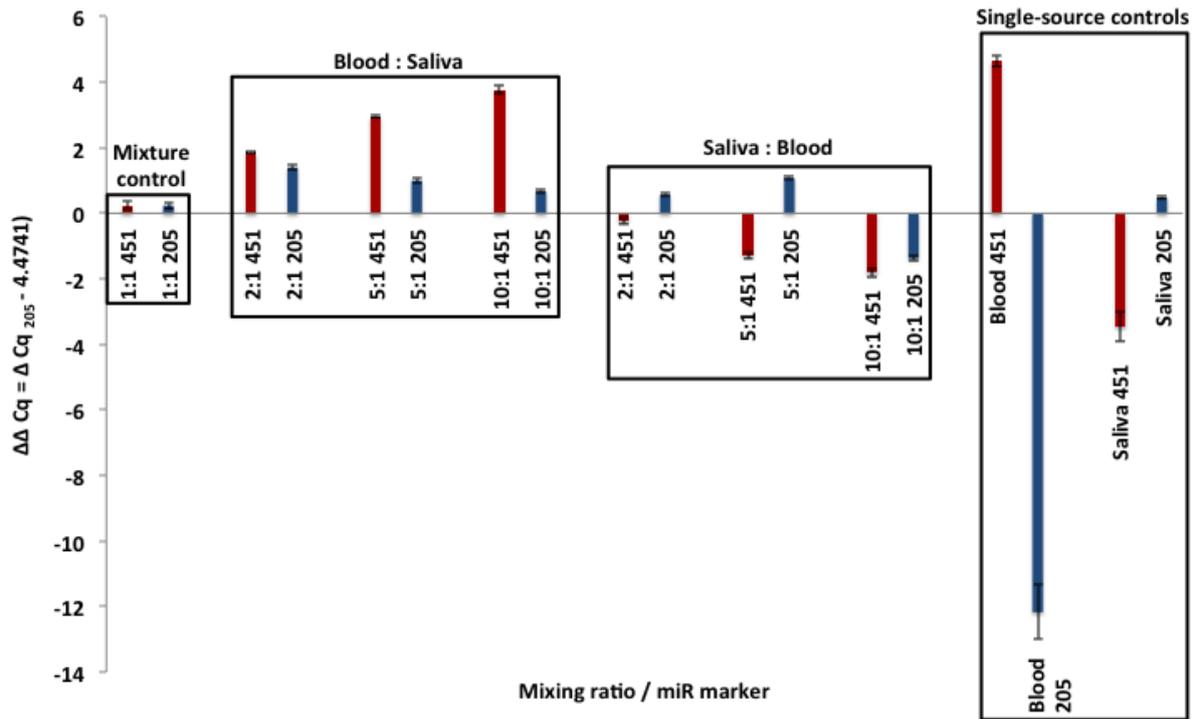
Figure 42. Showing the expression of RNU44 in the blood and saliva (swab) mixtures when normalised with the 1:1 mixture control and RNU44. Mixing ratios of 1 blood:1 saliva, 2 blood:1 saliva, 5 blood:1 saliva, 10 blood: 1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva: 1 blood were used in this study. For clarity, blood is shown in red and saliva is shown in blue (n=3).

Figure 42 shows the expression of miR-451 and miR-205 after sample normalisation with reference gene RNU44. Both single source controls performed as expected. The 1:1 mixture showed similar levels of miR-451 and miR-205. All major and minor components of each body fluid type could now be identified e.g. where blood was the major component and saliva was the minor component, miR-451 showed more expression in blood than miR-205. Conversely the same pattern was observed where saliva was the major component and blood was the minor component. The mixing ratios however were not maintained.
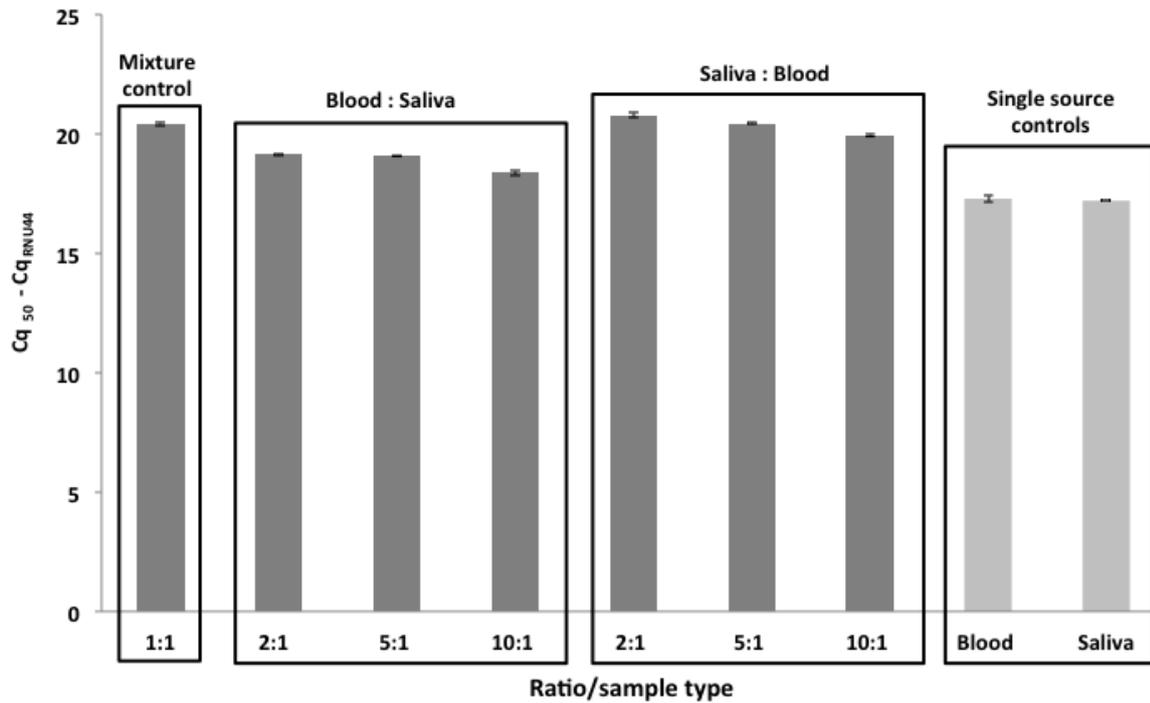
Figure 43. Showing the expression of RNU44 in blood and saliva (swab) mixtures when presented on its own. Mixing ratios of 1:1, 2:1, 5:1 and 10:1 were studied. For clarity, single-source body fluids were highlighted in light grey, while mixed body fluids were highlighted in dark grey (n=3).

To understand this further the expression of the reference gene was presented on its own (shown in Figure 43). No difference was seen between the RNU44 expression in the single-source blood control or the single-source saliva control. All mixtures showed more expression of RNU44 than in the single-source controls for blood and saliva.

Discussion

The aim of this work was to establish whether miRNA analysis could resolve mixtures: 1 blood:1 saliva, 2 blood:1 saliva, 5 blood:1 saliva, 10 blood:1 saliva, 2 saliva:1 blood, 5 saliva:1 blood, 10 saliva:1 blood.

In this study both the major and minor components of the mixtures were identified. A brief study by Courts and Madea (2011) on mixed blood and saliva (e.g. no mixing ratios explored) support these findings that both body fluids could be identified. However mixing ratios in this study were not maintained. This may have been a result of sample preparation. Samples in this study were prepared according to volume rather than concentration to more closely reflect casework samples. Since the DNA concentration of our single-source blood and saliva samples averaged at about 0.1 ng/µl and 5 ng/µl respectively, it was not unreasonable to assume that the copy numbers of miR-451 and miR-205 would vary within these body fluids.

Limited studies have been performed on miRNA copy numbers in these body fluids. For instance, there appears to be no studies published on the copy number of miR-205 within saliva. However there have been a couple of studies on the copy number of miR-451 within blood found that miR-451 had an average copy number of 1972 copies per cell in blood [308, 309].

The copy number within a mixture can affect the expression of the overall result, especially if a higher copy number is inputted into a reaction. However, this variable along with not knowing the composition of a mixture is going to be present in many casework samples.

Therefore, caution needs taken when interpreting mixed body fluids when using a miRNA BFID test.

In terms of casework applications, this study has shown the potential for incorporating miRNA analysis into current framework. Both the major and minor components have been identified. It may be possible to identify body fluid mixing ratios provided the starting input is know. Thus strengthening the overall ability to associate a miRNA analysis with current DNA profiling methods.

Mixed body fluids are commonly encountered in cases of sexual assault e.g. where issues of consent may occur. The identification of vaginal material and semen may not be difficult with the capabilities of DNA profiling available to distinguish between male and females. However it may be useful when there are low levels of these body fluids present, especially when a substantial amount of time has passed before collecting the body fluids such as in cases of non-consensual intercourse.

Other scenarios where the identification of mixtures may be useful include scenarios such as in a kidnap or abduction. The suspect may claim that the presence of the victims DNA may be from casual contact e.g. skin or saliva at the bar. Then it may be important to know if the body fluid origin is from skin or saliva or something else, such as vaginal material.

Conclusions

This study demonstrates the first comprehensive study on mixtures using miRNA analysis. At the very minimum this test has allowed identification of the major and minor components of body fluids. It also has shown the potential to identify mixing ratios of body fluids and its use on casework samples.

Chapter 6

Discussion

6.1 Discussion

The aim of this study was to explore the forensic applications of RNA analysis in the context of body fluid identification. Several aspects were included in this study, including the development of co-isolation and single isolation strategies, screening and selecting RNA markers, and finally applying the characterising of RNA markers within various sample conditions such as those commonly encountered in forensic casework. Such conditions include low-level amounts of samples, non-human samples, degraded samples, and mixed body fluid samples.

Co-isolation of a sample has shown to be an important step when analysing forensic casework to ensure that both the DNA profile and RNA profile are not being compromised and to avoid sample wastage. The latter is particularly important in low-trace samples. Initially efforts were focused on developing a novel technique for the co-isolation of DNA and mRNA utilising magnetic beads. Six isolation/extraction techniques were explored, two of which were commercially available and four of which were modifications. The principle behind the modifications was that silica-like beads were used for isolating DNA and oligo-dT coated beads were used for isolating mRNA with a poly-A tail. A combination of both beads in an extraction mix should allow for the isolation of both. Of the six techniques, it was demonstrated that the silica-beads isolation technique was the best one for the recovery of both DNA and mRNA. This was supported by Bowden *et al* (2011) who isolated DNA before isolating total RNA from the lysis fraction during DNA isolation from Promega's magnetic bead technology (DNA IQ system).

