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**Evaluating an mRNA based body fluid  
identification test using SYBR green fluorescent  
dye and real-time PCR**

Jo-Ann Clair Connolly BSc. (Hons)



**A thesis submitted to the University of Huddersfield**

**in partial fulfilment of the requirements for**

**the degree of Master of Philosophy**

**The University of Huddersfield**

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## **Abstract**

The requirement to have more definitive and wider ranging body fluid identification (BFID) tests has resulted in a range of mRNA based real-time PCR BFI assays utilising Taqman fluorescent dye. An attempt to make a reliable and cost effective BFI test utilising the alternative SYBR Green fluorescent dye was carried out. RNA was extracted from blood and saliva stains from both male and female donors, this was then reverse transcribed using M-MLV and random hexamers. Using real-time PCR, relative quantitation of blood and saliva specific markers was carried out on the cDNA from the blood and saliva samples using the SYBR<sup>®</sup> Green fluorescent dye. Melting curve analysis was also performed immediately following PCR amplification. The relative quantitation values were calculated using the formula  $2^{-\Delta\Delta C_T}$  and all samples were normalised to reference gene 18s rRNA. The results revealed good specificity for a number of markers using this chemistry, however some markers were undetected. Blood markers NCF2, SPTB, PBDG and saliva specific markers HTN3, SPRR1A, KRT4 and KRT13 were investigated. In the SYBR green studies, the most specific markers were NCF2, KRT4, KRT13 and SPRR1A, showing reproducible results in a number of studies. Blood marker SPTB also appeared to be specific to blood however the melt curve data for this marker in each study was questionable given the low melting temperature for the amplified products. Blood specific marker PBGD, and saliva specific marker HTN3 were not detected using SYBR Green and saliva marker STATH was detected however in each case appeared to be non-specific in nature when their melt curves were analysed. Analysis of the 18s rRNA Ct values showed a higher expression in saliva than in blood in almost all instances, this may be due to collection of a higher number of cells when using a buccal swab, coupled with the inability to accurately quantify the RNA extracts before reverse transcription. Taqman assays were run on all markers as an additional test, to compare with the SYBR

green data. All markers except SPTB showed very good specificity for their respective body fluids. SPTB, like in the SYBR green studies was detected in blood more than saliva, however detection was never consistent in each sample. It can therefore be said that real-time PCR using SYBR Green dye was capable of identifying specific mRNA markers blood and saliva however, the lack of specificity for this type of assay makes its use as a routine identification of body fluids in forensic casework not suitable. The main aim of this study was to develop a more cost effective BFID and as such involved the use of SYBR Green as a cheaper alternative to TaqMan. However, throughout these studies, it appeared to be quite costly in terms of validating a SYBR Green experiment, as more reagents were required in the long run due to vast amount of no template controls required per experiment. It therefore would appear that while SYBR Green is cheaper to buy, the cost to validate these type of experiments can be quite high, due to the non-specific nature of the dye itself. The SYBR Green studies were also much more time consuming with regards to data interpretation as post analysis of the amplification plot and melt curves is a necessity with this detection chemistry to ensure successful interpretation of the data.

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*“Life is not about waiting for the storm to pass, it’s about learning to dance in the rain”*

*Vivian Greene*

*This thesis is dedicated to my parents, Steven Connolly and Michelle Connolly.*

*Thank you both for your love and support over the years, you have helped me in so many ways, and without you both I wouldn’t be where I am today.*

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## **Publications**

### **Conference Abstracts**

Connolly, J. and Williams, G. (2012) *The Use of Forensic Messenger RNA (mRNA) Analysis to Determine Stain Age*. (Poster), In: *Proceedings of the American Academy of Forensic Sciences Annual Meeting*. American Academy of Forensic Sciences, P320, Atlanta, Georgia, USA

Connolly, J., Clarke, D. and Williams, G. (2011) *The Use of Forensic Messenger RNA analysis to determine stain age*. (Oral), In: *7th National FORREST Conference 2011 (FORnsic RESearch and Teaching)*. Nottingham, UK: The Higher Education Academy UK Physical Sciences Centre in conjunction with Nottingham Trent University, P.26

Connolly, J., Williams, G. and Clarke, D. (2010) *The development of an mRNA body fluid identification test utilising real-time PCR and the SYBR green fluorescent dye*. (Oral), In: *6<sup>th</sup> National FORREST Conference 2010, 29<sup>th</sup> – 30<sup>th</sup> June 2010*, Coventry, UK

### **Manuscripts**

Connolly, J. and Williams, G. (2011) *Evaluating an MRNA based body fluid identification test using sybr green fluorescent dye and real-time PCR*. *International Journal of Criminal Investigation*, 1 (4). pp. 177-185. ISSN 2247-0271

## **Abbreviations**

<b>Abbreviation</b>	<b>Description</b>
AQ	Absolute Quantification
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
FAM	Fluorescein Amidite
FRET	Fluorescence Resonance Energy Transfer
gDNA	Genomic DNA
GOI	Gene of Interest
hnRNA	Heterogeneous RNA
MGB	Minor Groove Binder
miRNA	Micro RNA
mRNA	Messenger RNA
NFQ	Non Fluorescent Quencher
NTC	No Template Control
PCR	Polymerase Chain Reaction
qPCR	Real Time Polymerase Chain Reaction

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RNA	Ribonucleic Acid
RNA Pol II	RNA polymerase II
RNases	Ribonucleases
RQ	Relative Quantification
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription endpoint PCR
qRT-PCR	Reverse transcription real-time PCR
S	Svedburg unit
snoRNA	Small Nucleolar RNA
snRNA	Small Nuclear RNA
snRNPs	Small Nuclear Ribonucleoprotein Particles
ssDNA	Single Stranded DNA
T <sub>m</sub>	Melting Temperature
tRNA	Transfer RNA

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## **Chapter 1**

### **Introduction**

## **1.1 Why is body fluid identification necessary**

Although DNA profiling is a powerful technique employed by forensic scientists to identify an individual from body fluid evidence encountered at crime scenes, it is often necessary in forensic casework to identify the body fluid from which the DNA was obtained from. In casework, a DNA profile can be used to identify the suspect and/or victim and even exonerate an innocent person who has been wrongly convicted. However, this evidence alone does not reveal the circumstances by which it got transferred. For investigators, the ability to identify of the type of body fluid from which a DNA profile originated could provide an important link between the donor of the sample and how the biological material was transferred, this contextual information could therefore greatly increase the evidential value of a DNA profile. For example, a DNA profile originating from skin cells could have a different meaning in a case than a profile that has originated from spermatozoa.

## **1.2 Conventional methods of body fluid identification**

The most commonly encountered body fluids at a crime scene are blood, saliva, semen, vaginal secretions and menstrual blood. Over the years a number of methods have been developed for the identification of body fluids in biological stains, these include chemical and immunological tests, protein catalytic activity tests, spectroscopic methods and microscopy (Kobilinsky, 2012). These conventional methods are however subject to a number of limitations; the majority are presumptive in nature i.e. they can produce false positive and negative results, they lack specificity i.e. cross-reaction with other species or tissues, and they also lack sensitivity.

## **1.3 Identification of Blood**

### **1.3.1 Kastle-Meyer and LMG Test**

The most commonly used body fluid identification tests for blood are Kastle-Meyer (KM) also known as phenolphthalein and Leucomalachite Green (LMG). The KM test relies on the peroxidase-like activity of haemoglobin in blood to catalyse the oxidation of phenolphthalein. The peroxidase-catalysed oxidation changes the oxidation state of phenolphthalein, and as a result the substrate changes from colourless to a bright pink colour, thus indicating the possibility of blood being present. Compared to the luminol test, an earlier presumptive test for blood, the reactions are based on a colour change rather than chemiluminescent emission (Webb et al., 2006, Counsil and McKillip, 2010). The LMG test is based on the same principle as Kastle-Meyer and performed under acidic conditions, the oxidation reaction changes the colour of the substrate from colourless to blue/green. The sensitivity of this test is similar to that of the Kastle-Meyer. Both of these tests are prone to false positive results i.e. oxidative and peroxidase false positives, and are therefore said to be indicative rather than definitive (Cox, 1991).

### **1.3.2 The Luminol Test**

The luminol test, has been around for over forty years (Barni et al., 2007), and used to be the most commonly used blood identification test, until recent years where forensic scientists predominantly use the LMG and KM test for the detection of blood. This was due to its higher sensitivity over other presumptive screening methods. It's classified as a chemiluminescent test and like the LMG and KM test, relies on the peroxidase-like activity of haemoglobin in blood. Investigators spray a suspected area with an aqueous solution of luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) and an oxidant, usually hydrogen



peroxide ( $\text{H}_2\text{O}_2$ ), and in the presence of a catalyst i.e. peroxidase or in the case of blood stains haemoglobin, oxidation of luminol occurs. This oxidation reaction changes the oxidation state of luminol, resulting in its electronic excitation and the emission of blue/green chemiluminescence (An, 2012, Webb et al., 2006, Johnston et al., 2008). Although the luminol test was a popular choice of presumptive test, it is not without its drawbacks. There are a number of interfering substances that can catalyse the chemiluminescence of luminol as effectively as haemoglobin, which lead to false positive results (Barni et al., 2007, Quickenden and Creamer, 2001, Creamer et al., 2003, Nilsson, 2006). These substances commonly occur in our everyday environment for example, disinfectants, soil, metal objects, plants, fruit and vegetables and domestic and industrial bleaches to name a few. The latter of these substances are highly problematic to forensic investigators when interpreting the results of a luminol test, as perpetrators have been known to use bleach to clean up a crime scene in an attempt to remove any traces of blood (Quickenden and Creamer, 2001). Grass stains are also commonly encountered at a crime scene.

### **1.3.3 Fluorescein and Bluestar Forensic**

Two other chemiluminescent tests available to forensic investigators for the presumptive identification of blood are the fluorescein method and Bluestar® Forensic (Barni et al., 2007). The latter method is a luminol-based formulation, which was developed in an attempt to improve on traditional luminol spray (Barni et al., 2007). Both of these tests are similar to the luminol test in that they rely on the peroxidase-like activity of haemoglobin, for example, in the presence of a peroxidase-catalyst, oxidation of fluorescein to fluorescein occurs in hydrogen peroxide. Albeit as effective as the luminol test, fluorescein as a choice of presumptive test is still less favourable, the main reason for this being, stains that are sprayed with fluorescein require additional exposure to an ALS to be visualised, whereas

luminol does not (Garofano et al., 2006). Studies have also shown that both of these tests are not detrimental to potential DNA evidence (Barni et al., 2007, Garofano et al., 2006).

#### **1.3.4 Benzidine, ortho-toluidine and TMB test**

Another chemical test that relies on haemoglobin in blood is the benzidine test, which was originally one of the most widely used presumptive tests, when blood reacts with the ethanol/acetic acid solution, a deep blue precipitate is formed (Webb et al., 2006). Like the majority of presumptive tests, this test lacks specificity and produced false positives, and due to the carcinogenic properties of benzidine, this test was discontinued. Replacement of this test resulted in the development of a further two chemical based presumptive tests for blood, the first is the ortho-toluidine (*o*-toluidine) test and the second is the tetramethylbenzidine (TMB) test. Both of these reagents are derivatives of benzidine, however *o*-toluidine was also largely discontinued after the discovery that this chemical, like its parent molecule benzidine, had carcinogenic properties and the use of TMB became more popular. TMB (3,3', 5,5'-tertramethylbenzidine) is the main reagent used in the Hemastix® test, this presumptive test was originally a field test designed for the identification of blood in urine but has since been used to detect blood stains on surfaces. In comparison to other chemical tests, this method produces a higher number of false positive results and is therefore a less popular method of choice for forensic investigators (Virkler and Lednev, 2009).

#### **1.3.5 Takayama Test**

Blood identification is can also carried out by crystal tests, the most common test being the Takayama test. When a dried blood stain is heated in the presence of pyridine and glucose, formation of haemochromogen occurs, resulting in needle-like crystals. This test has

decreased sensitivity in comparison to other presumptive tests, but it considered to be confirmative for the presence of blood.

## **1.4 Identification of Semen**

In sexual offence cases, forensic investigators are often faced with the challenge of identifying and recovering biological material that will link a perpetrator to the complainant. The ability to definitively identify semen in such cases could provide crucial probative evidence when reconstructing the crime. The majority of sexual offence crimes are unwitnessed therefore this evidence type is often required to corroborate the alleged crime, which in turn could have a major impact on the outcome of a case.

### **1.4.1 Composition of Semen**

Normal semen is comprised of two major components; seminal fluid, which is a mixture of fluids secreted largely by the prostate gland, seminal vesicles and Cowper's gland, and spermatozoa - these are the cellular components of semen which contain the male's genetic information. The spermatozoa count in healthy males has been reported to range between  $10^7$  and  $10^8$  (spermatozoa/mL of semen), the average male ejaculate containing 3.5 mL of seminal material.(Mitchell, 2012, 2012) Abnormalities in this spermatozoa count can be observed in some males, for example, oligzoospermia, which is a condition where males have an abnormally low spermatozoa count (usually less than 20 million spermatozoa per mL of semen), or azoospermia, which is a medical condition characterised by zero spermatozoa in semen (Skinker et al., 1997). Azoospermia can also be observed in males following a vasectomy, which is a surgical operation to render a male sterile or infertile; this procedure prevents spermatozoa from reaching the distal portions of the male reproductive tract by

ligating and cutting the vas deferens. Vasectomised males and males with abnormal spermatozoa conditions are still able to secrete seminal fluid (Kobilinsky, 2012, Harvey, 2010).

Forensic identification of semen often begins with the visual examination of evidence utilising alternative light sources. Once a suspected stain has been detected and located, after appropriate preparation, presumptive assays are then employed to indicate the presence of semen; these assays are based on the detection of enzymes present in seminal fluid. The presence of semen is then confirmed by performing confirmatory assays, these include the microscopic examination of spermatozoa and immunochromatographic assays that utilise antibody-detecting antigens present in seminal fluid.

There are a number of presumptive screening tests available to investigate the possible presence of semen. The most popular is the presumptive test for seminal acid phosphatase (SAP), which has been used by investigators for a number of years (Kind, 1957, Virkler and Lednev, 2009)

#### **1.4.2 Seminal Acid Phosphatase Test**

The SAP test is reliant on the reported high concentrations of the water-soluble enzyme acid-phosphatase in seminal fluid (Lewis et al., 2013, Redhead and Brown, 2013). There are two types of sample collection methods for the AP test that forensic investigators will employ when detecting semen, depending on the type of stain encountered; these are direct and indirect methods. The former direct method involves an intimate swab being moistened with water and rolled or transferred onto filter paper, or an extract of a possible semen stain will be dropped and transferred onto a filter paper. The latter indirect method is used for detection of possible semen stains on larger items or surface areas, where filter paper

is placed over the area/item, moistened with water and pressed firmly to transfer semen onto the filter paper. In both cases, a chemical reagent is applied to the filter paper, there are a number of variations in the chemical reagents used (Virkler and Lednev, 2009), however a more commonly used reagent consists of; sodium acetate, acetic acid,  $\alpha$ -naphthyl phosphate disodium salt and brentamine fast black K salt (Redhead and Brown, 2013, Lewis et al., 2013). If acid phosphatase is present, the organic phosphatase hydrolysis of  $\alpha$ -naphthyl to  $\alpha$ -naphthol will occur, which then reacts with the brentamine fast black K salt, the result is a colour change from orange to purple (Lewis et al., 2013, Redhead and Brown, 2013). Over the years it has been routine procedure to allocate two minutes for this reaction to occur, after which, if no reaction has occurred the result is reported as negative. This cut-off time however has recently been challenged in a study which reported two minutes was insufficient to detect the majority of stains. It reported that after 15 minutes, diluted stains using both the direct and indirect method could still be detected (Redhead and Brown, 2013).

Albeit the preferred choice of presumptive test for the detection of semen for investigators, the AP test is still not completely reliable; it produces false positives due to acid phosphatase being present in other biological materials such as vaginal material (Virkler and Lednev, 2009, Redhead and Brown, 2013). It has also been reported that a common multipurpose detergent can have inhibitory effects on AP tests (Vennemann et al., 2014). Seminal acid phosphatase levels also vary between males, therefore detectability is reliant on the concentration of AP in a suspected stain (Redhead and Brown, 2013). Due to the presumptive nature of the AP test, further confirmatory testing is required to confirm the presence of semen. For semen containing spermatozoa, this is done microscopically and for cases where azoospermic semen is suspected, tests such as the Choline, Laurell Rocket Electrophoresis and Prostate Specific Antigen (PSA) are usually employed (Davidson and Jalowiecki, 2012, Redhead and Brown, 2013).

### **1.4.3 Histological staining and Microscopic Identification of Spermatozoa**

When a presumptive test provides a positive result indicating the presence of semen, investigators will then perform further confirmatory tests. The most commonly used confirmatory test is the microscopic observation of spermatozoa cells (the cellular component of semen) following histological staining. Spermatozoa have three unique and distinct characteristics; a DNA containing head, a midpiece, and a tail (Mitchell, 2012). Prior to microscopic analysis, stains are swabbed or smeared onto microscopic slides and stained with one of a selection of dyes, to enable spermatozoa if present, to be visualised under the microscope.

### **1.4.4 Conventional staining techniques**

One traditionally used stain is the haematoxylin-eosin stain, which dyes the DNA heads in spermatozoa a purple colour. However, a more popular stain is the ‘Christmas Tree’ stain, reported to be more effective and reliable over haematoxylin-eosin, and was given its name due to its distinctive colour pattern of red and green (Allery et al., 2001, Davies and Wilson, 1974, Virkler and Lednev, 2009, Allard, 1997, Allard et al., 2007, Mitchell, 2012, Allery et al., 2003, Romero-Montoya et al., 2011). This stain is comprised of two reagents; picroindigocarmine and Nuclear Fast Red (also known as Kernechtrot). The picroindigocarmine reagent stains the neck and tail portions of spermatozoa a green/blue colour, and the Nuclear Fast Red reagent stains the DNA heads differentially, in that the head is stained a red colour, and the acrosomal caps (the tips of the heads) a pink colour. Other stains have been used and tested for the microscopic detection of spermatozoa, such as Papanicolaou, Baechi, May-Grunwald-Giemsa, Wright’s stain and acridine orange, these stains are however a less popular choice as they are less effective than the ‘Christmas Tree’ stain (Virkler and Lednev, 2009, De Moors et al., 2013).

#### **1.4.5 Disadvantages of microscopic analysis**

Although microscopic analysis of spermatozoa is a confirmatory test it has a number of disadvantages. Firstly, if the donor of the sperm is oligospermic i.e. the seminal stain is from a male with a low sperm count, or azoospermic, where the stain is from a male who produces no spermatozoa (either due to natural causes or a vasectomy) then little or no spermatozoa will be present, thus making a positive identification of semen difficult/impossible using microscopic analysis (Virkler and Lednev, 2009, Skinker et al., 1997, Miller et al., 2011). In such situations where false negative results are produced, i.e. when the presumptive test indicates the presence of semen, yet the microscopic analysis yields no detectable spermatozoa, further tests are required to determine the presence semen. One marker used for the identification of semen when no spermatozoa is present, is the glycoprotein P30, which is a prostate-specific antigen (PSA) secreted by the epithelial cells of the prostate gland, and is present in seminal fluid. It is produced independently from the generation of spermatozoa and can therefore be used for both spermic and azoospermic samples. The most common PSA test is the ABACard® p30 test (Hadi et al., 2010, Boward and Wilson, 2013).

Identification of semen may also be difficult in cases where there is little evidence and/or the quality is low (Wasserstrom et al., 2013). Spermatozoa biologically degrades over time, the tails are susceptible to damage and usually degrade first, separating easily from the sperm heads after ejaculation. The surrounding environment and the condition of the stain are vital factors which affect the degradation of spermatozoa (Mitchell, 2012). Another disadvantage of microscopic analysis is the lack of specificity when a stain is contaminated, i.e. when there are high levels of epithelial cells, bacteria and cellular materials present. The staining methods mentioned above are not specific to human sperm cells, and therefore interpretation can be difficult and extremely time-consuming (Mitchell, 2012, Miller et al.,

2011). Examination of the smear slides is a non-automated procedure, which must be performed by an experienced and skilled forensic analyst who has the ability to distinguish between spermatozoa and other cell types (Miller et al., 2011, Christoffersen, 2011).

One study however, has recently attempted to address these time-consuming issues and demonstrated the use of an automated microscope/detection system called the KPICS SpermFinder™. The results of this study showed that the automated system, which had an algorithm designed to locate spermatozoa on a ‘Christmas tree’ stained microscope slide, located more spermatozoa than manual examination (Mitchell, 2012).

Recently, a new stain, the SPERM HY-LITER™ kit has been developed by researchers in an attempt to standardise and improve the efficiency of the microscopic screening of semen evidence. It is a fluorescent based assay that uses mouse monoclonal antibodies to specifically target human spermatozoa. In comparison to conventional staining methods, this assay is reported to be more robust, and reliable, producing fewer false positive and negative results. It also offers higher specificity and sensitivity, and is a much faster and simpler technique (Christoffersen, 2011, De Moors et al., 2011, De Moors et al., 2013, Miller et al., 2011, Silva et al., 2007)

## **1.5 Identification of Saliva**

The ability to definitively identify human saliva in forensic casework could provide investigators with important probative evidence when reconstructing a crime, especially in sexual assault cases which often involve oral activity and the deposition of saliva. This type of evidence is a potential source of DNA due to the epithelial cells present in saliva, and could aid in corroborating a victim’s allegation by demonstrating contact between the complainant and the perpetrator. For example, saliva on the skin, bite mark evidence or even



mixed stains of saliva and semen in the cases of oral intercourse (Breathnach and Moore, 2013, Auvdel, 1986, Soukos et al., 2000, Silva et al., 2007).

### **1.5.1 Composition of saliva**

Human saliva is a colourless fluid comprised of more than 99% water and <1% proteins and salt. It is produced and secreted into the oral cavity mostly from three major salivary glands; the parotid, the submandibular, and the sublingual glands. Saliva is required for the process of mastication and its primary function is to aid in the initial processes of digestion. A normal individual on average produces between 500ml and 1500ml of saliva a day, at a rate of approximately 0.5ml/minute (Old et al., 2009, Chicharro et al., 1998, Chiappin et al., 2007). Human salivary  $\alpha$ -amylase is the major protein component in human saliva which catalyses the initial step in the digestion of starch, a main source of carbohydrates in the diet (Pang and Cheung, 2008, Butterworth et al., 2011). Amylase in humans is expressed by two separate genetic loci in humans on chromosome 1; AMY1, the salivary locus and AMY2, the pancreatic locus (Breathnach and Moore, 2013, Butterworth et al., 2011, Virkler and Lednev, 2009). There is an abundance of AMY1 salivary amylase in saliva, and lower concentrations in breast milk and perspiration. The AMY2 pancreatic amylase is found in urine, blood, semen, faeces and vaginal secretions (Breathnach and Moore, 2013, Virkler and Lednev, 2009, Myers and Adkins, 2008). In comparison to all other body fluids, saliva has been reported to have the highest concentration of  $\alpha$ -amylase (Breathnach and Moore, 2013, Kipps and Whitehead, 1975, Myers and Adkins, 2008). Because of this, presumptive tests for saliva were developed using amylase, however such tests remain preliminary, as even though saliva produces a high concentration of amylase it is still found in other biological fluids and can therefore not be confirmative (Nakanishi et al., 2011, Virkler and Lednev, 2009, Nakanishi et al., 2009).

### 1.5.2 Starch-iodine Test and Phadebas<sup>®</sup> Amylase Test

Most of the conventional methods for the identification of saliva rely on the detection of the activity of salivary enzyme  $\alpha$ -amylase (Akutsu et al., 2010, Auvdel, 1986, David and Judy, 2010, Feia and Novroski, 2013, Hedman et al., 2011, Keating and Higgs, 1994, Kipps and Whitehead, 1975, Martin et al., 2006, Myers and Adkins, 2008, Olsén et al., 2011, Old et al., 2009, Virkler and Lednev, 2009, Breathnach and Moore, 2013, Butterworth et al., 2011, Pang and Cheung, 2008). This type of presumptive test has been used for over thirty years (Hedman et al., 2011). The  $\alpha$ -amylase enzyme hydrolyses  $\alpha$ -1-4 glycosidic linkages in polysaccharides such as starch. The most common presumptive tests for the detection of amylase in saliva are the starch-iodine assay or the Phadebas<sup>®</sup> amylase test. In the starch-iodine test, a sample is incubated in starch solution to initiate  $\alpha$ -amylase activity. This is then followed by the addition of iodine, where starch in the presence of iodine gives a characteristic deep blue colour. In the presence of amylase, starch will break down and a positive result for amylase is indicated by the disappearance of the blue colour. The Phadebas<sup>®</sup> reagent is one of the most common commercial products, it utilises a water insoluble starch that is covalently linked to a blue dye. A positive result is based upon the  $\alpha$ -amylase activity in the suspected saliva sample hydrolysing the starch-blue dye bonds which lead to a release of blue dye into solution that can be quantitated (Myers and Adkins, 2008). In addition to the tube test a Phadebas<sup>®</sup> Forensic Press Test has also been developed to locate saliva stains on surfaces, in such tests the reagent is applied to filter paper which is then applied to the region of interest (Olsén et al., 2011).

One of the main disadvantages of these activity-based detection methods is these tests cannot identify between the two types of  $\alpha$ -amylase or between the many nonhuman sources of this enzyme i.e. bacterial, pancreatic, fungal or nonhuman saliva (Old et al., 2009), they also lack specificity and sensitivity.

### **1.5.3 RSID-Saliva and SALIgAE-Saliva**

Recently, the RSID-Saliva immunochromatographic test and a colourimetric test called SALIgAE-saliva have been developed and both demonstrated higher specificity and sensitivity over the Phadebas<sup>®</sup> test (Akutsu et al., 2010, Old et al., 2009, David and Judy, 2010).

## **1.6 Alternative Light Sources**

One of the simplest identification methods for blood, semen and saliva stains at a crime scene is the alternative light source, (ALS). This method is a presumptive screening test often used to detect and locate stains at a crime scene prior to performing further presumptive chemical analyses. This preliminary test is ideal for investigating large forensic areas for stains that are not visible to the naked eye, providing a more specific location for collection, rather than testing large areas unnecessarily. Once a stain has been located using this simple and non-destructive method, photographs of the enhanced stain can be taken and further tests are then usually performed to determine the presence of a body fluid.

### **1.6.1 Blood**

The most common light source used for the detection of blood is the Polilight<sup>®</sup> which produces intense bands of light at wavelengths between 310 and 650 nm (Vandenberg and Oorschot, 2006), blood is said to have a strong absorption band around ~ 415 nm (Stoilovic, 1991). The main disadvantages of this test however, are the lack in specificity and sensitivity, one study showed the maximum detectable dilution of blood stains using a Polilight<sup>®</sup> was 1/1000, in comparison to a chemical based method such as luminol which

showed a higher degree of sensitivity upto 1/5000000 (Webb et al., 2006, Vandenberg and Oorschot, 2006, Jackson and Hadi, 2007).

### **1.6.2 Semen**

There are a number of commercial light sources available to forensic investigators, these include the Woods Lamp, Bluemaxx™ BM500 and the Polilight® (Virkler and Lednev, 2009, Vandenberg and Oorschot, 2006, Stoilovic, 1991)

The Wood's Lamp (WL) emits light in wavelengths between 320 and 400nm (Gupta and Singhi, 2004), this light source has been used over a number of years for the detection of semen at crime scenes, however studies have since reported on its lack of sensitivity and specificity (Santucci et al., 1999). The WL is unable to differentiate between semen and other commonly found substances producing false positive results, and it has recently been reported that at 360 nm wavelength semen does not fluoresce using the WL (Nelson and Santucci, 2002, Virkler and Lednev, 2009). The Bluemaxx™ BM500 is another commercial light source that emits light in wavelengths between 390 and 500 nm and offers greater sensitivity and specificity over the WL (Virkler and Lednev, 2009). Another common yet more expensive light source is the Polilight®, which emits light in wavelengths between 415 and 650 nm, this light source offers greater specificity over other light sources, as it emits wavelengths in a narrow band (Wawryk and Odell, 2005). One study reported that the sensitivity of the Polilight® was comparable to the chemical presumptive test acid phosphatase test (AP) (Vandenberg and Oorschot, 2006). One of the main drawbacks to this light source is its lack of portability due to its size, making field testing more difficult.(Wawryk and Odell, 2005).

### **1.6.3 Saliva**

Alternative light sources are also used to detect saliva stains, when viewed under UV light, saliva appears a light blue colour. Commercial light source Polilight<sup>®</sup> has been reported for use in detection of saliva stains (Vandenberg and Oorschot, 2006)

## **1.7 Identification of Vaginal Material**

Vaginal material is not as commonly encountered at crime scenes like blood, saliva and semen, however when this evidence is available the ability to identify such stains could play an important role in sexual assault cases. Vaginal material can be found in the underwear of rape victims, penile swabs, condoms and other objects that may be used in a sexual assault. Vaginal secretions are a complex fluid and contain a large amount of epithelial cells, very similar to buccal cells. The ability to distinguish between buccal cells and vaginal epithelial cells could provide important probative evidence. For example a perpetrator could claim buccal cells are present due to oral activity i.e. drinking from a bottle; whereas the victim's allegation could differ dramatically claiming the buccal cells present on the bottle are a result from penetration in a sexual assault.

### **1.7.1 Lugol's Iodine Method**

There are currently no reliable tests for the identification of vaginal material; one proposed test was the Lugol's Iodine Test, which was based on the detection of glycogen in vaginal material (Hausmann et al., 1994, Rothwell and Harvey, 1978). This method proved to be highly susceptible to false positives and lacked specificity for vaginal material, and as such was reported too unreliable for use in forensic casework.

## **1.8 Sensitivity and Specificity of current presumptive tests**

In the current literature, differing sensitivities and specificities are reported for the various presumptive blood tests. A recent study by (Vennemann et al., 2014) investigated both the KM and LMG test, and reported that the KM test was higher in both sensitivity and specificity compared to LMG. Dilution factors in this study ranged from  $10^{-1}$  to  $10^{-7}$ , both tests showed a positive result for each dilution factor. However, 100 % positive results, which in this study was 57/57 tests, were only reported up to  $10^{-2}$  for LMG and up to  $10^{-3}$  for KM, this percentage decreased for each test with each further dilution.

In the same study, the influence of ascorbic acid, hypochlorite and ferrous sulphate on both the KM and LMG tests were investigated. The addition of ascorbic acid, which is an anti-oxidizing reagent commonly found in fruits, vegetables or juices, caused both presumptive blood tests to fail and as such, this reagent was reported as a strong inhibitor for both tests, with the LMG test being more affected over KM. The addition of hypochlorite also had an inhibitory effect on both tests and it was reported that the LMG test was more prone to false positive reactions compared with KM. Hypochlorite is a strong oxidizing reagent known to produce false positives in presumptive blood tests. It is a component found in many household cleaning agents and washing powders, and the effects of this reagent should therefore be considered very carefully when interpreting presumptive blood test results from swabs collected from bathroom or kitchen surfaces and from fabrics and clothes, all of which may have been subjected to hypochlorite ions. False positive results were also reported for both tests with the addition of 10% ferrous sulphate solution in the absence of blood, with a higher percentage of false positives observed in the KM test compared with LMG. Ferrous sulphate is a metal salt commonly found in sinks, showers and baths due to old eroding pipes, a false positive reaction for swabs taken in or around these areas could lead

to misinterpretation of the results and as such all or any presumptive blood test results would require careful consideration in a forensic investigation.

An earlier study by (Tobe et al., 2007) also investigated the sensitivity and specificity of six presumptive tests for blood; three of which were the most commonly employed tests by police and forensic scientists throughout the world; luminol, KM, and LMG, and three newer tests/reagents; Hemastix<sup>®</sup>, Hemident<sup>™</sup> and Bluestar<sup>®</sup>. The authors of this particular study had written about the contradictory findings reported in previous literature on the sensitivity of the luminol, KM and LMG tests, where luminol dilution factors ranged from  $5.0 \times 10^{-3}$  to  $10^{-8}$ , LMG from  $5.0 \times 10^{-3}$  to  $10^{-5}$  and KM from  $5.0 \times 10^{-4}$  to  $10^{-7}$ , and as a result based their study on testing and determining the sensitivity and specificity limits of newer tests/reagents in comparison to the older traditional tests. The specificity was investigated and compared by subjecting each new and old test to substances that are commonly known to interfere with the traditional reagents, or that are often misconstrued in blood spatter analysis (Table 1) and the sensitivity was investigated by subjecting each test to five different dilution factors (Table 2). The findings showed that the LMG test had a sensitivity of  $10^{-4}$ , whereas the remaining tests were able to detect blood to a dilution factor of  $10^{-5}$ , these results contradicted those reported by (Vennemann et al., 2014), where blood was detected at a dilution factor of  $10^{-7}$  for both the LMG and KM tests. With regards to the specificity of the six presumptive blood tests, each test produced a false positive for more than one common household substance. Luminol was reported to be the most specific test, however the author explained that this was a contradictory result compared with other previous literatures, and reported that the possible reason for this was most probably due to the substances drying time of 18hr minimum before testing. The high numbers of false positives reported in this study demonstrates that results from presumptive blood tests can be easily misinterpreted due to common household items.

