An Evaluation of Genetic Markers for Forensic Identification of Human Body Fluids

By

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Abstract

Body fluids are commonly recovered from crime scenes by forensic investigators and their identification are necessary part of forensic casework study. Current body fluid identification techniques rely on enzymatic tests, which have limited sensitivity and specificity, they require large amount of template, use separate assays for various body fluids, and are prone to contamination. Various genes are expressed in different body fluids that could be used as genetic markers for body fluid identification, and are used in forensic investigations.

The aim of this study was to use mRNA markers to identify human body fluids, which included blood, semen, saliva, vaginal secretion and menstrual blood. Initially, ten reference genes (UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and S15) were studied to establish an appropriate reference gene in body fluid identification. These are constitutive genes used for normalisation of gene expression data and control of variations in experiments. qRT-PCR efficiency, sensitivity and limit of detection (LOD) were investigated using SYBR Green and Taqman probes. The results of the SYBR Green efficiency test experiment displayed five markers, UCE, TEF, ACTB, B2M, and RPS29 with 90-110% efficiency with a slope -3.33 ± 10%.

Subsequently, Taqman probes were designed for the five markers and then used for the Taqman probe experiment. Reference gene stability test was carried out on body fluid samples stored up to 6 months at room temperature using the designed Taqman probe assays. The results established **ACTB** and **UCE** as best candidate reference gene markers in this study as both were most stable in samples stored for 6 months. Furthermore, to identify each of the five body fluids, thirty-two (32) body fluid specific mRNA markers were evaluated, optimised and validated. The experiment was initially carried out with non-fluorescent makers to determine the specificity of the markers. These were analysed using agarose gel electrophoresis. Further optimization was then carried out using fluorescently labelled markers. This was done in five separate multiplexes for each body fluid; –semen-plex, saliva-plex, vaginal secretion-plex, menstrual blood-plex and blood-plex. An attempt was made to combine all the five-separate multiplex into a single multiplex. All body fluids were identified unambiguously with no cross-reactions of non-target body fluids using the combined multiplex assays.

Following further evaluation and validation tests, a total of 14 markers were selected and a capillary electrophoresis (CE) based, multiplex assay was developed to identify blood, saliva, semen and vaginal secretion samples simultaneously. The markers in the developed multiplex assay included **ALAS2** and **PF4** (blood), **STATH** and **HTN3** (saliva), **PRM1**, **TGM4**, **MSMB**, **NKX3-1** (semen), **ACTB** and **UCE** (reference genes), **CRYP2B7P1**, **SFTA2**, **MUC4** and ***L. crispatus*** (vaginal secretion). Extensive validation, which include sensitivity, specificity, reduced volume reactions, degradation, reproducibility, mixtures, cycle number and mastermix, was carried out in accordance with the guidelines detailed in Scientific Working Group in DNA Analysis (SWGDAM). The 14-marker CE-based assay displayed high specificity and sensitivity. Each body fluid was detected down to 1:3000 dilution of mRNA except vaginal secretion that was detected down to 1:1500 dilutions of sensitivity. Specificity experiments showed no cross reactions of the assay with non-target body fluids. Reproducibility study displayed similar results reported from an independent laboratory. All body fluids exposed to environmental insult were identified up to at least day 30 of 51, with blood being identified up to day 51. In the mixture study, all body fluids were identified unambiguously using the developed multiplex assay.

In conclusion, the results of this study have led to the development of a new and novel capillary electrophoresis-based mRNA marker assay for forensic body fluid identification, demonstrating its compatibility with forensic laboratory workflows. The use of this assay to profile forensic casework samples for body fluid identification would be a future application of this work.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
PCR `	Polymerase chain reaction
RT	Reverse transcription
EPG	Electropherogram
LOD	Limit of detection
LOQ	Limit of quantitation
CE	Capillary electrophoresis
HBB	Haemoglobin-beta chain
CD93	Cell surface and membrane protein-93
AMICA1	Adhesion molecule-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
β –SPEC	Beta spectrin
SPTB	Beta spectrin
ALAS2	Aminolevuninate δ synthase-2
PPBP	Pro-platelet basic protein (chemokine (C-X-C motif) ligand-7)
HBA	Haemoglobin-alpha locus
PBGD	Hydroxymethylbilane synthase, also known as HMBS
CD3G	Gamma molecule (CD3-TCR complex)
ANK1	Ankyrin-1
AQP9	Aquaporin
GLYCOA	Glycophorin A
PRF1	Perforin
PF4	Platelet factor-4

FDCSP	Follicular dendritic cell secreted protein
PRB1	Proline-rich protein BstNI, subfamily-1
STATH	Statherin
HTN3	Histatin-3
SMR3B	Submaxillary gland androgen regulated protein 3B
PBR4	Proline-rich protein BstNI, subfamily-4
MUC7	Mucin-7
HBD1	Human beta defensin-1
MUC4	Mucin-4
MSLN	Mesothelin
STFA2	Surfactant-associated protein 2
FUT6	Fucosyltransferase-6
DKK4	Dickkopf homolog-4
MYOZ1	Myozenin1
CRP2B7P1 pseudogene1	Cytochrome P450, family2, subfamily B, polypeptide7,
SEMG1	Semenogelin1
NKX3-1	Homeobox protein Nkx-3.1
PRM	Protamine
TGM4	Transglutaminase-4
MSMB	Microseminoprotein, beta-
KLK3	Kallikrein-3
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MMP	Matrix metallopeptidase
MSX1	msh homeobox-1
SFRP4	Secreted frizzled-related protein-4

LEFTY2	Left-right determination factor-2
Hs202072e	Uncharacterised LOC100505776
LOR	Loricrin
CDSN	Corneodesmosin
KRT	Keratin
SPRR2A	Small proline-rich protein 2A
ACTB	beta (β)-actin
TEF	Transcription elongation factor
UCE	Ubiquitin conjugating enzyme
B2M	β2 microglobulin
RPS-29	Ribosomal Protein S29
OAZ1	Ornithine decarboxylase antizyme
B-ACTIN	beta-actin
S-15	Ribosomal protein-15
18S rRNA	18S ribosomal RNA
Ct	Cycle threshold

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''...a crime cannot be solved without evidence. Confessions and circumstantial evidence sometimes prove untrue, but physical evidence can provide an airtight case against a suspect.''

''…we all think of death as the ending. For forensics, it's just the beginning.''

-Nancy Haley

CHAPTER ONE

GENERAL INTRODUCTION/ LITERATURE REVIEW

1.0 Introduction

Forensic science encompasses the application of scientific knowledge and its techniques for the purposes of law. This includes evidence recognition, collection, processing and its interpretation for legal purposes (Lee *et al.,* 2001). Physical evidence is of great importance to the courts of law. Indeed, such evidence can be more valuable than an eyewitness' account in particular cases (Wells *et al.*, 2003; Spellman and Tenney, 2010). Physical evidence commonly encountered and recovered from crime scenes may include human body fluids like blood, semen, saliva, menstrual blood and vaginal secretions. Appropriate handling of such samples is necessary as environmental factors such as temperature/humidity and effects of microorganisms could have adverse effects on the recovery of these body fluids (Jackson and Jackson, 2011). Saferstein, (2006) reported the use of physical evidence (semen samples) in the apprehension of a serial killer in the United States of America. Following the discovery of bodies of five young females along Green river in Seattle, Washington, over a period of 6 months, the police in the area commenced an investigation into the cause of the murders. After another four years, it was discovered that the number had increased to forty; a development that made the police focus on a prime suspect - Gary Ridgeway. Because of lack of physical evidence, this case was followed up for over a decade until DNA profiling became popular. Ridgeway was arrested and made to undergo a lie detector test, which he passed. However, a profile of semen samples recovered from three of his victims and a reference sample of his saliva had a match. Other evidence that included paint particles on his victims clothing also had a match with the paints collected from his workplace. With these evidences in place, Ridgeway confessed to the murder of 48 women.

The process of human identification has witnessed a significant advancement over years (Schiro 2000, Roewer, 2013). Genetic profiles generated from recovered evidentiary items are invaluable in forensic casework. However, current processes of genetic profiling cannot identify the cellular source of evidential samples (Lindenbergh *et al.,* 2012).

Adequate identification and establishment of the nature and origin of stains complement each other. Therefore, during forensic casework, the identity of body fluids and recovery of evidential samples is normally undertaken before submission for genetic profiling. The traditional techniques of body fluid identification are mostly non-specific and laborious. In addition, they sometimes need a significant portion of the materials and might be destructive in nature, affecting the genetic profiling process downstream. Over the past decades, there has been a steady rise in the number of crime related cases that solely rely on DNA evidence and thus genetic profiling techniques have become central in most crime related casework. However, the identification of body fluids has not developed at the same pace. It is therefore imperative that body fluid identification methods are developed that match in sensitivity and specificity, to the current genetic profiling methods for human identification.

1.1 Body fluids

Body fluids like blood, semen, saliva, menstrual blood, vaginal secretion, sweat, urine and contact traces are frequently encountered by forensic investigators at crime scenes (Virkler and Lednev, 2009; Lindenbergh *et al.,* 2012). Most of these specimens pose challenges during identification as traditional serological methods are presumptive in nature (Ablett, 1983; Mokashi *et al.*, 1975; Kipps *et al.,* 1978). These are only sometimes complemented with confirmatory tests.

1.1.1 Body fluid composition

Each body fluid is made up of specific, unique and varied components that are dependent on their site of secretion and function within the body. Their general functions include excretion (urine and sweat), transportation (blood), lubrication and protection (vaginal secretion), and digestion, lubrication, host defence and tooth mineralisation and mastication or mixing of food (saliva). Some of these body fluids have identical components in varying proportions. These relative variations in their components determine the type of identification test that might be required for them (Table 1.1).

Table 1.1: Human body fluid components (Taken from Virkler and Lednev, 2009; Wilson 2005)

Blood	***Saliva***	***Semen***	***Urine***	***Sweat***	***Vaginal fluid***
Haemoglobin	Glycoprotein	Acid phosphatase	Urea	Urea	Mucins
Fibrinogen	Bicarbonate	PSA	Sodium	Lactic acid	Albumin
Erythrocytes	Chloride	Spermatozoa	Chloride	Chloride	Ig
Albumin	Potassium	Choline	Potassium	Sodium	Transferrin
Glucose	Sodium	Spermine	Ammonium	Potassium	Lactoferrin
Ig	cAMP	Putrescine	Phosphate	Ig	Glucose
	Amylase	Spermidine	Calcium		Glycogen
	Ig	Cadaverine	Creatinine		Mannose
	Urea	Semenogelin	Uric acid		Glucosamine
	Phosphate	Zinc	Bicarbonate		Fructose
	Lysozyme	Citric acid	Chlorine		Neutral lipids
	Calcium	Lactic acid	Glucose		Phospholipids
	Amino acids	Fructose			Urea
	Ammonia	Urea			Lactic acid
	Uric acid	Ascorbic acid			Acetic acid
	Lipid	Ig			Butanoic acid
	Thiocyanate				Propanoic acid
	Glucose				Amino acids
	Peroxidise				Sodium
	Citrate				Potassium
					Chloride

1.2 Traditional techniques of body fluid identification

Forensic body fluids are still being identified using traditional methods. These are mostly enzyme based, require a large amount of sample, prone to contamination and currently not available for all the body fluids. In addition, they do not have the ability to multiplex, which is a major drawback in forensic body fluid identification. The body fluids and their traditional tests also require confirmatory tests following initial presumptive tests. These are discussed in sections 1.2.1 – 1.2.5.

1.2.1 Saliva

Saliva is commonly deposited at various crime scenes by unsuspecting perpetrators. It may be found as 'spits', around the openings of used cans and bottles, cigarette butts and chewing gum, and around bite marks. Due to its colourless form and absence of solid particles, it is not easily detectable. Traditional methods of identification include Alternative Light Source (ALS) (Gaensslen, 1983; Jones Jr., 2005; Vandenberg and Oorschot, 2006) and testing of Amylase 1 (AMY1) activity (the starch-iodine test). AMY1 is present in other body fluids as well; therefore, a further test is necessary to identify saliva to prevent results with false positives (Greenfield and Sloan, 2003). In addition, ELISA is an immunological test that has been used to identify the presence of saliva in a stain. This technique tests a-amylase activity in saliva stains by combining monoclonal antibodies with horseradish peroxidase conjugate (Komuro *et al.,* 1995).

1.2.2 Semen

Just like saliva, semen is not easily detected by the naked eye due to its colourless nature. ALS has been used to detect the presence of semen in sexual assault cases. This technique was reported not to be 100% sensitive to semen stains (Virkler and Lednev, 2009; Gaensslen, 1983) and other body fluids could not be differentiated, thereby giving false positive results (Santucci *et al.,* 1999; Nelson and Santucci, 2002). Seminal Acid Phosphatase (SAP) is also commonly used. However, false positives with Vaginal Acid Phosphatase (VAP) have been recorded (Gaensslen, 1983). With these two body fluids commonly recovered in sexual assault cases as mixtures, a more reliable test is needed to distinguish SAP from VAP. Isoelectric focusing and acrylamide gel electrophoresis have been employed for this purpose, with little success (Virkler and Lednev, 2009;

Toates, 1979; Stolorow *et al.,* 1976). Adequate measures should be undertaken when carrying out the tests as long exposure of the enzyme to heat, mould or other chemicals could cause rapid degradation. Other presumptive tests available for semen identification give false positives for human body fluids, vegetables and faeces and most of these tests have not been validated on forensic casework samples thereby limiting their usage (Virkler and Lednev, 2009).