The same principle was explored for miRNA and DNA isolation using spin gel membrane columns; however, it was quickly shown that miRNA was still present within the eluent following isolation. The precise reason is unknown, but it was felt that it was either down to the miRNA being 'tangled' with the bound DNA, or that it was binding to the silica membrane whilst the miRNA was still double-stranded. During the biogenesis of miRNA, the miRNA strands is a stem-loop structure for a relatively long time until just before it is fully mature. Thus it is quite possible that primary or precursor miRNA strands that are being isolated rather than mature miRNA. Whilst this finding was somewhat unexpected, it is considered to be beneficial to forensic casework. Firstly, it requires no modification of the extraction step, meaning that all forensic laboratories already have the DNA extraction procedures laid down and validated.

Secondly, it brings in cold-case capability. If a specific specialised technique is required for the isolation of miRNA, then that means sample that have previously been extracted cannot undergo miRNA analysis. Consequently, this finding is of enormous impact. It does not necessarily means that DNA extraction should automatically be done for miRNA analysis. It may well be that a specific miRNA extraction kit (such as Qiagen's miRNeasy kit, Life Technologies mirVana™ kit) would be better, but what this study demonstrates is that it is possible to go back to the DNA extract and carry out miRNA analysis. This does not only apply to cold-cases, but in cases where the requirement for body fluid identification was not determined at the pre-assessment stage (mainly due to lack of a defence hypothesis or staged reporting). Consequently, on the back of this research, subsequent miRNA analysis has been carried out on DNA extracts. As far as is known, no other research group has published work relating to the miRNA analysis on DNA extracts.

The next stage of this study was the screening and evaluation of miRNA markers. At the time, there were minimal studies in this area, with much of the marker screening being conducted by Hanson *et al* (2009), Zubakov *et al* (2010), Courts and Madea (2011) and Wang *et al* (2013). In addition, such miRNA marker screens had been carried out with total RNA or miRNA isolation kits, rather than DNA isolation kits. Thus, it was necessary to conduct a search for the appropriate markers.

Following an extensive search of literature in forensic genetics and cancer research using the microRNA registry miRBase, a large panel of markers were selected (shown in Table 4) [221, 223, 306, 310]. A total of 6 different miRNA markers were then selected based on identification and differentiating capability for blood (miR-451 and miR-194), semen (miR-891a), saliva swabs and saliva deposits (miR-205) and vaginal material (miR-224 and miR-335). No miRNA markers were identified for skin (shown in Table 8).

Blood	Saliva swabs	Saliva deposits	Vaginal material	Semen
miR-451	miR-205	miR-205	miR-224	miR-891a
miR-16			miR-335	

Table 8. Showing the body fluid specific miRNA markers and differentiating capabilities in blood, semen, saliva swabs and saliva deposits and vaginal material. Green boxes indicate identification and differentiation to all other body fluids. Orange boxes indicate partial identification and differentiation to the other body fluids.

One major observation is that due to the nature of the samples, it was very difficult to find a marker that could definitively identify the presence or absence of a particular marker, with the exception of miR-891a for semen. However, it was much easier to find markers that could differentiate between body fluids. In most cases a combination of markers allowed

differentiation of body fluids. For instance the significantly higher expression of miR-451 and moderate expression of miR-194 could be used to say that a stain was most likely blood rather than saliva, skin, vaginal material or semen.   Similarly, the combination of miR-224 and miR-335 to identify and differentiate vaginal material from the other body fluids tested. Skin was the only body fluid, which could not be identified or differentiated from the 13 microRNA markers tested. Alternatively it was thought that the absence of marker miR-16 in skin and presence in all of the other body fluids could be used as a potential marker for skin.   However this is not ideal given the quality and quantity or nature of forensic casework samples.   Thus further studies on developing a panel of miRNA markers is currently being explored by other members of the University of Huddersfield Forensic Genetics Research Group.

Furthermore since the overall aim of this work is to develop a microRNA BFID test for use in forensic casework, in addition to miRNA markers, suitable reference genes are currently being explored. Research by another member of the University of Huddersfield Forensic Genetic Research Group has identified three potential reference genes for blood, saliva, semen and vaginal material: small nucleolar RNU7, RNU44 (which was also identified in this research as a potential reference gene for abundant and mixed body fluids e.g. blood and saliva) and RNU47.

After the identification of markers for blood and saliva (miR-451 and miR-205, respectively), these were subject of further study. The main reason behind this was the relative ease of obtaining such samples. It was much more difficult to obtain semen and vaginal material sample, due to the intimate nature of such samples. Therefore, to further assess the validity of miRNA analysis in forensic casework, just blood and saliva were used.

A number of factors were explored to assess the applications of this test in forensic casework, including sensitivity. The issue of sensitivity is an important as it should perform at least with equal sensitivity as current tests. 10-fold serial dilutions of blood and saliva deposits samples conducted and it was identified that the sensitivity range of the enzymatic tests were lower than that of the miR tests. However, it was subsequently identified that miR-451 was still being detected in blood at much lower levels (1 in 100 and 1 in 1,000) than the KM and LMG test 1 in 100 and 1 in 10,000 and the same for miR-205 (1 in 100 and 1 in 1,000) in saliva deposits than the Phadebas® test 1 in 100 and 1 in 10,000.

One of the main limiting factors in miRNA analysis was the reference gene (RNU44). The sensitivity range of RNU44 in blood was between a dilution of neat and 1 in 10 while the sensitivity range of RNU44 in saliva deposits was between a dilution of 1 in 10 and 1 in 100. Although there are couple of research groups who are currently searching for reference genes for miRNA analysis (e.g.. Gomes *et al* (2013) and Sauer *et al* (2014)) none have explored the sensitivity of their chosen reference genes. Consequently, it was established that the identification of the correct miRNA reference gene was a crucial stage. Thus other members of the University of Huddersfield Forensic Genetics Research Group are currently conducting efforts to identify highly abundant reference genes for low-level samples. The initial findings suggest that once an appropriate reference gene has been identified, then the miRNA based BFID test should have a lower sensitivity than the current enzymatic tests.

Another important area to explore was specificity. As mentioned previously, the transcript length of mature microRNA (18-25 nt) is shorter than mRNA (1500 to 2000 nt). The

shorter sequence length of miRNA may mean that the total number of possible targets for mRNAs is higher. In forensic casework, this can pose challenges in the interpretation of human body fluids if other animal body fluids are present e.g. in crimes that have taken place in a kitchen. A study exploring the species specificity of miR-451 in blood from seven different animals (e.g. cattle, chicken, deer, herring, human, pheasant and pig) showed lack of specificity in all animals. Life Technologies (2014) and Zubakov *et al* (2010) supported this finding. Interestingly in this study, RNU44 exhibited specificity towards human blood than in blood from the other animal types. The human specificity exhibited in RNU44 may be especially useful in cases where blood from these animal tested in this study may also be present.

Another factor to explore was stability. Stability is the ability of the RNA molecule to stay intact over a period of time. Messenger RNA has had a notorious reputation for being unstable, however, this has been shown to be a minor issue in forensic casework. The main reason is that the crime scene stains are usually dry and as such all intra-cellular processes (such a ribonuclease activity) have stopped. At a theoretical level, miRNA should be more stable then mRNA, due to its short size. A limited study was conducted on a 1-year-old blood-stain, and it was shown that the levels of miR-451 in the one year old stain were not significantly different from the fresh blood stain.

Whilst not unexpected, this study offered an element of reassurance that the miRNA marker can at least survive the transit from the crime scene to the laboratory without any loss of material. It should be noted that the 1-year-old stain tested was dry and stored under periods of light and dark. It is possible that different conditions may affect the stability of the miRNA marker. However, as long as the stain is dry, then it should not have a

detrimental effect. In addition, RNU44 also showed stability in bloodstains 1-year-old bloodstains, which is beneficial for casework samples that are 1-year-old. It is important to note that the use of RNU44 must be used with caution in low-level blood and saliva samples as demonstrated previously in the sensitivity work. Other members of the University of Huddersfield Forensic Genetics Research Group are exploring the stability of miRNA and RNU44 further.

One major issue with BFID and one that is becoming increasingly problematic with increasing sensitive DNA profiling kits, such as NGM SElect and further with GlobalFiler and Promega Fusion is the one of mixtures. DNA mixtures are common and will become increasingly common with the adoption of 'DNA17' (NGM Select kit) [3]. Consequently, the impact of mixed body fluids upon miRNA analysis was explored. The absolute minimum criterion for a BFID test is that it should be able to identify the presence of a mixture. It is very important to establish as in worst cases a mixture may be mistaken as a single source body fluid, which would consequently render this test unusable. Thus, a series of blood/saliva mixtures were prepared from a 1:1 through to a 10:1 mixture. Single source controls were included. When the BFID test was performed, it was quickly realised that the incorporation of a reference gene was crucial. In this case RNU44 was used. It was then realised that normalisation with a mixture containing equal volumes of blood and saliva extracts were required. This control (along with the reference gene) was used to normalise the expression levels and takes into account the variations in amount of genetic material between different body fluids.

Once completed, it could be seen that not only was the BFID test capable of identifying body fluid mixtures, it was also capable of identify the major body fluid and the minor body

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fluid. The mixing ratios were not maintained post-amplification, however, this is considered to be a minor issue at this stage. It is first important to establish whether the miRNA markers identified for single-source body fluids can be applied to mixtures.

As stated earlier, it was also obvious that the presence of an endogenous control was a crucial step, thus it maybe that a more appropriate endogenous control could allow for the 'mirroring' of mixing ratios between DNA mixtures and body fluid mixtures.

6.2 Novel work

The majority of the work performed in this study could be argued as being novel as no one else has carried out this miRNA work using DNA extracts. However, the main novel aspects were defined into three areas.

The first novel aspect in this work was the exploration of different magnetic bead ratios for co-isolation of DNA and mRNA and the co-isolation of miRNA and DNA using a single DNA isolation kit. The second novel aspect was the discriminative panel of body fluid specific miRNA markers for blood (miR-451 and miR-194); saliva (miR-205); vaginal material (miR-224 and miR-335) and semen (miR-891a).