Table 1: Substances investigated for false positive reactions in six presumptive blood tests, N represents no reaction and Y represents a positive reaction (Tobe et al., 2007)

Substance	Presumptive blood test					
	Luminol	LMG	KM	Hemastix	Hemident	Bluestar
Saliva	N	N	N	Y	N	N
Semen	N	N	Y	N	Y	N
Potato	N	Y	Y	Y	N	Y
Tomato	N	N	N	Y	N	Y
Tomato	N	N	Y	N	N	N
Sauce						
Tomato	N	N	N	Y	N	Y
Sauce with						
meat						
Red Onion	N	Y	Y	Y	Y	Y
Red Kidney	N	N	Y	N	N	Y
Bean						
Horseradish	N	N	Y	N	N	Y
0.1M	N	N	Y	N	Y	N
Ascorbic						
Acid						
5 % Bleach	N	N	Y	N	Y	Y
10% Cupric	Y	Y	Y	Y	Y	Y
Sulfate						
10% Ferric	Y	Y	Y	Y	Y	Y
Sulfate						
10% Nickel	Y	Y	Y	Y	Y	N
Chloride						



Table 2: The reported sensitivity of six presumptive tests for blood with dilution factors ranging from 10<sup>-4</sup> to 10<sup>-7</sup>, where N represents no reaction observed and Y represents a positive reaction (Tobe et al., 2007)

Test	Dilution Factor				
	1.E-04	1.E-05	1.E-06	2.E-07	1.E-07
Luminol	Y	Y	N	N	N
LMG	Y	N	N	N	N
KM	Y	Y	N	N	N
Hemastix	Y	Y	N	N	N
Hemident	Y	Y	N	N	N
Bluestar	Y	Y	N	N	N

Another recent study by Petersen et al (2014), investigated the specificity of phenolphthalein (the reagent used in the KM test) for false positive reactions from legume root nodules. The nodules of leguminous plants contain a protein called leghemoglobin, which has a similar function and structure to haemoglobin. The nodules of six common legume types including pea, bean, red clover, white clover, soyabean and alfalfa were all tested, along with five common garden fruits; strawberry, watermelon, tomato, blackberry and raspberry. Each fruit or legume nodule was crushed to create a reddish bloodstain like red-brown stain and subjected to the KM presumptive blood test. The common fruits or berries produced negative phenolphthalein reactions. The crushed nodules however from all tested legume plants yielded phenolphthalein false-positive reactions, all of which were reported to be indistinguishable from true bloodstains both in colour quality and in the time it took for the colour to develop. In this study, leguminous nodules were also crushed onto clothing and other material, air dried and then frozen. Each stain yielded a false-positive reaction for phenolphthalein after four years. The bloodlike staining caused by leghemoglobin-containing nodules when crushed, combined with the false-positive reactions for phenolphthalein, even several years after exposure on some materials or clothing, shows

there is potential to mislead investigators with regards to the origin of DNA profiles obtained from stained evidentiary items.

Vennemann et al, 2014 also studied the specificity of the acid phosphatase test. The study involved the analysis of a variety of teas for false positive reactions, camellia sinensis teas both the darjeeling and assamica (green and black) produced false positives for AP, as did two garden plants; c japonica camellia and erica heather. A common multipurpose detergent known as Teepol was also shown to be inhibitory to the AP test.

Another study that investigated the specificity and sensitivity of a direct and indirect (aerosol) acid phosphatase test was (Lewis et al, 2013), direct AP testing was reported to be of a higher sensitivity than the indirect, and each test subjected to a number of household products to check for specificity too, these ranged from green beans, broccoli, horseradish, laundry soap to lemon juice. In total there were 22 common household products listed. AP tested positive for a number of products in this study, the direct test showing quicker reactions to false positives than the indirect. The results from this study show that the AP test can like other presumptive tests provide a large number of false positives for many commonly found substances which can mislead investigators in casework.

For saliva, a study by (Casey and Price, 2010) investigated the sensitivity of the RSID™- saliva test and the Phadebas test, the results showed that the RSID test had a greater sensitivity than that of the Phadebas, it also has no cross over with other human forensically relevant body fluids. The Phadebas test has been reported to have a sensitivity of 1:100 when the sample was incubated at room temperature and 1:200 at 37°C (Hedman et al, 2008)

Real-time PCR in comparison to these conventional methods is a much more sensitive technique with sensitivities down to pg measurements, the limit of detection being the defined as the lowest amount of target that was amplified, real-time PCR offers much more sensitivity

given that it is able to analyse amplification in real time. If mRNAs are present and detected at such low values this offers much greater sensitivity over current methodologies.

### **1.8 mRNA body fluid identification methods**

Based on current the methodologies, it is clear there is a requirement for a more definitive body fluid identification (BFID) test. Conventional methods for all their advantages are prone to quite a number of false positive reactions, which could potentially mislead a forensic investigator when dealing with evidentiary items, they are also performed separately to DNA analysis, which can be problematic when there is limited sample available for the investigator to use – small stains sizes are common in forensic casework. An ideal BFID test therefore, would be one that identifies the origin of a DNA profile in the same assay, for example, a genetic test that will coincide with DNA profiling. Messenger RNA could be play a vital role in such a genetic test. The central dogma states that genetic expression starts with the DNA, which is transcribed into mRNA and subsequently translated into proteins. Proteins are present in all biological material, some of which will be specific to a particular body fluid and as such it is these proteins that will enable us to specifically identify body fluid types. Isolation and thus identification of the specific mRNAs that are responsible for the production of a particular protein in certain body fluids could play a vital role in the development of a mRNA body fluid identification test for use in forensic casework.

In recent years, RNA profiling has been adopted and proposed as a supplement to conventional BFID tests. RNA was once thought to be too unstable to use in forensic analysis, hence the major advances in DNA analysis over the past few decades. A number of recent studies however have contradicted the inherent instability of RNA, showing that in

actual fact RNA isn't quite as unstable as what was originally thought (Vennemann and Koppelkamm, 2010, Kohlmeier and Schneider, 2012, Juusola and Ballantyne, 2003, Setzer et al., 2008). This breakthrough has encouraged researchers worldwide to delve in to the world of RNA and explore its possible uses in the identification of body fluids. The RNA profiling methods employed in the literature over the past decade are based on the analysis of forensically relevant tissue-specific genes, and at the start of this study, the majority of methods focused on messenger RNA (mRNA) gene expression analysis.

One study by (Juusola and Ballantyne, 2003) reported the basis of a prototype RNA based assay that could later be developed as a supplement to conventional BFID methods. In this study, mRNA from housekeeping genes S15,  $\beta$ -actin and GAPDH was detected in blood semen and saliva stains. This method employed the use of reverse transcriptase-polymerase chain reaction (RT-PCR). A number of potential saliva-specific genes were also reported to be detected in this study, these included statherin (STATH), histatin 3 (HTN3) and PRB1, PRB2 and PRB3, mRNA from each of these genes were detectable in saliva but not blood or semen.

Another study around that time by (Bauer and Patzelt, 2003a) proposed a method for simultaneous RNA and DNA isolation from dried blood and semen stains. It is understood that DNA analysis takes precedence in routine forensic laboratory work when dealing with a limited sample amount, and Bauer and Patzelt therefore suggested a technique that would isolate RNA and DNA from the same sample, thus being able to identify to origin of the DNA profile. Bauer reported that in previous literature tissue-specific mRNAs had been used to determine the origin of forensic stains by reverse transcription polymerase chain reaction (RT-PCR) and as such used mRNA in this study (Bauer and Patzelt, 2003b, Bauer and Patzelt, 2002). The results demonstrated a co-isolation technique was possible when the sample size does not allow separate RNA and DNA isolation, and they were able to analyse

both the tissue-specific gene expression and the genetic profile of the same donor. However, a decreased sensitivity was observed for this co-isolation method compared with direct DNA isolation. Therefore in cases where maximum sensitivity was required i.e. due to a very small amount of sample or the age of a stain, direct DNA isolation should be the method of choice to achieve the best possible results. A number of mRNA and DNA co-isolation techniques have been described over recent years, (Alvarez et al., 2004, Haas et al., 2011c, Haas et al., 2014, Haas et al., 2013, Watanabe et al., 2014). Alvarez reported an optimised method, which included a number of body fluids, including blood, saliva, semen, and semen free vaginal secretions. This was again done using RT-PCR and the results were analysed using gel electrophoresis, this optimised method showed greater sensitivity and robustness. (Bowden et al., 2011) describes another mRNA and DNA co-extraction method using the Promega DNA IQ™ system. Semen, blood, saliva, vaginal fluid and menstrual blood were analysed in this study utilising the following mRNA specific genes; blood GlucoA, menstrual blood MMP11, saliva HTN3 and STATH, semen PRM2 and TGM4 and vaginal fluids CRIS and GASS. Two successful methods were demonstrated in this study, Bowden et al developed a method that recovered mRNA from a previously discarded by-product of the DNA IQ™ reaction and potential mRNA profiles were developed from all casework samples extracted using both a manual and automated DNA IQ™ method. There was no impact on the DNA extraction process using the DNA IQ™ as the DNA and RNA were separated into two components during the extraction process.

From 2011 to 2014 the European DNA profiling group EDNAP performed a number of collaborative exercises on mRNA profiling for the identification of body fluids all of which employed reverse transcription, endpoint PCR and capillary electrophoresis and in the later studies, multiplexing was also employed. The first study (Haas et al., 2011a) involved the detection of blood-specific genes HBB, SPTB and PBGD in blood stains. The results

showed that HBB was the most abundantly expressed gene followed by SPTB and PBGD. All but one out of 16 laboratories were able to isolate and detect RNA in dried bloodstains. This study, due to its high reproducibility and sensitivity, demonstrated the potential of mRNA profiling in forensic casework with regards to supplementing or even being an alternative to current serological methods. The subsequent EDNAP exercises were all based on the co-isolation of RNA and DNA; in 2012 a second collaboration reported a reproducible co-isolation of RNA and DNA in bloodstains (Haas et al., 2012), this was followed by a third study in 2013 which involved the evaluation of mRNA markers for saliva and semen (Haas et al., 2013) and subsequent to this in 2014, a study investigating dried menstrual blood and vaginal secretions was reported (Haas et al., 2014). Each of these studies gave a positive outcome with regards to supporting RNA profiling as a reliable body fluid identification method and one that can be easily combined with STR typing technology.

The EDNAP exercises along with a large number of other studies are the most recent developments in RNA profiling, at the time of this study there were a number of studies in the literature on mRNA profiling. Various body fluids were reported to be investigated including, blood semen, saliva, vaginal mucosa, semen, and menstrual blood. In 2005, a multiplex mRNA profiling study by (Juusola and Ballantyne, 2005) investigated a number of tissue-specific markers for various body fluids, including HTN3 and STATH (saliva) and SPTB and PBGD (blood), PRM1 and PRM2 (semen) HBD-1 and MUC4 for vaginal secretions. This method used again involved RT-PCR (end-point PCR) and capillary electrophoresis, In subsequent years a number of multiplexing/singleplex RT-PCR using end point PCR methods have also been described (Haas et al., 2008, Cossu et al., 2009, Haas et al., 2009b, Richard et al., 2012, Fox et al., 2014, Roeder and Haas, 2013, Lindenbergh et al., 2013). In 2008 and 2009, a whole genome amplification technique was described by (Zubakov et al., 2008, Zubakov et al., 2009) used to identify stable RNA markers for the

identification of blood and saliva, these included saliva-specific markers SPRR1A, KRT4 and KRT13 and NCF2 markers for blood. In 2013, a multiplex high resolution melting mRNA profiling assay was also described (Hanson and Ballantyne, 2013b).

In 2006, (Nussbaumer et al., 2006) reported a mRNA profiling method that employed real-time PCR, the body fluids tested were blood, semen, vaginal fluid and saliva and Taqman probes were employed. This paper demonstrated the usefulness of real-time PCR assays for identifying the origin of a biological stains, due to its stability and sensitivity; RNA profiling of various body fluids were shown to be possible, even on samples stored at ambient temperatures for a long period of time. Another study that year also employed the used of real-time PCR (Fang et al., 2006), again Taqman gene expression assays were used and specific assays targeting mRNA in saliva, semen, vaginal secretions and blood were identified. A number of studies subsequent to this reported the use of multiplexing and real-time PCR utilising Taqman probes (Ballantyne and Juusola, 2007b, Patel and Peel, 2008, Fleming and Harbison, 2009, Sakurada et al., 2009, Haas et al., 2009a, Lindenberg et al., 2012, Park et al., 2013, Hanson and Ballantyne, 2013b, Xu et al., 2014).

Since the start and throughout this study there has been a vast increase in the level of interest and the amount of research undertaken on the identification of body fluids utilising mRNA. From 2011 onwards there were a vast number of papers published investigating forensically relevant body fluids, with the identification of skin becoming a popular area of research in BFI studies (Sakurada et al., 2011a, Gomes et al., 2011, Hanson et al., 2011, Visser et al., 2011, Parker et al., 2011, Hadžić et al., 2011, Wobst et al., 2011, Haas et al., 2011b, Hanson et al., 2012, van den Berge et al., 2014, Gomes et al., 2013). One particular study also investigated the identification of nasal blood and compared the expression levels of target genes specific to saliva, nasal secretions and blood; including menstrual blood (Sakurada et al., 2012), nasal blood is suggested to be one of the most difficult types of blood

to identify. The results of this study showed that real-time PCR was used and able to detect nasal blood, however the stability of gene expression in nasal blood stains decreased over time, a disadvantage for older evidentiary stains in forensic casework.

In 2013 and 2014, there were a number of studies which focused on vaginal material and menstrual blood (Hanson and Ballantyne, 2013a, Jakubowska et al., 2014, Jakubowska et al., 2013), and the most recent study to-date in the world of mRNA profiling is a study by (Akutsu et al., 2015) which investigates the suitability of two common extraction methods for RNA profiling; silica column-based membranes and automated magnetic bead extraction, this study was done using RT-PCR. The results of this study suggest an automated system could be more effective over manual extraction, the sensitivity was reported to higher in the automated method.

Based on previous literature, it can be seen that real-time PCR is a very sensitive technique for the detection and quantification of nucleic acids and a vital tool in the development and progression of mRNA profiling in forensic casework, many research groups have employed the use of this method. However, at the time of this study, the majority of research being undertaken involved RT-PCR and analysis of the products either via capillary or gel electrophoresis. Real-time PCR studies were also underway around that time in a number of research groups, however these studies all employed Taqman chemistry. Assays utilising TaqMan chemistry are considered a costly method of analysis, an ideal mRNA BFID test that can be used in practice by forensic scientists would ideally be cost-effective to warrant the use of such a test in forensic casework. With this in mind, SYBR Green chemistry, an alternative to Taqman was considered.



## 1.9 Aims

Previous literature has outlined a requirement for a more definitive body fluid test, researchers worldwide are investigating the expression of mRNA specific genes in body fluids so that the origin of a DNA stain can be identified in forensic casework. At the time of this study, it was noted that researchers employing real-time PCR for mRNA profiling were utilising Taqman chemistry.

Taqman assays are the preferred choice of assay by many researchers in previous literature, most probably due to their specific nature and complex design. However, these type of assays are very costly and it was suggested that, to establish an mRNA profiling test that can be routinely used in forensic casework, a more cost effective test may be required.

The purpose of this study therefore was to find an alternative to Taqman assays to minimise the cost of an mRNA BFID. This was done by utilising real-time PCR with SYBR Green chemistry and unlabelled PCR primers. SYBR green lacks specificity compared to Taqman, it will bind to any double stranded DNA, however it was thought that it may be possible to validate such a chemistry and utilise melting curve analysis to positively identify mRNA specific genes in body fluids.

A number of tissue-specific genes for blood and saliva were investigated in this study, they were adopted from previous literatures. At the time of this study (Zubakov et al., 2008, Zubakov et al., 2009) reported the identification of mRNA specific genes in blood and saliva stains up to 16 years and 6 years respectively. These reports therefore proposed the use of such markers in forensic casework due to their reported stability and sensitivity. As such, saliva-specific markers SPRR1A, KRT4 and KRT13 were used in this study, along with the blood specific marker NCF2. Blood-specific markers SPTB and PBGD along with saliva

specific markers STATH and HTN3 were also used in this study, all these markers having been reported in a number of forensic mRNA analysis studies and showed good specificity in each study.. (Kohlmeier and Schneider, 2011, Haas et al., 2011c, Ballantyne and Juusola, 2007a, Juusola and Ballantyne, 2005, Sakurada et al., 2009, Richard et al., 2012, Juusola and Ballantyne, 2003, Haas et al., 2008, Fleming and Harbison, 2009, Sakurada et al., 2011b). Table 3 shows a list of the mRNA specific genes investigated in this study and their description.

Table 3: Body fluid specific markers; symbol, name and description

Body Fluid	Gene	Gene Name	Description
Blood	NCF2	Neutrophil Cytosolic Factor 2	Encodes p67(phox), an essential component of the multi-protein NADPH oxidase complex in phagocytic leukocytes (Gauss et al., 2006, Zubakov et al., 2009, Zubakov et al., 2008)
	SPTB	Beta-spectrin	A subunit of the major protein component of the erythrocyte membrane skeleton (Chu et al., 1994, Amin et al., 1993)
	PBDG	Porphobilinogen deaminase	An erythrocyte-specific isoenzyme of the heme biosynthesis pathway (Gubin and Miller, 2001)
Saliva	KRT4	Keratin 4	Type II cytokeratin expressed in differentiated layers of the mucosal and esophageal epithelia (Viaene and Baert, 1994, Zubakov et al., 2009, Zubakov et al., 2008)
	HTN3	Histatin 3	Histidine-rich protein involved in the nonimmune host defence in the oral cavity (Sabatini et al., 1993)
	KRT13	Keratin 13	Type I cytokeratin expressed in the differentiated layers of the mucosal and esophageal epithelia – pairs with KRT4 protein (Viaene and Baert, 1994, Zubakov et al., 2009, Zubakov et al., 2008)
	STATH	Statherin	Inhibitor of the precipitation of calcium phosphate salts in the oral cavity (Sabatini et al., 1990)
	SPRR1A	Small proline-rich protein 1A	Proline-rich salivary protein (Zubakov et al., 2009, Zubakov et al., 2008)
Reference gene	18s rRNA		Ribosomal gene family, the structural RNA for the small ribosomal subunit in eukaryotic cytoplasmic ribosomes

## **Chapter 2**

### **Materials and Methods**

## 2.1 Materials

Absolute ethanol solution, ethylene oxide sterile wooden swabs with cotton tips supplied in a tube and sterile disposable scalpels were purchased from Fisher Scientific UK Ltd, Loughborough, UK. Unistik<sup>®</sup> 3 Comfort lancet needles were from The Medical Shop, Owen Mumford Ltd, Oxfordshire, UK. Silverline disposable facemasks were from Toolbay Direct Ltd, Yorkshire, UK. Disposable nitrile exam gloves were from TopMedic. MicroSol 3+ Decontaminant Spray was purchased from Anachem Ltd, Bedfordshire, UK. All blood and saliva samples were donated by myself and/or other fellow colleagues working in the Forensic Genetics Laboratory, School of Applied Sciences Department, University of Huddersfield, UK. TURBO DNA-*free*<sup>™</sup> Kit, DEPC-Treated Water, RNase-free Microfuge Tubes, PCR tubes and RNaseZap<sup>®</sup> RNase Decontamination Solution were purchased from Ambion<sup>®</sup> Invitrogen<sup>™</sup> Life Technologies Ltd, Paisley, UK. Eppendorf<sup>™</sup> epReference<sup>™</sup> Pipettors and epDualfilter T.I.P.S<sup>®</sup> were purchased from VWR International Ltd, Leicestershire, UK. The RNeasy<sup>®</sup> Mini Kit was from Qiagen, Crawley, UK. BioPerformance Certified sterile-filtered water was purchased from Sigma-Aldrich Company Ltd, Dorset, UK. The M-MLV Reverse Transcriptase and 10X First Strand Synthesis Buffer, Random Hexamers and RNase Inhibitor (Cloned) 40 U/μl were all purchased from Ambion<sup>®</sup> Invitrogen<sup>™</sup> Life Technologies, Paisley, UK. The 10 mM dNTP mix was from Promega UK, Southampton, UK. All unlabelled PCR primers were purchased from Eurofins MWG Operon, Ebersberg, Germany. Fast SYBR<sup>®</sup> Green Master Mix, 2x TaqMan<sup>®</sup> Fast Universal PCR Master Mix - no AmpErase<sup>®</sup> UNG, TaqMan<sup>®</sup> Gene Expression Assays, Total RNA Control (Human), Eukaryotic 18s rRNA Endogenous Control (FAM<sup>™</sup>/MGB probe, non-primer limited) and MicroAmp<sup>®</sup> Fast Optical 96-Well Reaction Plate 0.1 mL were all purchased from Applied Biosystems<sup>®</sup> Life Technologies, Paisley, UK.

## **2.2 Sample Collection**

Blood and saliva samples were collected from two healthy volunteers (male and female) under informed consent before their inclusion in this study. The number of volunteers was low due to the time constraints of the project with regards to seeking the correct approval to expand the sample set. One individual provided two samples; a blood and saliva, two individuals were assigned on one plate depending on the number of markers being tested, and each sample was replicated three times.

All samples were collected in a separate room to the laboratory. Fresh blood was obtained using lancet needles to prick the finger of the individual (the needles gauge were 28G and the average blood volume for this type of needle was 5 $\mu$ l – 30 $\mu$ l), the blood was then spotted onto sterile filter paper to produce a blood stain. After staining, the filter paper was then sealed in an air tight bag in preparation for RNA extraction. Fresh saliva samples were collected in the form of sterile buccal swabs; this involved the volunteer swabbing the inside of their cheek several times to collect epithelial cells onto the cotton swab, the swab was then immediately placed back inside the supplied tube. All samples were extracted immediately after collection.

## **2.3 Sample Preparation**

All working surfaces and instruments/equipment were wiped clean with Microsol decontamination solution and then sprayed with RNaseZap, to prevent/limit the possibility of any contamination and to destroy any RNases present in the laboratory. Note deep cleans were performed on a weekly basis in the laboratory. As standard procedure, gowning up in a separate room prior to entering the laboratory took place, again to limit any contamination;

facemasks, gloves and howie lab coats were worn each time, gloves and masks were replaced out of the laboratory regularly and when necessary.

Saliva samples were prepared by removing the buccal swab from its tube and using a scalpel to remove the cotton tip, the saliva stained tip was then placed in a 1.5 ml microfuge tube and the lid sealed immediately. The scalpel, wooden shaft and tube were then disposed of immediately. Blood samples were prepared by cutting with a scalpel, three equal size small squares of filter paper stained with blood and placing them in a 1.5 ml microfuge; the lid was sealed immediately. The scalpel and filter paper were disposed of immediately. Note one scalpel was used per sample to prevent cross-contamination between samples and the 2ml microfuge tubes were supplied with the RNA isolation kit.

## **2.4 RNA Isolation**

The RNeasy<sup>®</sup> Mini Kit (Qiagen, Crawley, UK) was used to isolate total RNA from all samples. All solutions were made up to the manufacturer's specification and all consumables were supplied with the kit, with the exception of absolute ethanol, which was sourced from Fisher Scientific Ltd, Loughborough, UK.

Due to the forensic nature of the samples in this study, i.e. small sample sizes thus a limited number of cells, the protocol: *Purification of Total RNA from Animal Cells using Spin Technology*, was modified according to a method described by Zubakov *et al.* (2008), which involved a 1 hour incubation period at 4°C in RLT buffer solution prior to extraction. It was important to mimic the low amount of sample that could be realistically recovered from a crime scene, in order to establish if experiments could be accomplished and results obtained from such a low sample size.

All samples were added to the 1.5 ml microfuge tubes (as previously mentioned), then according to the modifications described by Zubakov *et al.* (2008), 350 µl of RLT buffer was added to each sample, and these were then briefly vortexed and incubated at 4°C for one hour. After this incubation period, the samples were removed to the bench top and the protocol was then followed from step 4. Note that when the samples were removed to the bench top, it was observed that the cotton tips had absorbed and retained a large amount of RLT buffer solution, resulting in a lower amount of lysate in the collection tube compared to the blood samples. This was overcome by pressing the cotton tips with the pipette to aid the release of more lysate from the swab into the tube. 350 µl of 70% ethanol was added to each sample, the ethanol solution was prepared parallel to each experiment using absolute ethanol (Fisher Scientific, Loughborough, UK) and nuclease-free water (Sigma-Aldrich Company Ltd, Dorset, UK), each sample was then mixed well by pipetting. 700 µl of each sample was transferred to an RNeasy spin column placed in a 2 ml collection tube (supplied with the kit), the tubes were centrifuged at 10,000 x g for 15 s. After centrifugation, the flow-through solutions were carefully discarded and the collection tubes were then re-used in the next step. 700 µl of the washing buffer RW1 (supplied with the kit), was then added to each spin column, to wash the silica-membranes. The tubes were centrifuged at 10,000 x g for 15 s. After centrifugation, the flow-through solutions were discarded from the 2 ml collection tubes, and again the tubes were reused in the next step. 500 µl of washing buffer RPE (supplied with the kit), was then added to each spin column, again to wash the silica-membranes. The tubes were then centrifuged at 10,000 x g for 15 s. After centrifugation, the flow-through was carefully discarded again and the collection tubes were reused in the next step. Another 500 µl of washing buffer RPE was added to each spin column. The tubes were then centrifuged at 10,000 x g for 2 minutes; this long centrifugation step dried the silica-membranes, ensuring there was no carryover of ethanol in the next step. The flow-through



solutions were carefully discarded again, and the 2 ml collection tubes were this time disposed of. The spin columns were then transferred to clean 2 ml tubes and centrifuged at full speed for 1 minute. This step was to eliminate any possible carryover of the washing buffer RPE, or any residual flow-through that remained on the outside of the spin columns after the last washing step. The collection tubes, including any flow-through were discarded and the spin columns were transferred to a clean 1.5 ml microfuge tube (supplied with the kit), and 50 µl of RNase-free water was then added directly to the centre of each spin column silica-membrane. The spin columns were centrifuged at 10,000 x g for 1 minute, the RNA eluted and the spin columns were discarded. A NanoVue™ Plus Spectrophotometer was used to determine the purity and concentration of the RNA samples (Section 2.5). All RNA samples were either used immediately or stored at -20°C.

## **2.5 DNase digestion of RNA extracts**

Each RNA extract was subjected to a DNase digestion treatment to remove any unwanted contaminating DNA using the TURBO DNA-free™ Kit (Ambion® Life Technologies Ltd, Paisley, UK). A 0.1 volume of 10X TURBO DNase Buffer and 1 µl of TURBO DNase was added to the RNA extract and mixed gently. The extract was then incubated at 37°C for 30 minutes on a heat block. After incubation, a 0.1 volume of resuspended DNase Inactivation Reagent was then added to the extract and mixed well. The extract was then incubated for 5 minutes at room temperature and mixed occasionally by flicking the tubes to redisperse the inactivation reagent. A low room temperature could affect the inactivation of the TURBO DNase, thus leaving residual DNase in the RNA extract. Therefore the room temperature was monitored throughout this incubation step to ensure the temperature did not fall below 22 – 26°C. After incubation, the RNA extract was then

centrifuged at 10,000 x g for 1.5 minutes, forming a pellet of DNase Inactivation Reagent. The supernatant containing the RNA was then carefully transferred into a fresh 0.5 ml microfuge tube. Careful measures were taken to ensure the inactivation reagent pellet was not mixed or transferred with the supernatant, as this reagent sequesters divalent cations and changes buffer conditions, and could thus affect any downstream enzymatic reactions. The DNase digested RNA extracts were either used immediately or stored at -20°C.

## **2.6 RNA Quantification/Purity**

The amount of RNA in a sample was quantified using the NanoVue™ Plus Spectrophotometer. The ultraviolet (UV) absorbance was measured at 260 nm ( $A_{260}$ ) and the concentration of RNA was calculated using the following formula:

$$\text{Concentration} = A_{260} * 40$$

The number 40 was the instruments default factor for RNA samples, due to a 40 µg/ml RNA solution of a typical synthetic Oligonucleotide having an optical density of 1.0 A in a 10 mm pathlength cell.

The NanoVue™ Spectrophotometer also gave an estimation of the purity of the RNA sample using the following absorbance ratio ( $A_{260}/A_{280}$ ). Proteins, which absorb light in the UV spectrum, accompany nucleic acids extracted from cells; extensive purification is required to remove any protein impurity. A pure RNA sample gives an absorbance ratio of  $\geq 2.0$ .

## **2.7 Reverse Transcription of RNA**

All RNA extracts were converted to complimentary DNA (cDNA) by reverse transcription for use in quantitative real-time PCR. A recombinant DNA polymerase;

Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase enzyme (Ambion<sup>®</sup> Life Technologies Ltd, Paisley, UK) was used to synthesise a complementary DNA strand from the primer hybridised single-stranded RNA template. 4 µl of RNA extract, 4 µl of random hexamers (Ambion<sup>®</sup> Life Technologies Ltd, Paisley, UK) and 4 µl of nuclease-free water (Sigma-Aldrich Company Ltd, Dorset, UK) were added to a 0.2 ml microfuge tube and vortexed, the tube was then briefly centrifuged and incubated on a 2720 Thermal Cycler (Applied Biosystems<sup>®</sup> Life Technologies, Paisley, UK) for 3 minutes at 75°C. Due to the inability to accurately quantify the RNA extract using the nanovue instrument described previously, it was decided that 4µl of RNA would be input every time so that at least a consistent amount was added to every reaction. The extraction techniques and sample collection were kept as accurate as possible with every run to help minimise any RNA concentration variances in the cDNA.

After incubation, the tube was removed to ice immediately, then centrifuged briefly again and then replaced back on the ice. Keeping the tube on ice, 1 µl of RNase Inhibitor (Cloned) 40 U/µl, 1 µl of M-MLV Reverse Transcriptase (Ambion<sup>®</sup> Life Technologies, Paisley, UK), 2 µl of 10X First Strand Synthesis Buffer (supplied with the M-MLV reverse transcriptase) and 4 µl of 2.5 mM dNTP mix (Promega UK, Southampton, UK), in that order, were added to the tube. Note the 10 mM dNTP was diluted using nuclease-free water (Sigma Aldrich Company Ltd, Dorset, UK). The tube was then gently vortexed and centrifuged briefly. After centrifugation, the tube was then incubated at 43°C for 1 hour, followed by 92°C for 10 minutes (to inactivate the M-MLV Reverse Transcriptase) on the 2720 Thermal Cycler. Reverse transcription negative controls followed the same protocol, except that the M-MLV reverse transcriptase was replaced with 1 µl of nuclease-free water. These negative

controls were used in quantitative real-time PCR to test for any DNA contamination. All samples were stored at -20°C until required.

## **2.8 Relative quantification real-time PCR**

### **2.8.1 SYBR<sup>®</sup> Green Chemistry**

Relative quantification (comparative  $\Delta\Delta C_t$  method) real-time PCR was performed to investigate the expression of specific mRNA markers in blood and saliva samples. Real-time PCR was performed on an Applied Biosystem<sup>®</sup> 7500 Fast Real-Time PCR System using the unlabelled gene-specific primers listed in Table 4 and Fast SYBR<sup>®</sup> Green Master Mix (Applied Biosystems<sup>®</sup> Life Technologies, Paisley, UK). The unlabelled gene-specific sequences were adopted from (Ballantyne and Juusola, 2007a, Juusola and Ballantyne, 2003, Haas et al., 2009a, Zubakov et al., 2008) Haas et al (ref) and Zubakov et al (Table 4)

Real-time PCR reactions were carried out at a final volume of 20  $\mu$ l with the following reaction mix: 2  $\mu$ l of cDNA sample/BPC grade water (negative controls), 1  $\mu$ l of forward and reverse primers, 10  $\mu$ l of Fast SYBR<sup>®</sup> Green Master Mix and 6  $\mu$ l of BPC grade water. The run method comprised of an activation step at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 30 s, 60°C for 45 s and 72°C for 30 s. This was followed immediately by a melt curve stage, where the temperature was increased to 95°C for 15 s, followed by 60°C for 1 min, the temperature was then increased again at a rate of 1°C to 95°C for 15 s, followed by 60°C for 15 s.

SYBR Green is an intercalating dye that fluoresces in its own right, in solution the unbound dye exhibits very little fluorescence. However, in the presence of double stranded

DNA (dsDNA), the dye intercalates with the DNA double helix (binds to the minor groove), altering the structure of the dye and thus significantly increasing its fluorescence. (Dragan et al, 2012) Therefore, in a PCR reaction, an increase in amplified DNA results in an increase of fluorescence. Unlike hydrolysis probes, intercalating dyes are non-specific i.e. they will bind to and report on any dsDNA that is formed during the PCR reaction regardless of what it is. Therefore, additional analysis, involving melting curve analysis and analysis of amplification plots is essential with this type of detection chemistry, to ensure correct interpretation of results.