The presence of semen can be confirmed by microscopic identification of sperm cells on a slide stained with Christmas Tree stain in non-azoospermic male individuals (Greenfield and Sloan, 2003). This, however, cannot be employed for azoospermic individuals (Virkler and Lednev, 2009). Other confirmatory tests include prostate-specific antigen (PSA) (Greenfield and Sloan, 2003); immuno-electrophoresis and ELISA (Tsuda *et al.,* 1984). In addition, Seratec® has employed PSA test in their PSA semi quant kit (www.seratec.com).

1.2.3 Blood

Bloodstains are frequently encountered at crime scenes. ALS such as ultraviolet light shows high specificity with the stain on a dark background. Though it can reveal stains covered by paints, an exposure of 30s at about 255 nm light could damage the DNA and render it too degraded for amplification (Shaler, 2002; Vandenberg *et al.,* 2006; Andersen and Bramble, 1997). Luminol test is often used and has been reported to be the most sensitive of all the presumptive tests (Gaensslen, 1983; Spalding, 2003). Luminol can reveal the bloodstain even when concealment has been attempted. Other tests include Phenolphthalein test also known as Kastle-Meyer (KM) test (Spalding, 2003); Leucomalachite green test (LMG) (Shaler, 2002) and Bluestar1, (Luczak *et al.,* 2006; Blum *et al.,* 2006). Although they have all been reported to yield very good results in bloodstain identification, a confirmatory test is however, still needed for unambiguous identification.

Specific confirmatory tests that have been reportedly used for blood identification and they include direct visualization of blood red and white blood cells in liquid blood (Shaler, 2002); Scanning electron microscope (SEM) using energy dispersive X-ray analyser (EDX) (Dixon *et al.,* 1976) and Teichman and Takayama crystal tests (Spalding, 2003) being the most popular. Recently used confirmatory tests include isozyme analysis

which indicates the presence of blood by monitoring the isozyme pattern of different fluids, comparing the differences in their lactate dehydrogenase (Divall and Ismail, 1983); Enzyme-Linked Immunosorbent Assay (ELISA) (Komuro, *et al.,* 1995), and Thin Layer Chromatography (TLC) (Gaensslen, 1983).

1.2.4 Vaginal secretion

Vaginal fluid can be important evidence in cases of sexual assault. It is often recovered as a mixture with semen especially when penile penetration is involved. The components of the vaginal secretions change depending on the menstrual cycle of the female (Gaensslen, 1983). Virkler and Lednev, (2009), described the use of periodic acid Schiff (PAS) that reports the detection of glycogenated epithelial cells. The test gives false positives especially with saliva as both secretions contain mucosal cells (Wilson, 2005). In addition, glycogenation in females fluctuate depending on menstrual cycle. Other reported traditional methods include the use of vaginal peptidase (Divall, 1984); monoclonal antibodies by immune-histochemical techniques to detect the presence of oestrogen receptors (Hausmann, *et al.,* 1996); comparison of lactic and citric acid ratio in vaginal secretion either singly or in mixtures (Martin and Chesire, 1986).

1.2.5 Menstrual blood

Menstrual blood is a complex mixture of circulatory blood, vaginal secretions, microbes, cells and tissues associated with menstrual cycle (Divall and Ismail, 1983). There is no known presumptive test for menstrual blood due to its composition. However, this body fluid has been successfully differentiated from other body fluids using molecular approaches (Lindenbergh, *et al.*, 2012; Harbison and Fleming, 2016).

The various techniques of body fluid identification carried out using traditional serological methods are non-specific and presumptive in nature (Virkler and Lednev, 2009; Mokashi, *et al.*, 1975; Kipps, *et al.,* 1978; Ablett, 1983). The methods require considerable amount of sample; and are prone to sample consumption and contamination. Furthermore, they are not available for all body fluids (Bauer, 2007) and based on the tests carried out and storage conditions, further tests might be required for mixtures and aged samples (Vitali *et al.*, 2012; Myers and Adkins, 2008).

1.2.6 RNA suitability in new methods of body fluid identification

Unlike traditional methods, new techniques using RNA have been reported to be suitable, unique and specific for body fluid identification. These include messenger RNA (mRNA) profiling (Alvarez, *et al.*, 2004; Juusola, and Ballantyne, 2007; Haas, *et al.,* 2009; Bowden, *et al.,* 2011; Haas, *et al.,* 2011; Roeder and Haas, 2013), micro RNA (miRNA) profiling (Hanson, *et al.,* 2009; Bentwich, *et al.,* 2005; Berezikov, *et al.,* 2005) and techniques such as cDNA next generation sequencing (Ogawa, *et al.,* 2013), multiplex high resolution melt analysis (HRM) (Hanson and Ballantyne, 2014), RNA Suspension Fluorescent in situ hybridization (RNA S-FISH) (Williams, *et al.,* 2013), Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Donfack and Willey, 2015), and massively parallel sequencing (MPS) (Lin *et al.* 2015) have been described in the literature. These have gained popularity due to the specificity of the molecular markers used, ability to multiplex such markers in a PCR assay and the potential for distinguishing the components of a heterogeneous mixture of human body fluids in a single assay (Alvarez, *et al.,* 2004; Juusola, and Ballantyne, 2003; Juusola and Ballantyne, 2005; Zubakov, *et al.,* 2010; Vandewoestyne *et al.,* 2009; Robertson, *et al.,* 2009).

1.3 Ribonucleic acid (RNA)

Ribonucleic Acid (RNA) is present in all living cells playing an essential role in protein synthesis. Numerous RNA families have been reported (Vennemann and Koppelkamm, 2010). They are not only distinguishable by their configuration and secondary structure; they also have different half-lives and carry out distinctive functions. While most RNAs regulate the process of gene expression, messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) are all directly involved in protein biosynthesis. The transfer of genetic information from DNA to RNA and protein synthesis is shown in Figure 1.1.

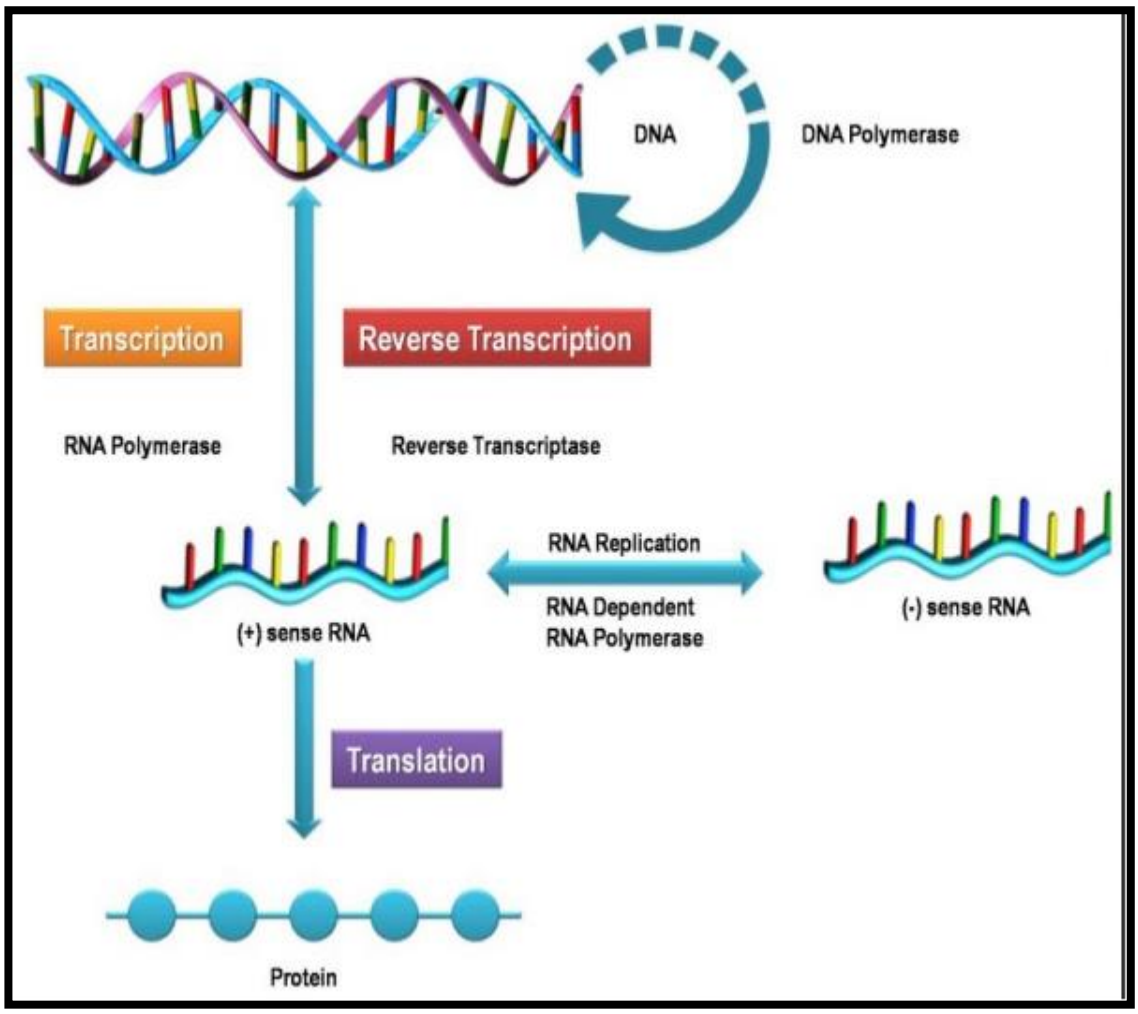

Figure 1.1: Schematic representation of the Centra Dogma showing the flow of genetic information from DNA through RNA to protein synthesis (Taken from Saxena and Chitti, 2016).

Oehmichen and Zilles, (1984) first reported the use of RNA in forensic science, describing post-mortem RNA synthesis. It was nearly another decade before a new report of RNA use in this research area was published (Phang, *et al.,* 1994). Subsequently, there was no significant advancement until another six years when the Reverse Transcriptase PCR (RT-PCR) technique was described (Bauer, 2007). Following this report, there has been a steady growth in the practical use of mRNA techniques in forensic science. These include determinations of age of wound, stains, post-mortem interval and body fluid identification.

The stability of RNA remains a major concern in forensic science because of the ubiquitous ribonucleases, which rapidly degrade RNA (Bauer, 2007). A number of reports have established the stability of mRNA under various environmental conditions

thereby indicating their suitability for use in gene expression studies (Bauer, *et al.,* 2003; Setzer, *et al.,* 2008; Zubakov, *et al.,* 2009). Bauer *et al.,* (2003) could detect 106 tested bloodstains stored up to 15 years at room temperature and protected from sunlight. More body fluids were assayed in a similar study by Setzer, *et al.,* (2008). The authors reported the stability of RNA prepared from blood, semen, saliva and vaginal secretion that were exposed to a range of environmental conditions (light sources, temperature, and exposure to rain) over a period of 1-547 days. For each type of body fluid, total RNA quantitation and reverse transcriptase polymerase chain reaction (RT-PCR) using seven different tissue-specific and a reference gene mRNA transcript monitored the stability of RNA. mRNA detection varied across each sample based on the effect of environmental conditions. Samples protected from direct rain impact displayed detection up to 180 days; those exposed to direct rain were only detected up to 7 days while mRNA was detected in only a few samples stored at room temperature for at least 547 days. In a similar study using different sets of markers, Zubakov, *et al.,* (2009) also reported identification of blood and saliva stains stored under ambient temperature (away from dust and humidity), for up to 16 years and saliva samples stored up to 6 years.

1.4 Applications of RNA in Forensic Science

Gene expression patterns of mRNA have also been utilised in forensic science in many areas that are discussed below.

1.4.1. Determination of circumstances leading to death

It is essential to diagnose the cause of death by careful examination of both functional and morphological variations that occurred in cells shortly before the time of death. Bauer, (2007) reported that though most circumstances leading to death do not reveal morphological changes within the cells of a tissue; the functional quality in the cell's mRNA pattern is, however, affected. Consequently, the cell responds rapidly, thus leading to a change in proportions of specific mRNAs due to the effects of external factors on the body (Vennemann, and Koppelkamm, 2010). Different gene expression patterns were observed in various types of death depending on the markers evaluated (Matsuo *et al.,* 2009; Takahashi, 2008). Ikematsu *et al.,* (2006) established that post-

mortem gene expression activities continue for at least 30 minutes after death. In their study, the authors investigated gene expression patterns of four candidate markers in mouse skin cells that were killed by either decapitation or compression of the neck. The markers in these two groups could differentiate the nature of death in the experimental rats, which indicated their possibility of use in decapitation or compression of neck-related deaths. Also, Takahashi, (2008) described mRNAs for catecholamine biosynthetic markers in adrenals and anterocervical ganglia of mice as potential candidates to determine acute response to contusion stress. Furthermore, methamphetamine-related-deaths were investigated by Matsuo *et al.,* (2009) where the authors studied immediate early genes (IEGs) which are expressed almost immediately after death in the mouse heart over a period of 4 weeks. Similarly, hypoxia-inducible factor 1 (HIF-1), erythropoietin (EPO) and vascular endothelial growth factor (VEGF) have been described by Zhao *et al.,* (2006) as robust mRNA transcripts in the kidney which could predict cause of death due to hypoxia. All these studies have established the possibility of determining the nature of death by monitoring the expression patterns of various gene transcripts.