The final novel aspect of this work was in the area of casework applications. The sensitivity of miR-451, miR-205 and RNU44 was explored in bloodstains and saliva deposits. The species specificity of miR-451 and RNU44 was explored in different animal bloodstains. The stability of miR-451 and RNU44 in aged bloodstains exposed to light and dark environments was also explored. Lastly a BFID strategy for resolving body fluid mixtures was developed.

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6.3 Future work

It is clear that there is a substantial amount of work to be carried out in the application of miRNA analysis body fluid identification. Whilst a lot of issues have been touched upon and explored in this study, further work is certainly required.

New miRNA markers are being identified on a regular basis, leading to the regular expansion of the miRNA repository, miRBase. Consequently, continual miRNA marker screening is required. One set of markers that require particular attention is the identification of an appropriate endogenous control as described earlier in this chapter. Other areas that need to be explored include the sensitivity of the selected miRNA markers. In the findings from the sensitivity results it was thought that the sensitivity of miRNA and reference genes was limited by both the TaqMan chemistry and the abundance within a sample types. Future work exploring the sensitivity of the miRNA markers and reference genes is needed in order to use this test in forensic casework. The stability of miRNA and reference genes was another area that was studied. It was demonstrated in this work that miRNA and reference genes were stable in 1-year-old bloodstains exposed to both light and dark. It would be interesting to establish at the point which the stability in both miRNA and reference genes is lost, particularly when additional or other environmental factors are present such as bacteria, RNases, humidity and temperature.

Finally, since mixed body fluids form a large part of samples recovered at crime scenes it would be useful to explore a different combination of body fluid mixtures e.g. vaginal material and semen, vaginal material and saliva; and menstrual blood and trauma blood as

the main application or purpose of developing this test is to be incorporated into forensic casework.

In this study a highly discriminative microRNA body fluid identification test was developed for blood, saliva, semen and vaginal material. The exploration of different co-isolation strategies showed that this method could be integrated not only into current DNA methodologies but also be applied to a range of casework samples including cold-case, low level, different animal species, aged (1-year-old) and mixtures. Thus demonstrating that a miRNA BFID is a powerful tool for forensic casework.

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Appendices

Supplementary information – mature miRNA sequences

miR-451
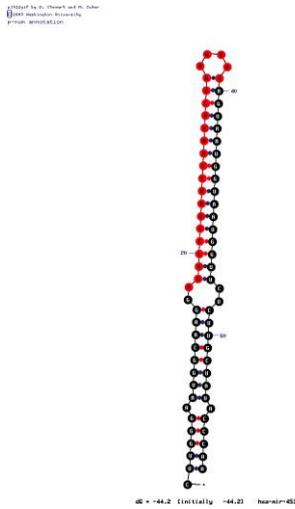
miR-16

Figure 44. Showing the predicted mature miRNA sequences highlighted in red for miR-451 (left) and miR-16 (right) highlighted in red. Images taken from miR map [311].

miR-205

miR-658
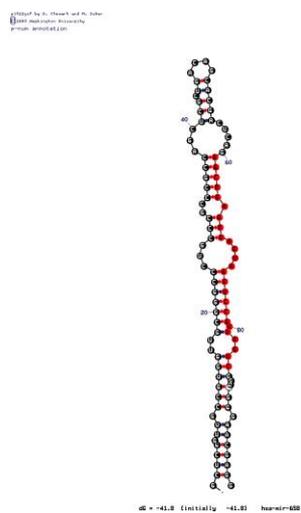
Figure 45. Showing the predicted mature miRNA sequences for miR-205 (left) and miR-658 (right) highlighted in red. Images taken from miR map [311].

miR-203
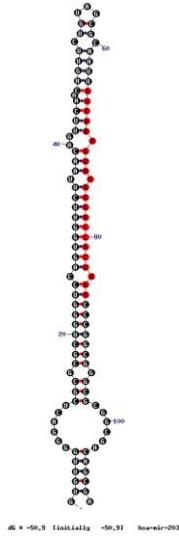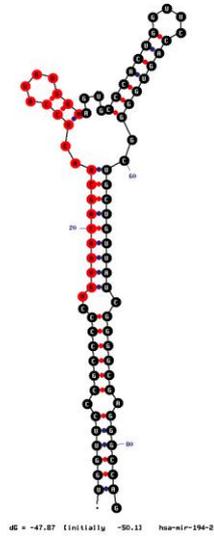
miR-194

Figure 46. Showing the predicted mature miRNA sequences for miR-203 (top left), miR-194 (top right) and miR-224 (bottom left) highlighted in red. Images taken from miR map [311].

miR-224
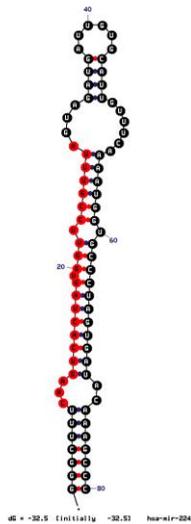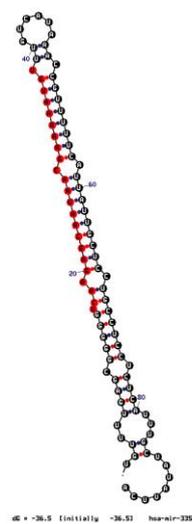
miR-224

Figure 47. Showing the predicted mature miRNA sequences for miR-224 (left) and miR-335 (right) highlighted in red. Images taken from miR map [311].

miR-617
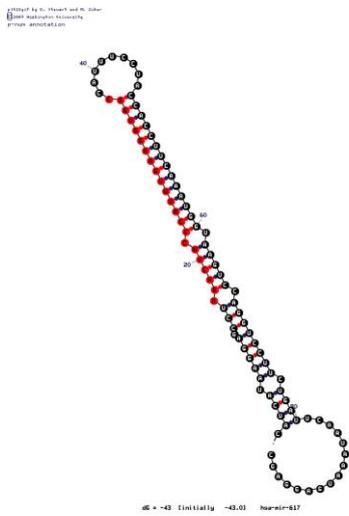
miR-372
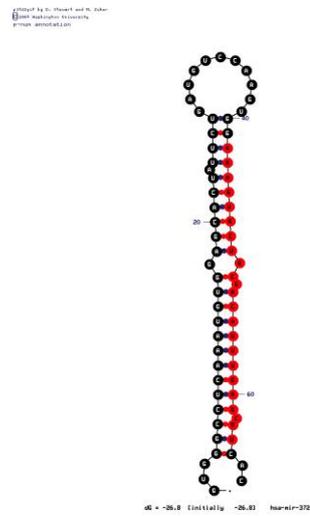
Figure 48. Showing the predicted mature miRNA sequences for miR-224 (left) and miR-335 (right) highlighted in red. Images taken from miR map [311].

miR-124a
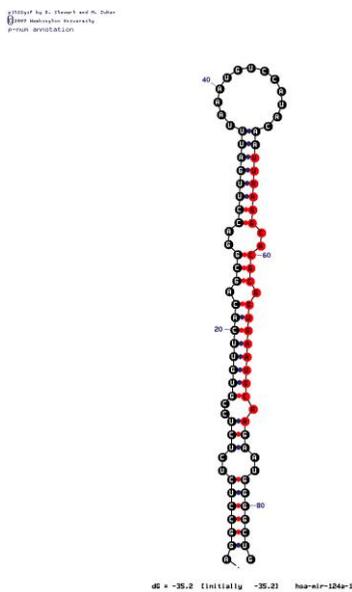
miR-588

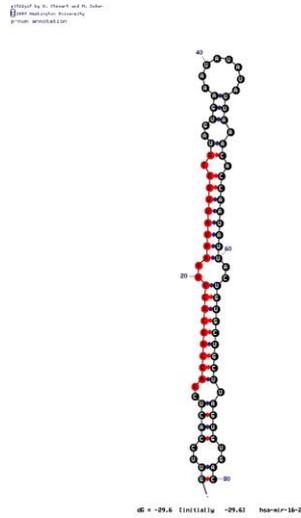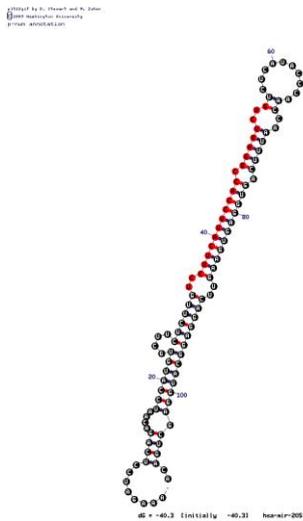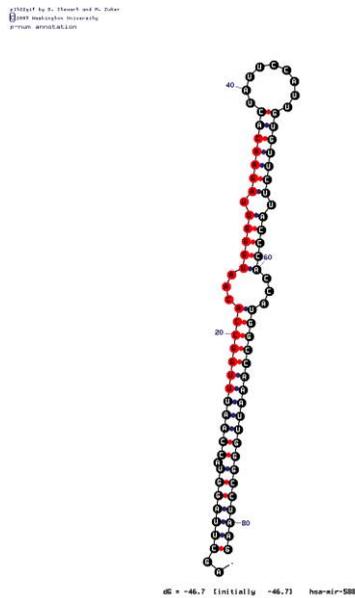*Figure 49. Showing the predicted mature miRNA sequences for miR-124a (left) and miR-588 (right) highlighted in red. Images taken from miR map [311].*

Supplementary information – miRNA species expression

	Homo sapiens (human)	Cyprinus carpio (carp)	Felis catus (cat)	Bos primingeous (cattle)	Gallus gallus (chicken)	Canis familiaris (dog)	Taeniopygia guttata (Zebra finch)	Sus scrofa (pig)
miR-451	X					X		X
miR-16	X					X		X
miR-205	X	X		X	X	X		X
miR-658	X							
miR-203	X					X		
miR-194	X	X		X	X	X	X	
miR-224	X							
miR-335	X			X		X		
miR-588	X							
miR-617	X							
miR-372	X							
miR-124a	X							
miR-891a	X							

Table 9. Showing the species specificity of the 13 miRNA markers used for screening. The letter X indicates expression for a particular species. Data provided by the Life Technologies website [300].