*Table 4: Unlabelled gene-specific PCR primers used in real-time PCR; (F) forward primer, (R) reverse primer, reference gene. All PCR primers span at least one exon junction to prevent gDNA contamination.*

Body Fluid	Gene	Primer Sequence		Size (bp)
Blood	NCF2	F - attacctaggcaaggcgacg	R - tctgggtggaggctcagct	100
	SPTB	F – aggatggcttggcctttaat	R – actgccagcaccttcatt	247
	PBDG	F - tggatccctgaggaggcagaag	R - ctgtcccctgtggtggacatagcaat	177
Saliva	KRT4	F - aggaggtcaccatcaaccag	R - gctcaaggttttgcaggag	208
	HTN3	F – gcaaagagacatcatgggta	R - gccagtcaaacctccataatc	134
	KRT13	F – cagagcggtggaggctgacat	R - cctcctgttcagctctgcac	295
	STATH	F – ctctgtagtctcatcttg	R - tgggtgtgggtatagtgtgttc	198
	SPRR1A	F – tggccactggatactgaaca	R – cccaaatccatcctcaaatg	213
Reference gene	18s rRNA	F – ctcaacacgggaaacctcac	R - cgctccaccaactaagaacg	110

## 2.8.2 Melting Curve Analysis

Melting curve analysis is a post-amplification method used to determine the specificity of SYBR Green-based real-time PCR assays. The non-specific nature of the SYBR Green dye necessitates a melting curve stage, as a way of identifying and

distinguishing the PCR products in the reaction, i.e. confirming whether the products are specific amplicons, primer-dimers or other non-specific PCR products. The analysis is performed automatically as per a predefined program, immediately upon completion of amplification. A melt curve is generated by gradually heating the PCR products at a constant rate (1%) through a range of temperatures, ~ 60°C to 95°C. The fluorescence data is monitored and collected throughout the process. At the beginning of the melt curve stage the fluorescent signal is high as the DNA is double stranded and the SYBR Green dye is in its bound state. As the temperature increases and the dsDNA is denatured (melted) into ssDNA, the SYBR Green dye is released into solution, resulting in a decrease in fluorescence. Initially the fluorescence decreases slowly, however, when the melting temperature ( $T_m$ ) of the PCR product is reached, a sudden decrease in fluorescent signal is observed. The  $T_m$  is defined as 50% dissociation; where 50% of the product is dsDNA and 50% of the product is ssDNA (melted), and is dependent on the product length, the GC content (a higher  $T_m$  is observed in GC rich PCR products) and sequence content. The real-time PCR software presents the melt curve data as a negative first derivative of fluorescence with respect to temperature ( $-dF/dT$  vs.  $T$ ), this is the rate-of-change-of fluorescence rather than absolute fluorescence (raw data). Plotting the data in this way gives a clearer indication of the  $T_m$  as the melting curve is converted into a melting peak (Figure 2).

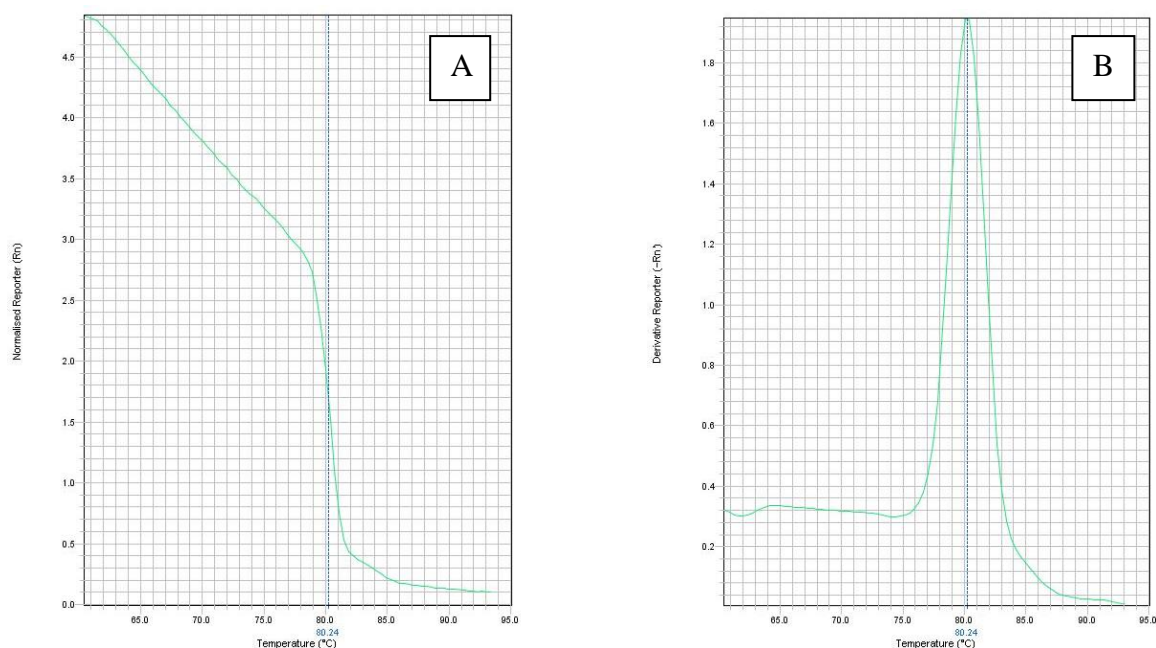


Figure 1: (A) Raw melt curve data, where the absolute fluorescence is plotted against the temperature. High fluorescence is observed at low temperatures as the DNA is double-stranded and the fluorophore is associated with the amplicon. As the temperature increases and the DNA dissociates into ssDNA, the fluorescent signal drops as the dye is released into solution. The vertical line represents the temperature at which 50% of the DNA has dissociated. (B) A negative first derivative melt curve plot, where the rate-of-change-of fluorescence ( $-dF/dT$ ) is plotted against temperature, this plot gives a clearer indication of the  $T_m$  of the PCR product; the inflection point on the melting curve is converted into a melting peak.

### 2.8.3 TaqMan® Chemistry

Following the SYBR Green based studies, relative quantification real-time PCR studies were performed to investigate the expression of specific mRNA markers in blood and saliva samples, this time utilising TaqMan chemistry. Real-time PCR was performed on an Applied Biosystem® 7500 Fast Real-Time PCR System using 2x TaqMan® Fast Universal PCR Master Mix- no AmpErase® UNG, Eukaryotic 18s rRNA Endogenous Control (FAM™/MGB probe, non-primer limited), and 20x TaqMan® Gene Expression Assays (Applied Biosystems® Life Technologies, Paisley, UK), listed in Table 5.

Real-time PCR reactions were carried out at a final volume of 20  $\mu$ l with the following reaction mix: 2  $\mu$ l of cDNA sample/BPC grade water (negative controls), 1  $\mu$ l of TaqMan® Gene Expression Assay/Eukaryotic 18s rRNA Endogenous Control, 10  $\mu$ l of

TaqMan<sup>®</sup> Fast Universal PCR Master Mix and 7 µl of BPC grade water. All assay probes were pre-designed and span an exon junction. The run method comprised of an activation step at 95°C for 20 s followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s.

*Table 5: All TaqMan<sup>®</sup> gene expression assays consisted of a pair of unlabelled PCR primers and a TaqMan<sup>®</sup> probe with a FAM<sup>™</sup> dye label on the 5' end, and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. The eukaryotic 18s rRNA endogenous control assay was also labelled FAM<sup>™</sup>/MGB.*

Body Fluid	Gene	Gene Name	Amplicon Length (bp)
Blood	NCF2	Neutrophil Cytosolic	106
		Factor 2	
Saliva	SPTB	Beta-spectrin	129
	KRT4	Keratin 4	61
	HTN3	Histatin 3	136
	KRT13	Keratin 13	75
	STATH	Statherin	90
Endogenous control	18s rRNA	Eukaryotic 18s ribosomal RNA	187

TaqMan<sup>®</sup> Gene Expression assays utilise hydrolysis probe-based detection chemistry. Just as in any PCR, TaqMan assays use an upstream and downstream primer with the addition of an internal probe that binds between the two primer-binding sites. The probes are fluorescently labelled sequence-specific oligonucleotides; covalently bonded to the 5' end of the probe is a fluorescent reporter signal (FAM<sup>™</sup>), and to the 3' end of the probe is a nonfluorescent quencher molecule. Probes differ to primers in that they are dual-labelled and lack a free hydroxyl group on the 3' end, thus preventing them from being extended by Taq polymerase. When a probe is intact, the reporter dye and quencher molecule are in close proximity, and as such the quencher greatly reduces the fluorescence emitted by the reporter molecule by Fluorescence Resonance Energy Transfer (FRET). A TaqMan probe will only



anneal between two primer sites if the target sequence is present, they are designed to anneal to one strand of the target sequence. Once annealed, the probe is then cleaved by the 5' exonuclease activity of Taq polymerase during primer extension, releasing the reporter molecule into solution and separating the reporter dye from the quencher. The probe is completely displaced as primer extension continues to the end of the template strand, and the permanent separation of the quencher and reporter molecule results in an increase reporter signal. Thus, with TaqMan probes, the level of fluorescence detected is directly proportional to the amount of amplified target in each PCR cycle. If no specific target sequence was present in the reaction, the fluorescent probe would be unable to hybridise, and as such the reporter molecule would remain quenched and no fluorescence would occur.

#### 2.8.4 Comparative $\Delta\Delta Ct$ Method

For all relative quantification studies, the  $2^{-\Delta\Delta Ct}$  method (Schmittgen and Livak 2001) was used to analyse the fold change of mRNA specific markers in blood and saliva samples. The 7500 Fast Real-Time PCR software v2.0.6 was used to calculate the threshold cycle ( $Ct$ ), which is defined as the number of PCR cycles required for the fluorescent signal to exceed background level. All genes of interest were normalised to the house keeping gene 18s rRNA, the results were expressed as a fold change ( $2^{-\Delta\Delta Ct}$ ) and were calculated as follows:

$$Ct_{GOI}^s - Ct_{norm}^s = \Delta Ct_{sample}$$

$$Ct_{GOI}^c - Ct_{norm}^c = \Delta Ct_{calibrator}$$

$$Ct_{sample} - Ct_{calibrator} = \Delta\Delta Ct$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Where, GOI represents gene of interest, norm represents normaliser/endogenous control, S represents sample and C represents calibrator. Results were also expressed as  $\Delta Ct$  to compare and illustrate the expression levels (normalised to the endogenous control) of specific markers in blood and saliva.

#### 2.8.5 Statistical analysis

All statistical analyses were performed using IBM® SPSS® Statistics v20 software. The results were expressed as mean  $\pm$  standard error of the mean (SEM) for ( $n$ ), where  $n$  represents the number of replicates. Statistical differences were calculated using the paired samples t-test and expressed as a  $p$ -value  $\leq 0.05$  (\*) and/or  $\leq 0.01$  (\*\*). Error bars in the gene

expression plots were based on the RQ minimum and RQ maximum data calculated by the real-time PCR software, the calculations for which are as follows:

### **RQ Minimum**

$$CI = m - t_{df}^{0.95} (sd/\sqrt{N})$$

### **RQ Maximum**

$$CI = m + t_{df}^{0.95} (sd/\sqrt{N})$$

Where CI represents confidence interval at 95%,  $m$  – average  $C_t$ ,  $t_{df}$  –  $t$  value for degrees of freedom,  $sd$  – standard deviation, and  $N$  – the number of replicates. Error bars in the  $\Delta C_t$  charts were based on  $\text{mean} \pm \text{SEM}$  for ( $n$ ).

Each results was presented as a comparison of male and female expression in each sample, the rationale behind this was simply that one volunteer was male and the other female consistently throughout the study, no previous literature has described any difference in gender however, it was thought it may be interesting given that it was a consistent factor throughout having both a male and female, to see if there were any distinct differences between genders.

### **Chapter 3**

## **Body Fluid Identification Utilising Fast SYBR® Green Master**

### **Mix and Real-Time PCR**

## Introduction

A series of qPCR relative quantitation analysis studies were undertaken, investigating the expression of mRNA specific markers in blood and saliva samples. Initial studies were based on the use of SYBR<sup>®</sup> Green detection chemistry utilising real-time PCR, these were then followed by a series of comparison studies utilising TaqMan<sup>®</sup> Gene Expression Assays. Due to the non-specific nature of the SYBR Green fluorescent dye, melt curve analysis was performed to assist in determining whether or not the target sequence had been amplified, and to distinguish this from primer-dimer amplification and other non-specific PCR amplification when necessary. Post amplification analysis was not possible or necessary for studies using TaqMan assays. Firstly, in a TaqMan reaction, the fluorophore does not remain associated with the amplicon post PCR and secondly, hydrolysis probes are highly specific in that a fluorescent signal is only detected when the primers and probe bind to the specific target, unlike SYBR Green that will bind to any dsDNA in the PCR reaction. As described in the materials and methods, typically for one study two volunteers provided two samples, a blood and saliva sample, these were then replicated three times on the plate and the number of specific markers varied per study.

Male blood was used as the calibrator in each result, this remained as such for consistency throughout the results and statistical analysis. However, this has no bearing on the result i.e. the calibrator sample could have been set to saliva for each study, the outcome would be the same only the graph would be reverse i.e. any saliva samples that were down regulated would now be up-regulated and vice versa, the same applies to the blood samples too; any down regulation observed in blood samples while blood was a calibrator, would change to up-regulation and vice versa if a saliva calibrator was chosen, the results are all relative.

Reference gene 18s rRNA was used in all studies, the expression levels of this gene in both blood and saliva were investigated and compared using the *Ct* values.

### 3.1 A quantitative comparison of blood and saliva specific markers in blood and saliva stains utilising SYBR<sup>®</sup> Green dye, and 18s rRNA endogenous control.

#### 3.1.1 Relative quantitation (RQ) and $\Delta C_t$ expression of specific markers PBGD, NCF2, SPTB, HTN3, STATH and KRT4

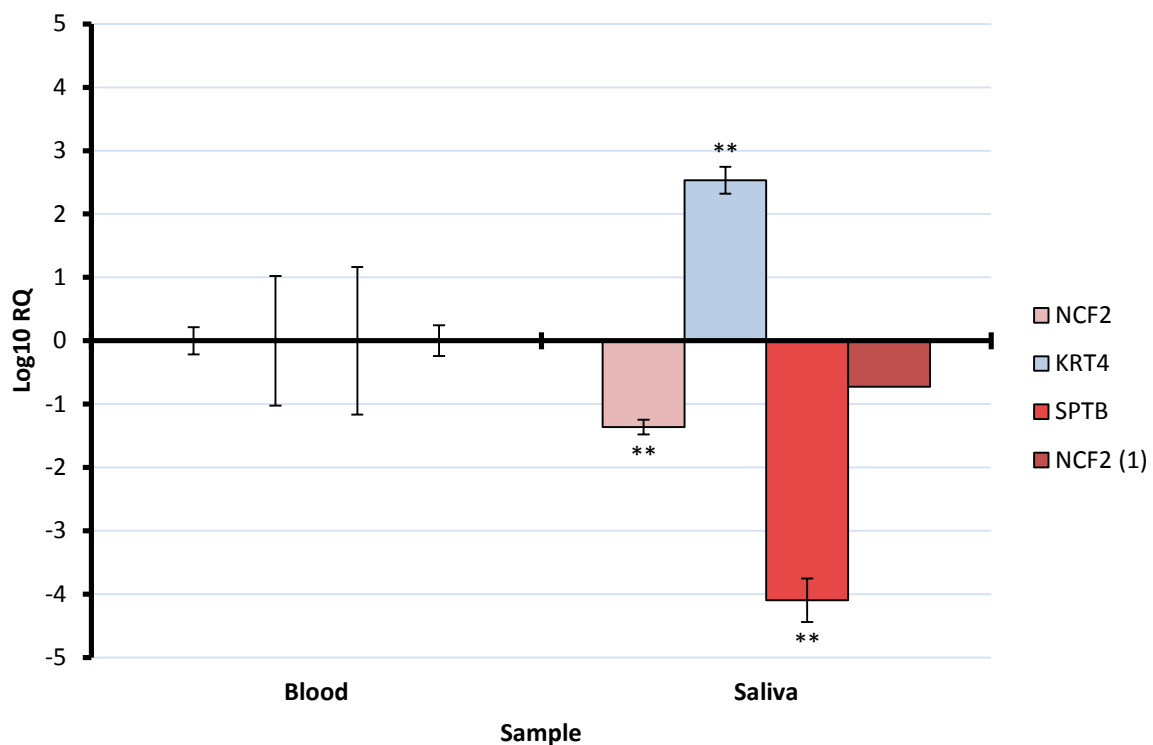


Figure 2: Relative expression of blood-targeted genes NCF2 and SPTB, and saliva-targeted gene KRT4 in blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference, the calibrator sample in each experiment was blood. Error bars based on RQ minimum and maximum data,  $n = 3$ . NCF2  $p \leq 0.01$ , KRT4  $p \leq 0.05$  and SPTB  $p \leq 0.01$ .

Figure 2 corresponds to three separate studies, the results shown are for the mRNA markers that exhibited detectable amplification in both blood and saliva samples. A list of the specific markers investigated in each study can be seen in Table 6. Blood specific marker PBGD and saliva specific marker HTN3 showed no detectable amplification in any study. KRT4 was detected in one out of two studies and STATH, a saliva specific marker, was

detected in two studies but only in one sample. As such, a comparative analysis was not possible for this marker in these particular studies; however the  $\Delta C_t$  values can be seen in Figure 4. Blood specific marker NCF2 was detected in both blood and saliva samples in two separate studies; the number (1) was therefore allocated to this marker in Figure 2 to show that this result was from a separate study.

Table 6: A list of the blood and saliva-specific markers investigated in each study. The specific-markers highlighted in red showed no detectable amplification, \* indicates markers that showed detectable amplification in one body fluid.

Study	Blood-specific marker	Saliva-specific marker
1	NCF2	KRT4
2	SPTB	STATH*
	PBGD	HTN3
3	NCF2	STATH*
	PBGD	HTN3
		KRT4

The results in figure 2 revealed an up-regulation for KRT4 in saliva (2.53), indicating an over expression of KRT4 in saliva compared to blood. In contrast, a down-regulation for NCF2 and NCF2 (1), was observed in saliva (-1.36 and - 0.72), and also SPTB (-4.09), indicating an under expression of both these markers in saliva compared to blood (Figure 2).

Statistical analysis demonstrated that the expression of NCF2 and SPTB was significantly lower in saliva than in blood ( $p \leq 0.01$ ; Table 7), and that the expression of KRT4 was significantly higher in saliva than in blood ( $p \leq 0.02$ ; Table 7).

A significant difference however, could not be obtained for NCF2 (1) in blood and saliva. This was due to the NCF2 marker in this particular study showing amplification in all replicates for blood but only one replicate for saliva, as NCF2 was not detected in two



replicates. Despite being able to do a statistical comparison, this didn't negate the results as NCF2 was detected in all blood replicates yet only one saliva replicate, thus supporting the specificity of this blood marker. Error bars are only seen on NCF2 (1) in blood and not saliva due to the lack of amplification in saliva.

Analysis of the  $\Delta C_t$  values for this data (the threshold cycle value post normalisation to the endogenous control) demonstrated there was a higher expression of NCF2 (1) in blood compared to saliva (Figure 3, Table 7), thus further supporting the specificity of NCF2 in blood. A low  $\Delta C_t$  value corresponds to a high expression level of specific mRNA.

As expected, the results in Figure 2 revealed high specificity for blood specific markers NCF2 and SPTB, and saliva specific marker KRT4. Each marker showed amplification in both body fluids however, the expression of blood-specific markers NCF2 and SPTB were significantly higher in blood than saliva, and the expression of saliva-specific marker KRT4 was significantly higher in saliva than in blood. For NCF2 (1), even though no significant difference was observed in that particular study, a higher expression was observed in blood compared to saliva based on the  $\Delta C_t$  values (Figure 3).

Unexpectedly, markers PBGD and HTN3 showed no amplification in either body fluid in any study. It was expected that each specific marker would show some level of expression in either the blood and/or saliva samples. However, this was not the case, and although KRT4 was detected in one study (Figure 2), a repeat study showed zero detectable amplification for this marker (Table 6).

STATH on the other hand was detected in both studies, in each study however, this marker was only present in one body fluid rather than both (Table 6). In one study, this marker was detected in blood and not saliva, and *vice-versa* in another study, the results can be seen in Figure 3. STATH detected in the blood sample had a  $\Delta C_t$  value of 13.67, this

result however was based on amplification occurring in one out of three replicates, (a positive result in that two replicates out of three showed zero amplification for this saliva specific marker in blood), thus giving an inaccurate  $\Delta Ct$  value. The raw  $Ct$  value ( $Ct$  being the threshold cycle value prior to normalisation) for this replicate was very high (42.07), an indication of very-low level expression, as the higher the  $Ct$  value the lower the expression. The melt curve data for this sample demonstrated typical primer-dimer characteristics, thus suggesting that the amplification in this sample was non-specific. STATH detected in the saliva sample in a separate study- denoted (1), had a  $Ct$  value of  $16.22 \pm 0.49$  (Figure 3, Table 7). This result initially indicated that STATH was expressed in all three replicates and showed specificity to saliva given that no expression had occurred in the blood sample. However, analysis of the melt curve demonstrated that the fluorescence detected in this sample was more than likely due to primer-dimer artefacts, exhibiting a small peak with a low melting temperature ( $T_m$ ) value  $\sim 69^\circ\text{C}$ . It can therefore be said that STATH was not expressed in either blood or saliva in any of these studies.

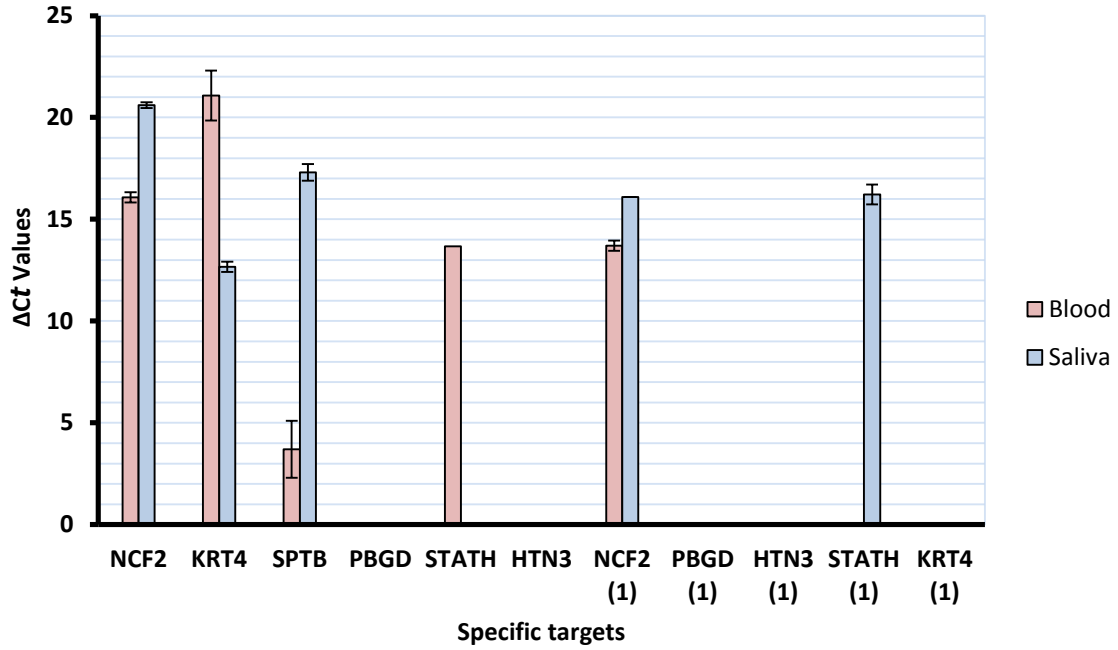


Figure 3: qRT-PCR  $\Delta C_t$  values for blood and saliva-specific markers in blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$  (except for STATH in blood and NCF2 (1) in saliva, where  $n = 1$ ). Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

Table 7:  $\Delta C_t$  values for blood and saliva targeted genes in blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$  (except for STATH in blood and NCF2 (1) in saliva, where  $n = 1$ ). Zero values correspond to no expression of target,  $p \leq 0.05$  for NCF2, KRT4 and SPTB.

Marker	$\Delta C_t$ value		$p$ - value ( $\leq 0.05$ )
	Blood	Saliva	
NCF2	16.07 $\pm$ 0.25	20.60 $\pm$ 0.14	0.01
KRT4	21.08 $\pm$ 1.26	12.66 $\pm$ 0.17	0.02
SPTB	3.70 $\pm$ 1.40	17.30 $\pm$ 0.41	0.01
STATH	13.67	0.00	-
NCF2 (1)	13.70 $\pm$ 0.25	16.10	-
STATH (1)	0.00	16.22 $\pm$ 0.49	-

### 3.1.2 Expression of endogenous control 18s rRNA in blood and saliva stains.

In theory, an ideal endogenous control should be expressed at a constant level in different tissues. However, this was not the case in these three studies, where the expression levels of 18s rRNA was different in blood and saliva. Statistical analysis demonstrated that in each study 18s rRNA was significantly higher in saliva than in blood ( $p \leq 0.05$ ; Table 8).

A possible explanation for this difference could be due to yield during the sample collection process, i.e. the amount of epithelial cells collected with a buccal swab compared to the number of cells collected from the finger prick method for the blood stains. Since 18s rRNA is a reference gene, a higher number of cells in the saliva sample would give a higher expression of the gene in that sample compared to blood.

Table 8: Raw Ct values for endogenous control 18s rRNA in blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$ . A low Ct value corresponds to a higher expression of the endogenous control,  $p \leq 0.05$  in all studies.

Study	18s rRNA Ct value		$p$ - value ( $\leq 0.05$ )
	Blood	Saliva	
1	18.05 $\pm$ 0.05	11.09 $\pm$ 0.07	0.00
2	28.40 $\pm$ 1.33	13.17 $\pm$ 0.27	0.01
3	27.06 $\pm$ 0.04	24.90 $\pm$ 0.35	0.02

### 3.1.3 Negative Control Amplification and Melt Curve Analysis

SYBR<sup>®</sup> Green is an intercalating dye which has non-specific binding properties, i.e. it is able to detect non-specific double-stranded reaction products. Melt curve analysis and analysis of the negative controls was therefore an additional test that could be used to validate

that the detected amplification was the result of a targeted amplicon rather than non-specific amplification or contamination.

The expression of STATH in the saliva sample in study 3 (Table 6) was a good example of this. Prior to melt curve analysis, the  $\Delta Ct$  values indicated good marker specificity – the saliva specific marker was detected in the saliva sample (amplification was observed in all three replicates) and not blood. However, analysis of the melting peaks for this amplification demonstrated that each replicate exhibited a low  $T_m$ , this along with the size of the peaks and their intensity, was characteristic for primer-dimer artefacts. Without the melt curve data, this result based on  $\Delta Ct$  values alone, may have been misinterpreted for specific amplification.

Amplification was detected in all 18s negative control samples (NTC) in each study. Analysis of the melt curve data demonstrated that the melting peak for 18s NTC in each study consistently peaked at the same  $T_m$  as the blood and saliva samples  $\sim 81^\circ\text{C}$ , an indication that the fluorescence detected in each sample (including the NTC) corresponded to specific amplification. The intensity of the NTC peaks however was much smaller than the blood and saliva samples (Figure 4; Table 9).

Table 9:  $Ct$  and  $T_m$  values for 18s rRNA negative control samples (NTC), and  $T_m$  values for 18s rRNA in blood and saliva samples in each study. Results are mean  $\pm$  SEM,  $n = 3$ .

Study	18s rRNA		18s rRNA $T_m$ value ( $^\circ\text{C}$ )	
	NTC $Ct$ value	$T_m$ value ( $^\circ\text{C}$ )	Blood	Saliva
1	30.71 $\pm$ 0.04	81.08 $\pm$ 0.06	81.40 $\pm$ 0.06	81.14 $\pm$ 0.11
2	39.82 $\pm$ 0.55	80.46 $\pm$ 0.00	80.40 $\pm$ 0.16	80.40 $\pm$ 0.12

Figure 4, illustrates a typical melt curve of this data, note that in each study the same melt curve was obtained for 18s.

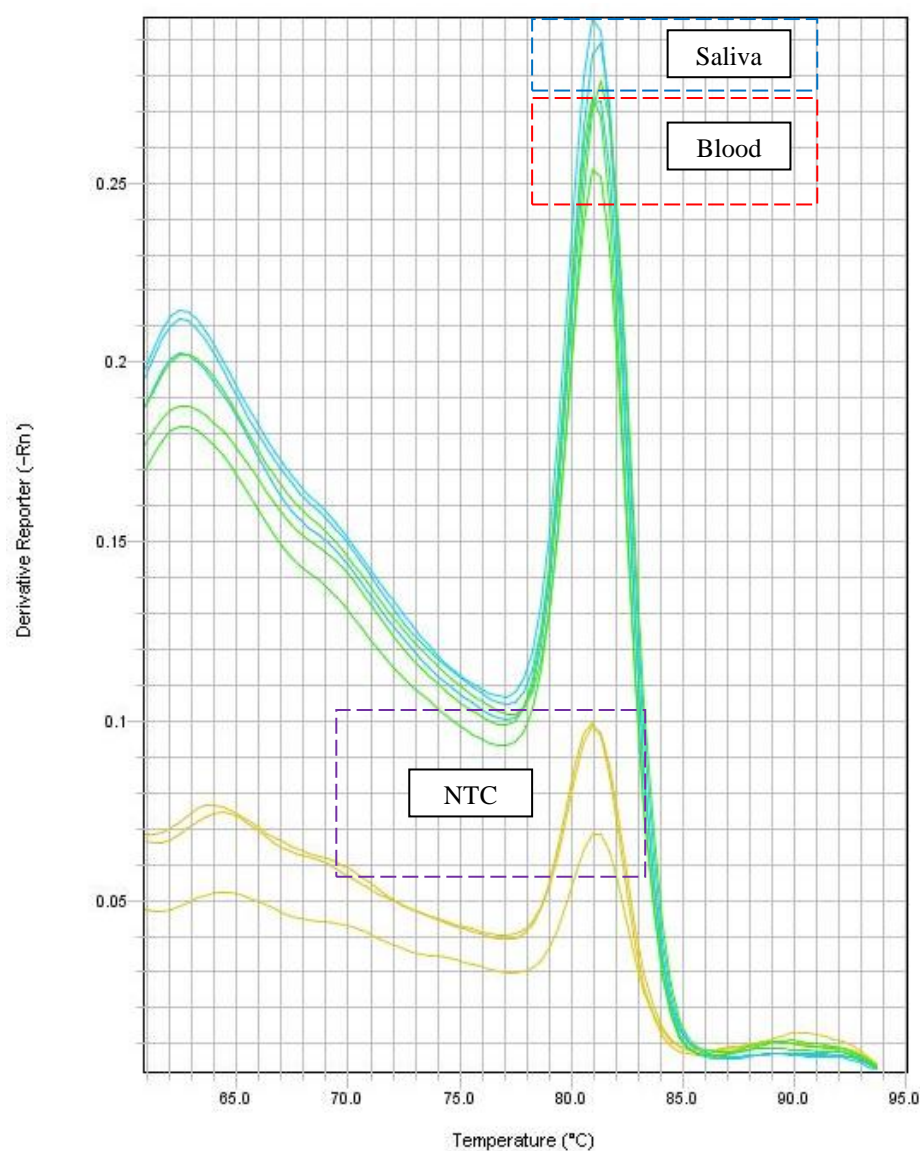


Figure 4: Melt curve analysis of endogenous control 18s rRNA in the negative control, blood and saliva samples. The fluorescence detected in all four samples corresponds to the target amplicon, indicating the expression of 18s rRNA in all samples, a lower intensity is observed for the NTC peaks.

Based on the  $C_t$  values, the expression that occurred in the 18s NTC samples was much lower compared to the blood and saliva samples in each study (Table 8 and 9). It was expected that some level of amplification would be observed in the NTCs for 18s rRNA, given its ubiquitous nature; ribosomal RNAs are highly abundant contributing up to >80% of total cellular RNA, this coupled with the lack of specificity of SYBR<sup>®</sup> Green could be a possible explanation for this detectable amplification.

It can be seen from Table 10 that NTC amplification was also observed in the KRT4 NTC sample in study 1, and the SPTB NTC sample in study 2. No amplification was detected in any other NTC sample.

*Table 10: Negative control  $C_t$  values for specific-markers in each study, the result = mean,  $n = 3$ .*

Study	Specific-marker	$C_t$ value for NTC
1	NCF2	-
	KRT4	39.28
2	SPTB	35.22
	PBGD	-
	STATH	-
	HTN3	-
3	NCF2	-
	PBGD	-
	STATH	-
	HTN3	-
	KRT4	-

Analysis of the raw  $C_t$  values for KRT4 in study 1 demonstrated that the amplification observed in the KRT4 NTC sample was very similar to that of KRT4 in blood ( $C_t$  values; 39.28 and 39.57 respectively). Both of which are high  $C_t$  values; the maximum number of

cycles in this study was 45 ( $C_t$  max), therefore indicating a low level of expression in both of these samples. For comparison purposes, the  $C_t$  value for KRT4 in saliva was much lower (23.75), this lower value supporting the specificity of this marker for saliva.

Analysis of the melt curves for these three samples demonstrated that the  $T_m$  values for the KRT4 NTC sample and KRT4 blood sample were also very similar to one another (73.21°C and 72.96°C respectively), and that the  $T_m$  value for the KRT4 saliva sample however, was significantly higher (82.54°C,  $p \leq 0.01$ ; Figure 5, Table 11).

*Table 11: Melting temperature ( $T_m$ ) values for saliva-specific marker KRT4 in the negative control, blood and saliva samples. Results are mean  $\pm$  SEM,  $n = 3$ ,  $p \leq 0.01$  \*\**

Sample	$T_m$ (°C)
KRT4 NTC	73.21 $\pm$ 0.23
KRT4 Blood	72.96 $\pm$ 0.44
KRT4 Saliva	82.54 $\pm$ 0.06 (**)

Such melting characteristics suggest that the fluorescence detected in the KRT4 saliva sample corresponds to the targeted amplicon. The KRT4 saliva peak shows a much higher intensity in comparison to the melting peaks observed for the KRT4 NTC sample and KRT4 blood sample, and also exhibits a higher  $T_m$  value (Figure 5). This melt curve data coupled with the RQ data, supports the specificity of this marker in this particular study.