1.4.2 Biological stain age determination of samples

The ability to determine the age of a biological stain can give an indication of the time a crime was perpetrated. Many studies have been carried out on approximate age of stain determination. Early methods included the use of HPLC chromatographs to measure alpha chain area relative to heme area in blood (Inoue et *al.,* 1991; Inoue *et al.* 1992). These studies displayed a linear decrease in the alpha/heme area ratio as the stain age increased. Although the results obtained indicated a direct correlation between the age of stain and haemoglobin deterioration, no similar sets of markers have been found for other forensically relevant body fluids. However, recent studies have focused on monitoring mRNA degradation to determine the approximate age of a biological stain. Bauer *et al.,* (2003) reported the identification of dried bloodstains using semi-quantitative duplex and competitive RT-PCR. In their experiment, blood samples were stored protected from sunlight at room temperature for up to 5 years. The effect of storage condition was examined in samples exposed to direct light, humidity and tubes containing EDTA over a period of 2 months to 2 years (Bauer *et al.,* 2003; Anderson *et al.,* 2005). They observed a significant correlation between RNA degradation and storage

interval; and this was reported for use as a possible indicator of age of stains. Subsequently, Anderson *et al.,* (2005) further described age of bloodstains identification using real-time RT-PCR. The authors showed that β-actin mRNA and 18S rRNA exhibited a linear change in ratio in dried human blood samples analysed over a period of 150 days under controlled conditions. These two transcripts were selected as they were widely acknowledged reference genes, and their RNA transcripts were likely recovered from any cellular material deposited at crime scenes. During the time course, a significant change in cycle threshold -Ct (cycle at which florescence signal crosses the threshold) value of β-actin but not 18 S was observed. Thus, there was an increase in relative ratio of 18S to β-actin over time. Using this phenomenon, the authors hypothesised that careful study of relative RNA ratios, which were consistent and predictable, could be used to determine the approximate age of bloodstains. In addition, since 18S and β-actin were broadly recognized as reference genes, the approach and analysis in this study might be useful for determining the approximate age of stains of other body fluids too.

1.4.3 Wound age determination

Wound-age determination is one of the key aspects in forensic pathology, especially in wound-related deaths. In humans and other animals, the skin shields the body against invasion from the surrounding environment, thereby making it susceptible to injuries (Masataka *et al.,* 2008). In order to determine the approximate age of wounds, a detailed knowledge of heterogeneous composition of cutaneous repair is important (Guler *et al.*, 2011). The mechanism of skin repair starts upon infliction of injury and this comprises three main phases viz inflammation, proliferation and maturation of newly formed tissues (Kondo, 2007). Early methods are still currently used to determine age of wounds include enzyme (phosphatase) activities and inflammatory cell dynamics (Betz, 1994; Hernandez-Cueto *et al.* 2000). However, with increased research and advance in technology, the use of mRNA markers has been reported (Masataka *et al.,* 2008; Guler *et al.,* 2011; Takamiya *et al.,* 2009; Palagummi *et al.,* 2013). Takamiya *et al.,* (2008) described the use of eight cytokines (interleukin (IL) 2, IL 4, IL 6, IL 8, IL 10, GM-CSF, IFN γ, and TNF α) to assess wound age in human dermal wounds. The expression of these cytokines was monitored at the early, middle and late stages of wound healing and the results analysed using quantitative multiplex bead-based immunoassay. IL 6, IL

8, IFN γ, and TNF α were reported to be significantly expressed during wound healing and thus recommended as candidate markers in wound age determination. Further study carried out by Takamiya *et al.,* in 2009 included nine more markers such as interleukin 1b (IL 1b), IL 5, IL 7, IL 12 p 70, IL 13, IL 17, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP 1), and macrophage inflammatory protein 1b (MIP 1b) which were together quantitatively monitored with the former eight cytokines. In this study, MCP1 was found to be most widely expressed. However, the authors proposed that quantitative analysis of all these markers combined could enhance the precision of age of wound determination. The relevance of tenascin and ubiquitin in wound age estimation have also been reported in wound age over 40 days (Guler *et al.,* 2011). Various types of wounds ranging between gunshot, blunt injuries, sharp weapon injuries and medical incisions were studied. While tenascin was negative in wounds over 40 days, ubiquitin was still observed in the fibroblasts. The expression of tenascin & ubiquitin was consistent irrespective of wound types. The authors concluded that semi-quantitative evaluation of both markers together would aid in estimating the age of the wound.

1.5 Environmental insults on sample degradation

Most forensic samples are usually recovered in environmentally compromised state. These are due to the effects of temperature, humidity and ultraviolet radiation (Barbaro *et al.,* 2008; Lund and Dissing, 2004; McNally *et al.,* 1989). In theory, DNA degrades rapidly, especially when exposed to extreme temperatures and absolute humidity. However, Lund and Dissing (2004) reported the stability of DNA in stains after they were exposed to 35 ^{0}C - 65 ^{0}C and 100% relative humidity. Although, the authors noticed a decrease in amplification of a few stains, which they reported to be because of microbial growth. Most of the samples, especially long DNA fragments were still reported to be amplifiable after being exposed over a month. A similar study was reported by Barbaro *et al.,* (2008) where the effects of high temperature was investigated on DNA typing from blood, saliva and semen. The temperature range included 50 ^{0}C - 200 ^{0}C. The authors established that it is possible to type these three body fluids with success after exposure to harsh conditions, although, they recommended analysis of mini STRs for an improved result. Fordyce *et al.,* (2013) in a recent study further explained the changes

in cell activities that could be a factor to their stability upon exposure to environmental insults. They stated that cells are usually dehydrated because of high temperature and humidity. During this process, microorganisms' and RNases' activities are inhibited, which in turn shields the nucleic acids from degradation. Furthermore, the presence of stronger N-glycosidic bonds and depyrimidation process in RNA molecules enables them resist hydrolytic depurination and prevention of phosphodiester bond hydrolysis. These were reported to be possible reasons why successful RNA/DNA typing could still be achieved upon sample degradation due to exposure to environmental insults.

1.6 Micro RNAs (miRNA)

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules (usually 18-24 nucleotides) which play an essential regulatory role for many cellular processes. Due to their relatively small size, they have been reported to be invaluable in molecular studies, especially in forensic body fluid identification where recovery of aged and environmentally compromised samples are common occurrence. These molecules were first described by Lee *et al.,* (1993) and to date, more than 700 miRNA markers have been discovered (Bentwich *et al.,* 2005; Berezikov *et al.,* 2005). miRNAs have been reported in embryonic development, cell proliferation and tumorigenesis and human disease studies such as cancer and viral infections *(*DeVincenzo *et al.,* 2010; Aten *et al.,* 2008). Hanson *et al.,* (2009) assessed the expression of 452 human miRNAs in dry semen, blood, saliva, vaginal secretion and menstrual blood. They reported most of the 452-human miRNA to be expressed in more than one body fluid or not expressed at all. This led to further screening of the miRNA transcripts and development of a 9-marker assay (miR451, miR16, miR135b, miR10b, miR658, miR205, miR124a, miR372, and miR412). The assay was reported to be highly sensitive and allowed the identification of blood, semen, saliva, vaginal secretions, and menstrual blood— with a minimum of 50pg total RNA input (Hanson *et al.,* 2009). Furthermore, specificity of the panel was studied and the researchers were able to distinguish each body fluid from other human tissues. Following the initial screening and enhancement of the specificity and sensitivity of the assay, the authors further assessed more miRNA (1198) transcripts and their expression in blood, semen, saliva, vaginal secretion and menstrual blood. Thirteen novel miRNA markers were identified with five from the initial assay, bringing the total number of

markers in the new assay to 18. In addition, a binary logistic regression statistical model that could provide exact indication of the presence of a forensic body fluid was developed. This assay, together with the statistical model, was reported to be specific in distinguishing forensically relevant body fluids of interest from other human tissues tested.

1.7 mRNA Based Identification of Body Fluids

Body fluids commonly encountered at crime scenes include blood, saliva, menstrual blood, vaginal secretion, and semen. Each body fluid is made up of unique cell types (Gaensslen, 1983; Greenfield and Sloan, 2003; Li, 2008) that have distinctive transcription and gene expression profiles. These distinctions are used to identify forensically relevant body fluids, especially in casework samples. Previously, studies of body fluid identification techniques have been limited on casework samples possibly due to limited sample recovery, lack of definitive assay to identify the body fluids and the impression that tissue identification might not be important to the case (Hanson and Ballantyne, 2013). Nonetheless, with advance in technology and ability to co-extract DNA and RNA, a few of these challenges have been curtailed (Alvarez *et al.,* 2004; Bauer and Patzelt, 2003).

Currently, body fluid identification is still being carried out using traditional serological methods; Saliva Phadebas amylase test, semenogelin test for semen (which involve the use of membrane strip test to identify human semenogelin), and tetramethylbenzinidine, Rapid Stain Identification (RSID) test for blood (Hedman *et al.,* 2008; Pang *et al.,* 2007; Garner, *et al.,* 1976). These methods are non-specific for human samples, presumptive and enzyme based. The techniques rely on basic colour change, which further requires a confirmatory test to analyse. Although they are still being used, their robustness is compromised when body fluids are mixed and contaminated – a scenario in which most forensic samples are recovered.

In addition to body fluids, skin cells are mostly left by the unsuspecting perpetrator of a crime and by the victims as well on various substrates. The recovery of skin cells from a crime scene could indicate the perpetrator's presence or contact with the crime scene. Previously used methods to identify skin cells include dactyloscopic fingerprint analysis

and other microscopic and immunological techniques (Lindenbergh *et al.,* 2012). These methods were not ideal as they were not applicable to all cell types (Schulz, *et al.,* 2010); could introduce contamination into the reaction (Van Hoofstat *et al.,* 1999) and were reported to have adverse effects on the nucleic acids in the skin cells (Grubwieser *et al.,* 2003).

Modern and alternative methods used to identify body fluids and skin cells have been reported and they include immuno-histochemical methods, Raman spectroscopy (Virkler and Lednev, 2008; Virkler and Lednev, 2010), miRNA and mRNA analysis (Alvarez, *et al.,* 2004; Hanson *et al.,* 2009; Haas *et al.,* 2015).

Recently, there has been increased research into the use of mRNA which could usher an era of replacement of traditional methods of body fluid identification with such methods (Courts and Madea, 2010; Mara *et al.,* 2012). This is due to the advent of automated technologies, ability of mRNA markers to identify broad spectrum of body fluids and also to simultaneously detect different body fluids through multiplex PCR systems comprising of mRNA markers (Juusola and Ballantyne, 2007; Haas *et al.,* 2009; Juusola and Ballantyne, 2005).

mRNA techniques also offer the potential of co-extraction of both DNA and RNA (Alvarez *et al.,* 2004; Bowden *et al.,* 2011; Chevillard, 1993; Triant and Whitehead, 2009; Xu *et al.,* 2008), thus increasing the possibility to independently carry out DNA and RNA profiling, especially when samples with limited biological materials are recovered (Alvarez *et al.,* 2004; Bauer *et al.,* 2003). Most importantly, decreased sample consumption and higher body fluid specificity give mRNA body fluid identification techniques an advantage over traditional methods (Juusola and Ballantyne, 2003). Sufficient quality and quantity of mRNA have been recovered in samples stored up to 15 years (Bauer *et al.,* 2003), 16 years (Zubakov *et al.,* 2009) and 23 years (Kohlmeier and Schneider, 2012), despite the challenging presence of RNAses' in the environment and storage conditions. Until multiplex PCR assays were developed, single-gene-based assays were used to study body fluid specific mRNA markers (Bauer, 2007; Juusola, and Ballantyne, 2005; Setzer *et al.,* 2008; Bauer and Patzelt, 2003; Ferri *et al.*, 2004). Subsequently, more markers were discovered and multiplex PCR assays were enhanced

(Haas *et al.,* 2011; Haas, *et al.,* 2012). Roeder and Haas, (2013) also reported a recent and comprehensive study, where the authors investigated the detection of body fluids utilising a minimum of five mRNA markers for each body fluid.

In all the above-mentioned mRNA body fluid identification studies, the markers reported were selected based on high expression in specific body fluids. Ideally, a single marker should detect the presence of a body fluid. However, physiological conditions, as well as the impact of environmental factors, could lead to significant variations in mRNA expression patterns. With much variability, false positive and negative results, including the possibility of one of the markers dropping out, cross reactivity with body fluids other than the target body fluid, the use of multiple markers have been recommended for forensic body fluid identification (Lindenbergh *et al.,* 2012; Juusola and Ballantyne, 2005).

Table 1.2: Body fluids and tissues based mRNA markers currently used for their identification in human materials

Blood	Saliva	Vaginal secretion	Semen	Menstrual blood	Skin	Mucosa
HBB	FDCSP	HBD1	SEMG1	MMP7	LOR	KRT4
CD93	PRB1	MUC4	PRM1	MMP11	CDSN	SPRR2A
AMICA1	PRB2	MSLN	PRM2	MMP10	KRT 9	KRT13
β-Spectrin	PRB3	SFTA2	TGM 4	MSX1		
SPTB	STATH	FUT6	MSMB	SFRP4		
ALAS2	HTN3	DKK4	KLK3	LEFTY2		
PPBP	SMR3B	MYOZ1	MCSP	Hs202072e		
HBA	PRB4	CRYP2B7P1				
PBGD	MUC7	*Lactobacillus gasseri*				
CD3G		*Lactobacillus crispatus*				
ANK1		ESR1				
AQP9		L. *jen*				
GlycoA						
PRF1						
PF4						

1.8 New techniques to identify forensic body fluids

Unlike the traditional methods of body fluid identification, several new techniques have emerged recently. Although, many of these are still in their early stages of use, however, they have been reported to be robust in unambiguous identification of forensic body fluids.