	Pan paniscus (Bonobo chimpanzee)	Pan troglodytes (chimpanzee)	Danio rio (Zebra fish)	Paralichthys olivaceus (Olive flounder)	Xenopus laevis (African clawed frog)	Xenopus tropicalis (western clawed frog)	Gorilla gorilla (gorilla)	Cricetulus griseus (Chinese hamster)
miR-451		X	X			X		
miR-16	X	X					X	X
miR-205	X	X	X		X	X	X	
miR-658								
miR-203	X	X						
miR-194		X			X	X	X	X
miR-224								
miR-335		X						
miR-588		X						
miR-617		X						
miR-372		X						
miR-124a				X				
miR-891a								

Table 9 continued: Showing the species specificity of the 13 miRNA markers used for screening. The letter X indicates expression for a particular species. Data provided by the Life Technologies website [300].

	Equus caballus (horse)	Petromyzon marinus (sea lamprey)	Anolis carolinensis (lizard)	Macaca nemestrina (southern pig-tailed macaaue)	Lagothrix lagotricha (brown woolly monkey)	Macaca mulatta (Rhesus monkey)	Ateles geoffroyi (Spider monkey)	Mus muculus (mouse)
miR-451			X			X		X
miR-16	X			X	X	X	X	X
miR-205	X		X	X	X	X	X	X
miR-658								
miR-203						X		X
miR-194	X		X	X		X	X	X
miR-224	X							
miR-335	X					X		X
miR-588								
miR-617								
miR-372						X		
miR-124a								X
miR-891a								

Table 9 continued: Showing the species specificity of the 13 miRNA markers used for screening. The letter X indicates expression for a particular species. Data provided by the Life Technologies website [300].

	Monodelphis domestica (grey short-tailed opossum)	Pongo pygmaeus (Bornean orangutan)	Ornithorhynchus anatinus (platypus)	Tetraodon nigroviridis (green spotted puffer)	Fugu rubripes (pufferfish)	Rattus rattus (rat)	Saquinus labiatus (white-lipped tamarind)
miR-451		X				X	
miR-16	X		X			X	X
miR-205	X	X		X	X		
miR-658		X					
miR-203		X				X	
miR-194		X	X			X	
miR-224							
miR-335		X				X	
miR-588		X					
miR-617							
miR-372		X					
miR-124a			X			X	
miR-891a							

Table 9 continued: Showing the species specificity of the 13 miRNA markers used for screening. The letter X indicates expression for a particular species. Data provided by the Life Technologies website [300].

Supplementary graphs – Chapter 3
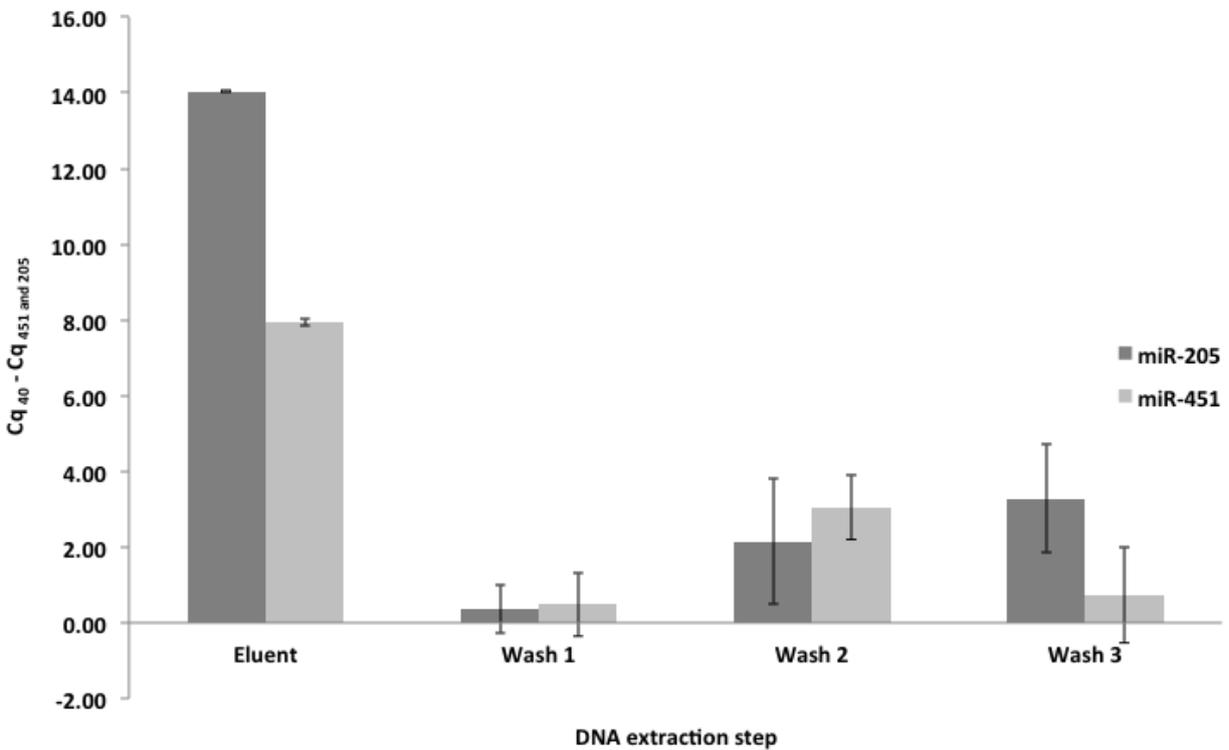
Figure 50. Showing the expression of miR-205 and miR-451 in saliva swabs using a DNA isolation kit (n=6).
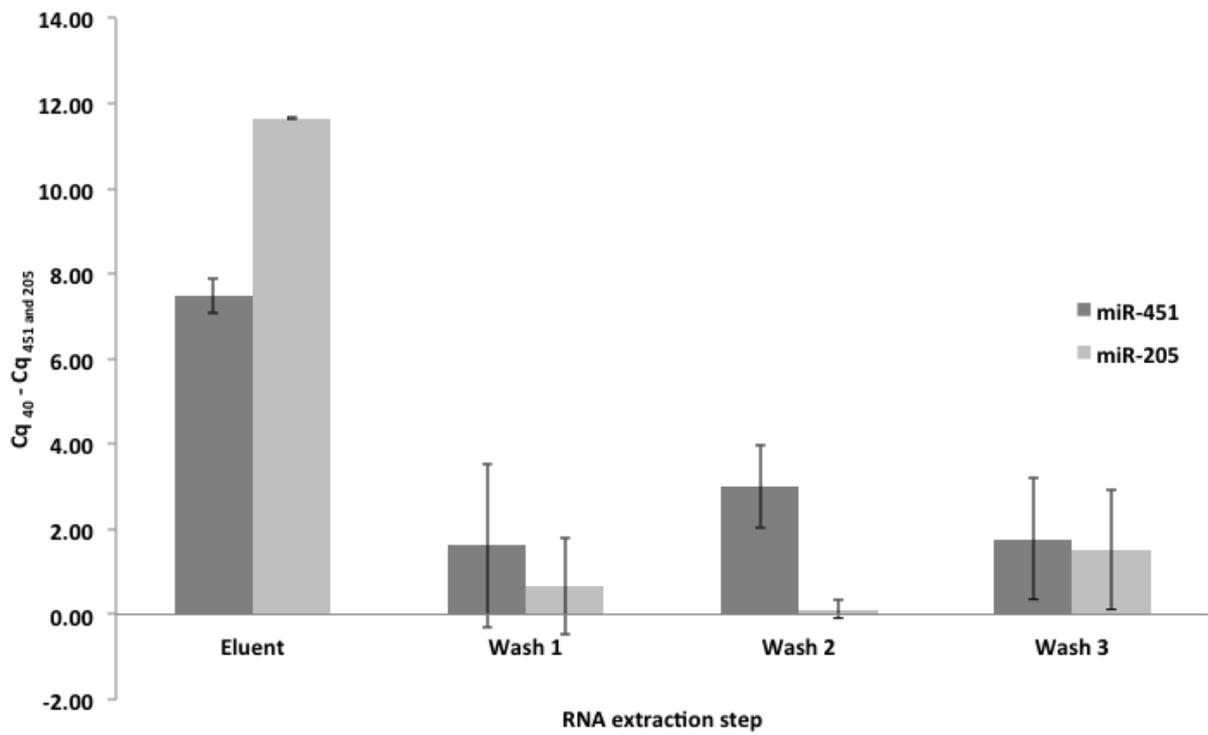
Figure 51. Showing the expression of miR-205 and miR-451 in saliva using the total RNA isolation method (n=6).