The melting peaks observed for the KRT4 NTC and KRT4 blood samples are much smaller in comparison to the saliva peak and exhibit lower melting temperatures, which suggests they are non-specific amplification products. The fluorescence detected in these samples is more than likely the result of primer-dimer artefacts, which in a melt curve are



usually observed by the presence of a smaller hump or shoulder to the left of the main peak (Figure 5). Primer-dimer artefacts are usually comprised of short nucleic acid sequences – shorter than targeted amplicons, and therefore denature at a lower temperature. The formation of primer-dimers in the NTC samples, and what looks like possibly the blood sample in this case too, most often occurs where there is an abundance of primer and no template. It is also possible again given the nature of SYBR<sup>®</sup> Green and its lack in specificity that this amplification is due to low level contamination, or a result of background fluorescence from the SYBR<sup>®</sup> Green chemistry.

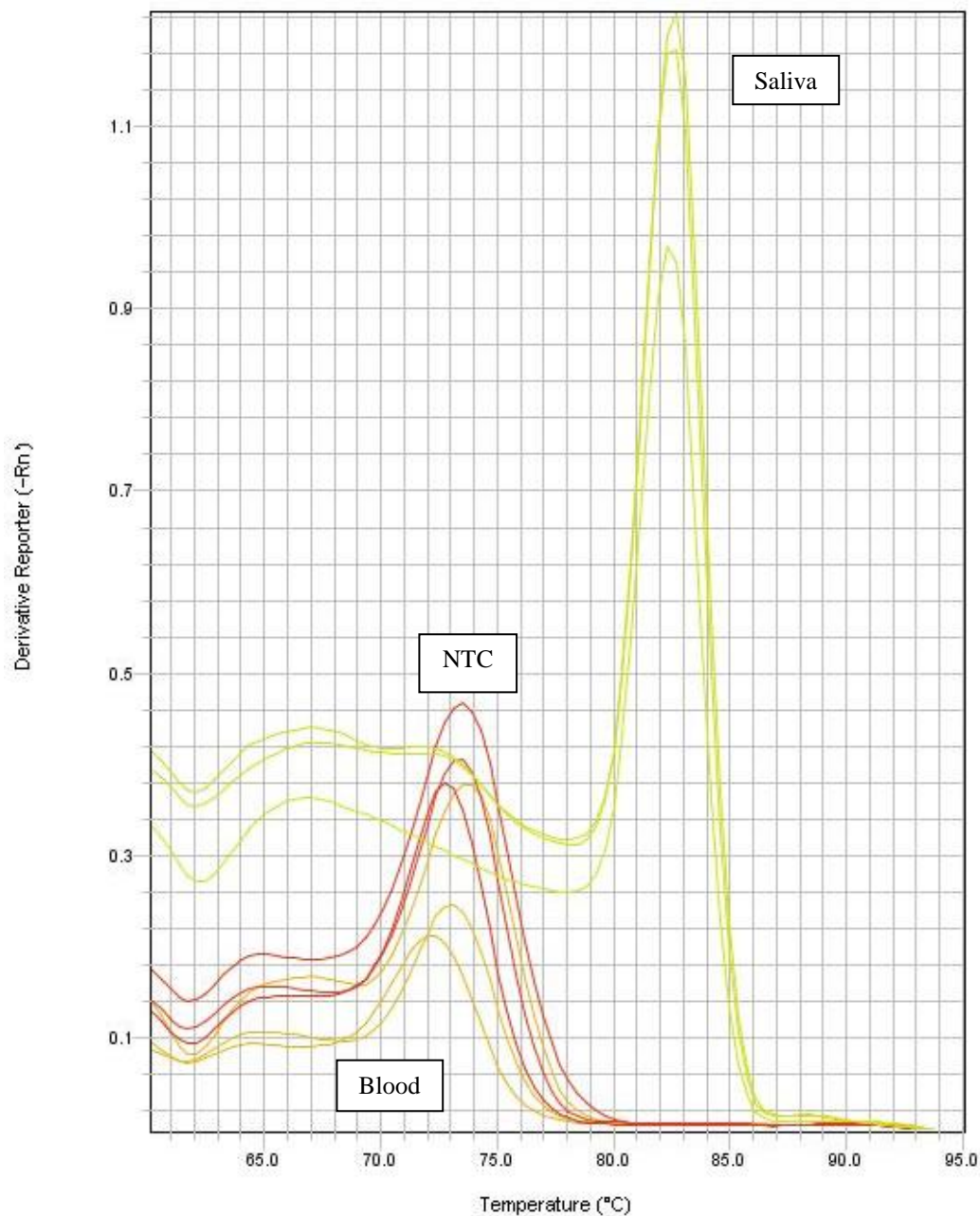


Figure 5: Melt curve analysis for saliva-specific marker *KRT4* in the negative control, blood and saliva samples.

The amplification observed in the SPTB NTC sample in study 2 (Table 10), gave a  $C_t$  value of 35.22. This was slightly higher than the  $C_t$  values observed in the SPTB blood and saliva samples (32.10 and 30.40 respectively).

The melt curve data for these three samples demonstrated that each sample (including the NTC sample) exhibited two peaks. The main melting peaks in each sample peaked at the

same  $T_m \sim 72^\circ\text{C}$ , an indication that the fluorescence detected in each sample may have been due to specific amplification. The second peak for each sample also peaked at the same  $T_m \sim 66^\circ\text{C}$  (Figure 6), a possible indication of primer-dimer artefacts. Unlike KRT4 in Figure 6, where the NTC amplification was shown to be non-specific, the SPTB NTC sample gave the same  $T_m$  as the blood and saliva melting peaks. This data could be an indication that low level contamination may have occurred in the sample, or it could be that non-specific amplification is being observed in all three samples, melt curve data is not definitive it only supports the RQ data and offers increased specificity for the SYBR<sup>®</sup> Green dye (Figure 6).

Even though no amplification was detected in the NCF2 NTC sample in either study (Table 10), analysis of the melt curves was still undertaken. The results demonstrated that the melt curves for NCF2 in blood and saliva were the same in both studies, and that in each case the data suggests that the amplification observed in the NCF2 samples was more than likely due to the targeted amplicon. These discrepancies in the NTC amplification (Table 8) i.e. zero amplification in some NTCs, yet amplification in others, could possibly be a result of the sensitivity of the real-time PCR reaction.

Figure 7 illustrates the typical melt curve for NCF2 marker in blood and saliva samples. Both samples exhibit two large peaks, showing high intensity with a  $T_m$  value of  $\sim 82^\circ\text{C}$  and smaller peak/hump to the left with a lower intensity and  $T_m$  value of  $\sim 68^\circ\text{C}$ . These melting characteristics suggest that the larger peaks correspond to the targeted amplicon NCF2, and that the smaller peaks more than likely correspond to primer-dimer artefacts. This result supports the RQ data by suggesting that NCF2 is present in both blood and saliva, and also supports the specificity of NCF2 given that a higher expression of this blood specific marker was observed in blood (Figure 7).

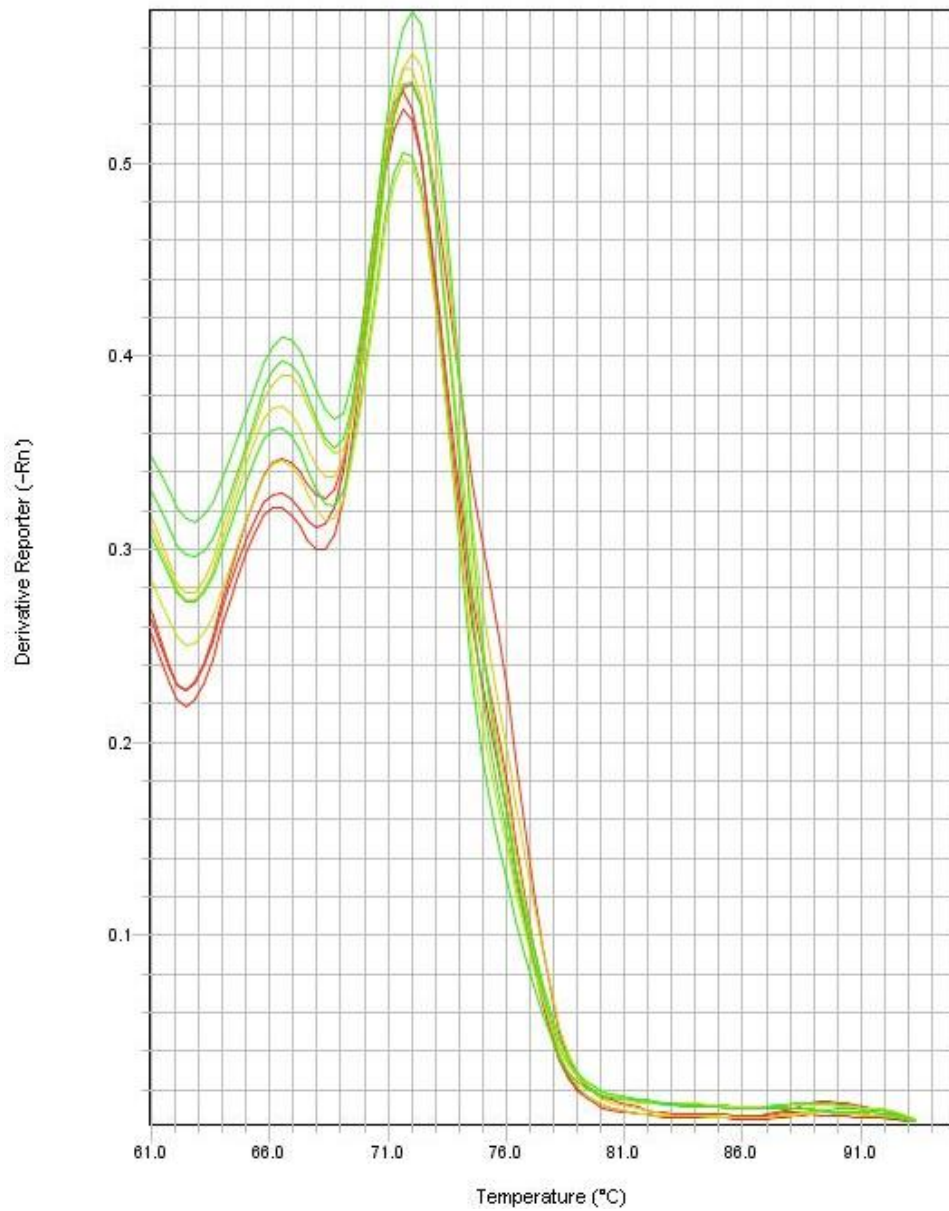


Figure 6: Melt curve analysis for blood-specific marker SPTB in the negative control, blood and saliva samples.

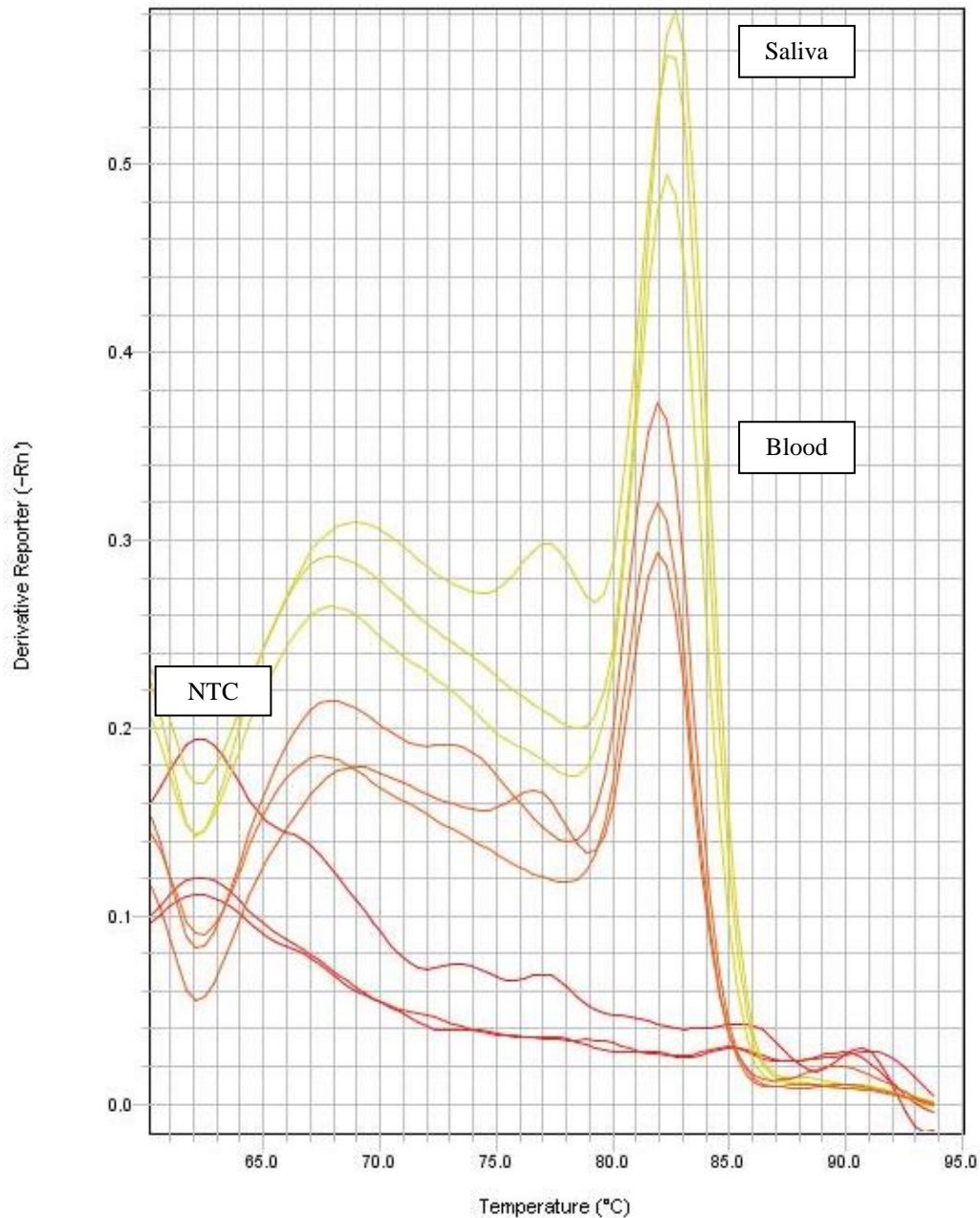


Figure 7: Melt curve analysis for blood-specific marker NCF2 in the following samples: negative control, blood and saliva.

### 3.1.4 Reverse transcription negative control amplification

Amplification was detected in 3 out of 18 reverse transcription negative control (RT NTC) samples across all three studies. In the first study, amplification was detected in the 18s blood RT NTC and NCF2 blood RT NTC. In the third study, amplification was detected in the STATH saliva RT NTC.

Analysis of the 18s blood RT NTC in study one revealed that the amplified product gave a similar  $C_t$  value (29.48) to the 18s NTC sample (Table 9), both of which were higher than the  $C_t$  values for 18s in blood and saliva (Table 8), indicating a lower level of expression. The melt curve data for this sample also demonstrated that the melting peak of the 18s blood RT NTC peaked at the same  $T_m$  as the 18s NTC, blood and saliva samples (Figure 4), indicating that the fluorescence detected in that sample was due to specific amplification of 18s rRNA. However, the peak had a much smaller intensity compared with all other peaks. This result may therefore be due to low level contamination or a result of background fluorescence.

Analysis of the NCF2 blood RT NTC in study one revealed that the amplified product gave a raw  $C_t$  value of 41.49. This value is much higher than the  $C_t$  values observed for NCF2 in the blood and saliva samples (34.57 and 31.69 respectively), and very close to the  $C_t$  max value of 45, indicating that the levels of expression observed in the RT NTC sample are very low. The melt curve data for this RT NTC sample suggested non-specific amplification, as the peak was smaller and exhibited a lower  $T_m$  value than the sample peaks (Figure 8). The amplified product in the NCF2 RT NTC sample is either due to primer-dimers artefacts or a result low level fluorescence, most likely due to the lack of specificity of the SYBR<sup>®</sup> Green. It is unlikely to be the result of a poor RNA extraction method as amplification would be seen in all RT NTCs and this was not the case.

It is worth noting that the number of PCR cycles ( $C_t$  max) was set to 45 in this particular study, had the cycle number been set to 40 (a standard PCR cycle number) no amplification would have been detected in the NCF2 blood RT NTC sample, thus indicating that there may be an issue with the test being too-sensitive.

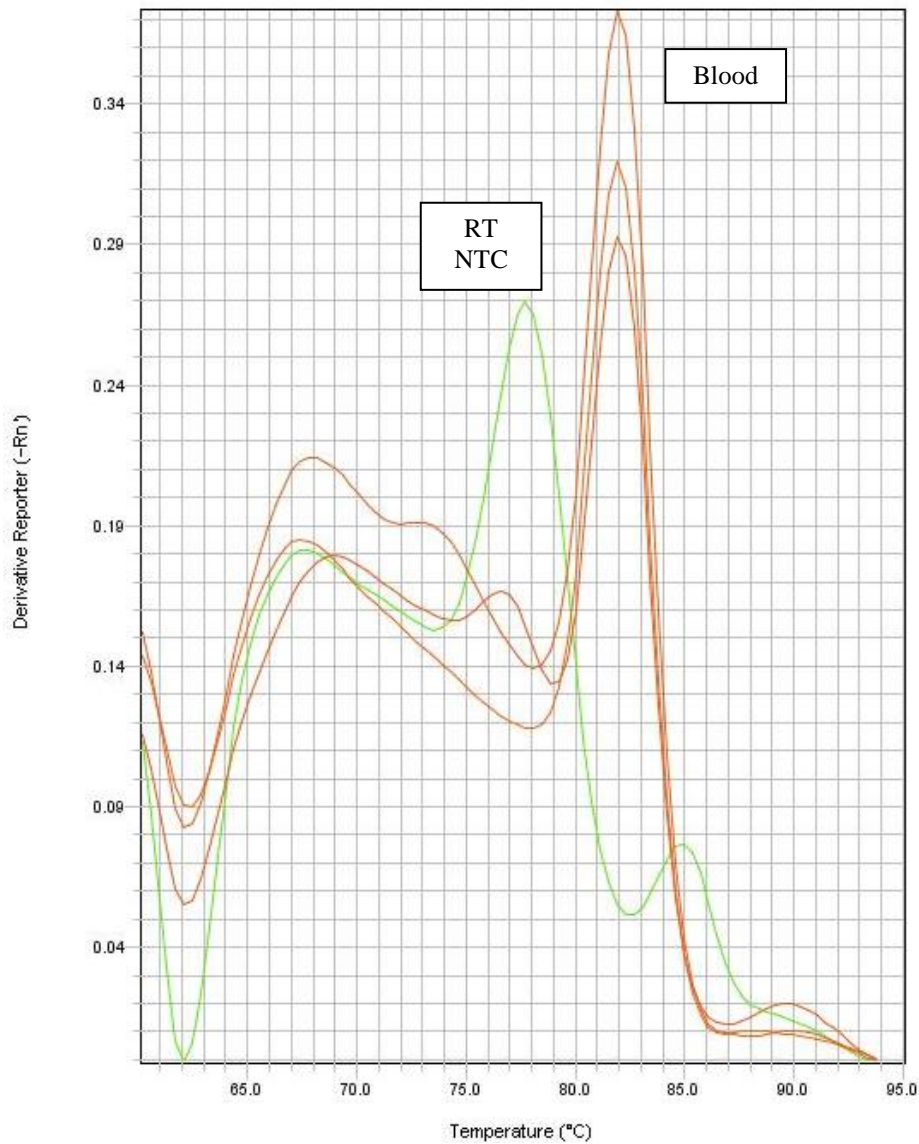


Figure 8: Melt curve analysis for NCF2 in the reverse transcription NTC samples compared to blood.

Analysis of the STATH saliva RT NTC in study three revealed that the amplified product gave a  $C_t$  value of 42.90, very similar to the STATH saliva sample which was revealed early on in this chapter to be primer-dimer amplification. The melt curve data for this RT NTC was also the same as the STATH saliva sample, the peak having a  $T_m$  value of  $\sim 69^\circ\text{C}$ . Thus all the data suggests primer-dimer formation in this particular sample.

No other sample in study three showed amplification in the RT blank NTCs, the NCF2 (1) RT NTC sample was also negative. Thus, strongly supporting the probability that

the amplification observed in the NCF2 blood and saliva samples was a result of amplifying the targeted amplicon and not due to contaminating DNA or non-specific amplification. This RT NTC data along with the melt curve and RQ data again supports the specificity of this blood-specific marker.



### 3.2 A quantitative comparison of blood and saliva specific markers in male and female blood and saliva stains, utilising SYBR<sup>®</sup> Green dye and 18s rRNA endogenous control

#### 3.2.1 Relative quantitation and $\Delta C_t$ expression of specific markers STATH and SPTB

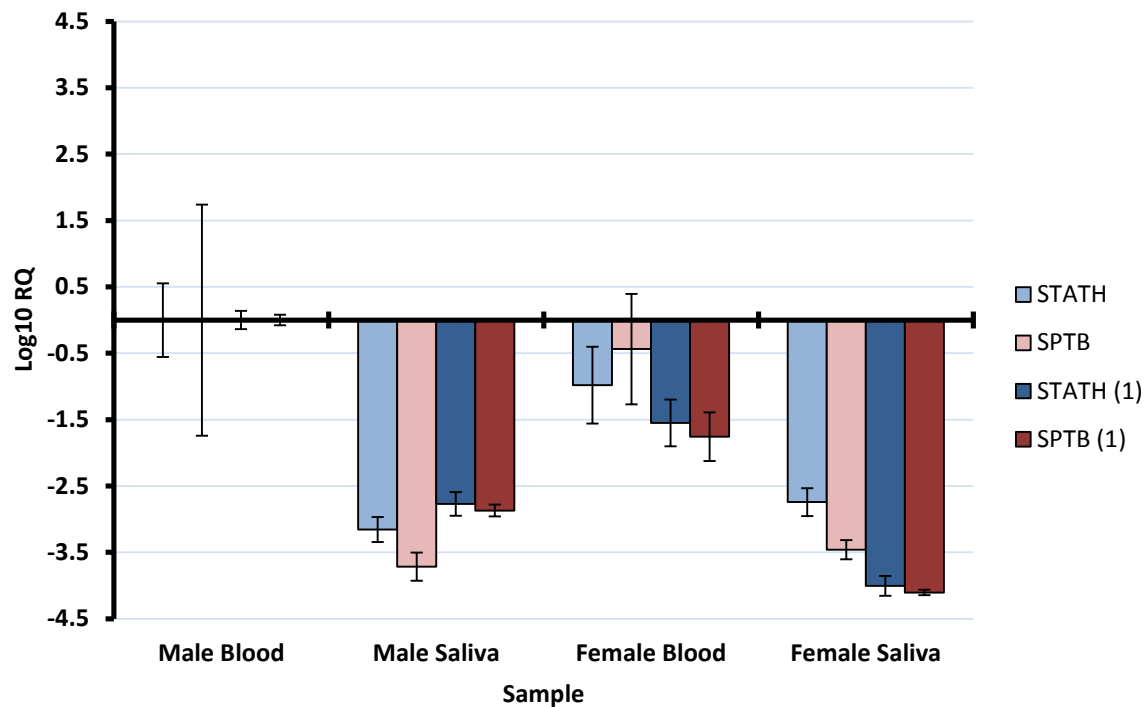


Figure 9: Relative expression of blood-targeted gene SPTB and saliva-targeted gene STATH in male and female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference, the calibrator sample in each experiment was male blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

In this part of the study, a number of quantitative analysis studies were performed to investigate the expression and specificity of blood and saliva specific mRNA markers in male and female blood and saliva stains.

The results in Figure 9 were based on two separate studies investigating the expression of saliva-specific marker STATH and blood-specific marker SPTB in both male and female blood and saliva stains (the second study denoted by the number 1 in Figures 9 and 10).

The results of the first study revealed a down regulation of STATH in all three samples compared to male blood. Thus, indicating that the saliva specific marker was over expressed in male blood. A down-regulation of SPTB was also observed in all three samples compared to male blood. Thus, indicating that the blood specific marker was over expressed in male blood. The results from the second study revealed the same results as the first; a down regulation of both markers in all three samples compared to the male blood sample.

For these both these markers it was expected that STATH would be over expressed in saliva given that it is saliva specific, and that SPTB would be over expressed in blood given that it is blood specific. The latter occurred in both studies, (a higher expression of SPTB in blood than saliva) thus suggesting a positive result for the specificity of SPTB. STATH however, based on these results, appeared to lack specificity for saliva; the results demonstrated there was more STATH present in the male blood sample than any other sample.

Statistical analysis demonstrated that the expression of SPTB and STATH was significantly lower in saliva compared to blood in both male and female samples ( $p \leq 0.05$ ; Table 12).

Interestingly, it was also observed that in both studies the expression of each marker was higher in the male samples compared to the female samples (Figure 10). Although in the first study there was no significant difference between the expression levels of SPTB in male and female blood samples.

Table 12:  $\Delta C_t$  values for blood targeted gene *SPTB* and saliva targeted gene *STATH* in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ )

Marker	$\Delta C_t$ Value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
SPTB	$-0.52 \pm 2.08$	$11.83 \pm 0.26^{**}$	$0.94 \pm 1.00$	$10.97 \pm 0.17^{**}$
STATH	$2.51 \pm 0.66$	$12.99 \pm 0.22^{**}$	$5.76 \pm 0.69$	$11.62 \pm 0.25^*$
SPTB (1)	$2.42 \pm 0.10$	$11.95 \pm 0.11^{**}$	$8.26 \pm 0.44$	$16.05 \pm 0.05^{**}$
STATH (1)	$3.40 \pm 0.17$	$12.61 \pm 0.22^{**}$	$8.56 \pm 0.42$	$16.71 \pm 0.18^{**}$

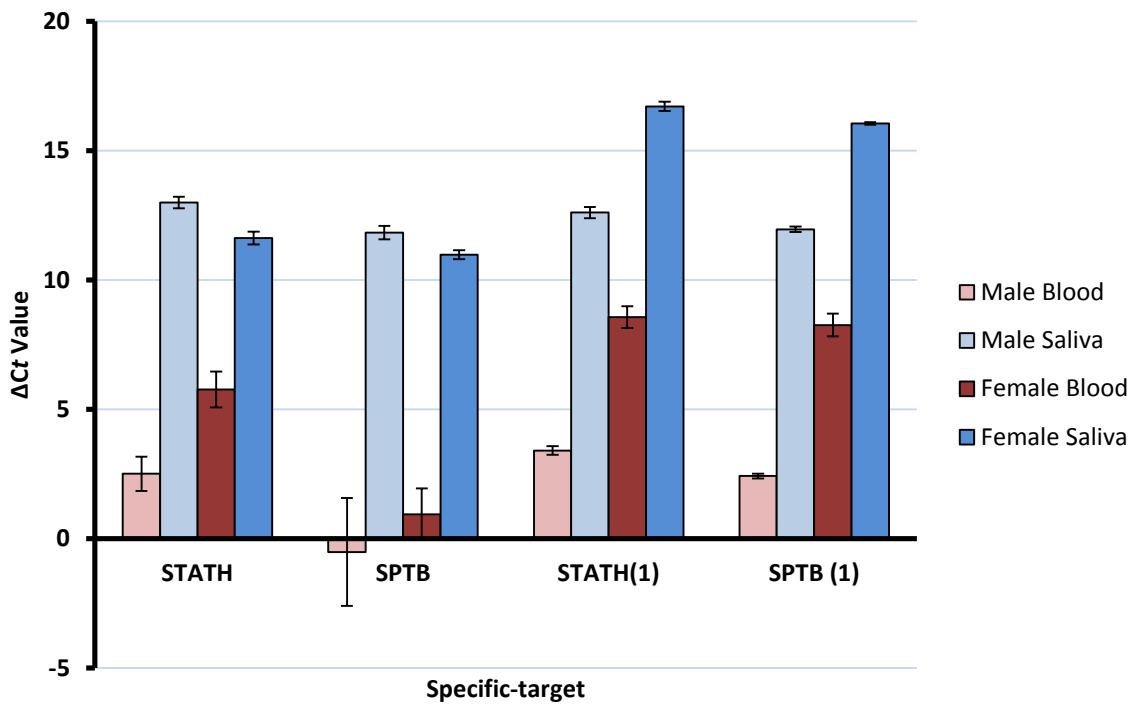


Figure 10: qRT-PCR for blood and saliva-specific markers in male and female blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$ . Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

Another noted observation included the consistent expression pattern of both markers in the male saliva samples in both studies, Figure 10 and the data in Table 12 illustrates the similarity in the  $\Delta C_t$  values. It can also be seen looking at the data for the second study, that

the expression levels of each marker in the male and female samples were almost identical. In both studies the expression of SPTB was also higher in the male sample than in female.

### **3.2.2 Negative control amplification and melt curve analysis**

In each study, a negative control sample was investigated for each marker including the 18s endogenous control. Amplification was detected in all these samples in both studies. The NTC expression levels for each target in study one were slightly higher than the expression levels observed in study 2 and statistical analysis demonstrated this difference to be significant. ( $p \leq 0.05$ ).

An individual analysis of the NTCs in both studies revealed that the  $C_t$  value for the STATH NTC was significantly higher than the  $C_t$  values observed in the corresponding test samples i.e. male and female blood and saliva in both studies, indicating a lower level of expression. However, analysis of the melt curve data demonstrated that in each study, all five samples peaked at almost the same  $T_m \sim 68^\circ\text{C}$ , and that a second peak was absent. Both indicators that the amplification observed in each of these samples were more than likely due to primer-dimer artefacts.

The  $C_t$  value for the SPTB NTC sample in the first study was quite similar to the test samples, with the exception of male saliva and female blood, which were significantly lower, thus indicating a higher level of expression in these two test samples. The  $C_t$  value for SPTB NTC in the second study was significantly lower in all test samples apart from the female blood sample. The melt curve data for SPTB was similar to the previous results in this chapter with regards to the  $T_m$  values. However the peaks appeared slightly different; no smaller peaks were observed in these studies. These results suggest that the amplification in the NTC sample is more than likely due to specific amplification.

Lastly, the  $C_t$  values for 18s NTC were significantly higher than all the corresponding test samples in each study, indicating that the expression in the 18s NTC sample was lower. The melt curve data for the 18s NTC samples in these two studies were almost identical, yet both melting profiles differed slightly in comparison to previous 18s NTC melting results in this chapter. The difference being that two peaks were observed in the profile for the 18s NTC samples as opposed to one. The main  $T_m$  peaks also had a slightly lower  $T_m$  value than previously reported  $\sim 79^\circ\text{C}$  and the second peaks appear to be  $\sim 71^\circ\text{C}$ ; possibly non-specific amplification. The melting peaks, like previous results, were much lower in intensity compared to test sample peaks. In any case, this melting curve analysis along with the negative control amplification (a higher  $C_t$  value observed in the NTC compared to test samples) suggests that the amplification observed in the 18s NTC samples in both studies was most probably due to low level specific amplification, as all the main peaks of the NTC samples peaked around the same  $T_m$  as the test sample. Again, low level specific amplification was expected due to the ubiquitous nature of reference gene 18s rRNA and the non-specific nature of SYBR<sup>®</sup> Green dye.

### **3.2.3 Reverse Transcription negative control amplification**

In the first study, amplification occurred in all RT NTC samples for STATH, except in the male blood sample, all RT NTCs in the second study also showed amplification. Analysis of the  $C_t$  values for this data demonstrated that in both studies the level of expression observed in the RT NTCs were similar to the expression observed in their corresponding test samples. For STATH RT NTC samples in both studies the melt curve data was almost identical; both demonstrated melting peaks at the same  $T_m$ , and in each melt curve the peaks for the RT NTCs peaked at the same  $T_m$  as the test samples. Indication that the amplification observed in these samples, (just like the STATH NTC and test samples) was non-specific and more likely the result of primer-artefact formation.

Unlike previous SPTB studies, amplification was observed in the SPTB RT NTC samples in both these studies. One SPTB RT NTC sample in the first study (male blood - the rest demonstrated zero amplification), and all SPTB RT NTCs in the second study. The  $C_t$  values for each of these RT NTC samples were also similar to the  $C_t$  values of the corresponding test samples. The melt curve data for all amplified SPTB RT NTCs across both studies were almost identical; in each case the RT NTC sample exhibited peaks at the same  $T_m$  as the test and NTC samples, thus casting doubt as to whether or not the amplification observed in the SPTB samples was due to specific amplification.

No amplification was detected for the 18s RT NTCs in the first study, however in contrast, all 18s RT NTCs in the second study showed detectable amplification. All  $C_t$  values for these RT NTCs were higher than their corresponding test samples, indicating a much lower level of expression. Melt curve analysis suggested that this amplification was specific to 18s, as the peaks in the RT NTCs peaked at the same  $T_m$  as the test samples, the intensity of the RT NTC peaks however was much smaller. This specific amplification could be due to the sensitivity of the real-time PCR coupled with the nature of the 18s rRNA gene and SYBR<sup>®</sup> Green dye.