1.8.1 RNA Suspension Fluorescent in Situ Hybridization (RNA S-FISH)

RNA Suspension Fluorescent in situ hybridization, a technology that aids specific regions of nucleic acids to be detected in situ, is a new method of body fluid identification proposed by Williams *et al.,* (2013) and Williams *et al.,* (2014) using locked nucleic acid probes (LNA). The method had been earlier reported by Vandewoestyne *et al.,* (2009) to distinguish male cells in male and female sample mixtures. FISH has also been described in embryo development (Arvey *et al.,* 2010), and molecular cytogenetic studies (Lourov *et al.,* 2005). Williams *et al.,* (2013) described a RNA suspension FISH (RNA S-FISH) to label epithelial cells. The authors designed and optimised locked nucleic acid probes (LNA) for KRT10 mRNA (present in vaginal and buccal epithelial cells) in fresh and aged samples. Leucocytes were utilised as a control for the KRT10 specific probes. As expected, fluorescent signals were observed for the KRT10 transcript and were reported to be expressed in buccal and vaginal epithelial cells but not in leucocytes (control). In addition, KRT10 signals were detected in their 10-month-old samples stored at room temperature. Furthermore, the authors investigated the possibility of generating DNA profiles from 150 buccal and vaginal RNA S-FISH labelled cells. Full DNA profiles were reported for the 150 fresh cells that were RNA S-FISH labelled. However, there was a significant decrease in peak heights between RNA S-FISH treated and untreated cells in both aged and fresh samples. This was suggested and it was due to likely degradation of DNA or PCR inhibition. To circumvent this, the authors recommended the use of more epithelial cells to generate a full DNA profile, whenever casework samples are to be analysed.

1.8.2 Next generation sequencing (NGS)

Next generation sequencing (NGS) is gradually gaining popularity in forensic science, especially in body fluid identification. With this new technology, Ogawa *et al.,* (2013) reported detection of miRNA markers in two different exosomes found in whole human saliva. Exosomes are membrane bound vesicles, originating from most living cells. They have been described to comprise proteins, lipids, RNAs, non-transcribed RNAs, microRNAs and small RNAs, which are peculiar to their cells of origin and thus can be used in their identification. Exosomes and markers derived from them have been reported to be viable in oral cancer (Li *et al.,* 2004) and ovarian cancer diagnoses (Beach *et al.,* 2014), although this was not done with NGS. With saliva being among body fluids commonly encountered at crime scenes, Ogawa *et al.,* (2013) described using NGS, the identification of 81 novel miRNA markers from exosomes I, II and whole human saliva. RNA was isolated from exosomes I, II and whole saliva and transcribed to cDNA. The cDNA was then sequenced using NGS; and 143 miRNA markers and other RNAs (piRNA, snoRNA) were discovered from both exosomes and whole saliva. Eighty-one miRNA out of these were reported to be novel, with 10 found to be mostly expressed in whole saliva and both exosomes II and I. These markers were proposed to be useful in biomarker research. Though there has been no report of a similar study with other body fluids, further optimization and validation of these miRNA markers could be a gold standard in saliva identification, especially when most importantly, when degraded samples are encountered (Harbison and Fleming, 2010; Roeder and Haas, 2013; Fleming *et al.,* 2013; Paterson *et al.,* 2006; Jakubowska *et al.*, 2013).

Lin *et al.,* (2015) described NGS methods as a useful technique to identify degraded forensic body fluids. Whole transcriptome sequencing (cDNA) of four body fluids (menstrual blood, vaginal secretion, oral mucosa/saliva and circulatory blood) that were aged two to six weeks was carried out. The RNA integrity was measured based on RNA integrity (RIN) score that was assessed throughout the time course of the experiment. In order to distinguish the body fluids, specificity and sensitivity was determined using fragments per kilobase of exon per million fragments mapped (FPKMs) values of the RNA transcripts (GYPA, MMP11, STATH, HTN3, UBE 2D2, TCEA1, G6PD) assayed. TopHat and Cufflinks analysis software (a tool for gene discovery and comprehensive analysis)

were used to generate FPKM values (Trapnell *et al.,* 2012). The authors reported that the FPKM values for body fluid specific markers corresponds to the target body fluid thus leading to their identification.

1.8.3 Multiplex high resolution melt (HRM) mRNA analysis

Another new and promising technique using a molecular approach (mRNA) to identify forensically relevant body fluids is the multiplex high resolution melt (HRM) analysis proposed by Hanson and Ballantyne in 2014. As current body fluid identification assays are capillary electrophoresis (CE) or quantitative RT-PCR (qRT-PCR) based. These authors were prompted to design a better analysis technique that is aimed at reducing the cost and time of analysis, which are both fundamental factors in CE and qRT-PCR, based assays. Previously, HRM has been reported for single nucleotide polymorphism (SNP) typing (Reed *et al.,* 2004), mutation and methylation analysis (Worm *et al.,* 2001), and copy number variant confirmation (Aten *et al.,* 2008). Post RNA isolation and cDNA synthesis, the technique was reported by the authors to require unlabelled PCR primers in addition to intercalating fluorescent dye (Eva green). The amplimers were slowly melted after amplification by increasing the temperature of the reaction medium. This generated fluorescent signals with the release of the bound dye into the solution. Each marker was thus identified based on the unique melt curve (peak) generated. In this study, single and multiplex assays were developed for forensically relevant body fluids - vaginal secretion, blood, menstrual blood, and semen. The assay had the capacity to distinguish each body fluid. However, some artefacts were reported for the semen/saliva assay. The authors suggested further optimization and/or replacement of these primers with more specific ones that would thus lead to unambiguous identification of the body fluids. Upon adequate validation and optimisation, the authors recommended the assay was a robust mRNA-based identification of body fluids, and a relatively cheaper alternative to CE and qRT-PCR based techniques.

1.8.4 Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)

This technique had been successfully utilised in nucleic acid research ranging from short tandem repeats analysis (STRs) (Krebs *et al.,* 2001); single nucleotide polymorphisms

(SNPs) detection; pharmacogenomics (Pusch *et al.,* 2002); and DNA/RNA sequencing (Edwards *et al.,* 2005). In order to establish an alternative method of mRNA/cDNA analysis of body fluid, while conserving time and cost of analysis, specifically in CE and qRT-PCR based methods where fluorescent dyes are used; Donfack and Willey, (2015) proposed a mass spectrometry based method (MALDI-TOF MS) which is useful in identifying body fluids. This is the first study that investigated the use of MALDI-TOF-MS to identify body fluids. The authors reported the technique to be based on the principles of single base primer extension assay with modified terminators as described by Oeth *et al.,* (2006). Primer (ALAS2, HBB, SPTB, HTN3, STATH, KRT16, KLK3, SEMG1, TGM4, B2M, GAPDH, and HMBS) specificity and sensitivity were tested in venous blood; semen and saliva with the body fluids were accurately identified with no cross reactivity observed. In addition, B2M (a reference gene used as a positive control) displayed a stable expression across the three body fluids tested. With 19 assays designed for this study, the authors recommended further study on other forensically relevant body fluids with adequate optimization, and validation of the assays. Besides, inclusion of more body fluid specific markers as proposed by the authors would likely increase the suitability of the assay as it could accommodate up to 36 plex.

1.9 Use of Reference Genes in mRNA Based Body Fluid Identification

Reference genes are constitutive genes that carry out maintenance of basic metabolic functions in a cell. Irrespective of the cells patho-physiological conditions (Butte, 2001; Pfaffl *et al.,* 2004). They were expected to be uniformly expressed. These unique characteristics make them ideal for use in calibration and normalization studies (Vandesompele *et al.,* 2002). Similarly, they were utilised as internal controls for some experimental studies (Robinson and Oshlack, 2010; Dheda *et al.,* 2004; Rubie, 2005). The use of reference genes has been extensively described in studies that involve qRT-PCR and capillary electrophoresis (CE) (Lindenbergh *et al.,* 2012; Roeder and Haas, 2013). Most body fluid identification assays using miRNA/mRNA attempt to incorporate them, as they were required as endogenous controls and for adequate normalisation of non-biological variances, which may include variations in sample loading, sample-to-sample variation and PCR efficiency. Their use could ascertain whether mRNA/miRNA had been successfully isolated from samples and validate true negatives. Usually the expression

of the target gene is compared to the reference gene, which thus aids the normalisation of data generated. Sauer *et al.,* (2014) in a recent study, assessed a panel of 13 reference genes (hsa-miR-93-5p, hsa-miR-191-5p, RNU6-1, RNU6-2, SNORA66, SNORA74A, SNORD7, SNORD24, SNORD38B, SNORD43, SNORD44, SNORD48 and SNORD49A) and investigated their suitability for use on saliva, venous blood, vaginal secretion, menstrual blood and semen. Expression of the markers in dry samples, mixtures was assayed, in addition to examining biological variation using five individual samples for each body fluid. GenEx software, which comprises geNORM and Normfinder algorithms, was used to select the reference genes. The authors reported SNORD24, SNORD38B and SNORD43 to be most stable across all the body fluids and, thus, recommended them for normalisation of qRT-PCR data analysis of forensic body fluids. Other reference genes of mRNA transcripts have also been reported (Dheda *et al.,* 2004, Pohjanvirta, *et al.,* 2006). Different researchers have outlined fundamental criteria in their choice of reference genes, based on their reported use in other studies. Though most experiments involving CE or qRT-PCR tend to have an identical sequence of workflow, however, the characteristics being investigated vary. With this in mind, it is worthy to note that the choice of a reference gene for any study should not be based on its traditional use in other studies but on adequate validation for the intended study (Meller *et al.,* 2005). Furthermore, since an ideal single reference gene capable of possessing all the required characteristics does not exist (Vandesompele *et al.,* 2002; Pohjanvirta *et al.,* 2006), and use of multiple reference genes has been proposed for normalization of mRNA/miRNA expression studies such as in body fluid identification (Sijen, 2015). Commonly used reference genes in forensic body fluid identification are listed in Table 1.3.

1.10 Contact Traces

Skin cells are mostly left by unsuspecting perpetrators of a crime and by the victims. The recovery of skin cells from a crime scene can give an indication of the presence of contact DNA. Previously, used methods to identify skin cells include dactyloscopic fingerprint analysis and other microscopical and immunological techniques (Lindenbergh *et al.,* 2012). These methods were not ideal as they do not apply to all cell types (Schulz *et al.,* 2010). However, they can introduce contamination into the reaction (Van Hoofstat *et al.,* 1999) and they were reported to have a degradative effect on the

nucleic acids in the skin (Grubwieser *et al.,* 2003). A recent development to circumvent this was reported by Visser *et al.,* (2011), Lindenbergh *et al.,* (2012) and Hall *et al.,* (2013). These authors reported the use of mRNA markers in multiplex reactions to identify skin cells.

Table 1.3: Names of markers and sequences of PCR primers for currently used reference genes for forensic body fluid identification

Marker names	**Forward primers (5'-3')**	**Reverse primers 5'-3'**	**References**
18S rRNA	CTC AAC ACG GGA AAC CTC AC	CGCTCC ACC AAC TAA GAA CG	(Lindenbergh *et al.,* 2012; Fleming, 2010)
ACTB	TGA CCC AGA TCA TGT TTG AG	CGT ACA GGG ATA GCA CAG	(Lindenbergh *et al.,* 2012; Ghani *et al.,* 2013)
B2M	GGCATTCCTGAAGCTGACA	AAACCTGAATCTTTGGAGTACG	(Hanson and Ballantyne, 2013a; Ghani *et al.,* 2013)
OAZ1	GGATCCTCAATAGCCACTGC	TACAGCAGTGGAGGGAGACC	(Hendrik *et al., 2*007)
RPS29	GCACTGCTGAGAGCAAGATG	ATAGGCAGTGCCAAGGAAGA	
S-15	TTC CGC AAG TTC ACC TAC C	CGG GCC GGC CAT GCT TTA CG	(Juusola and Ballantyne, 2003)
β-Actin	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	
TEF	TGG GCC ATC AAC TGA GAA AGA	TCT CCC TAC ACT TCA ACT GCA CA	(Fleming, 2010)
GAPDH	ATC ATC GTG GAG AAG CCC TTC	GTT CCA GAT GGG GCC GA	(Lindenbergh *et al.*, 2012; Park *et al.,* 2013)
UCE	AAT GAT CTG GCA CGG GAC C	ATC GTA GAA TAT CAA GAC AAA TGC TGC	(Fleming, 2010)

1.11 Methods involved in mRNA Profiling

Messenger RNA profiling follows an identical workflow as DNA profiling. The key stages include RNA isolation (Figure 1.2) and purification, quantitation, reverse transcription, polymerase chain reaction and capillary electrophoresis.

1.11.1 RNA extraction

RNA can be isolated from cell, tissues or organs of living organism. There has been reports of RNA isolation from materials that had come in contact with a human e.g. cigarette butts, can of drinks, clothing etc. (Zubakov *et al.,* 2009; Bauer *et al.,* 2003, Kohlmeier *et al.,* 2012). When isolating RNA, adequate care need to be taken to avoid DNA contamination. Few of the available extraction kits in this process include mirVana (Thermo Scientific (UK) Ltd), RNeasy mini plus kit (Qiagen Ltd), and guanidine isothiocynate-phenol chloroform (Thermo Scientific (UK) Ltd). All these studies are in similar manner with tissue lysis and homogenisation, genomic DNA (gDNA) elimination and silica membrane binding of RNA from lysed cells. Further purification includes the use of Turbo DNA-free kit (Thermo Scientific (UK) Ltd) or on-column DNase treatment (Qiagen Ltd). RNA degrades rapidly compared to DNA by ubiquitous ribonucleases (Bauer *et al.,* 2003; Setzer *et al.,* 2008). To circumvent this process, the isolated RNA is normally stored at -80 ^{0}C to stop all enzymatic activities, or converted to complementary DNA (cDNA) through reverse transcription and stored at -20 ^{0}C for further use.