Supplementary graphs – Chapter 4

miR-16
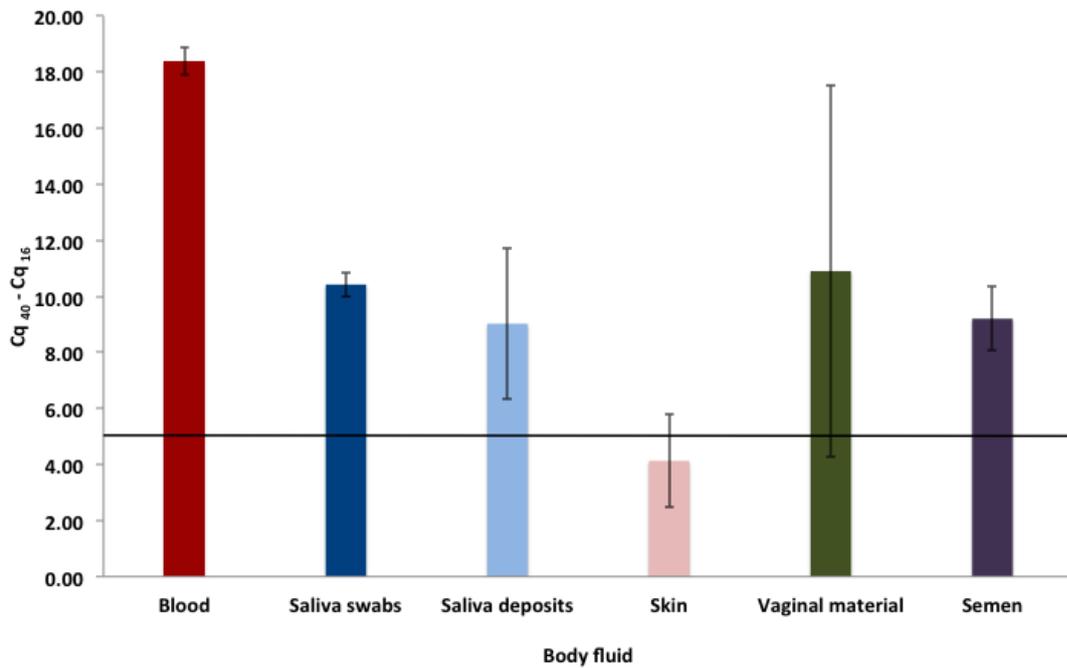
Figure 52. Showing the specificity of miR-16 in 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-658
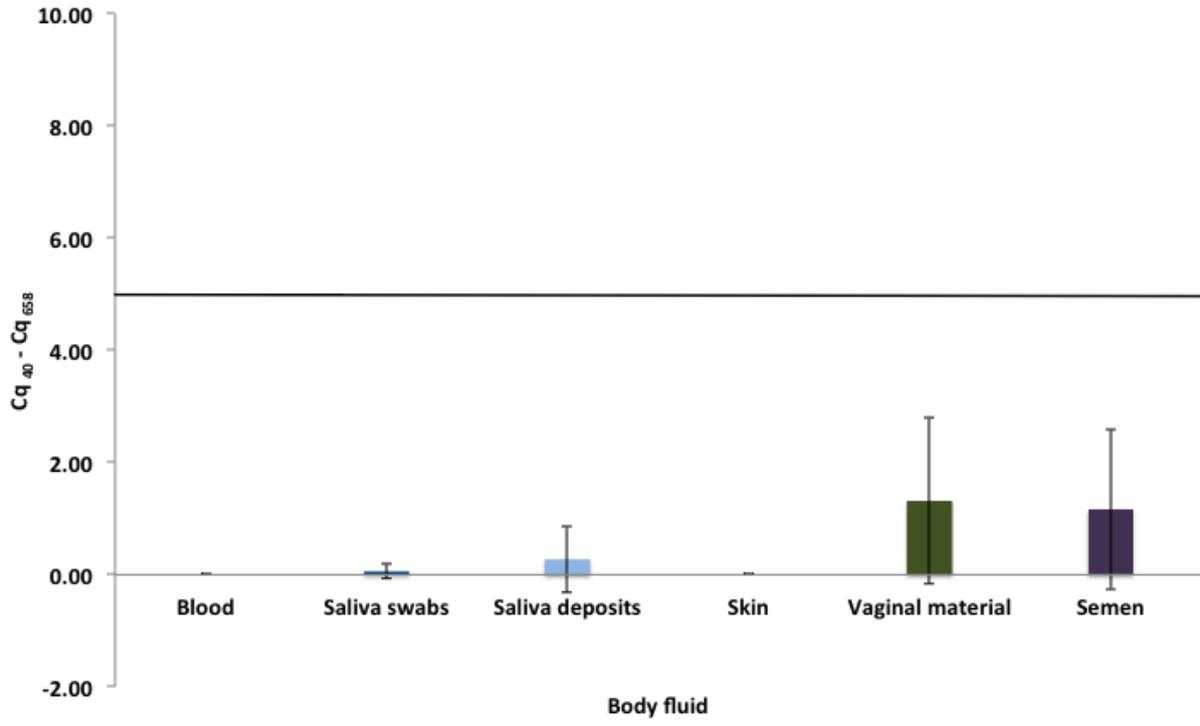
Figure 53. Showing the specificity of miR-658 in 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-203
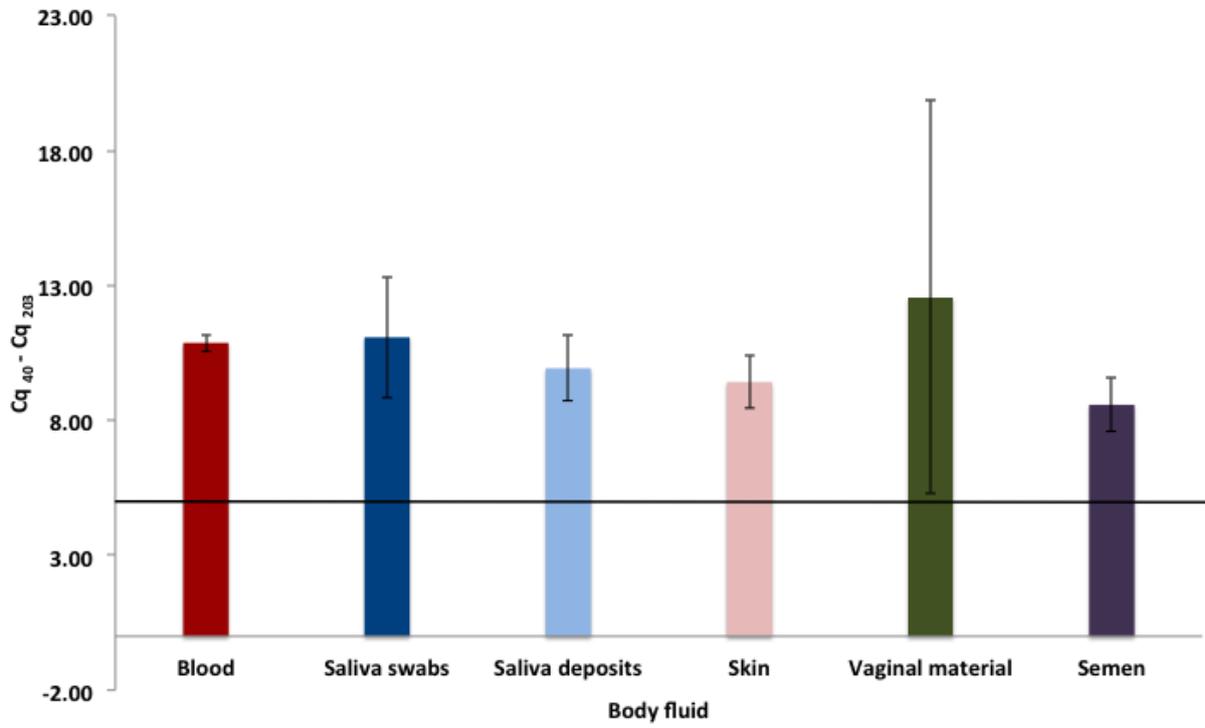
Figure 54. Showing the specificity of miR-203 in 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-617
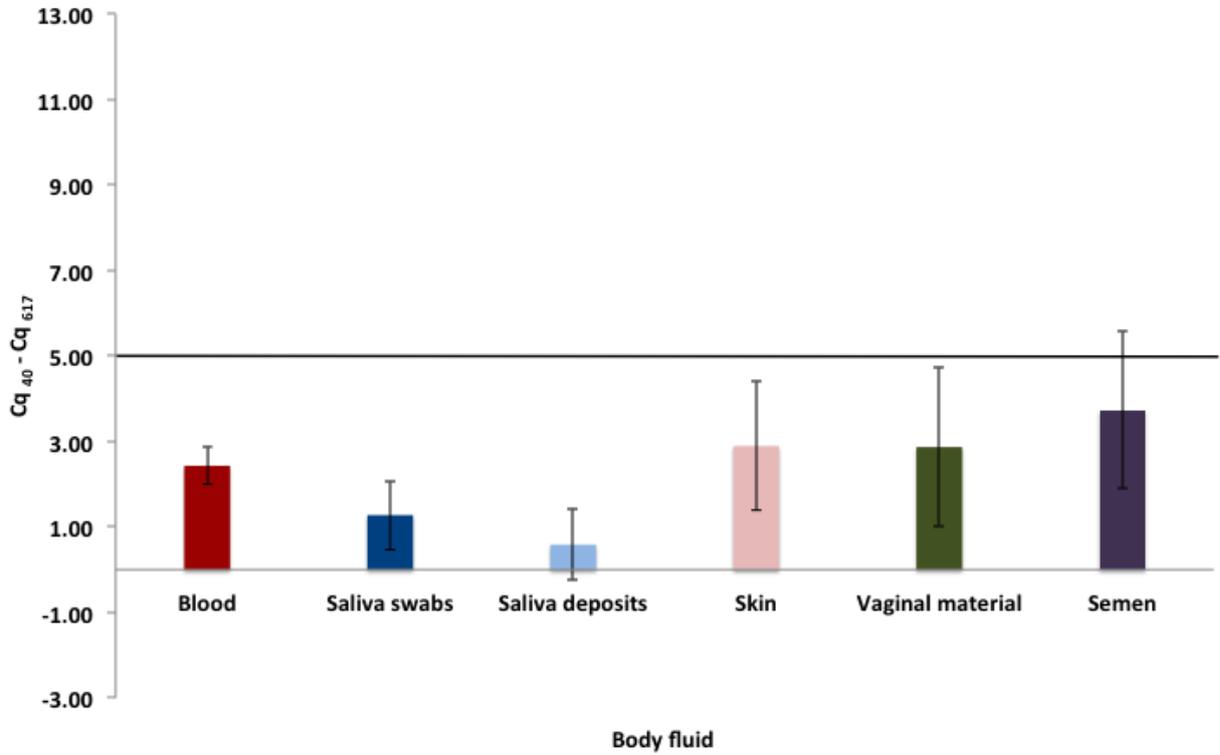
Figure 55. Showing the specificity of miR-617 in 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-372
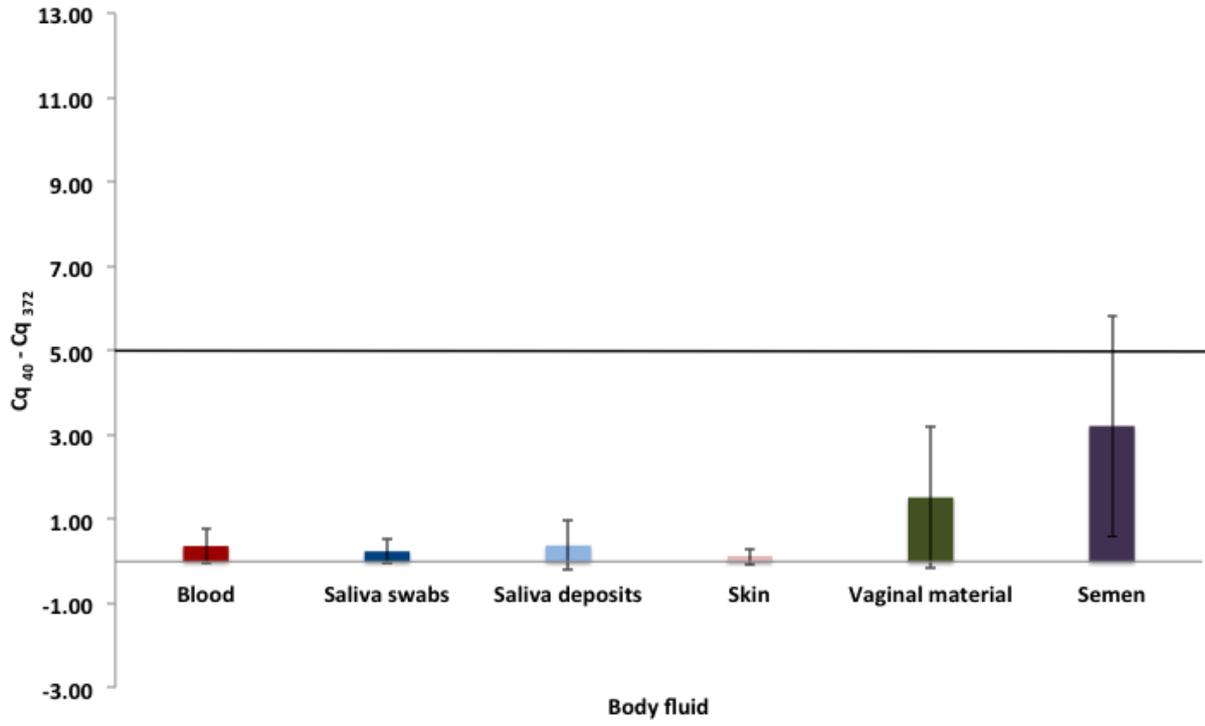
Figure 56. Showing the specificity of miR-372 in 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-124a
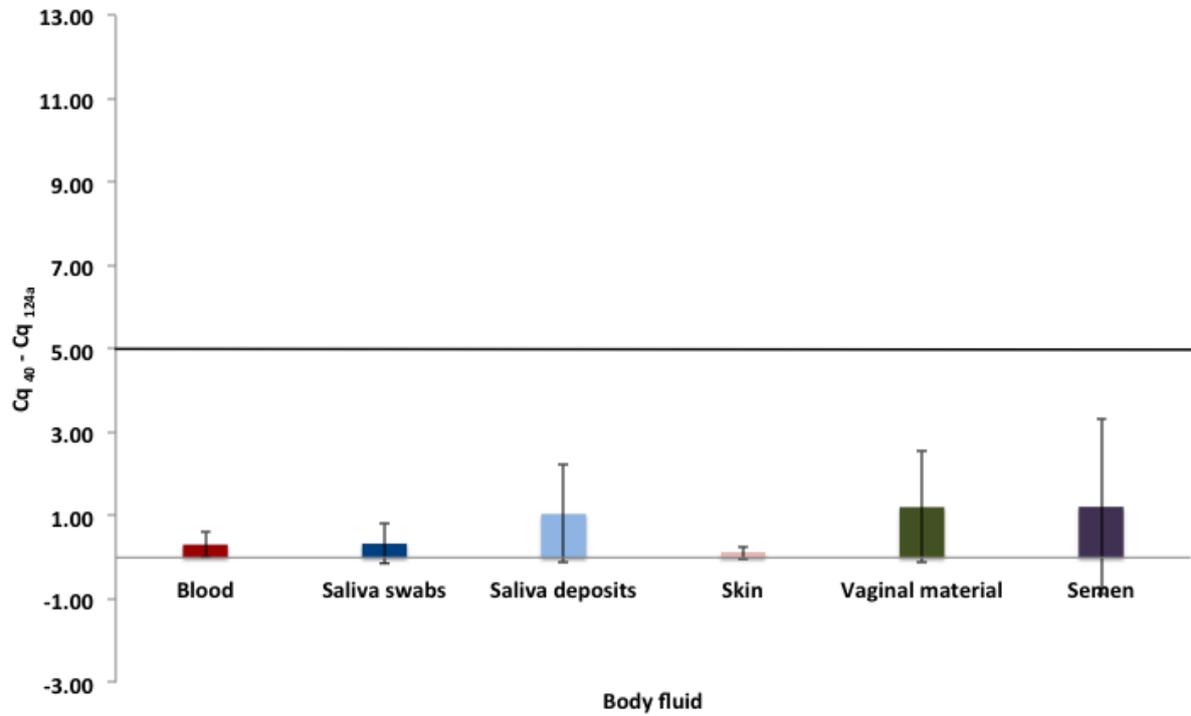
Figure 57. Showing the specificity of miR-124a using 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