### 3.2.4 Relative quantitation and $\Delta C_t$ expression of specific markers KRT13 and SPTB

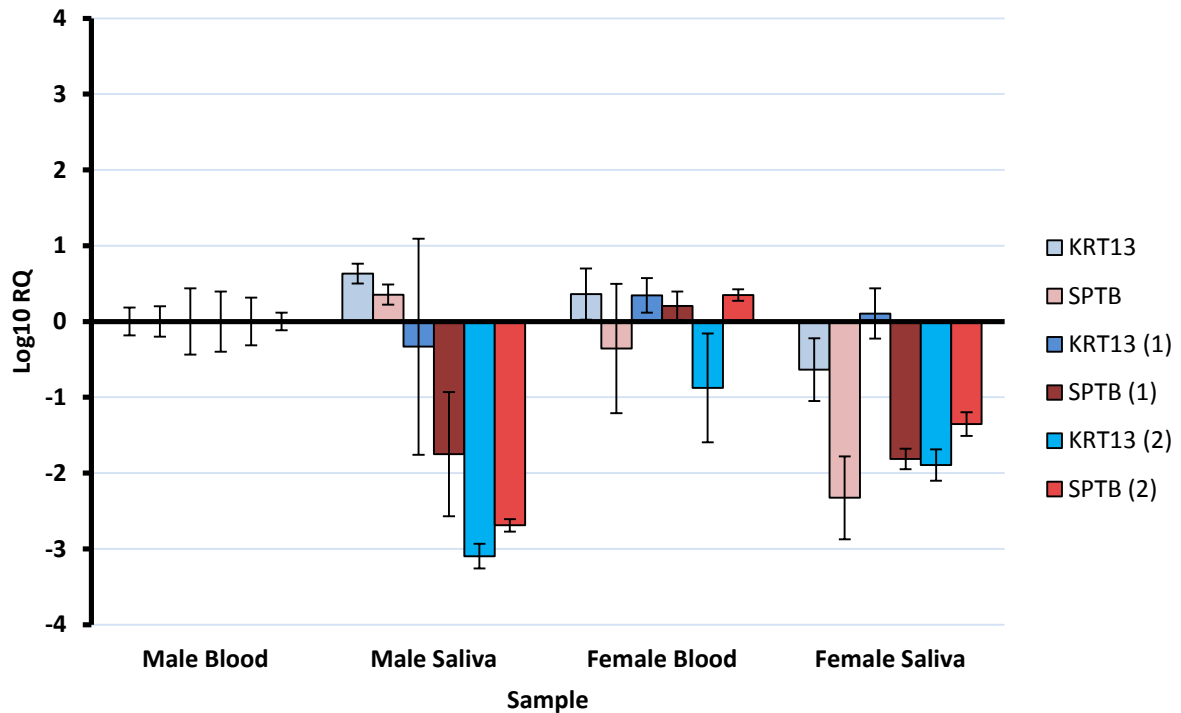


Figure 11: Relative expression of blood-targeted gene SPTB and saliva-targeted gene KRT13 in male and female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference. The calibrator sample in each experiment was male blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in Figure 11 were based on three separate studies investigating the expression of saliva-specific marker KRT13, and again blood-specific marker SPTB in both male and female blood and saliva stains (the second and third studies are denoted by the numbers 1 and 2).

The results of the first study in Figure 11 revealed an up-regulation of KRT13 in male saliva (0.63) and female blood (0.36), yet a down regulation in female saliva (-0.63). Thus, indicating that the saliva specific marker was under expressed in male blood compared to male saliva and female blood, and over expressed in male blood compared to female saliva. You would expect, given that the KRT13 marker is saliva-specific, an under expression of KRT13 in male blood compared to both male and female saliva, however this was not the case. Interestingly in this study, an up-regulation of SPTB was observed in the male saliva

sample (0.35), indicating that there was more SPTB present in male saliva compared to male blood. A result you would not expect given the SPTB is blood specific. In all previous studies, SPTB has been consistently under expressed in saliva, this was the first time an over expression of SPTB in saliva was observed. For the female blood and saliva samples, an under expression of SPTB was observed compared to the male blood sample (-0.35 and -2.32 respectively).

The results from the second study in Figure 11 revealed a down regulation for KRT13 (KRT13 1) in the male saliva sample this time (-0.33), and an up regulation in female saliva (0.35) – the opposite of study one. Suggesting that there was less KRT13 marker present in the male saliva sample compared to male blood, yet more present in the female saliva sample. You would ideally expect a higher expression of KRT13 in both male and female saliva samples compared to blood. An up-regulation was also observed for this marker in female blood (0.10). SPTB in this second study (SPTB 1) was under expressed in both male and female saliva samples compared to the male blood sample (-1.75 and -1.81 respectively), whereas an over expression was observed in the female blood sample (0.21). Indicating that there was more SPTB in male blood than both saliva samples, a result you would expect for a blood-specific marker, but less in the male blood sample compared to female blood, suggesting that the expression levels of this marker vary between donors and in this particular study were higher in the female sample.

The results from the third study in Figure 11 revealed KRT13 (KRT13 2) to be under expressed in male saliva, female blood and female saliva (-3.10, -0.88 and - 1.89 respectively), meaning a higher expression of KRT13 was observed in male blood. An unexpected result, as KRT13 is saliva-specific, therefore you would expect a higher expression in saliva samples compared to blood. The SPTB marker (SPTB 2) was again under expressed in both the male and female saliva samples compared to blood (-2.69 and -



1.35 respectively) , a positive result with regards to specificity for this blood marker as you would a higher expression of SPTB in blood. An over expression of SPTB was again observed in the female blood sample (0.35) just as in study 2 (SPTB 1), indicating a higher level of the SPTB blood marker in female blood in this particular study.

For these markers it was expected that KRT13 would be over expressed in saliva given that it is saliva specific, and that SPTB would be over expressed in blood given that it is blood specific. The expression of KRT13 varied among samples; in some cases it would be higher in saliva than blood and *vice-versa*, showing little specificity for saliva. For SPTB an over expression was observed once in male saliva, yet the remaining samples showed good specificity for blood, in fact two female blood samples showed a higher expression of SPTB than in the male blood sample.

In the first study, statistical analyses demonstrated that there was a significant difference in the higher expression of KRT13 in male saliva compared to male blood, there was also a significant difference in the higher expression levels of this marker in female blood compared to female saliva, no significant difference in the higher expression of SPTB in male saliva compared to male blood. SPTB however, was significantly lower in female saliva compared to female blood ( $p \leq 0.05$ ; Table 13).

In the second study, there was no significant difference in the expression of KRT13 (1) in either male or female blood and saliva samples. SPTB (1) however, was significantly lower in male saliva compared to male blood and in female saliva compared to female blood ( $p \leq 0.01$ ).

In the third study, the expression of KRT13 (2) was significantly lower in male saliva compared to male blood ( $p \leq 0.01$ ). This marker was also significantly lower in female saliva

compared to female blood ( $p \leq 0.05$ ). SPTB (2) was again significantly lower in male saliva compared to male blood, and in female saliva compared to female blood ( $p \leq 0.01$ ).

Table 13:  $\Delta C_t$  values for blood targeted gene SPTB and saliva targeted gene KRT13 in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ ).

Marker	$\Delta C_t$ Value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
KRT13	15.26 $\pm$ 0.22	13.17 $\pm$ 0.16**	14.06 $\pm$ 0.41	17.36 $\pm$ 0.49**
SPTB	11.91 $\pm$ 0.24	10.73 $\pm$ 0.16	13.09 $\pm$ 10.2	19.63 $\pm$ 0.65*
KRT13 (1)	12.18 $\pm$ 0.52	13.28 $\pm$ 1.71	11.03 $\pm$ 0.27	11.83 $\pm$ 0.40
SPTB (1)	6.81 $\pm$ 0.48	12.62 $\pm$ 0.98**	6.12 $\pm$ 0.22	12.83 $\pm$ 0.16**
KRT13 (2)	5.79 $\pm$ 0.38	16.07 $\pm$ 0.19**	8.70 $\pm$ 0.86	12.08 $\pm$ 0.25*
SPTB (2)	3.54 $\pm$ 0.14	12.48 $\pm$ 0.10**	2.38 $\pm$ 0.09	8.04 $\pm$ 0.19**

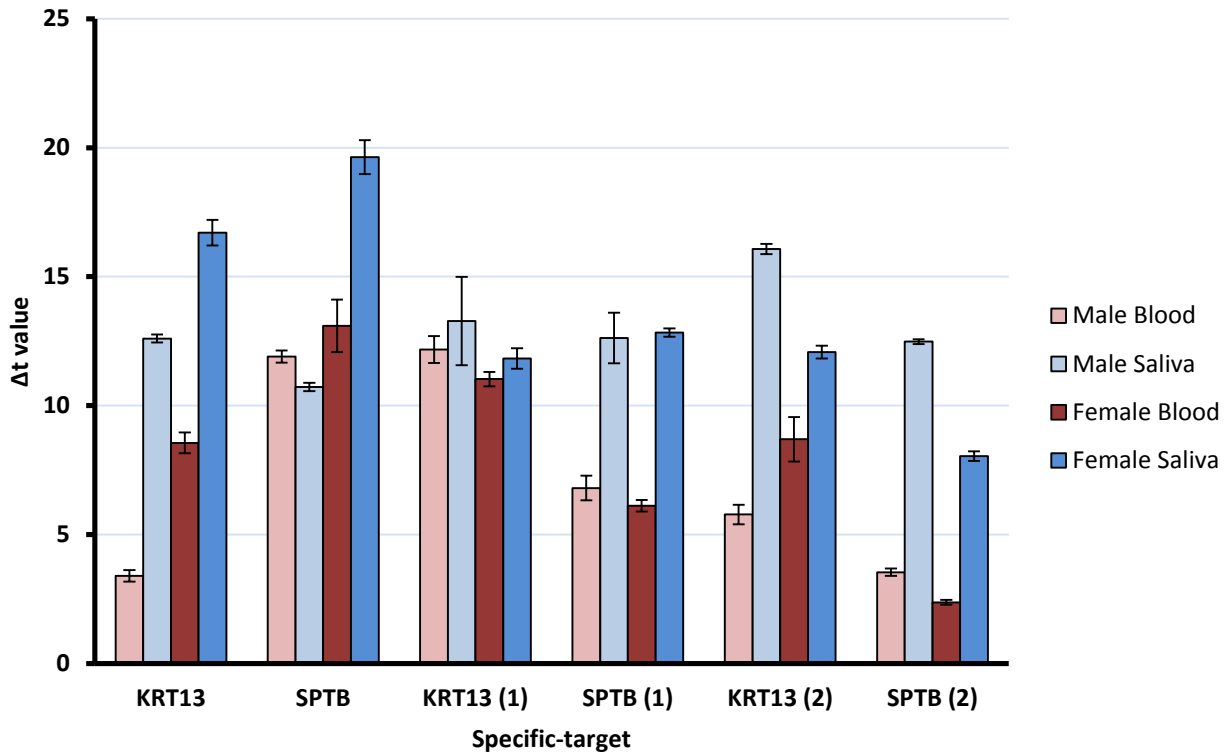


Figure 12: qRT-PCR for blood and saliva-specific markers in male and female blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$ . Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

Observation of the  $\Delta C_t$  values again demonstrates an almost consistent expression pattern for male saliva in each study for both markers (Figure 12), interestingly, there does however in each study, appear to be more SPTB present in male saliva than KRT13. The expression patterns for the other three samples however vary between studies.

In figure 12, comparison of the  $\Delta C_t$  values for male and female samples in the first study shows that there was a higher expression of KRT13 in male saliva than female saliva, and that this was *vice-versa* in the second and third study, an indication that KRT13 expression levels vary between individuals, which is something you would expect. However, according to the  $\Delta C_t$  results, even though expression was observed in the saliva samples for KRT13 which is saliva-specific, each study unexpectedly demonstrated a higher expression of KRT13 in all blood samples compared to saliva.

In each study, the expression levels of SPTB in both male and female samples were relatively close, variation in expression can be seen between donors i.e. a higher expression can be seen the male blood sample in study one, yet the expression is seen to be higher in female blood than male blood in studies two and three. In all but one instance, a higher expression of SPTB was observed in the blood samples compared to saliva – the exception being the first study where there appeared to be more SPTB in male saliva compared to both male and female blood.

### **3.2.5 Negative control amplification and melt curve analysis**

Again, in each study negative control amplification was observed for each marker including 18s.

Analysis of NTC  $C_t$  data in these studies showed that for each marker including the endogenous control, the NTC  $C_t$  values were more often than not, higher than the  $C_t$  values observed in the test samples, indicating a lower level of expression in the NTC samples. The melt curve analysis data for SPTB was consistent with the melt curves observed for SPTB in the previous SPTB and STATH study (section 3.2.2). Interestingly, the melt curve data for KRT13 in each of these studies showed that the amplification observed in the NTC and blood samples was more than likely due to non-specific amplification; only one peak was observed for these samples all exhibiting a  $T_m$   $\sim 74^\circ\text{C}$ . The KRT13 saliva samples in all studies however, each exhibited two peaks; one peak at  $\sim 74^\circ\text{C}$  (just like the blood and NTC) indicating primer-dimer artefacts, and then a main peak at  $\sim 82^\circ\text{C}$ , indicating specific

amplification. This melt curve data supports the RQ data and specificity of KRT13 for saliva. In Figure 12, there was a concern regarding the higher expression levels of KRT13 in all the blood samples compared to saliva. However, the melt curve data (Figure 13) suggests that the higher expression observed in these blood samples was due to non-specific amplification and that the expression ( $\Delta C_t$  values in Figure 12) observed in the saliva samples was due to amplification of the targeted sequence.

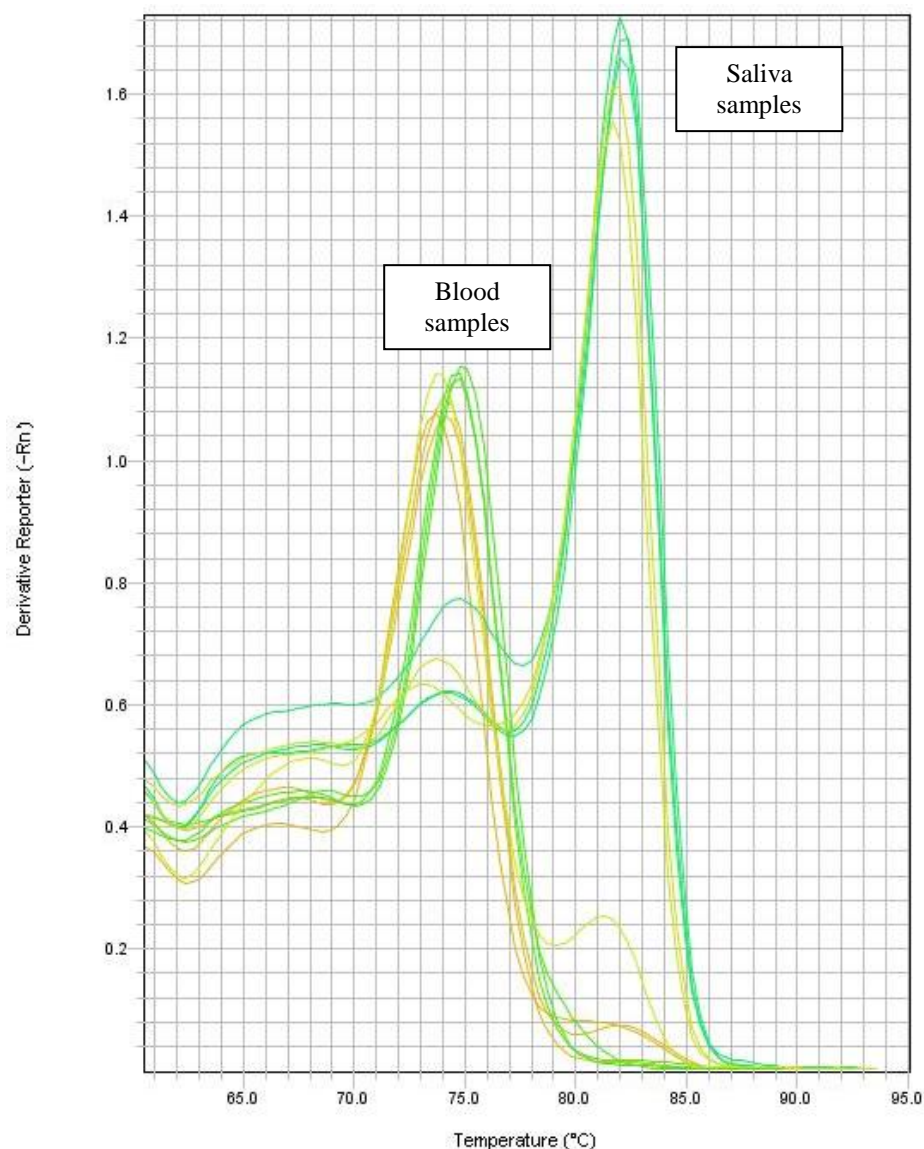


Figure 13: Melt curve analysis for KRT13 in blood and saliva samples. The lower  $T_m$  exhibited in the blood samples is characteristic of primer-dimer amplification.

The amplification detected in the 18s NTCs in the first and second study demonstrated typical melt curves observed throughout previous studies in this chapter, where one peak was observed which peaked at the same  $T_m$  as the test samples, indicating specific amplification (Figure 5; section 3.1.3). The  $T_m$  values were however slightly lower again  $\sim 78^\circ\text{C}$ , a similar value to the 18s NTCs in the STATH and SPTB study (section 3.2.2), and as in all studies the NTC peaks were smaller and lower in intensity than the test samples peaks.

In the third study however, the amplification detected in the 18s NTCs gave a different melt curve compared to previous studies, in that two peaks were observed and both these peaks were seen to peak at different and lower  $T_m$  values to the test samples (Figure 14), thus suggesting that the amplification in these NTCs was non-specific. The  $T_m$  value for the test samples  $\sim 80^\circ\text{C}$ .

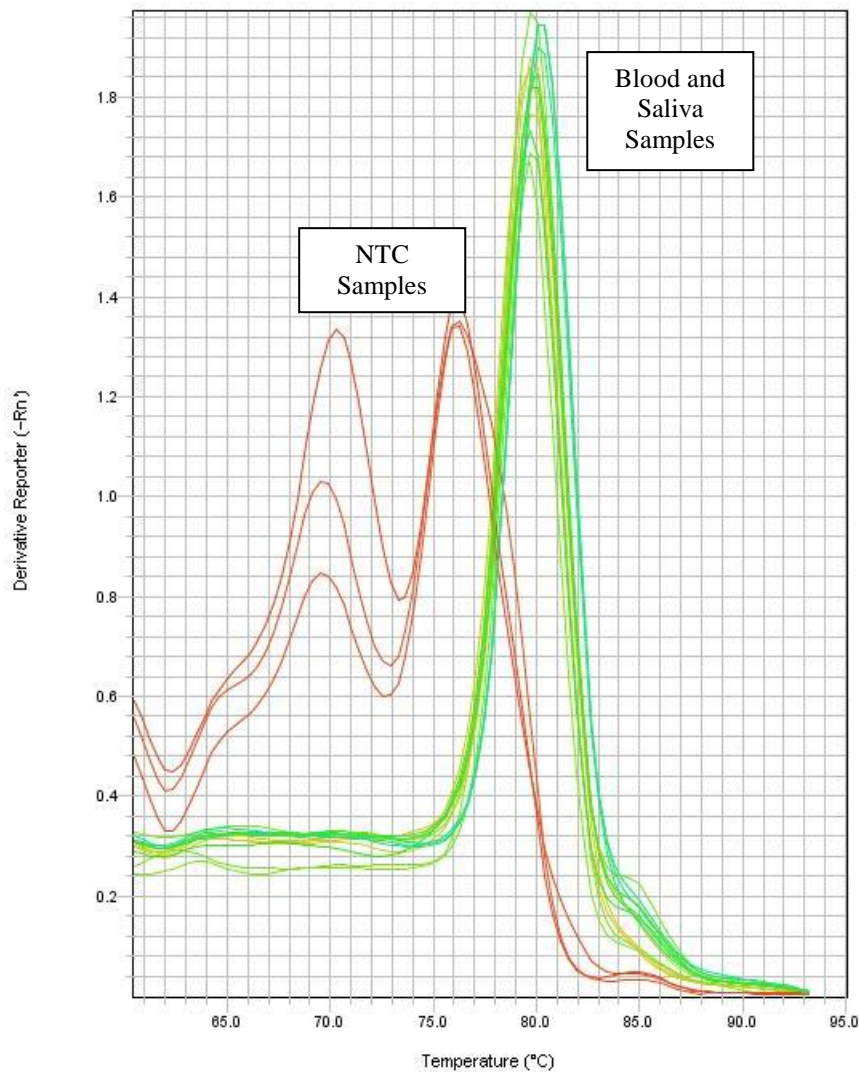


Figure 14: Melt curve analysis for 18s in NTCs and blood and saliva samples.

### 3.2.6 Reverse Transcription negative control amplification

Amplification was detected in all 18s reverse transcription negative control samples (RT NTC). The  $C_t$  values for these were higher than the  $C_t$  values observed in the test samples, indicating a lower level of expression, the  $T_m$  values were however  $\sim 79^\circ\text{C}$ , suggesting that the amplification may be specific yet very low level. The majority of all other RT NTC samples were negative, the odd replicate showing amplification in one or two samples. The  $C_t$  values of any amplified RT NTCs were never the same as the  $C_t$  values for

the corresponding test samples, an indication that some low level contamination may have occurred, most probably due to the lack of specificity of SYBR Green®, which will bind to any dsDNA and the ubiquitous nature of 18s rRNA.

### 3.2.7 Relative quantitation and $\Delta C_t$ expression of specific markers KRT4, SPTB, STATH and NCF2

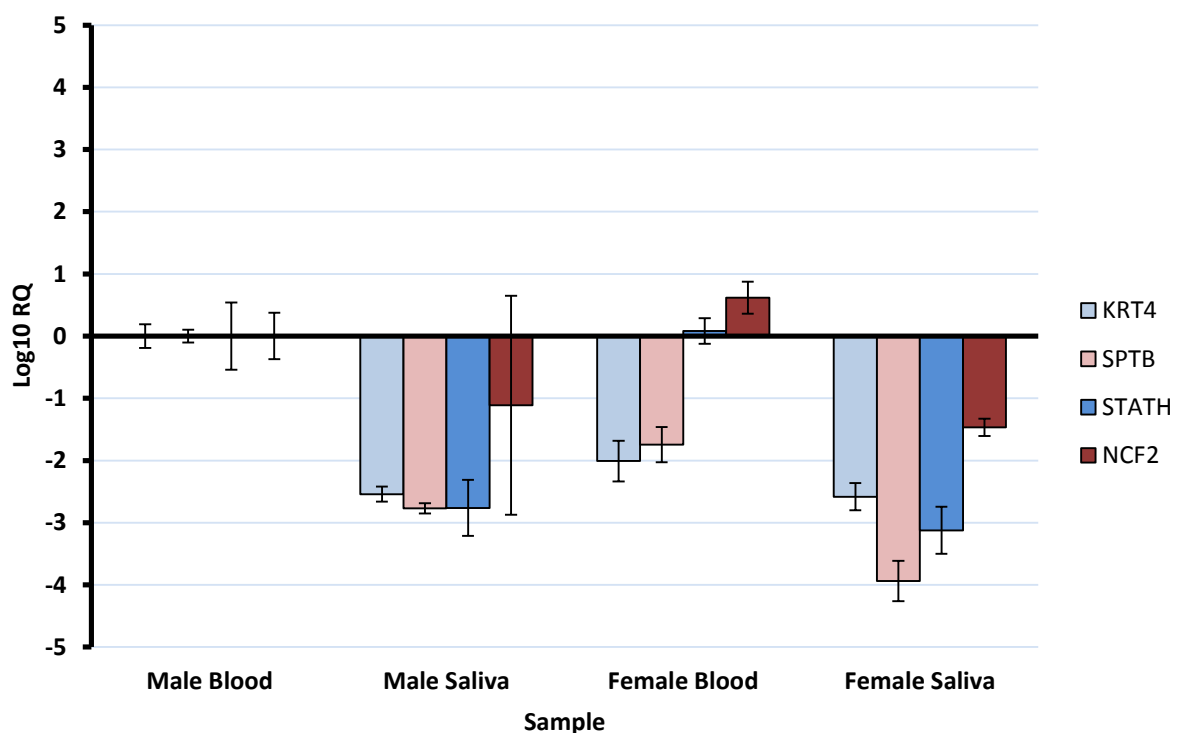


Figure 15: Relative expression of blood-targeted genes SPTB and NCF2 and saliva-targeted genes KRT4 and STATH in male and female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference. The calibrator sample in each experiment was male blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in Figure 15 were based on two separate studies investigating the expression of saliva-specific markers KRT4 and STATH and blood-specific markers SPTB and NCF2 in both male and female blood and saliva stains.



The results of these studies in Figure 15 revealed a down-regulation of KRT4, and SPTB in all three samples compared to male blood, indicating a higher expression of these markers in male blood. You would expect this for the SPTB marker given that it is blood-specific, but not KRT4 as the latter is saliva-specific. Overall a positive result for the SPTB marker with regards to the specificity.

A down-regulation of STATH was observed in both male and female saliva samples (-2.76 and -3.12 respectively), and an up-regulation (0.08) was seen in the female blood sample, compared to the male blood sample. This suggests that more STATH is present in the male blood sample than both saliva samples, and even more STATH present in female blood compared to the male blood, both results you would not expect given that STATH is saliva specific.

NCF2 was under expressed in both male and female saliva samples (-1.11 and -1.47 respectively), and an over expression of this marker was seen in the female blood compared to male blood suggesting that the levels of NCF2 were higher in the female donor in this particular study. This result again demonstrated good specificity for this particular marker as you would expect there to be less NCF2 in saliva than in blood.

Statistical analysis demonstrated that the expression of KRT4 was significantly lower in male saliva compared to male blood ( $p \leq 0.01$ ; Table 14). There was no significant difference observed for the lower expression of KRT4 in female saliva compared to female blood. SPTB again however was significantly lower in male saliva compared to male blood ( $p \leq 0.01$ ), and also significantly lower in female saliva compared to female blood ( $p \leq 0.05$ ). The expression of STATH was significantly lower in male saliva compared to male blood, with the same result for female saliva compared to female blood ( $p \leq 0.01$ ). No significant difference was observed for the lower expression of NCF2 in male saliva compared with male

blood. The expression of NCF2 in female saliva however was significantly lower compared to female blood ( $p \leq 0.01$ ).

*Table 14:  $\Delta C_t$  values for blood targeted genes SPTB and NCF2, and saliva targeted genes KRT4 and STATH in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ )*

Marker	$\Delta C_t$ Value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
KRT4	$3.99 \pm 0.23$	$12.43 \pm 0.14^{**}$	$10.66 \pm 0.39$	$12.57 \pm 0.26$
SPTB	$3.68 \pm 0.12$	$12.88 \pm 0.10^{**}$	$9.48 \pm 0.34$	$16.76 \pm 0.39^*$
STATH	$10.94 \pm 0.65$	$20.12 \pm 0.54^{**}$	$10.67 \pm 0.25$	$21.32 \pm 0.45^{**}$
NCF2	$8.41 \pm 0.45$	$12.10 \pm 2.10$	$6.36 \pm 0.31$	$13.28 \pm 0.17^{**}$

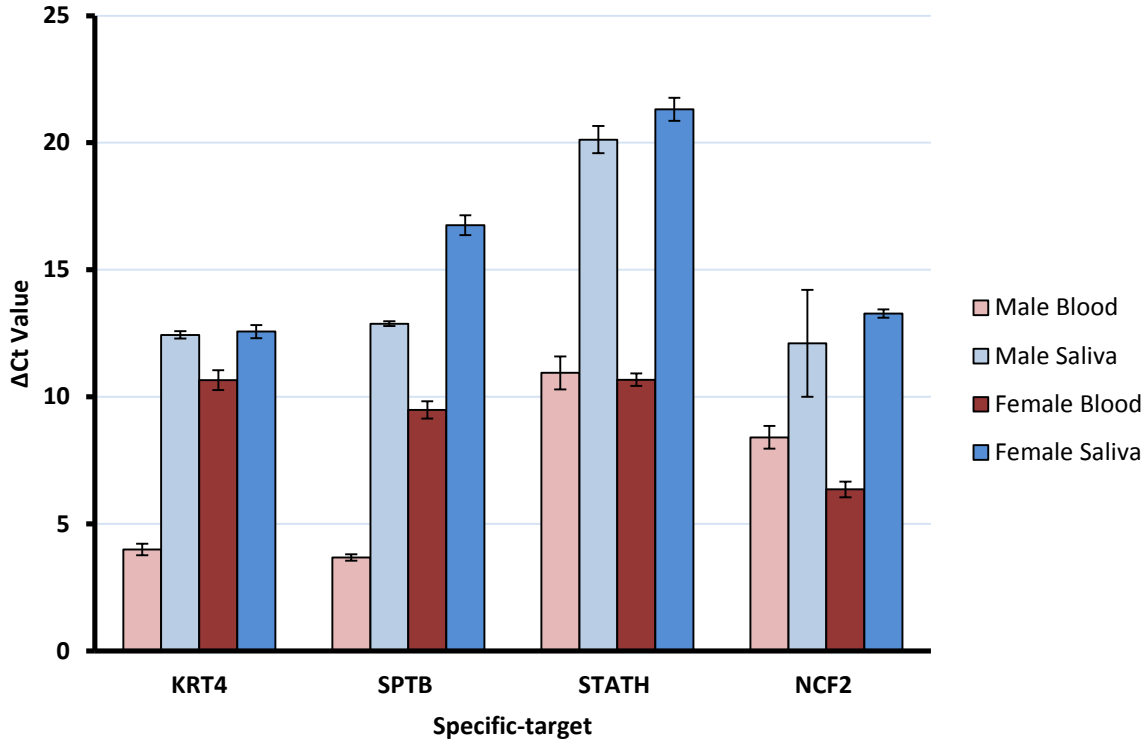


Figure 16: RT-PCR for blood and saliva-specific markers in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ . Low  $\Delta Ct$  values correspond to a high expression level of the specific mRNA.

Observation of the  $\Delta Ct$  values in these studies demonstrated an almost consistent expression pattern for each marker in female blood, with the exception of NCF2, which showed a slightly higher expression (Figure 16).

The  $\Delta Ct$  values in the first study show that the expression of KRT4 was higher in both male and female saliva than SPTB, and that the expression of SPTB was higher in both male and female blood compared to KRT4. Even though there appeared to be more KRT4 in the saliva samples than SPTB, the expression of KRT4 in both blood samples was higher than saliva in this study, a result you would not expect given that KRT4 is saliva-specific. A higher expression of SPTB was observed in the blood samples compared to saliva in this study, another positive result for SPTB showing good specificity for this marker.

The  $\Delta Ct$  values in the second study revealed that the expression of NCF2 was higher in both male and female blood samples compared to STATH, however in contrast, the

expression of STATH was lower in male and female saliva samples compared to NCF2. The blood marker NCF2 showed good specificity in this study, as there was a higher expression of this marker in both blood samples than saliva. The opposite was observed for saliva marker STATH, which demonstrated a higher expression in blood than saliva.

It can be seen in both studies that there is variation in the expression levels of different markers between individuals.

### **3.2.8 Negative control amplification and melt curve analysis**

Amplification was detected in all NTCs in both studies. In the first study, the SPTB NTC gave a higher  $C_t$  value than all the test samples indicating a lower level of expression. The melt curve data for SPTB was consistent with the latter two previous results for SPTB (Section 3.2.2 and 3.2.5) where one peak was observed rather than two and the  $T_m$  values were the same also.

The KRT4 NTC sample demonstrated a higher  $C_t$  value than both the male and female saliva test samples, indicating a lower expression in this NTC sample compare to saliva. In comparison to both the blood test samples the KRT4 NTC  $C_t$  value was very similar. Analysis of the melt curve data showed the same melting characteristics as previous KRT4 melt curves (Figure 5; Section 3.1.3). The data confirmed that the amplification observed in the KRT4 blood and NTC samples were non-specific, as both of these samples exhibited a small peak/hump to the left with a low  $T_m$  value, typical characteristics of primer-dimer artefacts. The main melting peak for KRT4 in saliva however was again much larger and exhibited a higher  $T_m$  value, suggesting that the amplification in this sample was specific to KRT4. Previous analysis of the  $\Delta C_t$  values in Figure 16 showed a higher expression of KRT4 in blood compared with saliva, casting doubt on the specificity of this marker.

However, the melt curve data suggests this was more than likely due to non-specific amplification; this information, coupled with the RQ data thus shows good marker specificity.

In the second study, the melt curve data for NCF2 demonstrated that specific amplification occurred in the NTCs, as peaks for these samples peaked at the same  $T_m$  value as the test samples  $\sim 82^\circ\text{C}$ , smaller peaks/humps were also observed an indication of primer-dimers. This amplification may be the result of low level contamination or possibly due to the high number of PCR cycles coupled with the background fluorescence of SYBR<sup>®</sup> Green dye.

Just as in previous studies, the melt curve data for the STATH NTCs demonstrated that the peak for these samples peaked at the same  $T_m$  as the test samples, which all exhibited a low  $T_m \sim 68^\circ\text{C}$ , characteristic of primer-dimer artefacts, thus indicating non-specific amplification in these samples.

In all cases, the 18s NTCs demonstrated a higher  $C_t$  value than the test samples, indicating a lower level of expression. The melt curve data in each study was similar to that observed in Figure 16; Section 3.2.5, where two peaks were observed, both of which peaked at a different  $T_m$  value to the test samples, thus suggesting that the amplification observed in these NTCs was non-specific. The  $T_m$  value for the test samples was consistent with all other studies  $\sim 80^\circ\text{C}$ .