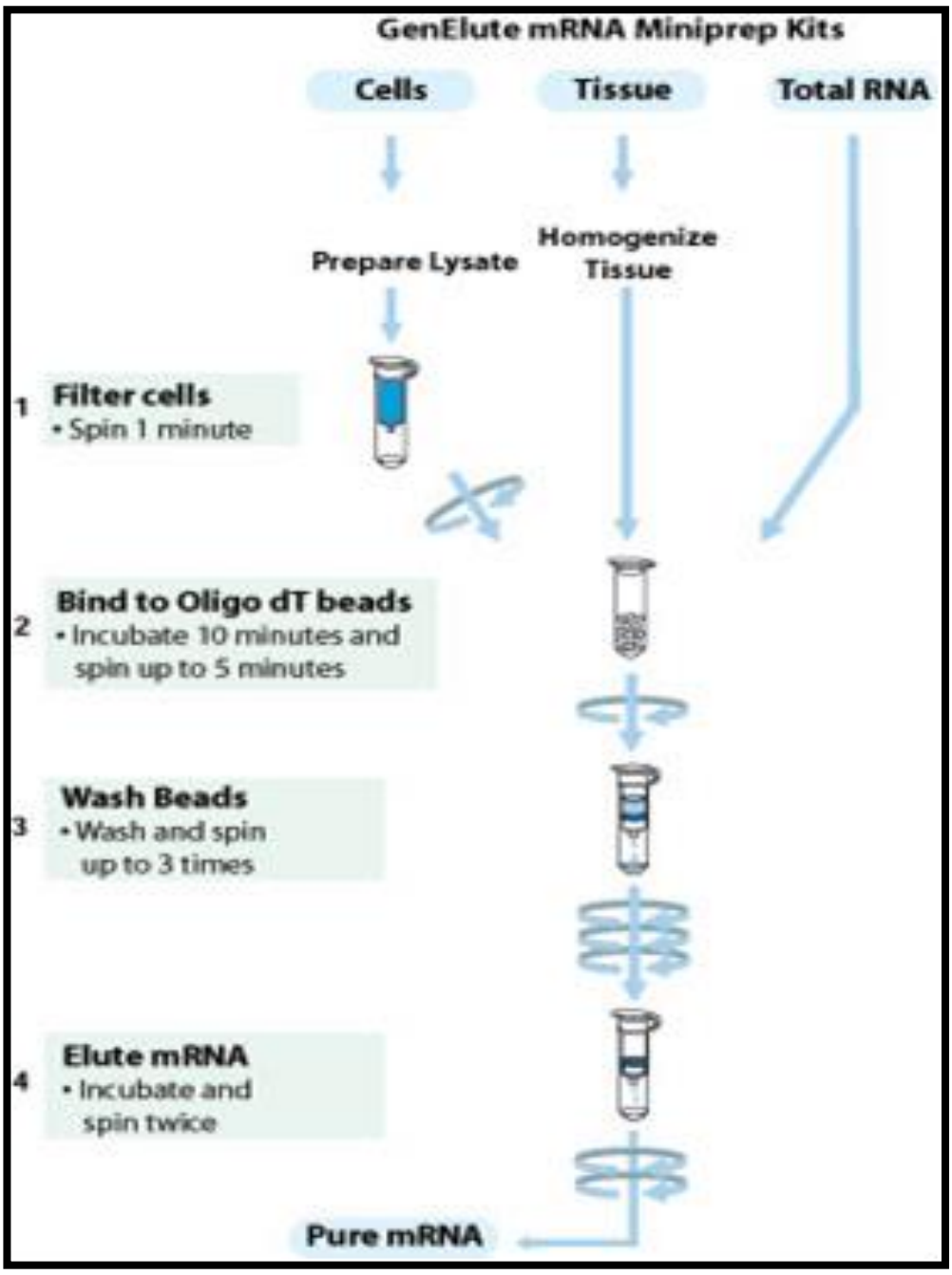

Figure 1.2: Schematic representation of stages involved in RNA isolation from cells and tissues. (www.sigmaaldrich.com. Accessed 03/14/16).

1.11.2 RNA quantitation and reverse transcription

It is important to know the quantity of RNA present in a given sample, especially where the downstream use of RNA involves sample normalisation studies (Vandesompele *et al.,* 2002). This is special to prevent overload of samples, which may result in PCR failure. Popular quantitation kits and equipment include Quant-IT RNA assay kit (Molecular

probes, Eugene); Qubit fluorometer (Thermo Scientific (UK) Ltd) and NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE).

Further, RNA can be converted into complementary DNA (cDNA) through reverse transcription. cDNA is stable and can be stored at 4 ℃ for further use.

1.11.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an enzymatic amplification of a specific region (loci) of DNA, to produce several million copies of the target region. It was first reported in the nineteen-eighties by Saiki *et al.,* (1985) and Mullis and Faloona, (1987). Initially, single regions of DNA were targeted but soon, multiplex PCR, which is the enzymatic amplification of two or more target sequence (loci) was evolved (Kimpton *et al.,* 1993; Edwards and Gibbs, 1994). These suited forensic genetics as minute amounts of template DNA could be studied for multiple loci in a single reaction. The technique also reduced cost and labour of analysis. PCR amplification takes place in the presence of reaction components such as magnesium chloride, DNA polymerase, deoxynucleotide triphosphates (dNTPs), PCR primers and template DNA. During a specific thermal cycling process that allows the PCR primers to anneal and extend, copy number increases exponentially (Garcia and Ma, 2005; Ruijter *et al.*, 2009). Successful amplification requires adequate optimisation of the PCR cycling parameters, especially annealing temperature as this allows the primer pairs to bind specifically to the complementary DNA sequence (Garcia and Ma, 2005) and primer concentrations to compensate for varying amplification efficiencies (Butler *et al.,* 2001). In addition, the number of cycles is a critical parameter that needs to be optimised; most reactions tend to show optimum amplification at 30-33 cycles.

1.11.3.1 Endpoint PCR and Real-time PCR

Endpoint PCR is a biochemical technique whereby a single or multiples copies of DNA is amplified to generate several thousand or millions of a specific DNA sequence. It is carried out either in one-step or two-step. One-step PCR technique combines both cDNA synthesis and PCR in a single reaction, thereby simplifying reaction setup, for easy processing. In addition, possibility of carry over contamination is minimised, as the tube is usually not opened between synthesis and amplification stages. Its usage is slightly

limited as it permits only sequence-specific primers (Wacker and Godard, 2005). Two-step PCR, however, involves reverse transcription of RNA into cDNA in the presence of reverse transcriptase, followed by transferring the cDNA into a different tube for PCR. It is advantageous over one-step PCR in that it gives the flexibility of both primer (oligo (dT), random hexamers or gene-specific primers) and DNA polymerase choice (www.thermofisher.com). The final PCR product from both steps can then be visualised on gels under UV light (for unlabelled primers) or genetic analysers using fluorescently labelled primers (See Figure 1.3)

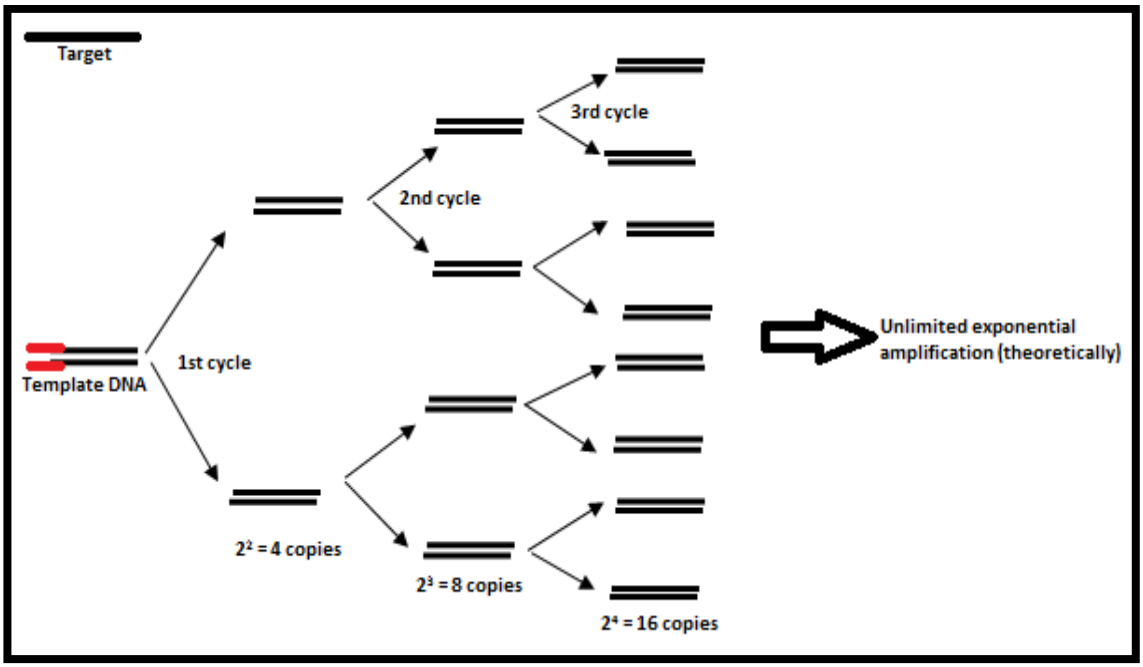

Figure 1.3: Schematic representation of exponential amplification that occurs during PCR (Drawn by hand).

1.11.3.2 Real-time PCR

Unlike end product PCR, real-time PCR allows amplification and profile efficiency to be studied 'real-time' at each cycle of amplification as reaction progresses. This increases the possibility of precise measurement of the amount of amplicons at each cycle, which in turn enables accurate quantitation of the amount of starting materials in samples (Deepak et al., 2007, Real-time PCR handbook, Thermofisher Scientific Ltd). The invention of PCR technology by Mullis and Faloona, (1987) led to the discovery of real time PCR that has since then witnessed a significant revolution in its usage. Its application has cut across many fields of study such as cancer, bacteriology in veterinary

and medical science; plant species identification, food microbiology and forensic science (Deepak *et al.*, 2007). Due to a rise in Reverse Transcriptase (RT-PCR), studies and data generated led to a guideline, which was developed by Bustin *et al.*, (2009). This was referred to as the minimum information required for publication of quantitiative real-time PCR Experiments (MIQE). The main aim of the guideline was to encourage consistency between laboratories, increase transparency in the RT-PCR experiments and highlight the minimum information that should accompany a RT-PCR experimental data to ensure its reliability for publication. These requirements are classified as either essential (E) or desirable (D) (see Bustin *et al.*, 2009).

Real-time PCR technique makes use of two detection chemistries viz non-specific intercalating dyes (SYBR Green) or sequence specific probes (e.g. Hydrolysis/Taqman probes) (Monis *et al.,* 2005; Kubista *et al.,* 2006). SYBR Green chemistry due to its non-specificity binds every double-stranded DNA (dsDNA) molecule in the reaction thereby reducing amplification efficiency. Sequence-specific probes, e.g. Taqman probes however, binds only specific sequence in the reaction medium, thus increasing amplification efficiency (Figure 1.4)

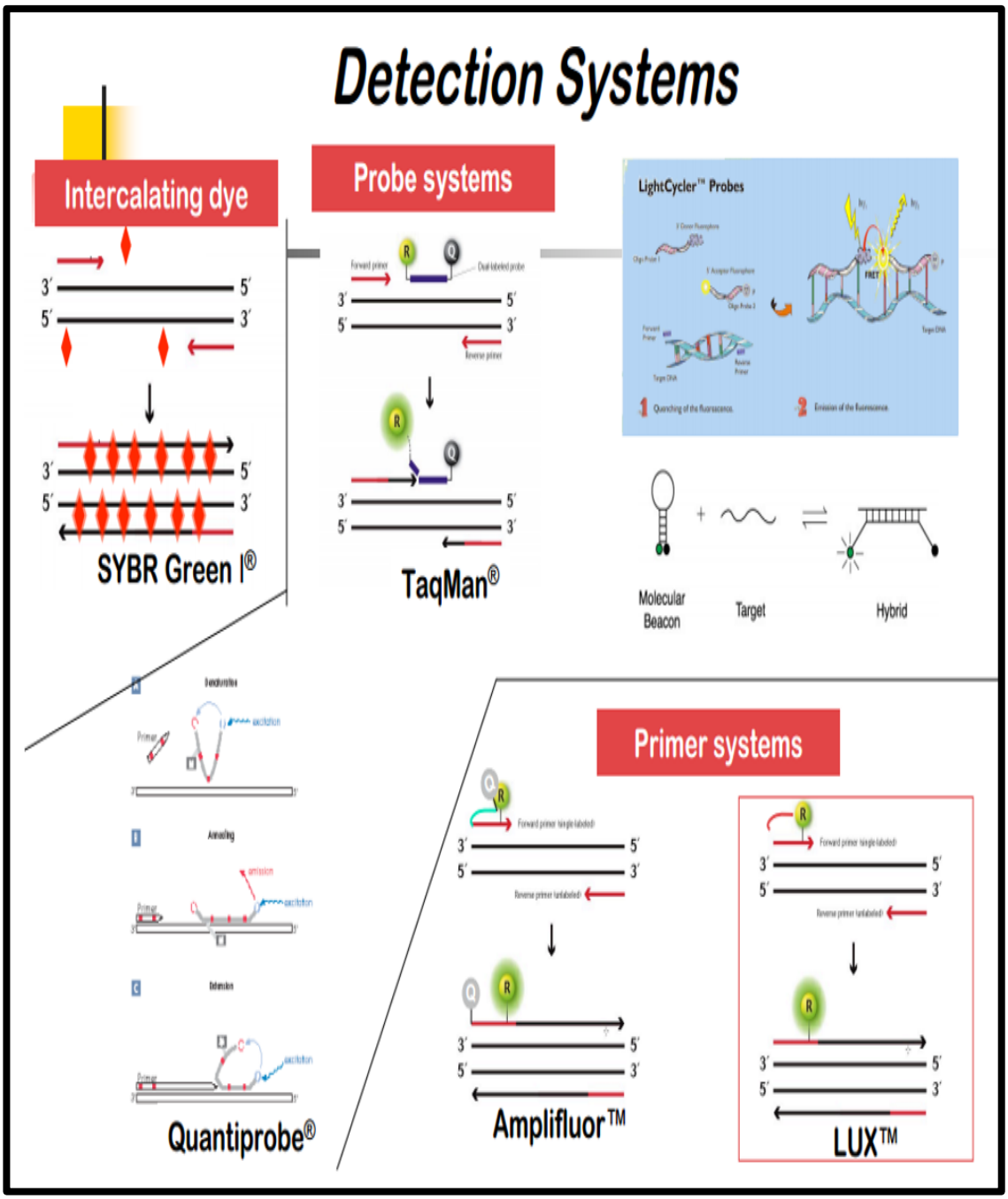

Figure 1.4: Schematic representation of SYBR Green and Taqman probes detection systems used in real-time PCR experiments. SYBR Green uses intercalating dyes while Taqman uses the probes (Adapted from PowerPoint presentation 07/12/2013, Thermofisher Scientific Ltd).