miR-588
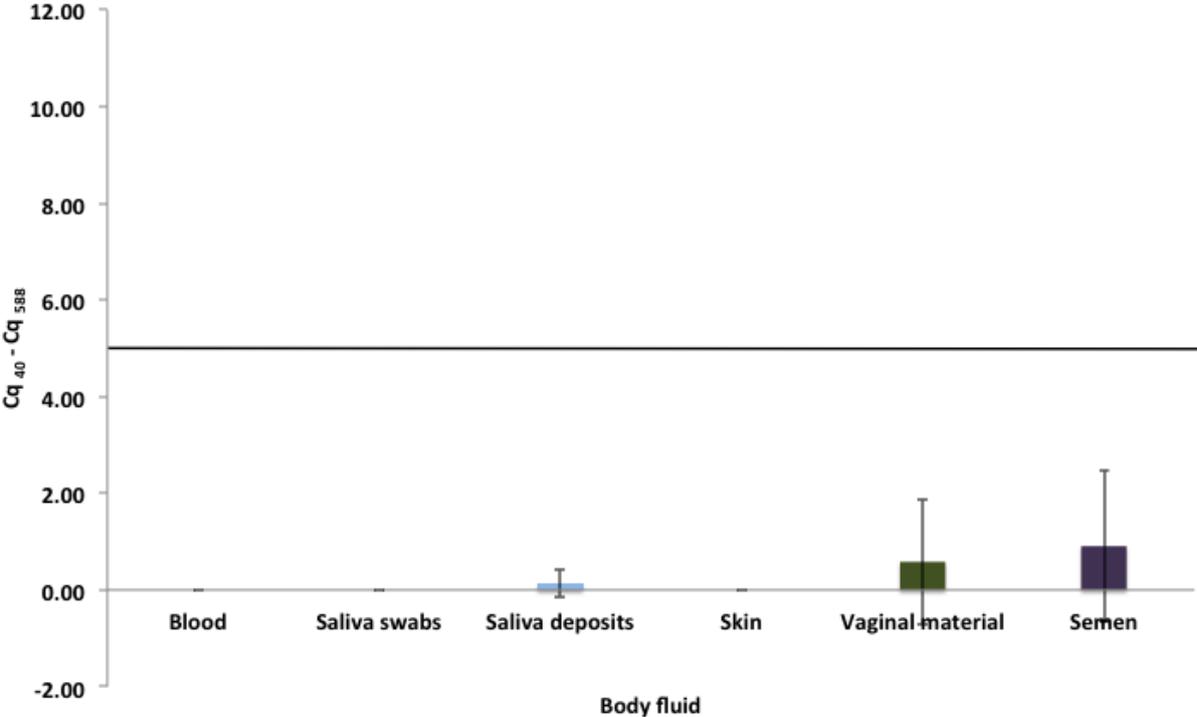
Figure 58. Showing the specificity of miR-588 using 6 different body fluids. The black line at ΔCq 5 represents a threshold. Values above this line are considered expression while values below this line are considered background amplification. Error bars represent one standard deviation (n=5).