### **3.2.9 Reverse Transcription negative control amplification**

In the first study, amplification was detected in the male blood 18s RT NTC sample and the female saliva 18s RT NTC, the other 18s RT NTCs (male saliva and female blood) were negative.

The  $C_t$  values for both the male blood and female saliva 18s RT NTCs were much higher than the  $C_t$  values observed in the corresponding test samples, thus indicating low level specific amplification may have occurred in these samples due to the lack of specificity of SYBR Green®. The melt curves for these samples also suggested specific amplification, with both peaks peaking at the same  $T_m$  as the test samples but at a lower intensity. All other RT NTC samples for markers SPTB and KRT4 were negative.

In the second study, amplification was seen in all the RT NTCs, and the  $C_t$  values were very similar to those observed in the corresponding test samples. Thus, casting doubt on the specificity of amplification observed in the NCF2 samples. It has already been established that STATH in this study did not appear to work; however the fact that similar amplification was observed in the RT NTCs for NCF2 as in the test samples suggests that the amplification may not have been mRNA and could possibly be DNA. Again a disadvantage of using SYBR Green dye is that will bind to any genomic DNA (gDNA) present in a sample, sometimes making it difficult to distinguish whether or not the amplification/ detected fluorescence is due to the targeted sequence of non-specific amplification.

### **3.2.10 Relative quantitation and $\Delta C_t$ expression of specific markers NCF2, HTN3, KRT4 and SPRR1A**

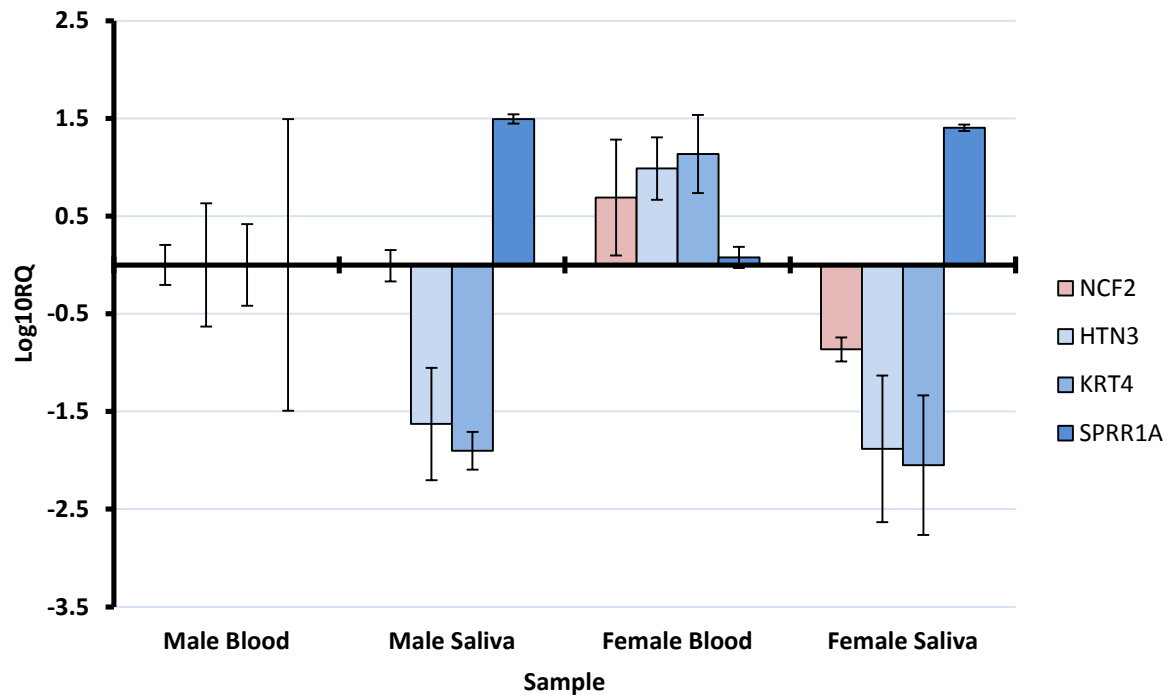


Figure 17: Relative expression of blood-targeted gene NCF2 and saliva-targeted genes HTN3, KRT4 and SPRR1A in male and female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference, the formula used to create the gene expression plot was  $2^{-\Delta\Delta CT}$  and the calibrator sample in each experiment was male blood. The error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in figure 17 were based on one study investigating the expression of saliva-specific markers HTN3, KRT4 and SPRR1A and blood-specific marker NCF2 in both male and female blood and saliva stains.

The results revealed a down regulation for HTN3 (-1.63 and -1.88) and KRT4 (-1.90 and -2.05) in both male and female saliva samples, indicating a higher expression of these markers in the male blood sample. An up-regulation of SPRR1A was observed in both male and female saliva samples (1.49 and 1.40 respectively); indicating a lower expression of this saliva marker in male blood, and a down regulation of NCF2 was observed in the female saliva sample (-0.86), indicating a higher expression of this marker in male blood.

Expression of NCF2 in male saliva did occur however the expression was so small (-0.01) that it could not be observed in the expression plot, this result supporting the specificity of NCF2 for blood.

Statistical analysis demonstrated that the expression of NCF2 was significantly lower in female saliva compared to female blood ( $p \leq 0.05$ ; Table 17). However, no significant difference was observed for the lower expression of NCF2 in male saliva compared with male blood. HTN3 was significantly lower in male saliva compared to male blood ( $p \leq 0.05$ ), and again in female saliva compared to female blood ( $p \leq 0.01$ ). KRT4 was also significantly lower in male saliva compared with male blood ( $p \leq 0.01$ ), and the again the same in female saliva and blood ( $p \leq 0.05$ ). There was no significant difference observed for the higher expression of SPRR1A in male saliva compared with male blood, however a significant difference was seen in the higher expression of SPRR1A in female saliva compared with female blood, in figure 17 a slight up-regulation was observed for SPRR1A in female blood.

Table 15:  $\Delta Ct$  values for blood targeted gene NCF2, and saliva targeted genes KRT4 and SPRR1A in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ ).

Marker	$\Delta Ct$ Value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
NCF2	10.05 $\pm$ 0.24	10.08 $\pm$ 0.19	7.76 $\pm$ 0.71	12.92 $\pm$ 0.15*
HTN3	9.97 $\pm$ 0.75	15.38 $\pm$ 0.69*	6.69 $\pm$ 0.38	16.23 $\pm$ 0.90**
KRT4	9.11 $\pm$ 0.86	15.43 $\pm$ 0.23**	5.34 $\pm$ 0.48	15.92 $\pm$ 0.85*



SPRR1A	$11.10 \pm 1.79$	$6.14 \pm 0.06$	$10.84 \pm 0.13$	$6.43 \pm 0.04^{**}$
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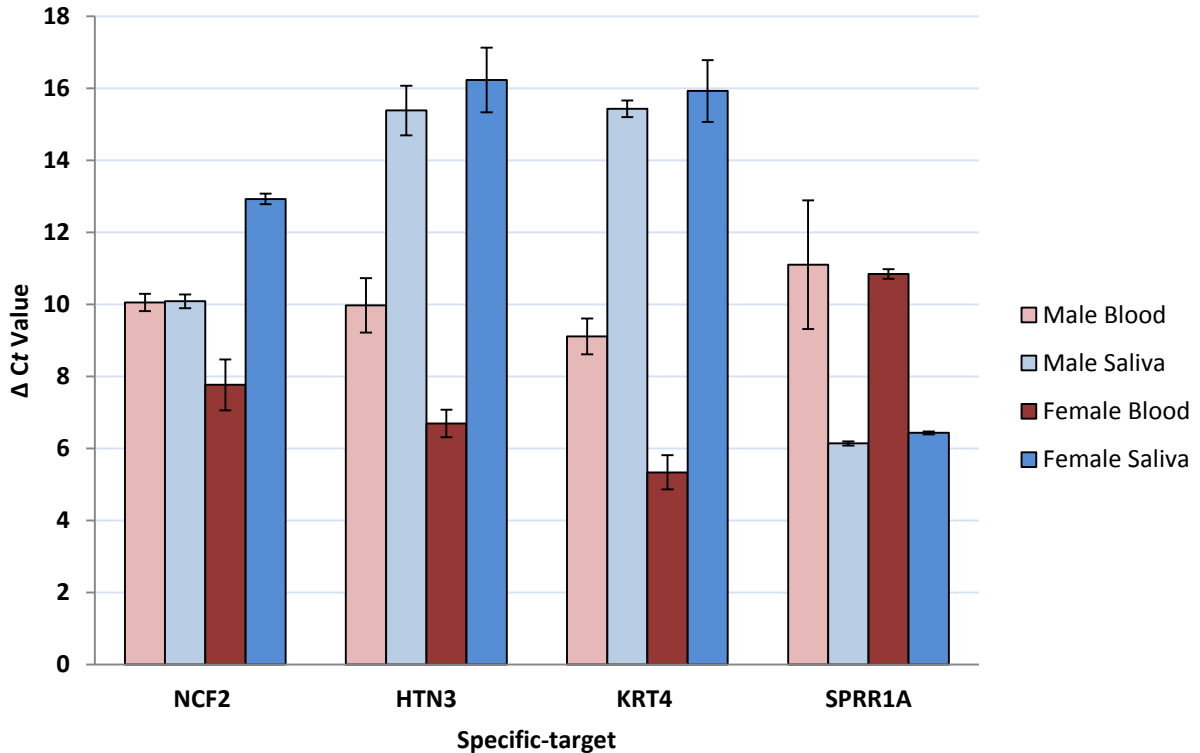


Figure 18: RT-PCR for blood and saliva-specific markers in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ . Low  $\Delta Ct$  values correspond to a high expression level of the specific mRNA.

Observation of the  $\Delta Ct$  values demonstrated a higher expression of NCF2 in the female blood sample compared to both the male and female saliva samples; however there appears to be the same expression of NCF2 in male blood as there is in male saliva.

Unexpectedly, a higher expression of HTN3 was observed in both blood samples compared to saliva, there even appeared to be more HTN3 in blood than NCF2. A similar expression of HTN3 was observed in male and female saliva samples, with the male sample showing the highest expression.

A higher expression of saliva-specific marker KRT4 according to the  $\Delta Ct$  values in Figure 19 was observed in both blood samples compared to saliva, again a result you would

not expect given that KRT4 is saliva specific. The expression of KRT4 in blood was also higher than the blood-specific marker NCF2 which you would not expect. The expression levels of KRT4 in male and female saliva were quite similar, again the male saliva sample showing a slightly higher expression, this result showing levels of expression of KRT4 can vary between individuals. In comparison to the saliva-specific marker HTN3, there appeared to be more KRT4 present in all samples, and unexpectedly, a higher expression of NCF2 in both male and female saliva was observed compared to both these markers.

A higher expression of SPRR1A was observed in both saliva samples compared to blood. A positive result for this marker given that it is saliva specific and you would expect the expression to be higher in saliva than in blood. There even appeared to be more SPRR1A marker in saliva compared to the blood marker NCF2, and also less SPRR1A in blood than NCF2. The expression of SPRR1A was much higher in saliva compared to the HTN3 and KRT4.

### **3.2.11 Negative control amplification and melt curve analysis**

Amplification was detected in all the NTCs in this study. The *Ct* value observed for the NCF2 NTC sample was similar to the *Ct* value observed in the male saliva test sample, but lower than the *Ct* values for the other three test samples, indicating a slightly higher expression level in the NTC sample compared to male blood, female blood and female saliva. Interestingly, the melt curve data for NCF2 differed to previous studies (Section 3.1.3 and 3.2.8); two peaks were observed for the NTC, the main  $T_m$  value being  $\sim 82^\circ\text{C}$  and the lower  $T_m$  value being  $\sim 75^\circ\text{C}$ , indication of specific amplification and primer-dimer artefacts. For both the male and female blood samples, one peak was observed  $\sim 76^\circ\text{C}$ , and for male and female saliva one peak was observed at  $\sim 82^\circ\text{C}$ . This result is therefore suggesting that in this

case the targeted amplicon was observed in saliva rather than blood, which you would not expect given that NCF2 is blood specific, and that specific amplification was observed in the NTC sample.

The *Ct* values observed for the HTN3 and KRT4 NTC samples were both lower than their corresponding test samples, indicating a slightly higher level of expression in the NTCs. The melt curve data for HTN3 demonstrated that the NTC sample peaked at the same  $T_m$  value as the test samples, and that these peaks had a  $T_m$  value of  $\sim 70^\circ\text{C}$ , so therefore are more than likely due to non-specific amplification, possibly due to primer-dimer formation. This result is suggestive that HTN3 may have failed to work in this study. The melt curve data for KRT4 demonstrated that the peak observed in the NTC had a  $T_m$  value of  $\sim 73^\circ\text{C}$ . This was the same  $T_m$  value observed in the male and female blood samples and the female saliva sample. For the male saliva sample, two peaks were observed, the main peak having a  $T_m$  value  $\sim 73^\circ\text{C}$ , and a smaller peak to the right exhibiting a  $T_m$  value of  $\sim 82^\circ\text{C}$ . This  $T_m$  value is almost the same as the  $T_m$  value observed for KRT4 in saliva in Figure 5, Section 3.1.3. However in this particular study, the peak with the larger  $T_m$  that corresponds to the targeted amplicon is much smaller and lower in intensity, indicating that the amplification of the targeted amplicon in this case may have been lower than the amplification of primer-dimers, this could explain why no second peak was observed in the female KRT4 saliva sample.

For the SPRR1A NTC sample however, a very high *Ct* value was observed  $\sim 44$ , this value is extremely close to the *Ct* max value 45 (where *Ct* max is the maximum number of PCR cycles), and is indication of very very low levels of expression. This *Ct* value was closer to *Ct* observed in the blood samples, in particular the female blood sample, but much higher than the *Ct* values observed in the saliva samples, thus supporting the specificity of this saliva marker. Analysis of the melt curve data showed that the peak for the SPRR1A NTC sample was small/low intensity, and exhibited a low  $T_m$  value of  $\sim 68^\circ\text{C}$ , characteristic

of primer-dimer formation. The test samples; male saliva and female saliva both exhibited two peaks, a smaller peak with a lower  $T_m$  value similar to the NTC, and a much a larger peak with a higher  $T_m$  value  $\sim 79^\circ\text{C}$ , suggesting that the amplification in these samples was due the targeted amplicon and specific to SPRR1A. The male and female blood test samples however, demonstrated one peak rather than two - similar to that of the NTC, an indication that the amplification in these samples was non-specific (Figure 19)

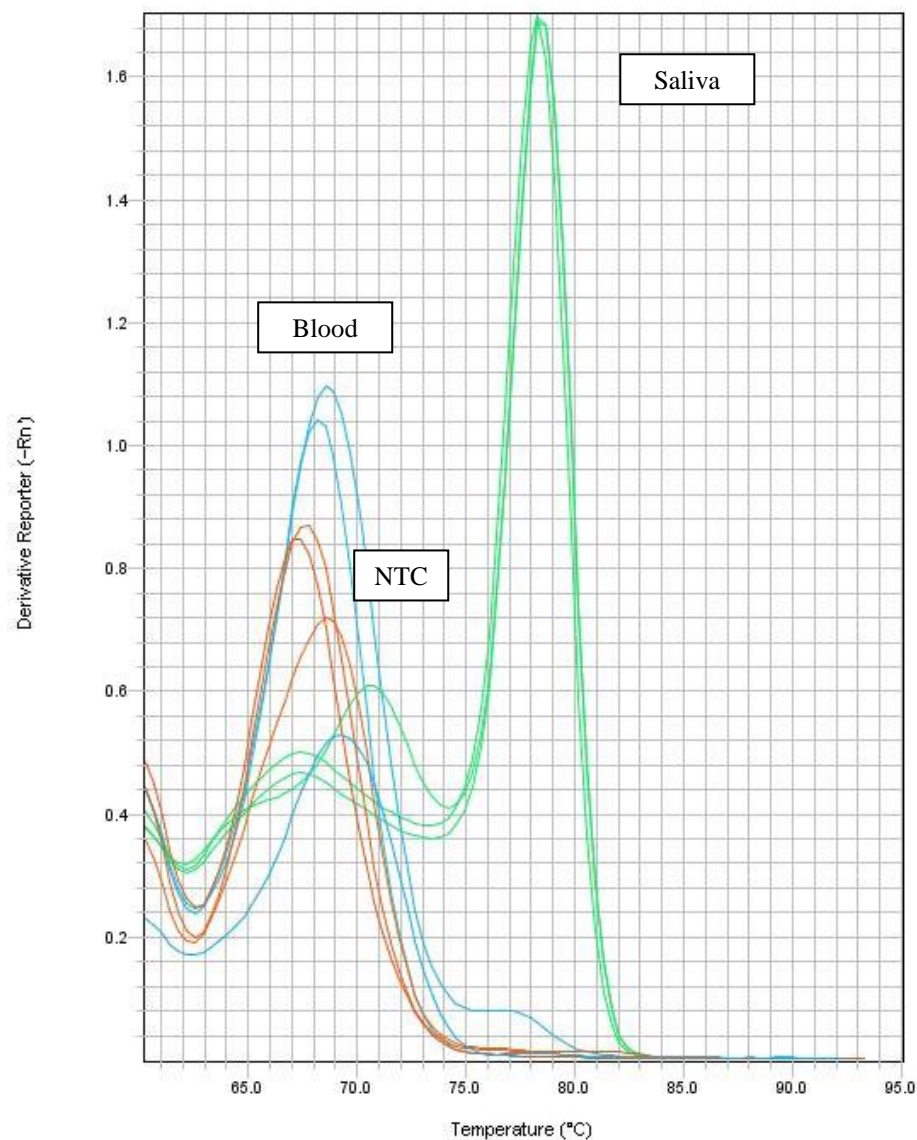


Figure 19: Melt curve analysis for saliva-specific SPRR1A in the negative control, blood and saliva samples.

In all cases, the 18s NTCs again demonstrated a higher  $C_t$  value than the test samples, indicating a lower level of expression. The melt curve data in this study was similar to that observed in Figure 16. Section 3.2.5, where two peaks were observed for the 18s NTC sample, both of which peaked at a different  $T_m$  value to the test samples, thus suggesting that the amplification observed in these NTCs was non-specific. The  $T_m$  value for the test samples was consistent with all other studies  $\sim 80^\circ\text{C}$  (Figure 20)

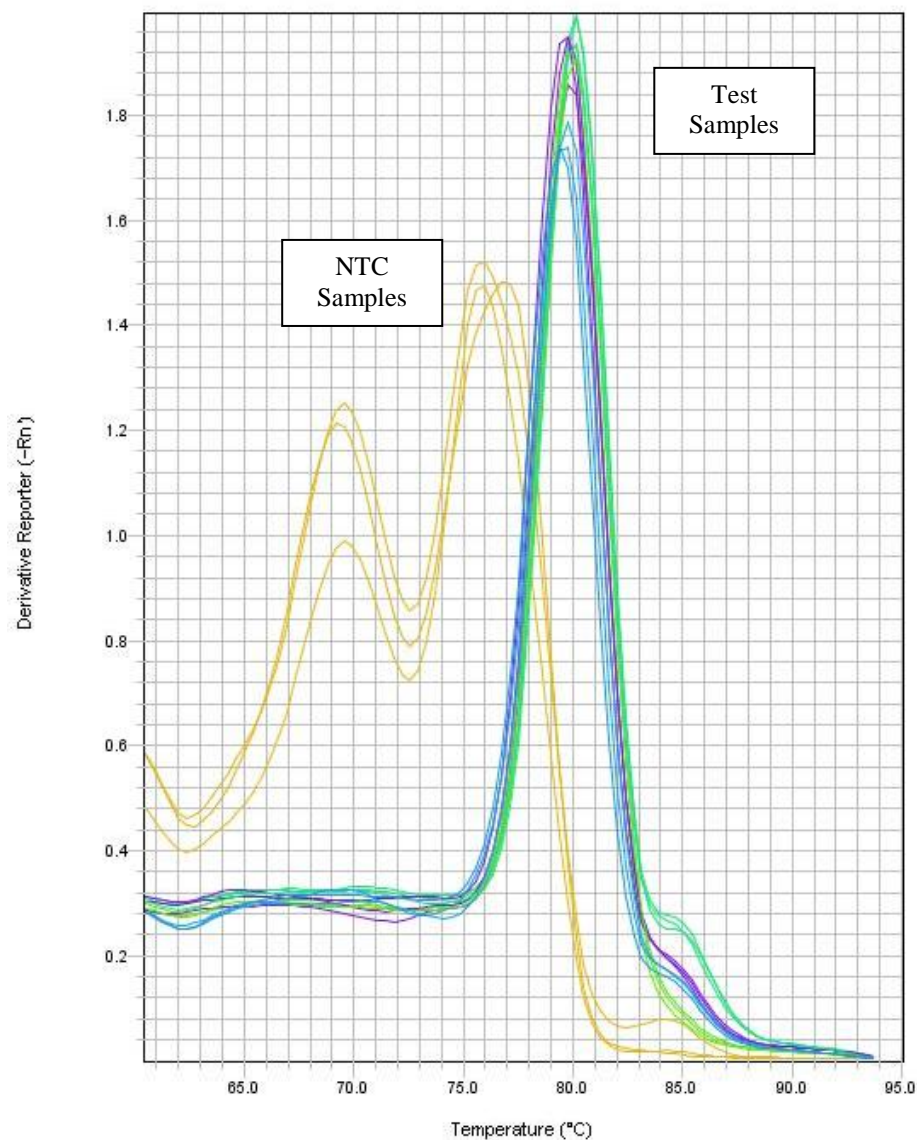


Figure 20: Melt curve analysis for 18s in the negative control and test samples

### 3.2.12 Reverse Transcription negative control amplification

Amplification was detected in the representative RT NTC samples, and analysis of the  $C_t$  values demonstrated that all the RT NTCs were similar to the corresponding test samples, again casting doubt on the amplification observed in the test samples. The melt curve data for these RT NTC samples also showed similar peaks to those observed in the test samples.

### 3.2.13 Relative quantitation and $\Delta C_t$ expression of specific markers NCF2, SPTB, KRT4 and SPRR1A

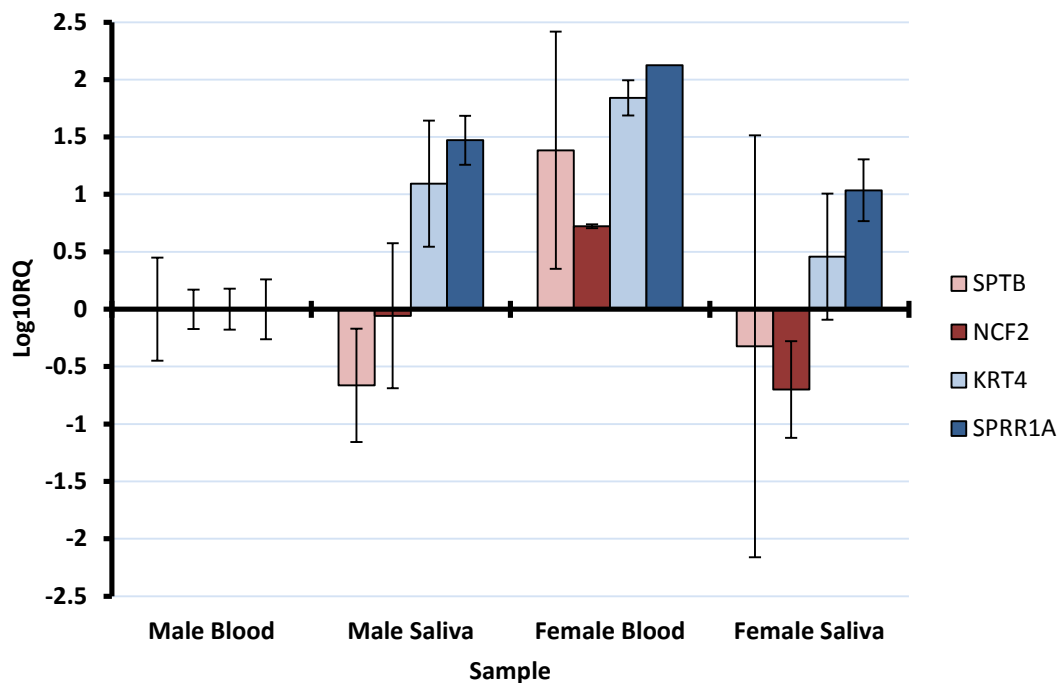


Figure 21: Relative expression of blood-targeted genes SPTB and NCF2 and saliva-targeted genes KRT4 and SPRR1A in male and female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference. The calibrator sample in each experiment was male blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in figure 21 were based on one study investigating the expression of saliva-specific markers KRT4 and SPRR1A and blood-specific markers SPTB and NCF2 in both male and female blood and saliva stains.

A down-regulation of blood markers SPTB and NCF2 was observed in both the male and female saliva samples, indicating a higher expression of these markers in blood. An up-regulation of saliva markers KRT4 and SPRR1A was observed in both male and female saliva samples, indicating a higher expression of these markers in saliva.

This result therefore shows good specificity for each marker in this study, where the blood markers were more present in blood and the saliva markers were more present in saliva.

Statistical analysis demonstrated that there was no significant difference observed in the lower expression of SPTB in either the male or female saliva sample compared with the corresponding blood samples. The under expression of NCF2 in male saliva compared to male blood was also not significant. In female saliva however, a significant difference was observed in the lower expression of NCF2 in this sample compared with female blood ( $p \leq 0.01$ ; Table 16). The higher expression of KRT4 in male saliva compared with male blood was significantly different ( $p \leq 0.01$ ), this was also the case for the higher expression of KRT4 in female saliva compared with female blood. SPRR1A was significantly higher in male saliva compared with male blood ( $p \leq 0.01$ ), however no significant difference was observed in the higher expression of this marker in female saliva compared with female blood.

Table 16:  $\Delta C_t$  values for blood targeted genes SPTB and NCF2, and saliva targeted genes KRT4 and SPRR1A in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ ). Result highlighted in red  $n=1$ .

Marker	$\Delta C_t$ Value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
SPTB	24.35 $\pm$ 0.54	26.55 $\pm$ 0.59	19.75 $\pm$ 1.24	25.42 $\pm$ 2.20
NCF2	19.52 $\pm$ 0.20	19.71 $\pm$ 0.76	17.12 $\pm$ 0.02	21.84 $\pm$ 0.50**
KRT4	22.19 $\pm$ 0.21	18.55 $\pm$ 0.66**	16.07 $\pm$ 0.18	20.67 $\pm$ 0.66**

SPRR1A	$23.46 \pm 0.31$	$18.57 \pm 0.26^{**}$	$16.39 \pm 0.00$	$20.02 \pm 0.32$
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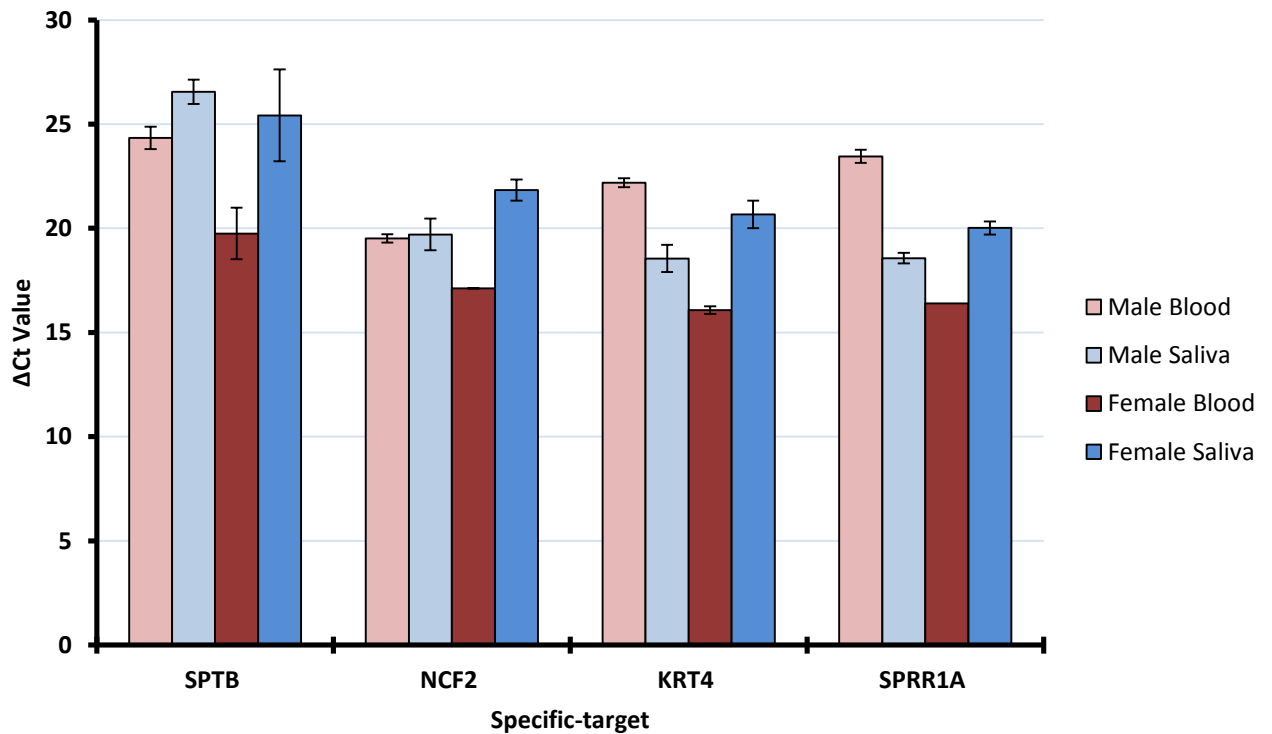


Figure 22: qRT-PCR for blood and saliva-specific markers in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$  (except SPRR1A in female blood where  $n=1$ ). Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

Observation of the  $\Delta C_t$  values demonstrated a higher expression of SPTB in female blood compared to male blood, there was also more SPTB present in blood than in saliva (Figure 22), indicating good specificity for this marker.

A higher expression of NCF2 was observed in both blood samples compared to saliva; however the expression observed in male blood was only slightly lower than the male saliva sample. These results also show that there was more NCF2 in both blood samples compared to SPTB.



Analysis of the  $\Delta C_t$  values for KRT4 demonstrates that the expression of this marker is higher in female blood than both male and female saliva samples, a result you would not expect given that this marker is saliva specific. The expression of this marker in male blood was lower than all samples, this sample therefore showing good marker specificity. According to these results there was more KRT4 present in female blood than both NCF2 and SPTB, and more KRT4 present in male blood than SPTB. Again the latter result you would not expect given that KRT4 is saliva specific. Analysis of the melt curve data will suggest whether or not this amplification is specific. The expression of KRT4 in both saliva samples was similar, again a slightly higher expression was observed in the male saliva sample.

The  $\Delta C_t$  values for SPRR1A were almost identical to that of KRT4 (Figure 22), expression of SPRR1A was higher in both saliva samples compared to the calibrator sample male blood; a result you would expect given SPRR1A is saliva-specific. However, Figure 22 shows that the expression levels of SPRR1A were higher in female blood compared to both the male and female saliva samples – melting curve analysis will suggest whether or not this amplification is specific. The expression levels of SPRR1A in both saliva samples were similar, the higher expression again observed in male saliva.

#### **3.2.14 Negative control amplification and melt curve analysis**

Amplification was observed in all the NTC samples. The  $C_t$  value for the SPTB NTC sample was lower than all the corresponding test samples but not by much. The melt curve data for SPTB was again consistent with previous studies for SPTB (Section 3.2.2, 3.2.5, and 3.2.8), where one peak was observed rather than two, and the  $T_m$  values were the same also, with exception for one replicate in the male SPTB blood sample that showed a peak at  $\sim 80^\circ\text{C}$ .

The  $C_t$  value for the NCF2 NTC sample was similar to both the male blood and saliva samples, but lower than the values obtained for the female blood and saliva samples. In this study, the melt curve data for the NCF2 NTC samples showed a large peak at  $\sim 83^\circ\text{C}$ . The test samples all showed two peaks, the smaller peaks exhibiting a lower  $T_m$  and the larger peaks exhibiting higher  $T_m$  value  $\sim 80^\circ\text{C}$ , suggesting that this amplification observed in the test samples was due to the targeted amplicon and the smaller peaks are probably due to primer-dimer artefacts. The fact that the NCF2 NTC sample exhibited one main peak at such a high  $T_m$  value suggests that this amplification is more than likely due to specific amplification too, thus indicating low level contamination may have occurred.

The  $C_t$  value for the KRT4 NTC sample gave a similar value to the male blood sample and both the female blood and saliva sample. The male saliva  $C_t$  value in this study however was much higher indicating a lower level of expression. Analysis of the melt curve data demonstrated that the KRT4 NTC sample exhibited a peak with a  $T_m$  value  $\sim 72^\circ\text{C}$ , this was also the case for male and female blood KRT4 test samples. In the KRT4 saliva samples for both male and female two peaks were observed, the smaller peak consistent with the  $T_m$  values observed for the NTC and blood samples, and a larger peak that exhibited a higher  $T_m$  value  $\sim 80^\circ\text{C}$ . This value was again very similar to the  $T_m$  values observed in previous melt curves for KRT4 in saliva (Figure 5, Section 3.1.3). This result therefore suggests that the amplification observed in the NTC and blood samples was non-specific, and that the amplification observed in the saliva samples was as result of specific amplification for the saliva marker KRT4, thus showing good specificity for this marker. In Figure 22, the  $\Delta C_t$  values indicated there to be more KRT4 in the female blood sample compared to both saliva samples, the melt curve data shows that the amplification observed in those blood samples was a result of non-specific amplification.