Reaction efficiency is calculated by the given formula:

$$\mathbf{E = 10^{(-1/slope)} - 1}.$$

For a reaction slope of **-3.32** reaction efficiency becomes **1** and this is given by the following equation:

$$\mathbf{y = x\,(1+ E)^{n}}.$$

When PCR efficiency is 100%, the template doubles after each cycle and the equation becomes:

$$\mathbf{y = x\,2^{n}},$$

Where:

y = number of PCR products;

x = initial target copy number;

E = Efficiency and

n = Number of PCR cycles (See Figure 1.5).

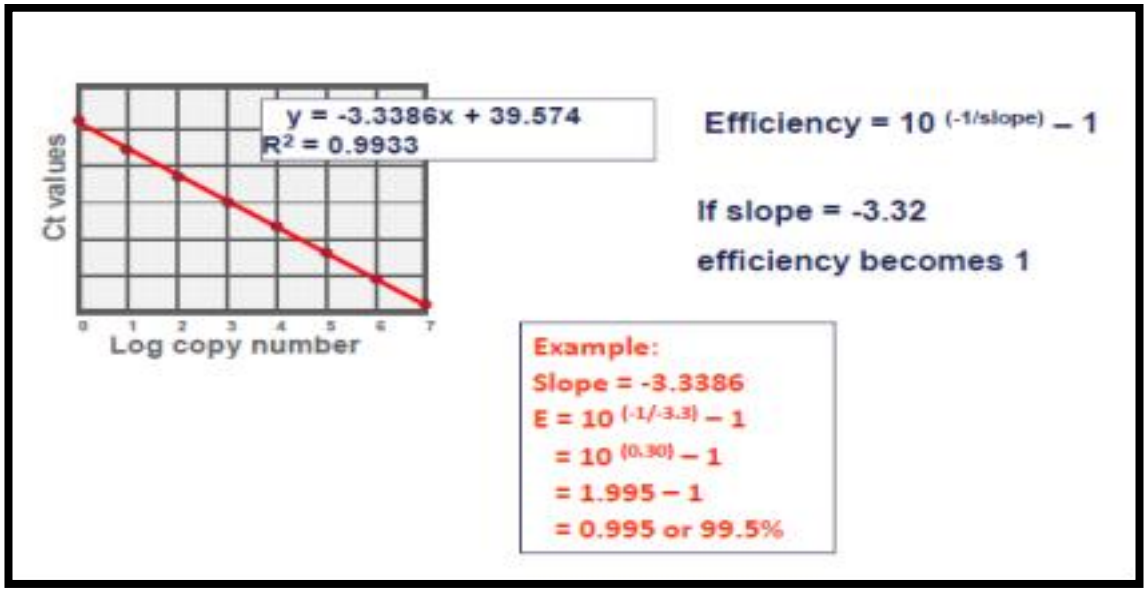

Figure 1.5: A typical A typical graph of Ct values; Log copy number; near 100% efficiency and slope of a real-time PCR reaction. A slope of -3.32 signifies an approximate 99.5% efficiency. (Taken from Life Technologies training PowerPoint presentation, November, 2013).

An amplification plot is generated and captured by a florescent detector in a real-time PCR equipment e.g. ABI 7500 (Thermo Scientific (UK) Ltd). Florescent signal increases with product. Unlike endpoint PCR, four main phases occur in real-time PCR: lag phase, exponential phase, linear and plateau phases. The lag phase is the first in real-time PCR amplification. PCR products increases, however, the florescence generated is too low for detection by the real-time PCR instrument. Most PCR efficiencies are attained at the exponential phase, which is also referred to as the geometric phase. All PCR components – dNTPs, primers, polymerase and template DNA are in abundance thus increasing PCR products generated, also, a direct proportion in signal to increase in PCR product is noticed. At the linear phase, one or more of the PCR components is being used up; this in turn decreases reaction amplification. A further decrease in amplification is noticed at this phase. The plateau phase is the final phase in real-time PCR where no more amplification is noticed because of all reaction components being used up. Florescence detection stops at this stage (Figure 1.6).

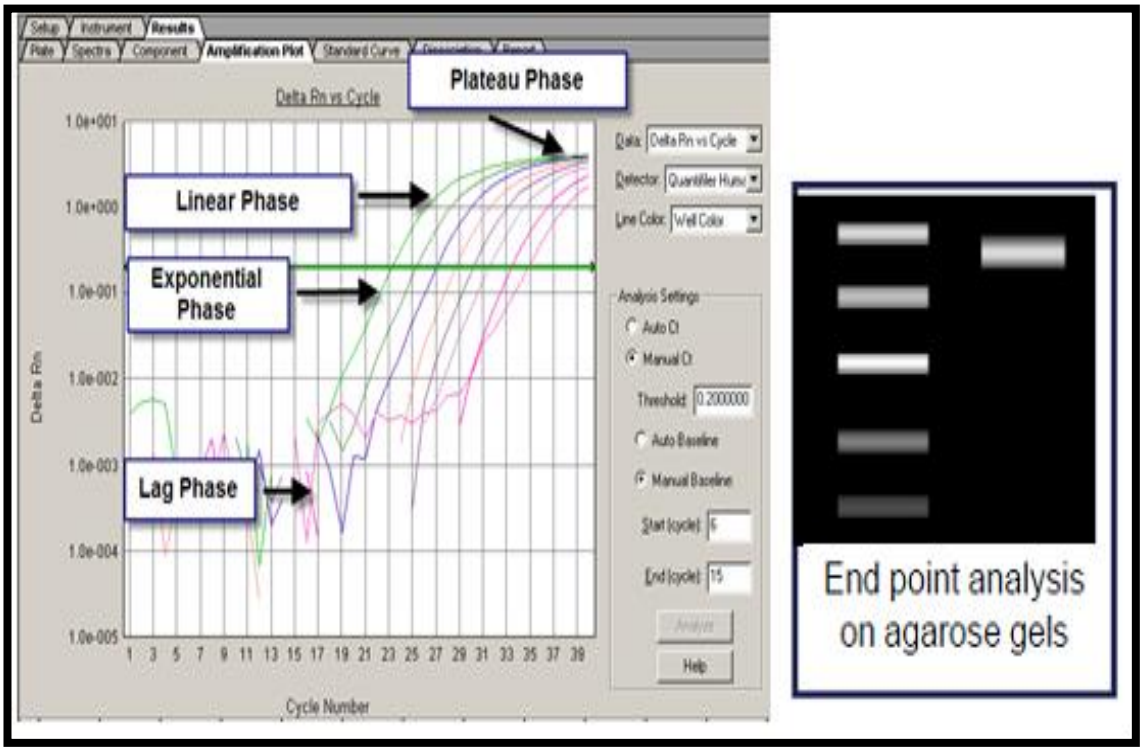

Figure 1.6: A screenshot of lag, exponential, linear and plateau phases - four main phases of real-time PCR experiments (as seen on ABI 7500 (Taken from Thermo Scientific (UK) Ltd) compared to end-product PCR visualization on agarose gels

Ideally, initial cycles 3-15 display an amplification with little or no florescence signal; this is referred to as the baseline. For the purpose of clarity, amplification signal needs to be differentiated from the 'background noise' and therefore, a threshold is set above the baseline. This is where signal level is statistically significant. A cycle threshold (Ct) is the cycle at which florescence signal crosses the threshold. Rn is the value of reported signal divided by reference signal (Figure 1.7).

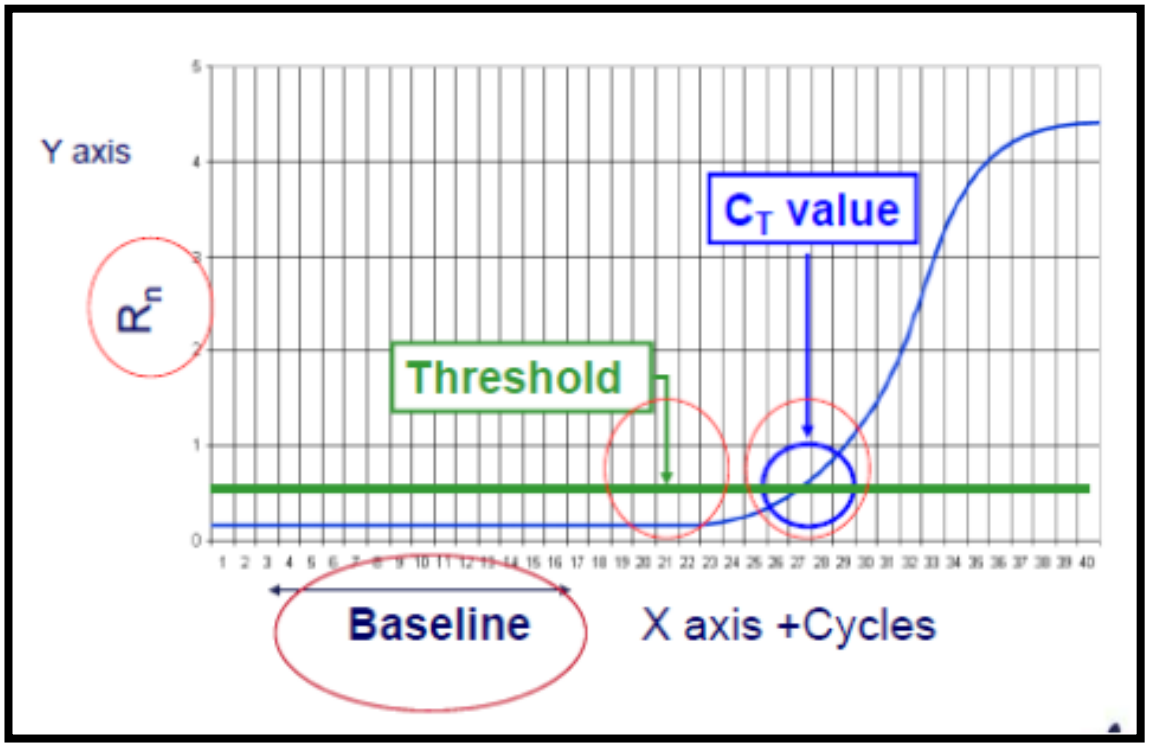

Figure 1.7: A figure showing various terms used in real-time PCR experiments; Ct values, threshold and baseline (Taken from Thermo Scientific (UK) Ltd).

Cycle threshold is inversely proportional to the amount of template added to the reaction. A high amount of template DNA in a reaction will result in low amplification cycle and vice versa. This principle enables DNA samples to be effectively quantitated using calibration curve of Ct value against the logarithm of initial DNA concentration (Kontanis and Reed, 2006; Wong and Medrano, 2005). Standard curves are generated from dilution series of known templates and are used to determine the amount of target DNA samples. In real time PCR experiments, amplifications are quantified either absolutely or relatively. An absolute quantitation makes use of dilution series of known templates to generate a standard curve; unknown templates are then quantified based on the standard curve generated. Relative quantitation however occurs when the

expression of a specific gene of interest (e.g. in treated samples) is compared to the same gene in untreated samples.

The use of taqman probes is preferred to SYBR Green for real time PCR experiments because of its specificity, less concerns about primer dimer formation, flexibility of multiplexing, and minimal optimization requirement. Also, most manufacturers have made available millions of pre-developed assays; however, the overall technique can be cost effective. SYBR Green is cheaper compared to Taqman probes but less specific thereby creating a need for optimization. It does not have the flexibility of multiplexing and they are not pre-developed as found in Taqman probes. In most instances, there is always a need to run melt curves in order to increase specificity of reactions (Real-time PCR handbook, Thermofisher Scientific Ltd). At the end of PCR cycles, the temperature of the reaction is raised, this make the dsDNA molecule dissociate thus releasing the incorporated dye. Different PCR products can then be differentiated from common artefacts (Figure 1.8).