Supplementary graphs – Chapter 5
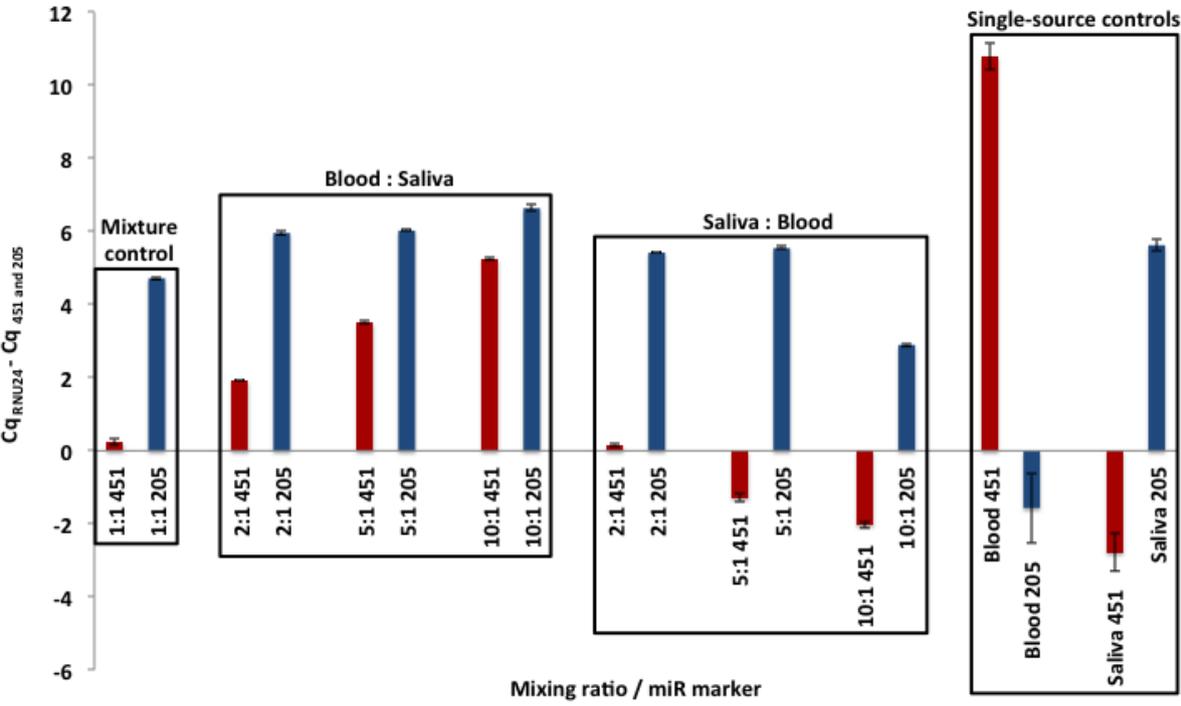
Figure 59. Showing the expression of miR-451 and miR-205 in 1 blood:1 saliva, 2 blood:1 saliva, 5 blood:1 saliva, 10 blood:1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva:1 blood mixtures when normalised with RNU24 (n=3).
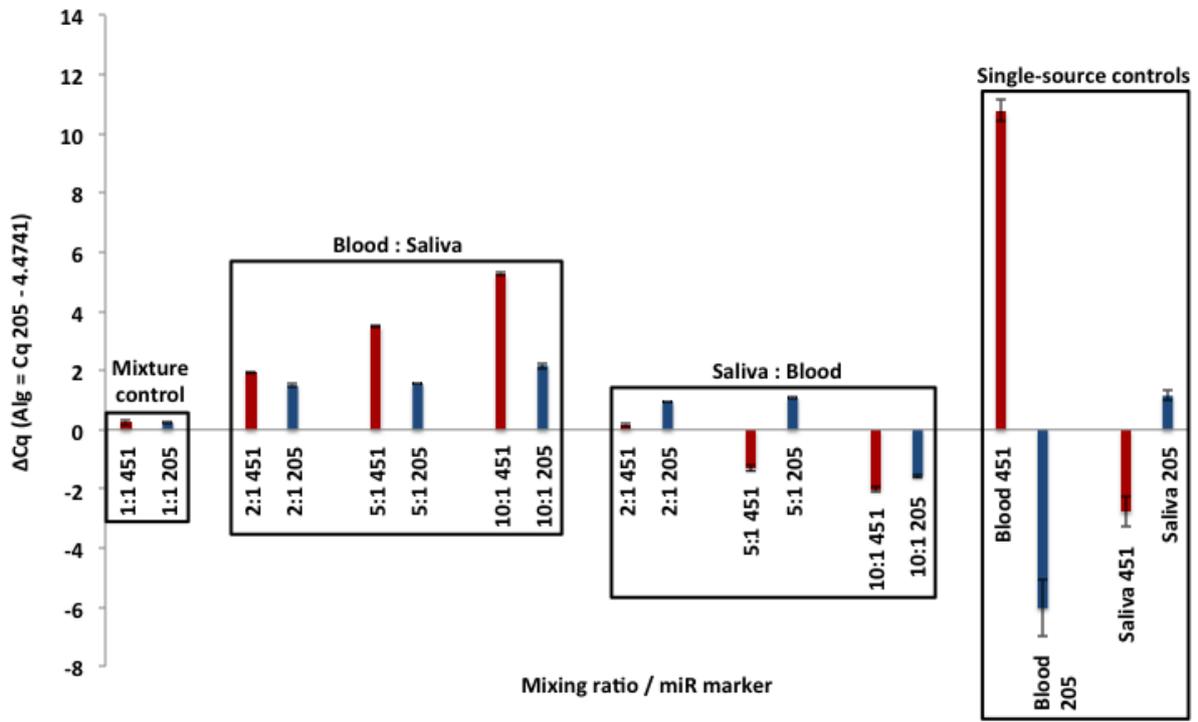
Figure 60. Showing the expression of miR-451 and miR-205 in 2 blood:1 saliva, 5 blood:1 saliva, 10 blood:1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva:1 blood mixtures when normalised with the RNU24 and the 1 blood:1 saliva mixture (n=3).
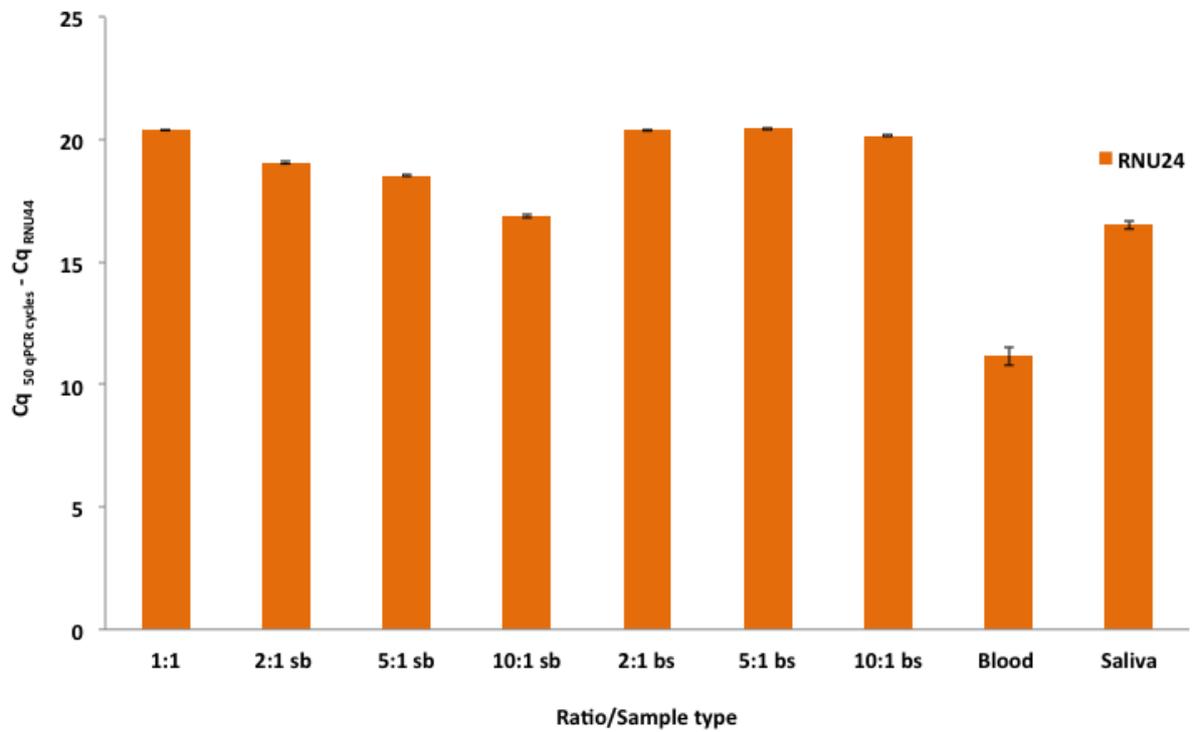
Figure 61. Showing the RNU44 expression on its own in 2 blood:1 saliva, 5 blood:1 saliva, 10 blood:1 saliva, 2 saliva:1 blood, 5 saliva:1 blood and 10 saliva:1 blood mixtures (n=3).

Supplementary data – miRNA panel

Blood

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Bloodstain A	18.30	18.06	8.94	0.00	0.49	1.05	2.41	0.00	0.00	8.82	10.77	0.89	4.11
Bloodstain B	17.89	16.70	8.01	0.00	0.00	0.08	2.59	0.00	0.00	8.72	11.36	0.00	3.19
Bloodstain C	18.84	20.98	5.69	0.00	0.68	0.43	2.61	0.00	0.00	8.94	10.95	0.71	4.95
Bloodstain D	17.94	16.18	3.79	0.00	0.00	0.18	2.84	0.00	0.00	8.26	10.53	0.00	3.41
Bloodstain E	18.94	18.12	5.95	0.00	0.38	0.09	1.71	0.00	0.00	7.35	10.75	0.25	4.83
Mean	18.38	18.01	6.48	0.00	0.31	0.37	2.43	0.00	0.00	8.42	10.87	0.37	4.10
SD	0.49	1.86	2.03	0.00	0.30	0.41	0.43	0.00	0.00	0.65	0.31	0.41	0.80

Table 10. Showing the ΔCq data from Chapter 4 miRNA screen in bloodstains

Saliva swabs

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Saliva swab A	11.07	8.61	9.45	0.28	0.63	0.11	2.57	0.00	0.00	1.59	12.45	6.76	1.42
Saliva swab B	10.10	6.58	9.08	0.00	0.00	0.51	1.36	0.00	0.00	0.56	12.47	6.17	1.15
Saliva swab C	10.39	6.98	10.16	0.00	1.04	0.59	1.16	0.00	0.00	0.98	13.18	7.46	1.41
Saliva swab D	9.98	7.34	8.09	0.00	0.00	0.00	0.39	0.00	0.00	0.02	8.77	7.01	1.20
Saliva swab E	10.57	7.13	8.78	0.00	0.00	0.00	0.89	0.00	0.00	0.00	8.54	7.33	0.67
Mean	10.42	7.33	9.11	0.06	0.33	0.24	1.28	0.00	0.00	0.63	11.08	6.95	1.17
SD	0.43	0.77	0.77	0.13	0.48	0.29	0.81	0.00	0.00	0.67	2.24	0.51	0.31

Table 11. Showing the ΔCq data from Chapter 4 miRNA screen in saliva swabs.

Saliva deposits

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Saliva deposit A	10.78	8.11	7.81	0.00	0.55	0.60	0.00	0.00	0.00	3.25	10.51	5.38	0.00
Saliva deposit B	11.24	9.06	7.65	0.04	2.51	1.31	0.00	0.00	0.00	3.18	11.29	5.30	1.28
Saliva deposit C	10.85	13.15	9.02	1.33	2.08	0.00	0.00	0.00	0.00	1.49	8.67	0.00	0.00
Saliva deposit D	6.80	10.95	7.81	0.00	0.00	0.00	1.12	3.29	0.66	1.43	10.55	0.00	0.00
Saliva deposit E	5.45	7.96	5.92	0.00	0.08	0.00	1.80	0.00	0.00	0.50	8.65	0.71	0.00
Mean	9.02	9.85	7.64	0.27	1.04	0.38	0.58	0.66	0.13	1.97	9.93	2.28	0.26
SD	2.70	2.20	1.11	0.59	1.17	0.58	0.84	1.47	0.29	1.20	1.20	2.81	0.57

Table 12. Showing the ΔCq data from Chapter 4 miRNA screen in saliva deposits.