Analysis of the  $C_t$  value for SPRR1A NTC demonstrated that this value was similar to the  $C_t$  values observed in both the male and female blood samples, and that both saliva samples exhibited a lower  $C_t$  value, indicating a higher level of expression in both these test samples compared to the blood and NTCs. This result supports the specificity of this marker, as you would expect the expression of SPRR1A to be higher in saliva than in blood, it suggests that the amplification observed in blood could be the same as the NTC and may be non-specific. The melt curve data was almost identical to Figure 19, Section 3.2.11, where two peaks were observed for the saliva samples and one peak was observed for the NTC and blood samples. The  $T_m$  values for each sample were also very similar, thus suggesting that specific amplification occurred in the saliva samples for SPRR1A, and that the amplification observed in the NTC and blood samples was more than likely non-specific amplification.

Again, the 18s NTC sample demonstrated a much higher  $C_t$  value than the test samples, indicating a very lower level of expression. The melt curve data for the 18s NTCs in this study was again similar to Figure 14, Section 3.2.5 and Figure 20, Section 3.2.11, where two peaks were observed for the NTC, both of which peaked at a different  $T_m$  value to the test samples, thus suggesting that the amplification observed in these NTCs was non-specific. The  $T_m$  value for the test samples was consistent with all other studies  $\sim 80^\circ\text{C}$ .

### **3.2.15 Reverse Transcription negative control amplification**

Amplification was detected in the representative RT NTC samples. The  $C_t$  values for the male blood SPTB RT NTC and female blood NCF2 RT NTC were similar to their corresponding test samples, indicating the same level of expression in both the test and RT NTC samples. The  $C_t$  values for the male saliva KRT4 RT NTC and female saliva SPRR1A

RT NTC were higher than their corresponding tests samples, thus indicating a lower level of expression in the RT NTCs.

Analysis of the melt curve data demonstrated that the peak for the male blood SPTB RT NTC sample peaked at the same  $T_m$  as the corresponding test sample. The melt curve data for the male saliva KRT4 RT NTC sample demonstrated one peak that was consistent with the smaller peak in the KRT4 male saliva sample i.e. primer-dimer artefacts, no second peak was observed therefore indicating that the amplification observed in this RT NTC sample was non-specific. The melting peak observed for the female blood NCF2 RT NTC sample also demonstrated one peak that peaked at the same  $T_m$  value as the smaller peak in the female blood NCF2 test sample. There was also no second peak in this RT NTC sample, thus indicating that the amplification was non-specific. Analysis of the melt curve for the female saliva SPRR1A RT NTC sample demonstrated one peak which corresponded to the smaller peak observed in the corresponding test sample. Thus indicating that this RT NTC was non-specific (Figure 23).

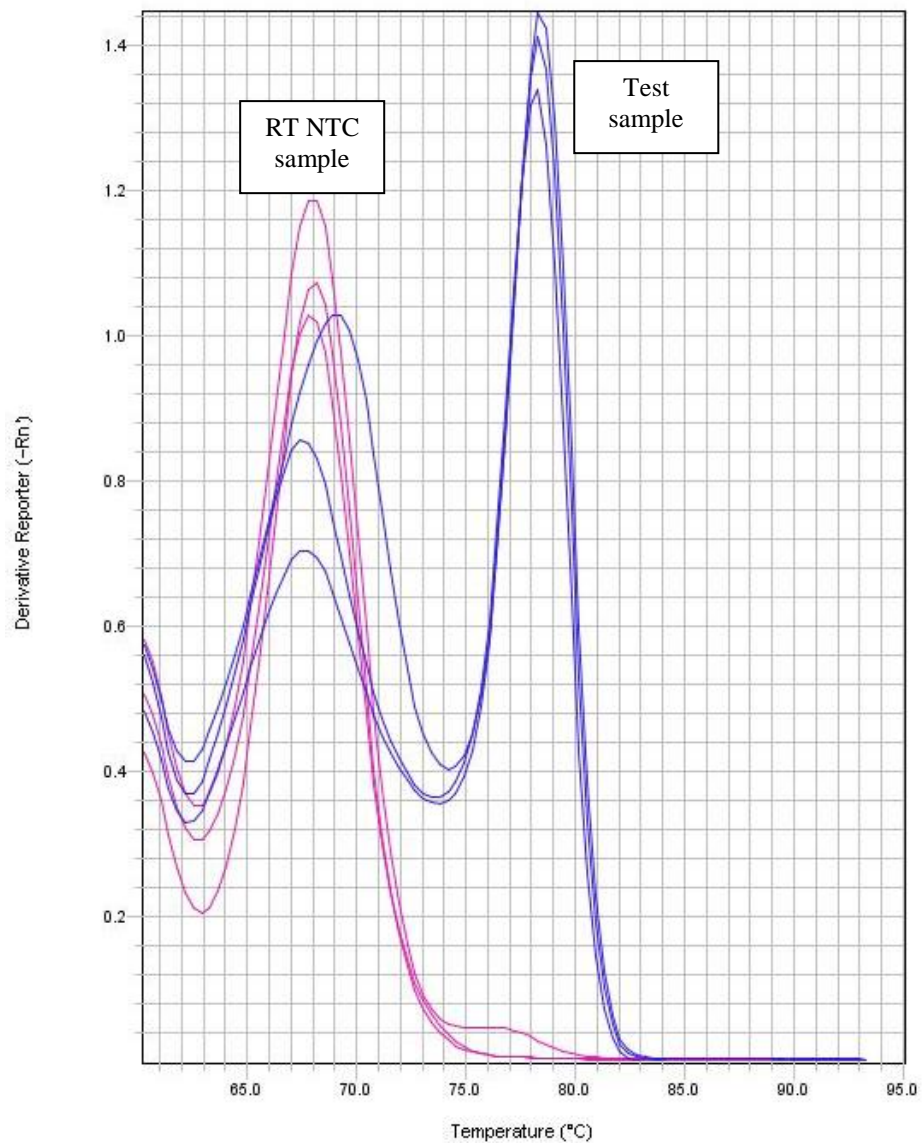


Figure 23: Melt curve analysis of the female saliva SPRR1A RT NTC sample compared to the female saliva SPRR1A test sample

### 3.2.16 Expression of endogenous control 18s rRNA in blood and saliva stains.

Table 17: Raw Ct values for endogenous control 18s rRNA in male and female blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$ , ( $p \leq 0.01^{**}$ ). The data highlighted red indicates zero significant difference indicating equal expression of 18s rRNA.

Study	18s rRNA Ct value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
1	27.03 $\pm$ 0.66	15.92 $\pm$ 0.02**	24.12 $\pm$ 0.15	17.42 $\pm$ 0.11**
2	26.82 $\pm$ 0.06	17.29 $\pm$ 0.10**	21.35 $\pm$ 0.41	12.90 $\pm$ 0.05**
3	16.16 $\pm$ 0.21	16.90 $\pm$ 0.07	18.60 $\pm$ 0.32	12.54 $\pm$ 0.13**
4	17.09 $\pm$ 0.44	11.39 $\pm$ 0.98**	18.00 $\pm$ 0.21	11.17 $\pm$ 0.08**
5	26.39 $\pm$ 0.09	14.12 $\pm$ 0.09**	26.64 $\pm$ 0.09	20.56 $\pm$ 0.02**
6	24.96 $\pm$ 0.10	15.54 $\pm$ 0.07**	19.54 $\pm$ 0.30	11.44 $\pm$ 0.20**
7	26.30 $\pm$ 0.27	15.83 $\pm$ 0.05**	28.12 $\pm$ 0.08	19.87 $\pm$ 0.04**
8	25.48 $\pm$ 0.017	18.28 $\pm$ 0.04**	29.12 $\pm$ 0.13	18.25 $\pm$ 0.01**
9	10.42 $\pm$ 0.13	7.87 $\pm$ 0.25**	15.78 $\pm$ 0.02	10.06 $\pm$ 0.10**

As mentioned earlier in this chapter (Section 3.1.2, an ideal endogenous control should be expressed at a constant level in different tissues, and in each study throughout this chapter, this has not been the case.

### 3.3 A quantitative comparison of blood and saliva specific markers in blood and saliva stains utilising Taqman<sup>®</sup> hydrolysis probes and 18s rRNA endogenous control.

#### 3.3.1 Relative quantitation and $\Delta C_t$ expression of blood and saliva specific markers

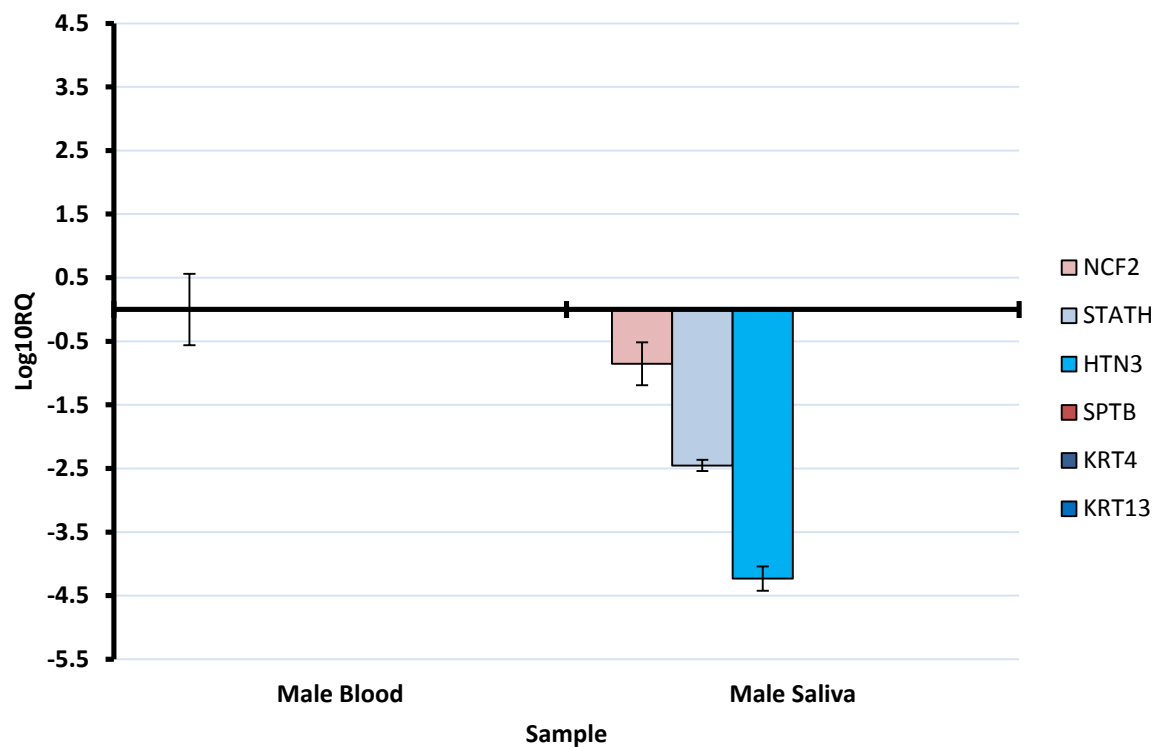


Figure 24: Relative expression of blood-targeted genes *NCF2* and *SPTB*, and saliva-targeted genes *STATH*, *HTN3*, *KRT4* and *KRT13* in male blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference. The calibrator sample in each experiment was male blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in Figure 24 were based on one study investigating the expression of saliva-specific markers *KRT4*, *KRT13*, *STATH* and *HTN3* and blood-specific markers *SPTB* and *NCF2* in both male and female blood and saliva stains, this time using Taqman<sup>®</sup> Gene Expression Assays rather than SYBR<sup>®</sup> Green dye.

A down-regulation was observed for *NCF2* (-0.85) in the saliva sample compared to blood, indicating a higher expression in blood than saliva. A down-regulation was also

observed for saliva specific markers STATH (-2.45) and HTN3 (-4.23) in saliva compared to blood.

In Figure 24, there is no RQ data for markers SPTB, KRT4 and KRT13; this was due to amplification occurring in one sample and not the other, preventing a comparative analysis. As a result, the data for these markers was expressed as  $\Delta C_t$  and is shown in Table 18 and Figure 25.

Statistical analysis demonstrated that the expression of NCF2 was significantly higher in blood than saliva ( $p \leq 0.01$ ; Table 15). Statistical comparison for other markers between body fluids was not possible, due to the fact that these markers were mainly detected in one body fluid over the other. However, the absence of amplification and the inability to do a comparative analysis does not negate a result; an absent result alone could be meaningful. For example in Table 15, the saliva specific markers KRT4 and KRT13 showed zero detectable amplification in the male blood samples compared to saliva, a result you would expect given that these markers are specific to saliva. Amplification did occur for STATH and HTN3 in the male blood samples however only one replicate showed detectable amplification, the other two replicates showed zero amplification, whereas in the male saliva samples, all replicates showed amplification for these markers. Zero amplification was detected for blood marker SPTB in male saliva, yet the result for SPTB in blood was based on one replicate showing detectable amplification, decreasing the value of this particular result.



Table 18:  $\Delta Ct$  values for blood targeted genes *NCF2* and *SPTB*, and saliva targeted genes *KRT4*, *KRT13*, *STATH* and *HTN3* in male blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ . Zero values correspond to no expression of target. Data highlighted red is based on amplification occurring in one replicate, ( $p \leq 0.01^{**}$ ).

Marker	$\Delta Ct$ value	
	Male Blood	Male Saliva
NCF2	12.20 $\pm$ 0.67	15.03 $\pm$ 0.40**
SPTB	17.67	0.00
KRT4	0.00	15.13 $\pm$ 0.08
KRT13	0.00	12.20 $\pm$ 0.08
STATH	4.54	12.68 $\pm$ 0.10
HTN3	3.09	17.14 $\pm$ 0.23

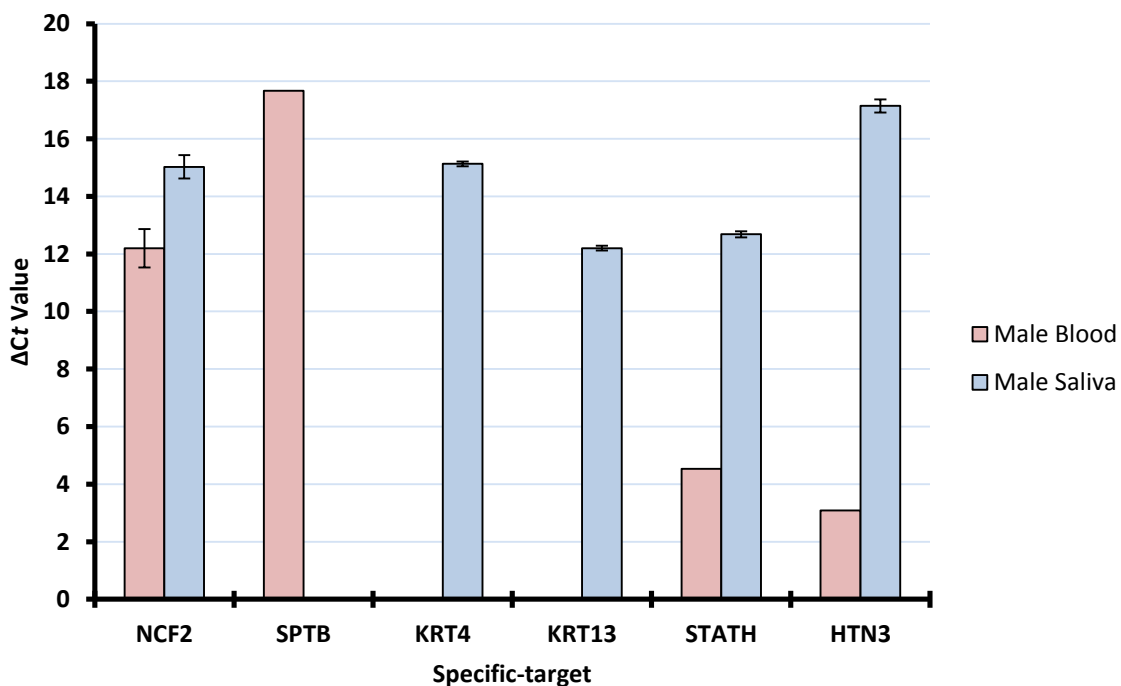


Figure 25: qRT-PCR for blood and saliva-specific markers in male blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$  (except in cases where  $n < 3$ ). Low  $\Delta Ct$  values correspond to a high expression level of the specific mRNA.

Analysis of the  $\Delta Ct$  values in Figure 25 demonstrated that the blood-specific marker *NCF2* was expressed in both blood and saliva, and that a higher expression was observed in

the blood sample, showing good specificity for this marker. Expression of the blood-specific marker SPTB was also observed in blood and not saliva, however this data was based on one out of three replicates showing amplification. Saliva-specific markers KRT4 and KRT13 were both expressed in saliva and not blood, thus both markers showing very good specificity to saliva – in this study there appeared to be more KRT13 present in saliva than KRT4. For the other saliva markers; STATH and HTN3, the  $\Delta C_t$  values show a higher expression was observed in blood than saliva, however this data is inaccurate as it was based on one out of three replicates showing detectable amplification. Amplification of these markers in the saliva samples however occurred in all replicates, thus from this data it can be suggested that STATH and HTN3 are more specific to saliva than blood.

### 3.3.2 Relative quantitation and $\Delta C_t$ expression of blood and saliva specific markers

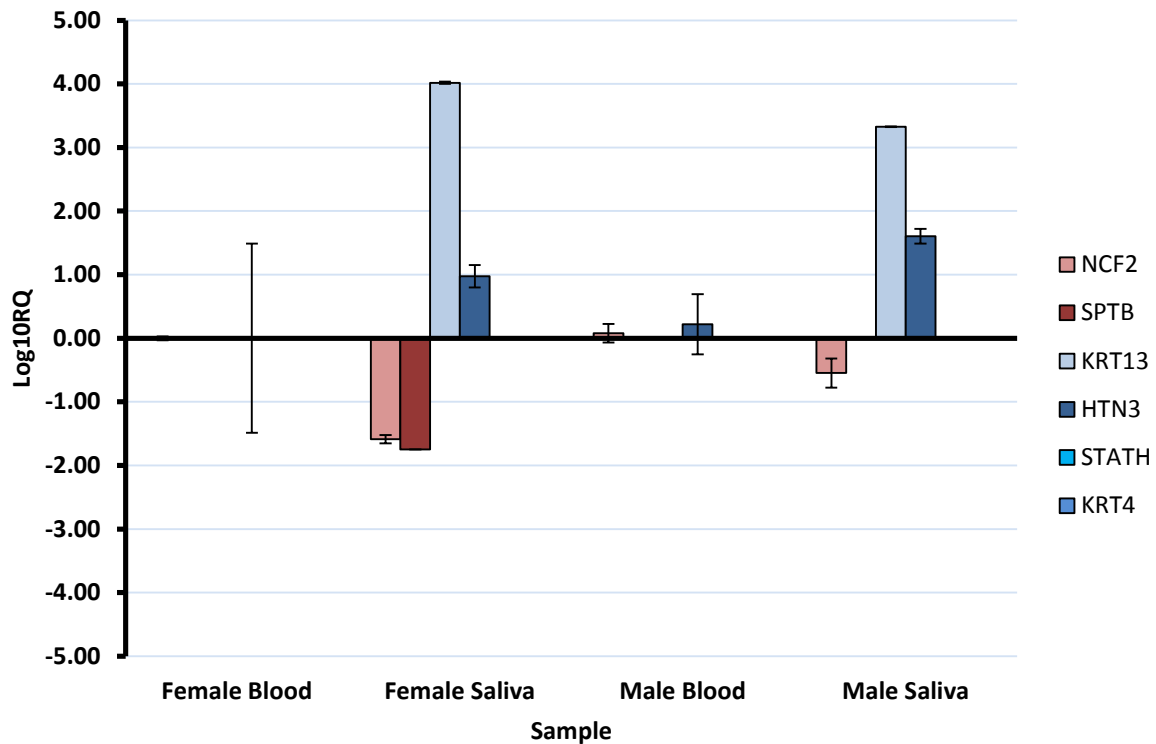


Figure 26: Relative expression of blood-targeted genes *NCF2* and *SPTB*, and saliva-targeted genes *KRT13*, *HTN3*, *STATH* and *KRT4* in male and female blood and saliva stains. The results were normalised to reference gene *18s rRNA* and expressed as a fold difference. The calibrator sample in each experiment was female blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in Figure 26 were based on one study investigating the expression of saliva-specific markers *KRT4*, *KRT13*, *STATH* and *HTN3* and blood-specific markers *SPTB* and *NCF2* in both male and female blood and saliva stains, this time using Taqman® Gene Expression Assays.

A down-regulation was observed for blood-specific markers *NCF2* (-1.59) and *SPTB* (-1.75) in female saliva, indicating a higher expression of these markers in female blood, *NCF2* was also under expressed in male saliva (-0.55) compared to female blood, no expression for *SPTB* was observed in the male saliva sample. These results all show good specificity for both blood markers. An up-regulation was observed for saliva-specific markers *KRT4* and *HTN3* in both male and female saliva samples (4.02, 0.97, 3.32 and 1.60

respectively), indicating a lower expression of these markers in the female blood sample. No expression data was observed in the RQ gene expression plot for markers STATH and KRT4. This was due to amplification occurring one sample rather than the both, therefore a relative comparison could not be made. The amplification for these saliva markers was expressed as  $\Delta Ct$  instead (Figure 27), and the results are listed in Table 17. For both of these saliva markers, zero amplification was detected in both male and female blood samples, showing good marker specificity.

In both male and female samples, statistical analysis demonstrated that the expression of NCF2 was significantly higher in blood than saliva ( $p \leq 0.05$ ; Table 19). There was however, no significant difference in the expression of HTN3 in both male and female blood and saliva samples. For all other markers a statistical difference between body fluids could not be established as amplification mainly occurred in one body fluid, this absence of data again does not negate the result, and is in fact meaningful with regards to the specificity of these markers.

Table 19:  $\Delta Ct$  values for blood targeted genes NCF2 and SPTB, and saliva targeted genes KRT4, KRT13, STATH and HTN3 in male and female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ . Zero values correspond to no expression of target. Data highlighted red is based on amplification occurring in one replicate, ( $p \leq 0.05^*$ ;  $p \leq 0.01^{**}$ )

Marker	$\Delta Ct$ value			
	Male Blood	Male Saliva	Female Blood	Female Saliva
NCF2	14.28 $\pm$ 0.17	16.37 $\pm$ 0.28*	14.55 $\pm$ 0.04	19.82 $\pm$ 0.08**
SPTB	0.00	0.00	17.50 $\pm$ 0.48	23.31
KRT4	0.00	10.97 $\pm$ 0.02	0.00	10.75 $\pm$ 0.01
KRT13	0.00	7.45 $\pm$ 0.01	18.50	5.15 $\pm$ 0.03
STATH	0.00	14.69 $\pm$ 0.14	0.00	16.43 $\pm$ 0.05

HTN3	16.25 ± 0.40	11.66 ± 0.14	16.98 ± 1.78	13.74 ± 0.21
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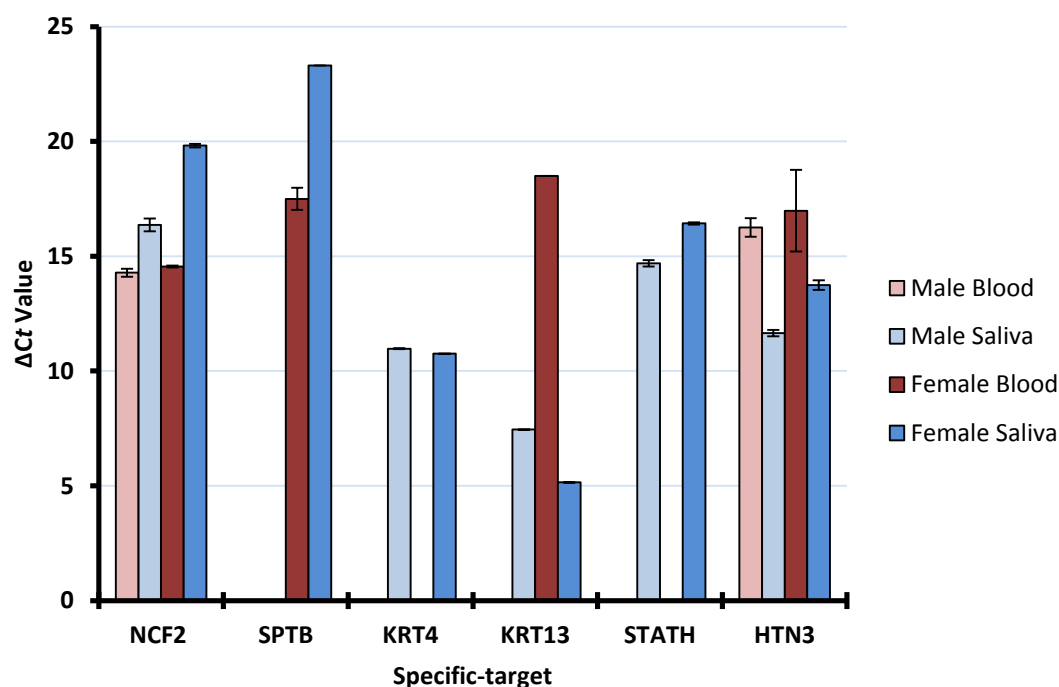


Figure 27: qRT-PCR for blood and saliva-specific markers in male blood and saliva stains. Results are mean ± SEM,  $n = 3$  (except in cases where  $n < 3$ ). Low  $\Delta Ct$  values correspond to a high expression level of the specific mRNA.

The  $\Delta Ct$  values in Figure 27 demonstrate that the expression of NCF2 was again higher in both male and female blood samples compared to saliva, just like previous results, therefore showing good specificity for this marker. SPTB was also higher in female blood than female saliva – the  $\Delta Ct$  value for the latter sample being higher than the blood, and based on one replicate showing amplification, thus showing good marker specificity. SPTB was not present in the male blood sample in this particular study, which you would expect given that SPTB is blood-specific, but this result is similar to the result in section 3.3.1, where only one replicate showed amplification for SPTB in the male blood sample and zero amplification occurred in the male saliva sample. It may be that SPTB was expressed higher in the female donor sample in this study.

Just as in the previous study, good specificity was shown for saliva marker KRT4, where zero amplification was detected in either the male or female blood samples but expression occurred in both the male and female saliva samples. The expression levels in these latter samples were also quite similar to one another, with female blood showing a slightly higher expression (Figure 27).

KRT13 was shown again to be saliva specific, the  $\Delta Ct$  values demonstrate a higher expression of this marker in both male and female saliva compared to blood, the highest expression observed in female saliva. No amplification was detected in the male blood sample for KRT13. This was a very positive result with regards to the specificity of this marker, as the zero amplification indicates an absence of KRT13 in that particular blood sample. The  $\Delta Ct$  value observed for KRT13 in female blood, which was higher than those observed in saliva, was based on one replicate showing amplification, the result therefore indicates a very low level expression of KRT13 in this female blood.

The  $\Delta Ct$  values for STATH were very similar in both the male and female saliva samples, with the male sample showing a slightly higher expression. Zero amplification was detected in either the male or female blood samples, showing excellent marker specificity for STATH given that it is saliva-specific.

Lastly, a higher expression of HTN3 was observed in both the male and female saliva samples than blood. The expression levels were again quite similar with the higher expression observed in male saliva. Even though statistical analysis showed there was no significant difference in the expression of HTN3 in saliva compared to blood, the results in figure 27 at least support the specificity of this marker.

### 3.3.3 Relative quantitation and $\Delta C_t$ expression of blood and saliva specific markers

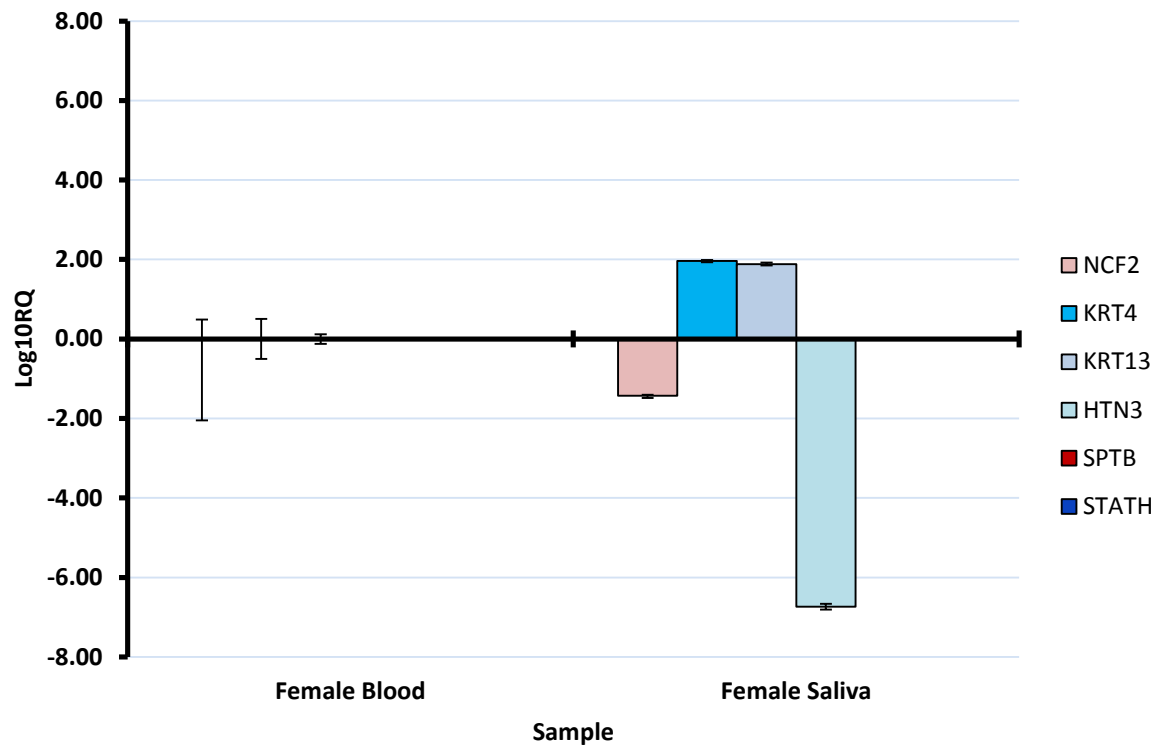


Figure 28: Relative expression of blood-targeted genes NCF2 and SPTB, and saliva-targeted genes KRT4, KRT13 and HTN3 in female blood and saliva stains. The results were normalised to reference gene 18s rRNA and expressed as a fold difference. The calibrator sample in each experiment was female blood. Error bars were calculated using the RQ minimum and maximum data,  $n = 3$ .

The results in Figure 28 were based on one study, again repeating the investigation of the expression of saliva-specific markers KRT4, KRT13, STATH and HTN3 and blood-specific markers SPTB and NCF2 in both male and female blood and saliva stains, this time using Taqman<sup>®</sup> Gene Expression Assays.

Just as in previous results, a down-regulation was observed for NCF2 (-1.43) in saliva compared to blood, and an up-regulation of KRT4 (1.96) and KRT13 (1.89) was observed in saliva compared to blood, these results therefore showing good specificity for these markers. In contrast to previous results, a down-regulation was observed for HTN3 in saliva (-6.73),

this result suggesting that there was more HTN3 present in blood. However having observed the raw  $\Delta Ct$  values for this data, it was discovered that the amplification observed in the blood sample for HTN3 was based on one replicate rather than three (Table 20 and Figure 29), thus skewing the data in the plot.

For markers STATH and SPTB, no expression data was observed in the RQ gene expression plot (Figure 28). This was due to amplification occurring in one sample rather than both, therefore a relative comparison could not be made. The amplification for these markers was expressed as  $\Delta Ct$  instead (Figure 30), and the results are listed in Table 20.

Statistical analysis demonstrated that the expression of NCF2 was significantly higher in blood than in saliva ( $p \leq 0.01^{**}$ ). The expression of KRT4 and KRT13 was also significantly higher in saliva than in blood ( $p \leq 0.01^{**}$ ). For all other markers a statistical difference between body fluids could not be established as amplification mainly occurred in one body fluid, this absence of data again does not negate the result, and is in fact meaningful with regards to the specificity of these markers. Markers SPTB and STATH show excellent specificity in Table 18, as zero amplification was detected in saliva for SPTB and in blood for STATH. The  $\Delta Ct$  result for SPTB in female blood is consistent with the previous results in Section 3.3.2.

*Table 20:  $\Delta Ct$  values for blood targeted genes NCF2 and SPTB, and saliva targeted genes KRT4, KRT13 and HTN3 in female blood and saliva stains. The results are mean  $\pm$  SEM,  $n = 3$ . Zero values correspond to no expression of target. Data highlighted red is based on amplification occurring in one replicate, ( $p \leq 0.01^{**}$ )*

Marker	$\Delta Ct$ value	
	Female Blood	Female Saliva
NCF2	16.17 $\pm$ 0.37	20.92 $\pm$ 0.17**
SPTB	18.56 $\pm$ 0.12	0.00
KRT4	17.55 $\pm$ 0.60	11.04 $\pm$ 0.03**



KRT13	15.49 ± 0.15	9.22 ± 0.04**
STATH	0.00	17.15 ± 0.03
HTN3	-9.75	12.61 ± 0.09



Figure 29: RT-PCR for blood and saliva-specific markers in male blood and saliva stains. Results are mean ± SEM,  $n = 3$  (except in cases where  $n < 3$ ). Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

Observation of the  $\Delta C_t$  values demonstrated that NCF2 was again higher in blood than saliva, and that SPTB was only expressed in blood – showing very good marker specificity. Note that SPTB was not detected in the male samples in previous experiments but was detected in all replicates in the female blood sample in this study and the previous study.