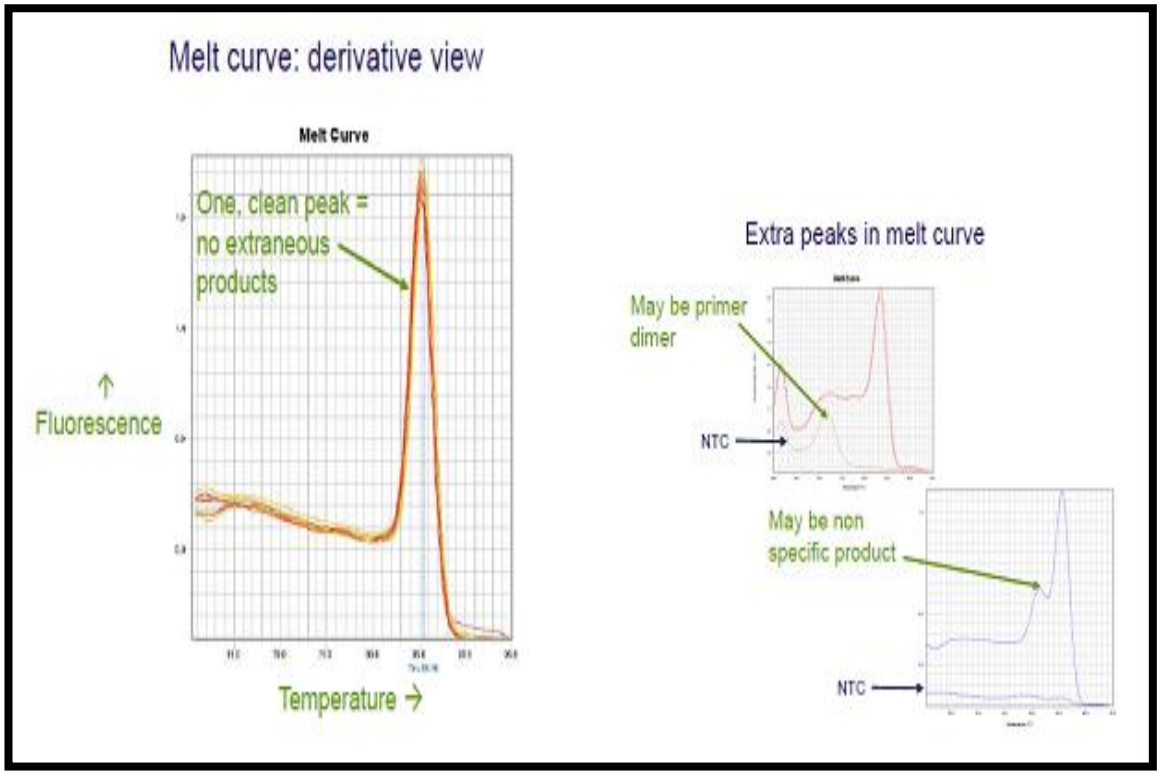

Figure 1.8: Figure showing derivative view of melt curve without extraneous products and with non-specific products which may be artefacts. Melt curves are generated by an additional cycle with increase in temperature. This separates the specific and non-specific products. (Taken from Life Technologies training PowerPoint presentation, November, 2013).

In summary, due to increasing rates of crime which involve body fluids and advancement in technology, there has been an urgent need to develop new techniques to identify forensic body fluids. As stated earlier, the developed assays consist of markers that were reported with cross reactivity with non-target body fluids. This is a major concern in forensic body fluid identification study. The unambiguous identification of body fluids with no report of cross-reaction of non-target body fluids is a major focus of this study.

1.12 Working Hypothesis

An evaluation of genetic markers is of paramount importance for the identification of human body fluid.

1.13 Research aims

Most research on body fluid identification using mRNA makers have reported the use of reference genes and body fluid specific genes for various purposes, which range from normalization to calibration etc. However, reports from these experiments have indicated various cross-reactions of their assays with non-target body fluids, and did not indicate adequate optimization and validation of the genes used.

Therefore, the aim of this project was to develop and validate a multiplex assay consisting of body fluid specific mRNA markers and reference genes, which could be used to identify various body fluids unambiguously.

1.13.1 Research objectives:

- To evaluate a minimum of 10 reference markers currently used in forensic body fluid identification studies.
- To establish the primer efficiencies using SYBR Green chemistry.
- To select the robust markers with 95-100% efficiency and analyze the selected markers using Hydrolysis/Taqman probes and selecting the best with minimal variation across the samples.
- To validate the selected housekeeping genes using fresh and aged samples.
- To develop an mRNA multiplex assay for semen, saliva, menstrual blood, vaginal secretion, and blood.

- To assess the integrity of RNA samples over a period under varying environmental conditions such as temperature, and ultraviolet radiations.
- To validate the developed multiplex assay using SWGDAM guidelines, a criteria used in forensic genetics.
- To assess the efficacy of the developed multiplex assay with the results in published literatures.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.0 General materials

This section described the general materials and methods that were used in all experiments described in this thesis. Other specific methods peculiar to each study was described in relevant chapters.

2.1 Reagents and consumables suppliers

RNeasy Mini Plus kit	(Qiagen Ltd)
Qiagen PCR multiplex mix	(Qiagen Ltd)
RNase-Zap1	(Ambion, Austin, TX, USA)
β- Mercapto ethanol (β-ME)	(Thermofisher Scientific Ltd)
RNase-free water	(Thermofisher Scientific Ltd)
Turbo DNA-free kit	(Thermofisher Scientific Ltd)
SuperScript VILO MasterMix	(Thermofisher Scientific Ltd)
XpressRef Universal RNA	(Qiagen Ltd)
SuperMix Express SYBR GreenER qPCR	(Thermofisher Scientific Ltd)
Taqman Gene Expression assay	(Thermofisher Scientific Ltd)
TaqMan Universal PCR Master Mix	(Thermofisher Scientific Ltd)
RNAse mix	(Thermofisher Scientific Ltd)
Platinum PCR singleplex mix	(Thermofisher Scientific Ltd)
Platinum PCR multiplex mix	(Thermofisher Scientific Ltd)
1.5 ml microcentrifuge tube	(Starlabs (UK Ltd)

0.5 ml PCR tubes	(Starlabs (UK Ltd))
Propylene seal	(Starlabs (UK Ltd))
96-well PCR plate	(Applied Biosystems Ltd)

2.2 Good Laboratory practice

Adequate precautions were taken throughout the course of this study in order to guarantee quality results. Before commencement of any experiment, workbench and pipettes were cleaned with 70% ethanol and RNAse-Zap1 (Ambion, Austin, TX, USA). Dedicated lab coats for both pre and post-PCR labs, and disposable gloves were worn throughout the procedure. Dedicated set of pipettes for RNA use and sterile filter tips were used to prevent cross-contamination. Unless otherwise stated, each batch of reactions was prepared on ice with both positive and negative control samples; all samples were run in duplicate.

2.3 Health and Safety; Ethics

All risk assessments, COSHH forms were completed before the commencement of lab work. Ethical approval was obtained from the Health, Safety and Ethics Committee of the University of Central Lancashire prior to sample collection and start of experiments (Appendix 1-2).

2.4 Equipment calibration

All major instruments used in this research was checked to ensure up-to-date calibration. Authorised personnel, usually a laboratory technician or a Field Engineer from the manufacturer carried these out regularly. Dedicated set of micropipettes used were sent to Star labs for calibration before the start of laboratory work.

2.5 Sample collection

Blood, saliva, semen, vaginal secretion and menstrual blood samples were collected from voluntary donors (recruited through poster advert) in the University of Central Lancashire, Preston, UK after an informed consent was obtained (Appendix 3). Blood sample was collected by two methods viz finger prick and by intravenous collection. For finger prick collection, the finger was cleaned with disinfectant wipes; a sterilized lancet

was then used to prick the finger. Blood was then collected on cotton swabs and left to dry at room temperature. Venous blood was collected into EDTA tubes without anticoagulant by a qualified phlebotomist within the University. The dry blood on sterile cotton swabs and those in EDTA tubes were then stored at -80 ^{o}C for further use. Saliva samples were collected using sterile cotton swabs and directly into 1.5 ml Eppendorf tubes. For cotton swab collection. The donors were encouraged to swab their inner cheek for at least 30 seconds before being left to dry at room temperature. A volume of 20 µL aliquots were prepared for saliva collected in Eppendorf tubes before being stored frozen at -80 ^{o}C together with those collected on sterile cotton swabs. Semen was collected in 1.5 ml Eppendorf tubes and 20 µL aliquots were made before being stored frozen at -80 ^{o}C. Menstrual blood and vaginal secretion samples were collected on sterile cotton swabs. For vaginal secretion, donors were also encouraged to swab the vaginal until the cotton swabs becomes damp. Likewise, for menstrual blood, donors were encouraged to press the cotton swabs tightly against the sanitary pad that contains the menstrual blood. They were then advised to leave the samples to dry at room temperature before submission for storage. Both samples upon collection were then stored at -80 ^{o}C. Further details regarding sample preparation and storage have been highlighted in relevant chapters.

2.5.1 Primer quality control

Prior to primer order, Basic Local Alignment Search Tool (BLAST) was used to obtain each gene sequence in GeneBank® (www.ncbi.nlm.nih.gov) and to check species specificity. These were then ordered from Life Technologies (UK) Ltd and Eurofins Genomics Ltd (See Tables 2.1 and 2.2)

Table 2.1: Primer sequences of 10 reference genes used in Real-time PCR assays

Oligo names	Forward sequences 5'-3'	Reverse sequences 5'-3'	References
TEF	TGGGCCATCAACTGAGAAAGA **Hs01896387_g1, 206bp****	TCTCCCTACACTTCAACTGCACA	(Fleming and Harbison, 2010; Roeder and Haas, 2013)
ACTB	TGA CCC AGA TCA TGT TTG AG **Hs03023943_g1, 75bp****	CGT ACA GGG ATA GCA CAG	(Ghani et al. 2013; Lindenbergh *et al.*, 2012)
UCE	AATGATCTGGCACGGGACC **Hs00366152_m1, 241bp****	ATCGTAGAATATCAAGACAAATGCTGC	(Fleming and Harbison, 2010; Roeder and Haas, 2013)
B2M	GGCATTCCTGAAGCTGACA **Hs00187842_m1, 120bp****	AAACCTGAATCTTTGGAGTACG	(Ghani et al. 2013; Hanson and Ballantyne, 2013)
18S rRNA	CTC AAC ACG GGA AAC CTC AC	CGCTCC ACC AAC TAA GAA CG	(Fleming and Harbison, 2010; Lindenbergh *et al.*, 2012; Haas *et al.*, 2009; Haas *et al.*, 2011).
OAZ1	GGATCCTCAATAGCCACTGC	TACAGCAGTGGAGGGAGACC	(Hendrik *et al.*, 2007)
S-15	TTC CGC AAG TTC ACC TAC C	CGG GCC GGC CAT GCT TTA CG	(Juusola and Ballantyne, 2003)
GAPDH	ATC ATC GTG GAG AAG CCC TTC	GTT CCA GAT GGG GCC GA	(Virkler and Lednev, 2009; Jakubowska *et al*. 2013; Nussbaumer *et al*., 2006)
β-Actin	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	(Juusola and Ballantyne, 2003)
RPS29	GCACTGCTGAGAGCAAGATG **Hs03004310_g1, 213bp****	ATAGGCAGTGCCAAGGAAGA	(Heinrich *et al.,* 2007*)*

****Genes selected for Taqman probe design.**

Table 2.2: Body fluid specific primer sequences:

Markers	Sequences (5'-3')	Sizes (bp)	References
PRM1	**Fw:** AGA CAA AGA AGT CGC AGA C **Rv:** TAC ATC GCG GTC TGT ACC	91	(Lindenbergh *et al.*, 2012)
TGM4	**Fw:** TGAGAAAGGCCAGGGCG **Rv:** AATCGAAGCCTGTCACACTGC	215	(Roeder and Haas, 2013)
MSMB	**Fw:** GGA GTT CCA GGA GAT TCA **Rv:** GTA GCA AGT GCA TGT CTC	105	(Park *et al.*, 2013)
NKX3-1	**Fw:** GTT GGA CTC TGA AAA CAC TTC **Rv:** GCC GAC AGG TAC TTC TGA	144	(Park *et al.*, 2013)
ALAS2	**Fw:** GCCAATGACTCTACTCTCTTCACCTTGGC **Rv:** GGAAGCATGGTTGCCTGCGTCTGAG	81	(Roeder and Haas, 2013)
PF4	**Fw:** GGTCCGTCCCAGGCACATCACCAGC **Rv:** CAGCGGGGCTTGCAGGTCCAAG	124	(Roeder and Haas, 2013)
STATH	**Fw:** TTT GCC TTC ATC TTG GCT CT **Rv:** CCC ATA ACC GAA TCT TCC AA	93	(Roeder and Haas, 2013)
HTN3	**Fw:** GCA AAG AGA CAT CAT GGG TA R**v:** GCC AGT CAA ACC TCC ATA ATC	134	(Roeder and Haas, 2013)
CRYP2B7P1	**Fw:** TCCTTTCTGAGGTTCCGAGA **Rv:** TTTCCATTGGCAAAGAGCAT	198	(Hanson and Ballantyne, 2013)
L. *crispatus*	**Fw:** GTATCCAGAGCAAGCGGAAGCACAC **Rv:** GCATCTCTGCATTGGGTTCCCCC	267,305	(Roeder and Haas, 2013)
MUC4	**Fw:** CTG CTA CAA TCA AGG CCA **Rv:** AAG GGA AGT TCT AGG TTG AC	141	(Lindenbergh *et al.,* 2012)
SFTA2	**Fw:** CGGGACTTCAGTGTCTCCTC **Rv:** TTCCAGGAAGCTGGACTCAT	183	(Hanson and Ballantyne, 2013)
UCE	**Fw:** AATGATCTGGCACGGGACC **Rv:** ATCGTAGAATATCAAGACAAATGCTGC	241	(Roeder and Haas, 2013)
ACTB	**Fw:** TGA CCC AGA TCA TGT TTG AG **Rv:** CGT ACA GGG ATA GCA CAG	75	(Lindenbergh *et al.,* 2012)

2.5.2 Preparation of RNA standards

Human XpressRef Universal RNA (Qiagen Ltd) was used as RNA standard. A 10 ng/µl aliquot was prepared from the stock by diluting with RNase free water. For reference genes experiment (*Chapter 3*), 1:10 and 1:5 serial dilutions of the standard (10 ng/µl) was prepared over five logs (0.1, 0.01, 0.001, 0.0001, and 0.00001) and (0.2, 0.04, 0.008, 0.0016, and 0.00032) respectively with RNase-free water. RNA isolated from blood, semen, saliva, vaginal secretion and menstrual blood were also prepared in both 1:10 and 1:5 serial dilutions over five logs.