Skin

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Skin A	2.66	8.36	6.00	0.00	0.22	0.14	2.99	0.00	0.00	0.25	8.24	0.00	0.00
Skin B	4.68	10.54	8.03	0.00	0.00	0.00	5.47	0.00	0.00	0.66	8.61	0.00	0.00
Skin C	4.72	10.58	7.89	0.00	0.34	0.43	2.09	0.00	0.00	0.00	9.64	0.20	0.00
Skin D	2.30	9.04	9.00	0.00	0.00	0.00	2.02	0.00	0.00	0.00	10.18	0.00	0.00
Skin E	6.30	12.18	7.76	0.00	0.00	0.00	1.90	0.00	0.00	0.00	10.44	0.00	0.00
Mean	4.13	10.14	7.74	0.00	0.11	0.11	2.89	0.00	0.00	0.18	9.42	0.04	0.00
SD	1.65	1.49	1.09	0.00	0.16	0.19	1.50	0.00	0.00	0.29	0.96	0.09	0.00

Table 13. Showing the ΔCq data from Chapter 4 miRNA screen in skin.

Semen

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Semen A	8.17	7.44	5.87	1.69	3.65	5.93	3.85	11.35	2.72	3.12	8.13	4.03	3.56
Semen B	8.90	9.88	7.01	0.00	0.00	0.71	1.77	8.35	0.00	3.84	9.09	2.50	2.66
Semen C	8.15	10.05	6.47	0.00	0.00	0.11	2.24	3.44	0.00	0.61	10.07	0.00	0.44
Semen D	10.21	7.22	6.38			4.59	4.36	10.26		6.07	7.84	3.63	4.07
Semen E	10.57	6.60	6.24	2.96		4.74	6.39	10.60		3.85	7.77	4.10	5.07
Mean	9.20	8.24	6.39	1.16	1.22	3.22	3.73	8.80	0.91	3.50	8.58	2.85	3.16
SD	1.14	1.61	0.41	1.44	2.10	2.62	1.84	3.20	1.57	1.96	0.98	1.72	1.75

Table 14. Showing the ΔCq data from Chapter 4 from the miRNA screen in semen.

Vaginal material

	miR-16	miR-451	miR-205	miR-658	miR-124a	miR-372	miR-617	miR-891a	miR-588	miR-194	miR-203	miR-224	miR-335
Vaginal material A	17.04	11.46	16.14	1.39	0.72	2.58	4.54	0.01	0.00	7.24	18.32	12.73	6.44
Vaginal material B	10.12	10.11	10.44	0.00	1.18	0.00	2.98	0.00	0.00	3.95	15.53	7.25	1.27
Vaginal material C	15.03	10.79	15.21	0.12	0.67	1.19	2.31	0.45	0.00	0.00	12.75	19.01	14.04
Vaginal material D	0.00	8.04	10.11	1.39	0.00	0.00	0.00	2.20	0.00	0.00	0.00	16.92	9.49
Vaginal material E	12.31	7.23	12.07	3.68	3.46	3.83	4.51	3.93	2.91	4.95	16.26	13.25	4.07
Mean	10.90	9.53	12.79	1.32	1.21	1.52	2.87	1.32	0.58	3.23	12.57	13.83	7.06
SD	6.64	1.81	2.75	1.48	1.33	1.67	1.87	1.72	1.30	3.18	7.31	4.50	4.94

Table 15. Showing the ΔCq data from Chapter 4 miRNA screen in vaginal material.

miR-451

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-451	18.06	8.61	8.11	8.36	11.46	7.44
	16.70	6.58	9.06	10.54	10.11	9.88
	20.98	6.98	13.15	10.58	10.79	10.05
	16.18	7.34	10.95	9.04	8.04	7.22
	18.12	7.13	7.96	12.18	7.23	6.60
Mean	18.01	7.33	9.85	10.14	9.53	8.24
SD	1.86	0.77	2.20	1.49	1.81	1.61

Table 16. Showing the ΔCq data from Chapter 4 miR-451 in 6 different body fluids.

miR-194

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-194	8.82	1.59	3.25	0.25	7.24	3.12
	8.72	0.56	3.18	0.66	3.95	3.84
	8.94	0.98	1.49	0.00	0.00	0.61
	8.26	0.02	1.43	0.00	0.00	6.07
	7.35	0.00	0.50	0.00	4.95	3.85
Mean	8.42	0.63	1.97	0.18	3.23	3.50
SD	0.65	0.67	1.20	0.29	3.18	1.96

Table 17. Showing the ΔCq data from Chapter 4 miR-194 in 6 different body fluids.

miR-205

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-205	8.94	9.45	7.81	6.00	16.14	5.87
	8.01	9.08	7.65	8.03	10.44	7.01
	5.69	10.16	9.02	7.89	15.21	6.47
	3.79	8.09	7.81	9.00	10.11	6.38
	5.95	8.78	5.92	7.76	12.07	6.24
Mean	6.48	9.11	7.64	7.74	12.79	6.39
SD	2.03	0.77	1.11	1.09	2.75	0.41

Table 18. Showing the ΔCq data from Chapter 4 miR-205 in 6 different body fluids.

miR-224

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-224	0.89	6.76	5.38	0.00	12.73	4.03
	0.00	6.17	5.30	0.00	7.25	2.50
	0.71	7.46	0.00	0.20	19.01	0.00
	0.00	7.01	0.00	0.00	16.92	3.63
	0.25	7.33	0.71	0.00	13.25	4.10
Mean	0.37	6.95	2.28	0.04	13.83	2.85
SD	0.41	0.51	2.81	0.09	4.50	1.72

Table 19. Showing the ΔCq data from Chapter 4 miR-224 in 6 different body fluids.

miR-335

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-335	4.11	1.42	0.00	0.00	6.44	3.56
	3.19	1.15	1.28	0.00	1.27	2.66
	4.95	1.41	0.00	0.00	14.04	0.44
	3.41	1.20	0.00	0.00	9.49	4.07
	4.83	0.67	0.00	0.00	4.07	5.07
Mean	4.10	1.17	0.26	0.00	7.06	3.16
SD	0.80	0.31	0.57	0.00	4.94	1.75

Table 20. Showing the ΔCq data from Chapter 4 miR-335 in 6 different body fluids.

miR-891a

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-891a	0.00	0.00	0.00	0.00	0.01	11.35
	0.00	0.00	0.00	0.00	0.00	8.35
	0.00	0.00	0.00	0.00	0.45	3.44
	0.00	0.00	3.29	0.00	2.20	10.26
	0.00	0.00	0.00	0.00	3.93	10.60
Mean	0.00	0.00	0.66	0.00	1.32	8.80
SD	0.00	0.00	1.47	0.00	1.72	3.20

Table 21. Showing the ΔCq data from Chapter 4 miR-891a in 6 different body fluids.

miR-16

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-16	18.30	11.07	10.78	2.66	17.04	8.17
	17.89	10.10	11.24	4.68	10.12	8.90
	18.84	10.39	10.85	4.72	15.03	8.15
	17.94	9.98	6.80	2.30	0.00	10.21
	18.94	10.57	5.45	6.30	12.31	10.57
Mean	18.38	10.42	9.02	4.13	10.90	9.20
SD	0.49	0.43	2.70	1.65	6.64	1.14

Table 22. Showing the ΔCq data from Chapter 4 miR-16 in 6 different body fluids.

miR-658

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-658	0.00	0.28	0.00	0.00	1.39	1.69
	0.00	0.00	0.04	0.00	0.00	0.00
	0.00	0.00	1.33	0.00	0.12	0.00
	0.00	0.00	0.00	0.00	1.39	
	0.00	0.00	0.00	0.00	3.68	2.96
Mean	0.00	0.06	0.27	0.00	1.32	1.16
SD	0.00	0.13	0.59	0.00	1.48	1.44

Table 23. Showing the ΔCq data from Chapter 4 miR-658 in 6 different body fluids.

miR-203

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-203	10.77	12.45	10.51	8.24	18.32	8.13
	11.36	12.47	11.29	8.61	15.53	9.09
	10.95	13.18	8.67	9.64	12.75	10.07
	10.53	8.77	10.55	10.18	0.00	7.84
	10.75	8.54	8.65	10.44	16.26	7.77
Mean	10.87	11.08	9.93	9.42	12.57	8.58
SD	0.31	2.24	1.20	0.96	7.31	0.98

Table 24. Showing the ΔCq data from Chapter 4 miR-203 in 6 different body fluids.

miR-588

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-588	0.00	0.00	0.00	0.00	0.00	2.72
	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.66	0.00	0.00	
	0.00	0.00	0.00	0.00	2.91	
Mean	0.00	0.00	0.13	0.00	0.58	0.91
SD	0.00	0.00	0.29	0.00	1.30	1.57

Table 25. Showing the ΔCq data from Chapter 4 miR-588 in 6 different body fluids.

miR-124a

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-124a	0.49	0.63	0.55	0.22	0.72	3.65
	0.00	0.00	2.51	0.00	1.18	0.00
	0.68	1.04	2.08	0.34	0.67	0.00
	0.00	0.00	0.00	0.00	0.00	
	0.38	0.00	0.08	0.00	3.46	
Mean	0.31	0.33	1.04	0.11	1.21	1.22
SD	0.30	0.48	1.17	0.16	1.33	2.10

Table 26. Showing the ΔCq data from Chapter 4 miR-124a in 6 different body fluids.

miR-372

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-372	1.05	0.11	0.60	0.14	2.58	5.93
	0.08	0.51	1.31	0.00	0.00	0.71
	0.43	0.59	0.00	0.43	1.19	0.11
	0.18	0.00	0.00	0.00	0.00	4.59
	0.09	0.00	0.00	0.00	3.83	4.74
Mean	0.37	0.24	0.38	0.11	1.52	3.22
SD	0.41	0.29	0.58	0.19	1.67	2.62

Table 27. Showing the ΔCq data from Chapter 4 miR-372 in 6 different body fluids.

miR-617

	Blood	Saliva swabs	Saliva deposits	Skin	Vaginal material	Semen
miR-617	2.41	2.57	0.00	2.99	4.54	3.85
	2.59	1.36	0.00	5.47	2.98	1.77
	2.61	1.16	0.00	2.09	2.31	2.24
	2.84	0.39	1.12	2.02	0.00	4.36
	1.71	0.89	1.80	1.90	4.51	6.39
Mean	2.43	1.28	0.58	2.89	2.87	3.73
SD	0.43	0.81	0.84	1.50	1.87	1.84

Table 28. Showing the ΔCq data from Chapter 4 miR-617 in 6 different body fluids.