The results for saliva specific markers KRT4 and KRT13 in this study differ from the two previous TaqMan<sup>®</sup> studies (Section 3.3.1 and 3.3.2). Previously, these markers either demonstrated zero amplification in blood, or amplification in just one replicate (KRT13 in female blood). In this particular study, KRT4 and KRT13 show detectable amplification in the female blood samples amplifying in all three replicates (Table 20). Thus, indicating that

the levels of these markers can vary in individuals at different times. The expression levels of KRT4 and KRT13 in these blood samples however, were still lower than the expression levels observed in saliva, indicating a higher expression in saliva, therefore supporting the specificity of these saliva-specific markers.

Good marker specificity was observed for STATH in this study also, as STATH was only expressed in saliva and not blood (Table 20)

Lastly, the  $\Delta Ct$  values for saliva-specific marker HTN3 demonstrated a higher expression in blood than saliva, thus showing decreased specificity for this marker. However, as mentioned previously, the  $\Delta Ct$  value for HTN3 in blood was based on one replicate showing amplification, whereas all replicates showed amplification in saliva, thus the latter may be a truer reading and thus support the specificity of the marker.

#### **3.3.4 Expression of endogenous control 18s rRNA in blood and saliva stains.**

Just as in previous SYBR® Green studies, the expression levels of eukaryotic 18s rRNA endogenous control in these studies was different in blood and saliva. Statistical analysis demonstrated that 18s rRNA in each study was again significantly higher in saliva than in blood ( $p \leq 0.01^{**}$ ; Table 21). A low  $Ct$  value corresponds to a higher expression level of the target.

Table 21: Raw Ct values for endogenous control 18s rRNA in male and female blood and saliva stains. Results are mean  $\pm$  SEM, n = 3, N/A corresponds to samples that were not investigated in that particular study. A low Ct value corresponds to a higher expression of the endogenous control, ( $p \leq 0.01^{**}$ ).

Study	18s rRNA Tm value			
	18s rRNA Tm value ( $^{\circ}\text{C}$ )			
	Male Blood	Male Saliva	Female Blood	Female Saliva
1	19.39 $\pm$ 0.03	16.76 $\pm$ 0.07**	N/A	N/A
2	21.08 $\pm$ 0.01	18.96 $\pm$ 0.00**	18.51 $\pm$ 0.02	14.96 $\pm$ 0.01**
3	N/A	N/A	18.22 $\pm$ 0.00	13.78 $\pm$ 0.03**

### 3.3.5 Other studies confirming the specificity of blood and saliva specific markers.

A further three Taqman® studies were repeated for the same markers, the results again showing good specificity for most markers, the exceptions being HTN3 and SPTB (Figure 31) – the separate studies denoted by a number 1 or 2. With regards to HTN3; firstly this marker was not detected in blood or saliva in the first study. Secondly, in the last study the expression of HTN3 was lower in saliva than blood, a result that showed good specificity, Comparison of the  $\Delta\text{Ct}$  values for HTN3 in the blood sample in the last study compared with the HTN3 in the saliva sample in the second study shows that these values are very similar, which decreases the specificity of this marker slightly, in that you would not expect to see the same level of expression of a saliva marker in both blood and saliva.

Analysis of the raw data however, demonstrated that in both blood samples that showed expression of HTN3, only two replicates showed amplification. All replicates were amplified for HTN3 in saliva and each exhibited a lower Ct value therefore supporting the specificity for saliva.

SPTB was only detected in two studies in two different blood samples, and even then analysis of the raw data showed that amplification did not occur in all replicates. This type of result is consistent throughout these Taqman<sup>®</sup> studies, where SPTB did not amplify in male blood; therefore the specificity of this marker seems good, but is not however always detectable in samples.

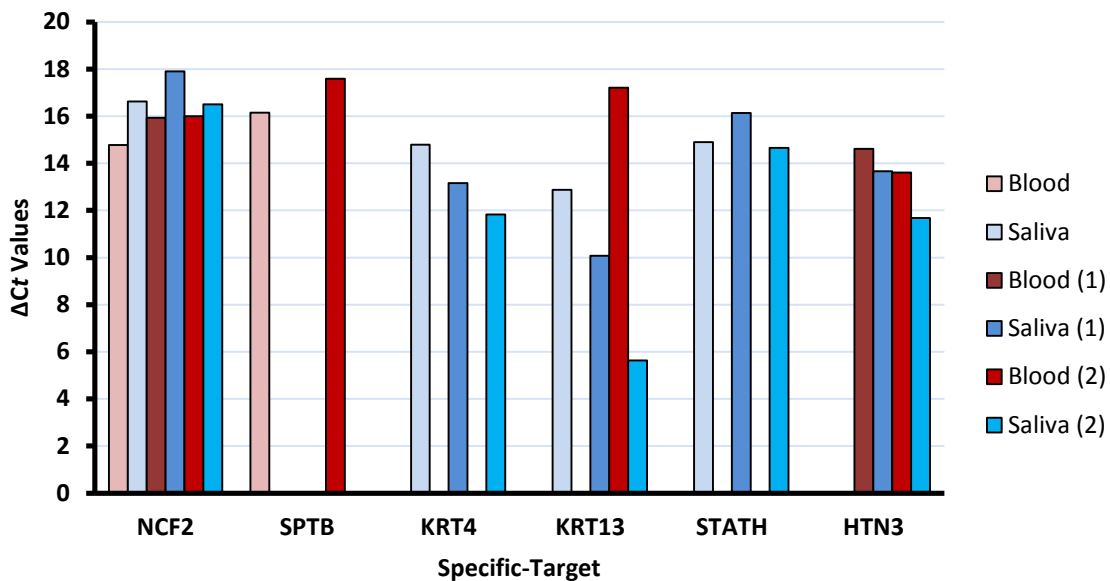


Figure 30: qRT-PCR for blood and saliva-specific markers in blood and saliva stains. Results are mean  $\pm$  SEM,  $n = 3$  (except in cases where  $n < 3$ ). Low  $\Delta C_t$  values correspond to a high expression level of the specific mRNA.

### 3.3.6 Negative control amplification using Taqman<sup>®</sup> hydrolysis probes

In SYBR<sup>®</sup> Green studies, where amplification was more often than not detected in the NTCs and the intercalating dye was non-specific, melt curve analysis was required and used to analyse the amplified product in the NTCs to confirm whether the amplification was specific. In contrast however, no amplification was detected in the negative control samples in the Taqman<sup>®</sup> studies, thus demonstrating the advantages of using Taqman<sup>®</sup> hydrolysis

probes in comparison to SYBR Green, and also showing no contamination had occurred (Figures 31 - 34).

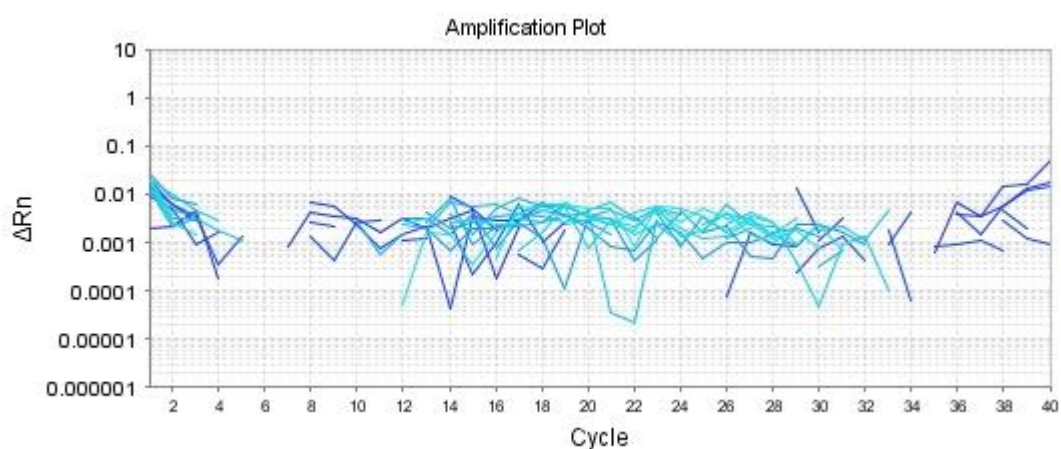


Figure 31: Real-time PCR results for reverse transcription negative controls using Taqman<sup>®</sup> hydrolysis probes.

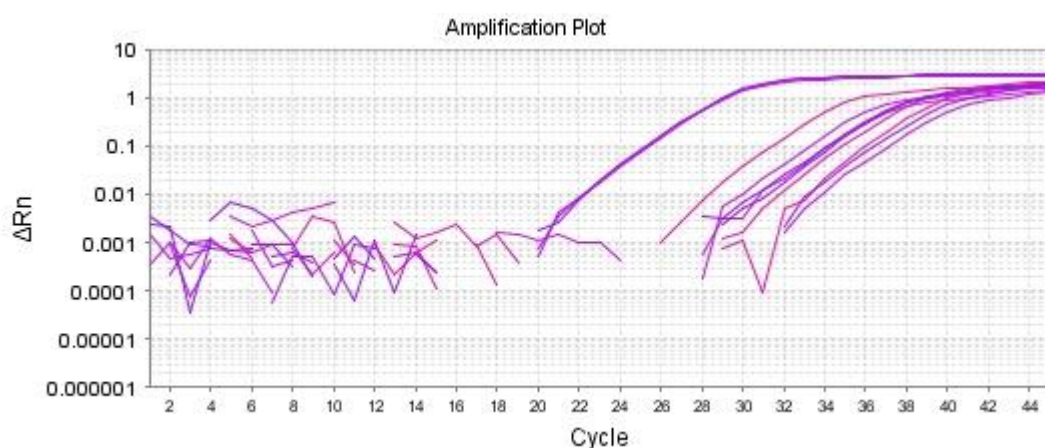


Figure 32: Real-time PCR results for reverse transcription negative controls using SYBR<sup>®</sup> Green fluorescent dye

A comparison of the reverse transcription negative control amplification samples (RT NTCs) in both Taqman<sup>®</sup> and SYBR<sup>®</sup> Green studies was undertaken.

Figure 31 shows a typical amplification plot for the RT NTCs in a Taqman<sup>®</sup> study, where zero amplification was observed. In contrast, Figure 32 shows an amplification plot from a SYBR<sup>®</sup> Green study where all RT NTCs showed significant amplification, more than

likely due to the non-specific nature of the SYBR<sup>®</sup> Green fluorescent dye, which will bind to any ds-DNA.

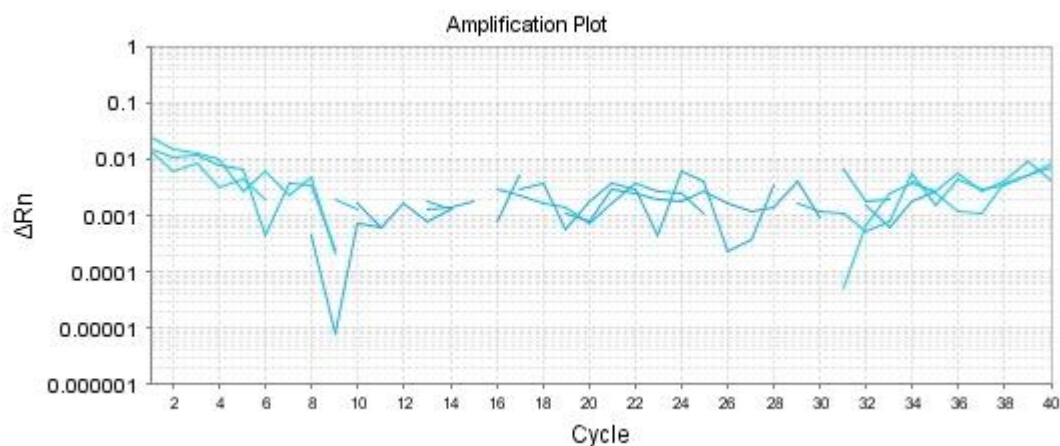


Figure 33: Real-time PCR results on negative control samples using Taqman fluorescent dye

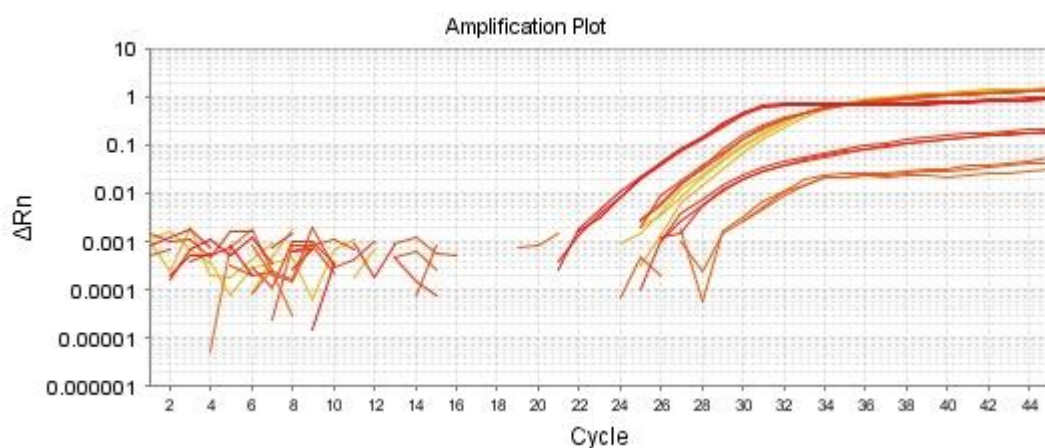


Figure 34: Real-time PCR results on negative control samples using SYBR Green fluorescent dye

A comparison of the negative control amplification samples (NTCs) in both Taqman<sup>®</sup> and SYBR<sup>®</sup> Green studies was also undertaken.

Figure 33 shows a typical amplification plot for NTC samples in a Taqman<sup>®</sup> study, where zero amplification occurred. Again in contrast, Figure 34 shows an amplification plot

from a SYBR Green® study, where some amplification was detected in the NTC samples, again demonstrating the non-specific nature of this type of chemistry

## **Chapter 4**

### **Discussion**



#### **4.1 Primer specificity using SYBR<sup>®</sup> Green**

The results from initial SYBR Green studies (three studies in total) revealed high specificity for blood-specific markers NCF2 and SPTB and saliva-specific marker KRT4. All three of these markers were present in both blood and saliva samples. NCF2 and SPTB were significantly under expressed in saliva signifying an over expression of these markers in blood. KRT4 was significantly over expressed in saliva signifying an under expression in blood. The specificity of these markers supports previous literature; markers NCF2 and KRT4 were reported to be highly specific and stable for use in forensic casework for the identification of blood and saliva, NCF2 being detected in 16 year old whole blood samples and KRT4 in 6 year old saliva stains (Zubakov et al., 2008, Zubakov et al., 2009). SPTB has also been reported as a specific marker for blood in a number of studies, (Haas et al., 2009a, Juusola and Ballantyne, 2005, Ballantyne and Juusola, 2007b, Haas et al., 2008, Haas et al., 2010, Haas et al., 2011a, Mindy et al., 2008, Haas et al., 2014, Patel and Peel, 2008). Blood specific marker PBGD and saliva specific marker HTN3 however, were not detected in either blood or saliva in these initial studies. This was an unexpected result given that previous literature reported good specificity for these markers. It is worth noting that even though a positive result was obtained for KRT4 in one study, when investigated in a second repeat study, this marker was undetected in blood and saliva, thus questioning the suitability of this marker with regards to sensitivity as well as specificity. NCF2 was also investigated in a second study, this marker was again detected in both blood and saliva samples and the result revealed an over expression in blood. This over expression however in this instance could not be subjected to statistical analysis due to the fact NCF2 was only detected in one out of three replicates in the saliva samples. Despite being able to statistically analyse this result, the NCF2 marker in this study again showed high specificity for blood, in that all three replicates in the blood samples showed expression for NCF2 in comparison to the saliva

sample that showed almost zero amplification. In these initial studies, two out of three investigated the expression of saliva-specific STATH in blood and saliva, it was initially thought that this marker was detected in blood in one study and saliva in another; however the melt curve data for these results indicated clearly that this amplification was non-specific and most likely due to primer-dimer formation. Thus, it can be said that in these studies, unexpectedly STATH was undetected in both blood and saliva samples, this result does not support previous literature which reported STATH to be a good mRNA marker for detection of saliva (Juusola and Ballantyne, 2003, Juusola and Ballantyne, 2005, Ballantyne and Juusola, 2007b).

Analysis of the  $\Delta C_t$  data confirmed the expression levels of the three markers that showed good specificity, in each case the blood specific markers NCF2 and SPTB exhibited lower  $\Delta C_t$  values in blood than in saliva, and the saliva specific marker KRT4 showed a lower value in saliva than in blood, a low  $\Delta C_t$  value indicates a higher expression of mRNA.

Interestingly, the melt curve data for saliva marker KRT4 in blood and saliva samples suggested that the amplification detected in the blood sample was due to non-specific amplification, and that the amplification observed in saliva was the result of targeting the sequence, thus revealing that KRT4 in this particular study was only detected in saliva, a positive result in terms of marker specificity. For NCF2, the melt curve data suggested that specific amplification occurred in both blood and saliva. Amplification of the targeted sequence in saliva was not a major cause for concern, since the expression levels observed in these studies revealed there was more NCF2 present in blood than in saliva. Thus, this melt curve data supports the real-time PCR result with regards to marker specificity, in that the as expected, NCF2 was detected in blood and at a much higher level than saliva. In the case of SPTB, the melt curve data proved more difficult to determine, the melting peaks suggested the amplification in blood and saliva may have been specific, as two peaks were observed; a

main peak and a smaller hump to the left. However, the peaks were not obviously separated like other melt curve data and the  $T_m$  of the main peak was quite low. Thus casting doubt as to whether or not the amplification was a result of targeting the sequence or due to non-specific amplification such as primer-dimer or low level contamination.

Amplification was detected in all 18s rRNA negative control samples (NTC) in these studies; this was expected given the ubiquitous nature of this reference gene and the non-specific nature of SYBR Green dye. This amplification also appeared to be specific according to the melt curve data however, the expression levels were very low according to the  $C_t$  values, and the melting peaks were of a much lower intensity in comparison to those observed for blood and saliva. All other negative control samples were negative with the exception of KRT4 NTCs (in the study that showed detectable amplification for KRT4) and SPTB NTCs. The melt curve data for KRT4 NTCs however suggested that this amplification was non-specific as the peaks exhibited typical primer dimer characteristics; in fact the peaks were almost identical to the peaks observed in the KRT4 blood sample. Albeit it not ideal having amplification in the NTC sample, the fact that two distinguishable peaks were observed for the saliva sample and NTCs supports the result in that the amplification in saliva was most probably due to targeting the KRT4 sequence. The melt curve data for SPTB NTCs suggested that the amplification observed in these samples was the same as the amplification observed in both blood and saliva, indicating that if the amplification observed in these samples were specific, then low level PCR product contamination may have occurred. Melting profiles are supportive and suggestive data used to increase the specificity of SYBR Green real-time PCR reactions, melting profiles on there own cannot be used as a sole way of validating the amplified products of a reaction, the negative control data and result of the real-time PCR reaction must also be taken in to consideration for successful interpretation of a result. As such, it can be said that in this particular study for SPTB, the data in its entirety is

suggestive that the correct amplified product may have been amplified however this could only be said with a low level of confidence due to the imperfect melt curves observed and the fact that the signals for the NTCs were not hugely different from the sample signals.

As the study progressed, further studies were performed to investigate the specificity of the specific markers mentioned above plus another two saliva-specific markers; KRT13 and SPRR1A. Again these markers were reported in the literature as stable and highly specific mRNA markers for the detection of saliva in body fluid identification tests in forensic casework (Zubakov et al., 2008, Zubakov et al., 2009). In these later studies, the number of samples increased; both male and female samples were subjected to analysis for all markers.

There results were illustrated in five different expression plots for clarity. In the first expression plot (Figure 9, Section 3.2.1), specific markers STATH and SPTB were investigated. The results revealed a significant under expression of SPTB in all saliva samples compared to blood. A positive result for the specificity of this marker as you would expect SPTB Under expression of STATH in all saliva samples also occurred compared with blood, this amplification later being confirmed by melt curve analysis to be non-specific, and as such it can be said that again this saliva-specific marker did not amplify in blood or saliva. Just as in previous studies, the melting profiles for SPTB could not confidently clarify whether or not the correct product had been amplified, this time only one peak was observed yet the  $T_m$  was still relatively low. Amplification also occurred in the NTC for SPTB. However, in one of these studies even though the same  $T_m$  was observed for the NTCs and samples, the  $C_t$  values for were notably different, thus suggesting that if the amplification in these samples was due to targeting the SPTB gene then low level PCR product contamination may have occurred. In this instance, if specific amplification has occurred, then the difference in these cycle numbers was big enough to be confident that the small amount of

product contamination was low enough to have little effect on the reliability of the sample signals. However, this was not the case in the other study for SPTB where the melting peaks for NTCs were the same as the sample peaks and the  $C_t$  values were also relatively close.

In the second gene expression plot (Figure 11; Section 3.2.4), SPTB was investigated again along with a saliva-specific marker KRT13. The results for SPTB again showed good specificity for blood in terms of the levels of expression observed in saliva samples compared to blood for both male and female samples, with the exception of one instance where SPTB was over expressed in a female saliva sample compared to male blood. The melt curve data however, was again very similar to previous results for this marker and amplification was again detected in the NTCs, it was therefore hard to distinguish whether or not specific amplification of the target had occurred. The expression levels between male and female samples also varied, in some cases more SPTB appeared to be present in male blood samples compared with female blood and *vice-versa*. Indicating that expression of this marker varies between individuals. The results for KRT13 revealed that in these studies, this marker showed very good specificity for saliva. According the gene expression plot, KRT13 initially appeared to over expressed in a couple of blood samples compared to saliva, however analysis of the melt curve data indicated that this was not the case and that the amplification observed in these samples was non-specific. The melting profile for KRT13 in saliva displayed typical characteristics for targeted sequence amplification; the peak was very distinctive to the blood sample peaks, being much larger in size and exhibiting a much higher  $T_m$  value. This data therefore supports data shown in the expression plot and is strongly indicates that KRT13 in this particular study was specific to saliva.

Negative control amplification was observed in the KRT13 NTCs but the data showed almost identical results to the amplification observed in blood, suggesting this amplification was non-specific and most likely due to primer-dimer formation. As expected, amplification

was again observed in all 18s NTC samples, in two out of three of these studies the 18s NTCs appeared to be specific just as in previous results, where the specific amplification was the result of very low levels of expression given the nature of the re gene and SYBR Green. In the third study however, the melting profiles gave a more positive result for 18s NTC amplification, as the peaks for these particular samples exhibited a lower  $T_m$  value than the test samples, thus indicating the amplification in these negative controls was more than likely due to non-specific amplification.

In the third gene expression plot (Figure 15, Section 3.2.7) markers NCF2, KRT4, STATH and SPTB were investigated. The results revealed good specificity for NCF2 and KRT4. NCF2 was under expressed in saliva compared to blood in all cases and analysis of the melt curve data suggested this amplification was specific to targeting the NCF2 gene. KRT4 initially appeared to be over expressed in blood samples compared to saliva, however analysis of the melt curve data indicated that the amplification in blood for this marker was due to non-specific amplification, thus showing good specificity for this marker in saliva. SPTB was again under expressed in all saliva samples compared to blood, a positive result in terms of specificity, however the melt curve data showed a low  $T_m$  value like previous melt curves for this marker making it difficult to be confident that the amplified product was specific. Saliva marker STATH showed expression data in the gene expression plot, however melt curve analysis again suggested this amplification was due to primer-dimer formation rather than specific amplification, thus it can be said that STATH was undetected in both blood and saliva samples in this particular study.

Amplification was observed in the NTCs for all markers in these studies including 18s NTCs. The melt curve data clarified that the SPTB NTCs were due to specific amplification however, importantly the expression levels were much lower than the test samples. The same was the case for NCF2 NTCs these result indicating that in these samples

low level PCR product contamination may have occurred. The melting peaks for the 18s NTCs and KRT4 NTCs showed these samples to be due to non-specific amplification.

In the fourth gene expression plot (Figure 19; Section 3.2.10), saliva specific markers HTN3, KRT4 and SPRR1A and blood marker NCF2 were investigated. The results revealed that again NCF2 was under expressed in saliva, initially showing good specificity, however the melt curve data appeared to show that the main peak in the melting profile was down to amplification in the saliva sample rather than blood a result you would not expect given that NCF2 is blood specific. . Also unexpectedly, KRT4 and HTN3 were under expressed in saliva; analysis of the entire data including the melt curve analysis demonstrated that the amplification observed for HTN3 was more than likely due to non-specific amplification indicating that this specific marker failed to amplify in all samples in these studies. In contrast to previous results, KRT4 in this study did not show positive data with regards to specificity for saliva,, the over expression of this marker in blood coupled with the melt curve data made it difficult to conclude whether or not specific amplification had taken place in these samples. Saliva specific marker SPRR1A however, gave a positive result in terms of specificity. This marker was over expressed in all saliva samples and the melt curve data suggested this amplification was due to specific amplification. Non-specific amplification was observed in the blood and NTCs for this marker, indicating these samples were the result of primer dimer formation, thus showing further specificity for this marker as it appears it was only detected in the saliva samples. The NTC amplification observed for this marker was also extremely low indicating very low levels of PCR product contamination.

Again all NTCs exhibited amplification in this study, some were a cause for concern as the demonstrated *Ct* values similar to test samples if not lower, with the exception of SPRR1A that showed the least level of expression in the NTCs. The 18s NTCs again showed non-specific amplification.

In the fifth gene expression plot (Figure 21, Section 3.2.13), specific markers SPTB, KRT4, SPRR1A and NCF2 were investigated. The results of this study revealed an over expression of SPTB and NCF2 in blood compared to saliva, and an over expression of SPRR1A and KRT4 in saliva than in blood, each of these markers showing good specificity for their respective body fluids. The melt curve data for SPTB again showed similar results to previous studies, however amplification in the NTCs for this marker showed the NTC  $C_t$  values were slightly lower than the test samples, indicating that if the amplification was due to the targeted sequence then a slightly higher expression of product was observed in the NTCs. The melt curve data for NCF2 marker suggested that the amplification observed in the blood and saliva samples was due to amplification of the targeted sequence. This was a positive result for the specificity of this marker, as the expression levels were much higher in blood than saliva. The melt curve data did however show that specific amplification occurred in the NTC sample for this marker, and that the  $C_t$  value was quite similar to the test samples thus questioning the reliability of this data. Like the majority of previous studies, the melt curve data for KRT4 supported the real-time PCR expression data for this marker and showed good marker specific. The amplification observed in the saliva samples appeared to be specific to KRT4, and the amplification observed in blood as well as the NTCs appeared to be non-specific amplification. A result you would expect given that KRT4 is saliva specific, ideally you would expect to see no primer-dimer formation or non-specific amplification in the blood and NTC samples however, given the nature of the detection chemistry this appears unlikely. Lastly, the melt curve data for SPRR1A was almost identical to the previous result; the data suggested specific amplification occurred in the saliva samples and non-specific amplification occurred in the blood and NTCs, indicating SPRR1A was only detected in saliva. Again, NTC amplification varied in samples some showed very low levels of expression and some showed levels of expression on par with the test samples, which isn't



ideal. Amplification was detected in all 18s NTC samples again, like previous studies, and again this amplification appeared to be non-specific.

Overall, the use of SYBR Green for the detection of these markers has proved quite challenging. Specific markers PBGD and HTN3 were undetected using SYBR Green dye and amplification for the STATH marker proved in all cases to be non-specific. For all other markers though it appeared amplification in most cases was specific to the targeted sequence, however some of the results were left to open to interpretation due to the non-specific nature of the SYBR Green dye. The most specific markers in these studies appeared to be NCF2, KRT4, KRT13 and SPRR1A showing reproducible results in a number of studies. Blood marker SPTB also appeared to be specific to blood however, the melt curve data for this marker in each study was consistently questionable given the low melting temperatures for the amplified products. It can therefore be said that real-time PCR using SYBR Green dye was capable of identifying mRNA specific markers in blood and saliva; however the specificity of these markers was not entirely reliable given the non-specific nature of SYBR Green.

## **4.2 Comparison studies using TaqMan<sup>®</sup> assays**

Following the SYBR Green studies a number of TaqMan<sup>®</sup> comparison studies were undertaken to assess the differences in primer detection and specificity using this detection chemistry. The markers investigated in these studies were NCF2, STATH, HTN3, SPTB, KRT4 and KRT13. Overall, NCF2 was consistently over expressed in blood compared to saliva, a positive result for NCF2 given that it is blood specific. KRT4, KRT13, and STATH were also consistently detected in saliva and sometimes in blood, more often than not there

was zero amplification for these markers in blood. HTN3 in most cases was detected in saliva than in blood, although on one occasion this marker was undetected in both blood and saliva. The results for blood marker SPTB revealed that SPTB was detected in blood samples more than saliva however the detection in blood for this marker was still few and far between, i.e. SPTB was mainly detected in female samples compared with male and in some instances amplification was only based on one replicate out of three amplifying.

Overall, the mRNA markers of choice appeared to show better specificity using TaqMan Gene Expression Assays over SYBR Green. For example, HTN3 showed no detectable amplification using SYBR Green, but was however detected in saliva on a number of occasions using TaqMan. The results obtained for blood marker SPTB using TaqMan<sup>®</sup> were however not fantastic, and reflected the results for this marker in the SYBR Green studies, thus question the suitability of this marker. STATH on the other hand showed very good specificity in these studies compared to SYBR Green where no detectable amplification was observed for this marker in any body fluid in any study.

Analysis of the negative controls using TaqMan assays also proved to be much better than SYBR Green studies, this includes the reverse transcription negative control samples too.

The results of the SYBR Green studies vs. TaqMan highlights the disadvantages of using intercalating dyes rather than hydrolysis probe based detected chemistry for detection of specific mRNAs in body fluids. The use of SYBR Green dye results in the amplification of non-specific PCR products which have demonstrated in this study to be quite problematic with regards to successful interpretation of the data. The use of a dual-labelled probe however to identify a specific target sequence, results in the amplification of a specific target only, non specific amplification does not occur using TaqMan chemistry due to the specific

nature of the probe. Thus, experiments using that latter chemistry are much less time consuming and require less validation than SYBR Green studies.

### **4.3 18s Endogenous Control Amplification**

It was noted that in all studies including both TaqMan and SYBR Green studies, the expression of 18s rRNA was consistently higher in saliva than in blood. It is thought that this was due to amount of cells collected on a buccal swab during sample collection. There are undoubtedly more cells collected on the swab during sample collection in comparison to cells from a fingerprick method when collecting blood, which would explain why there appears to be more 18s rRNA in saliva than blood as 18s is ubiquitous in nature.

### **4.4. Summary**

Originally, the aim of this study was to develop a more cost effective BFID test capable of distinguishing between blood and saliva, using SYBR Green fluorescent dye rather than TaqMan probe. This was achieved by comparing the expression levels of specific mRNA markers for both blood and saliva. The majority of markers demonstrated good specificity, however the reliability of the data was questionable on a number of occasions as low level contamination occurred in a number of samples. Throughout this study, increasingly vigorous anti-contamination procedures were implemented to try to eliminate the possibility of contamination in these studies. However, despite these attempts, the low-level amplification in the NTCs was never completely eliminated. Specific amplification occurred in a number of NTC samples throughout the SYBR Green studies, thus decreasing the specificity and reliability of the result. A vast amount of non-specific amplification also occurred, contributing to the on going challenge of interpretation.

SYBR Green dye was originally chosen as it was thought to be more cost effective than TaqMan chemistry. This is the case when purchasing reagents for a reaction, the primers are a lot cheaper than TaqMan probes, however one of the main disadvantages of using SYBR Green dye is that it will bind to any dsDNA, therefore the additional analyses required to validate and increase the specificity of the reaction due to the non-specific nature of this dye soon became quite costly. With SYBR Green tests, more no template controls are required and the experiments were often repeated due to the issues of possible low level contamination in the NTCs.

It can therefore be said that, SYBR Green dye utilising real-time PCR can be used to identify mRNA markers in blood and saliva, however TaqMan offers much greater sensitivity and specificity, and is less time consuming in terms of interpretation of the data (no post amplification analysis is required with hydrolysis probe based detection chemistry). After these studies, it is thought that the use of SYBR Green dye in the long run appears not to be as cost effective as originally thought, due to the vast amount of validation controls required per experiment. It is also a much more time consuming analysis compared with TaqMan chemistry.

#### **4.5 Further work**

Further work would include the analysis of additional mRNA specific markers described in the literature, to see if any offer better specificity than those described and used in this study. As well as blood and saliva, other forensically relevant body fluid markers would also be tested, such as vaginal secretions, menstrual blood and semen. Repeat experiments would be performed to increase the reproducibility of the results in each study. Further studies in to primer concentration and optimisation would also be performed to

reduce or possibly eliminate the amount of primer-dimer formation; this would greatly increase the specificity of the reactions. It is thought that if there was longer time to spend on this study then optimisation studies improving various experimental conditions could possibly result in a study that required less repeats and validation, thus bringing the cost down. A study in to the use of different endogenous controls would also be used to see if there were a more suitable control, the ubiquitous nature of 18s rRNA resulted in a vast amount of NTC amplification. Lastly, once a number of specific mRNA markers had been established and validated, future work could be carried out to assess the stability of these markers in body fluids over time, to assess whether or not body fluids of unknown age at a crime scene could be identified.

## **Chapter 5**

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