2.5.3 Preparation of body fluid samples

All fresh samples were preserved by storage at -80 ^{0}C in order to stop enzymatic activities. Ten samples for each body and positive control were prepared for reference genes study. A volume of 10 µL of blood, saliva and semen were aliquot onto sterile cotton swabs; 10 swabs each of vaginal secretion and menstrual blood were also collected from -80 ^{o}C storage. All body fluids were placed in a dark cupboard and left undisturbed at room temperature for a minimum of six months.

2.5.4 Preparation of RNA samples for degradation experiments

RNA samples were degraded using incubator and UV cross linker. More details of degradation experiments are stated in relevant sections and chapters in this thesis.

Table 2.3: Oligo names and sequences

Oligo names	Forward primers (5'-3')	Reverse primers 5'-3'	References
18S rRNA	CTC AAC ACG GGA AAC CTC AC	CGCTCC ACC AAC TAA GAA CG	(Lindenbergh *et al.,* 2012, Fleming and Harbison, 2010, Haas *et al.,* 2011, Haas *et al.,* 2009).
ACTB	TGA CCC AGA TCA TGT TTG AG	CGT ACA GGG ATA GCA CAG	(Lindenbergh *et al.,* 2012, Ghani *et al.,* 2013)
B2M	GGCATTCCTGAAGCTGACA	AAACCTGAATCTTTGGAGTACG	(Ghani *et al., 2*013, Hanson and Ballantyne, 2013)
OAZ1	GGATCCTCAATAGCCACTGC	TACAGCAGTGGAGGGAGACC	(Hendrik *et al.,* 2007)
RPS29	GCACTGCTGAGAGCAAGATG	ATAGGCAGTGCCAAGGAAGA	
S-15	TTC CGC AAG TTC ACC TAC C	CGG GCC GGC CAT GCT TTA CG	(Juusola and Ballantyne, 2003)
b-Actin	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	
TEF	TGG GCC ATC AAC TGA GAA AGA	TCT CCC TAC ACT TCA ACT GCA CA	(Fleming and Harbison, 2010)
GAPDH	ATC ATC GTG GAG AAG CCC TTC	GTT CCA GAT GGG GCC GA	(Park *et al.,* 2013, Lindenbergh *et al.,* 2012, Haas *et al.,* 2009, Virkler and Lednev, 2009, Jakubowska *et al.,* 2013) (Ghani *et al.,* 2013, Nussbaumer *et al.,* 2006)
UCE	AAT GAT CTG GCA CGG GAC C	ATC GTA GAA TAT CAA GAC AAA TGC TGC	(Fleming and Harbison,2010)

2.6 Efficiency of RT primers

The efficiencies of reference genes were evaluated using SYBR Green detection method. Genes with 90-110% efficiency and a slope of -3.33±10% were then selected and Taqman probes were designed for them (See Figure 3.1).

2.6.1 SYBR Green

The efficiency of 10 primers selected (Table 2.3) was determined using SYBR Green detection chemistry. Reverse transcribed human XpressRef Universal RNA (Qiagen Ltd) was used for efficiency testing. This sample was selected due to its source and industrial purification. Also, it reduced the doubts of getting false negative and positive results which could arise due to variations in the different body fluids.

A 10 ng/µL starting input RNA concentration was reverse transcribed. 1:10 and 1:5 serial dilution was prepared over 5 logs using 5 µL cDNA. Following manufacturers recommendations, a volume of 10 µL SuperMix Express SYBR GreenER qPCR (Thermofisher Scientific Ltd) and 2 µL cDNA from each serial dilution was prepared. Primer concentrations and water volumes were varied to make a 20 µL final reaction volume. Negative controls were used to assess any possible DNA contamination of the primer/enzyme mixes. Standard curve was generated for the samples using a thermal cycling condition of 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C for 15 sec, 60 ^{o}C for 60 sec. Melting curve stage was added for specificity in the target sample amplification. A software default instrument threshold of 0.133 was used for data analysis.

2.6.2 Taqman/Hydrolysis probe selection

Custom Taqman probes were designed for each body fluid markers that passed the qPCR efficiency test using SYBR Green detection chemistry. The primer sequences were copied into the Basic Local Alignment Search tool (BLAST) to get the gene sequences. These were then pasted into the Custom taqman assay design tool (Thermofisher Scientific Ltd) for taqman probe design. The probes were designed to span exon junctions so as not to amplify gDNA. The custom taqman assay design tool is a free and user-friendly online software (Thermofisher Scientific Ltd) that enables the user to design a custom taqman assay specific for an experiment. It displays the locations of

genes on the the chromosome and which makes it easier should a probe redesign be necessary (see Figure 2.1).

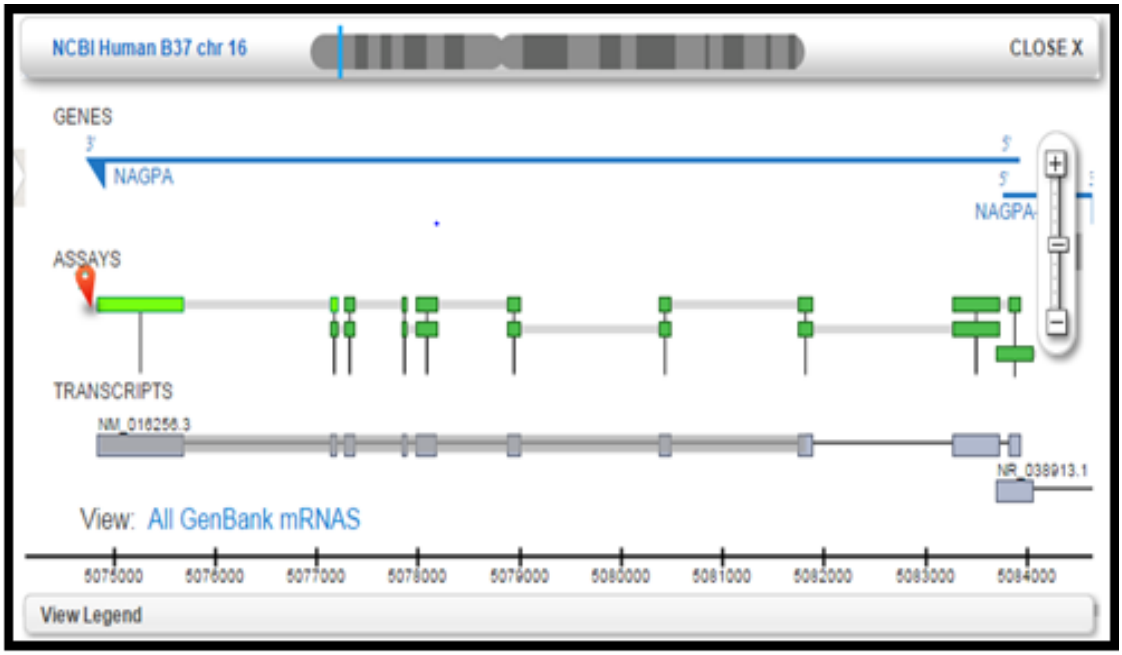

Figure 2.1: Screenshot of Taqman probe assay design tool (Thermofisher Scientific Ltd). This displays the location of specific genes on the chromosomes, should a possible redesign be necessary.

Table 2.4: Primer sequences selected for Taqman probe design.

Oligo names	Forward primers 5'-3'	Reverse primers 5'-3'
TEF	TGGGCCATCAACTGAGAAAGA	TCTCCCTACACTTCAACTGCACA
ACTB	TGA CCC AGA TCA TGT TTG AG	CGT ACA GGG ATA GCA CAG
UCE	AATGATCTGGCACGGGACC	ATCGTAGAATATCAAGACAAATGCTGC
B2M	GGCATTCCTGAAGCTGACA	AAACCTGAATCTTTGGAGTACG
RPS29	GCACTGCTGAGAGCAAGATG	ATAGGCAGTGCCAAGGAAGA

2.7 Limit of detection/ sensitivity testing of RT primers and probes

According to MIQE guideline (See Bustin *et al.,* 2009), LOD which is the concentration of a sample that can be detected with resonable certainty in a given experiment was calculated. As a rule, this is also required as one of the essential checklists for RT-qPCR experiments.

All samples that were selected for LOD/sensitivity testing were diluted to 1ng/µL. A volume of 2 µL of each sample was then taken and further diluted down to 25 pg and reverse transcibed. This was to identify the ideal starting RNA concentration that should be added to the realtime PCR experiment without inhibition. A volume of 2 µL cDNA from each concentration (25pg – 2ng) was then added to the qRT-PCR sample preparation.

Quantitative PCR (qPCR) reaction was performed using target-specific TaqMan Assays and TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies) following manufacturer's protocol. A final reaction volume of 20 µL comprised 10 µL Taqman Universal PCR Mastermix, a volume of 1 µL Taqman Gene Expression assay mix (20x), 2 µL cDNA and 7 µL RNase free water. All samples were run in duplicates for limit of detection (LOD) experiments. Standard curve which is the use of dilution series of known template concentration to determine the starting amount of target template in experimental samples was generated on ABI 7500 SDS software (version 1.4.1) (Thermofisher Scientific Ltd). The cycling conditions include the following: UNG activation of 2 min at 50 ^{o}C; initial step of hold, 10 min at 95 ^{o}C followed by 40 cycles of denaturation at 95 ^{o}C for 15 sec and final annealing/ extension at 60 ^{o}C for 1 min. See Chapter 2 for more details of statistical analysis.

2.8 Body fluid specific marker selection and Multiplex design

Multiplex PCR was first described in Chamberlain *et al.,* (1988). For successful amplification to take place, the role of each component – PCR reagents, primer concentrations, and cycling conditions must be clearly understood and put into consideration (Henegariu *et al.*, 1997). The markers selected (Tables 2.1 and 2.2) were aimed to amplify amplicon sizes of 76 bp – 304 bp with predicted annealing temperature of 54-62 ^{o}C. The G+C contents of each primer was in the range 40-65%.

At the start of the experiment, non-fluorescent primers were purchased and each of these was optimised in singleplex PCRs. Initially, reference genes were optimised using Platinum PCR multiplex mix (Life Technologies Ltd), Tm 54-62 ^{o}C, equimolar primer concentrations (10 µM) and cDNA (2 ng positive control RNA (Qiagen Ltd), (Table 2.5). The primer concentrations were then modified as required throughout the experiment. These were optimised at 20, 25, 30, 33 and 35 cycles, with modified extension times from 10 min to 8min, 5min and 2 mins, respectively. PCR products were assessed on Agarose gels. Subsequent experiments involved PCR optimisation using mRNA isolated from each specific body fluid – blood, semen, saliva, vaginal secretion and menstrual blood using Qiagen PCR multiplex kit (Qiagen Ltd), (Table 2.6). Following the results obtained from PCR optimization using non-florescent primers, markers that displayed positive amplification with target body fluids were labelled with fluorescein dye at the 5' end (Table 2.12). Again, these were optimized in singleplex PCRs before being combined into a multiplex assay.

The multiplex PCR combinations were carried out at first in five separate multiplexes, one for each body fluid. These comprised of four markers (**Glyco-A, ALAS2, PF4** and **SPTB**) for blood; three markers (**MSX-1, SERP** and **LEFTY**) for menstrual blood; five markers (**PRM1, PRM2, TGM4, MSMB,** and **NKX3-1**) for semen; four markers (**SFTA2, MUC4, L. *crispatus*,** and **CRYP2B7P1**) for vaginal secretion; and three markers (**HTN3, FDCSP** and **STATH**) for saliva. These five assays were then combined into a single multiplex, which was optimised in this chapter and validated (*Chapter 5*).

Table 2.5: PCR cycling parameters used for Platinum PCR SuperMix for Optimization of Reference Gene PCR

PCR stages	Temperatures		Times
Initial denaturation	94 ^{o}C		30 s
Denaturation	94 ^{o}C		30 s
Annealing	58 ^{o}C	**35 cycles**	30 s
Extension	68 ^{o}C		1 min
Final extension	68 ^{o}C		10min
Final hold	4 ^{o}C		∞

Table 2.6: PCR cycling parameters used for Qiagen multiplex kit for Multiplex PCRs of mRNA Markers for Blood, Saliva, Semen, Vaginal Fluid and Menstrual Blood

PCR stages	Temperatures	Times
Initial denaturation	95 °C	15 min
Denaturation	94 °C	15 s
Annealing	58 °C **33 cycles**	1 min
Extension	72 °C	1 min
Final extension	72 °C	20min
Final hold	4 °C	∞

2.9 Developmental validation sample preparation

The developmental validation was carried out using well-calibrated instruments and micropipettes. These were within the manufacturer's calibration settings where required (Section 2.4).

Samples used in this study were collected from voluntary donors from the University of Central Lancashire, Preston (Section 2.5). Degraded samples were sent in from Saudi Arabia to assess the robustness of the developed multiples assay. Unless otherwise stated, mRNA was isolated and quantified from human body fluid samples using the methods described (Sections 2.10-2.14).

2.9.1 Reaction components and thermal cycling conditions

Reaction components such as annealing temperature, cycle number and master mix were optimised in this study. For consistent and robust results, two different mastermix, Qiagen PCR multiplex mix (Qiagen Ltd) and Platinum supermix (Life technologies Ltd) were both optimised at 54 °C, 56 °C, 58 °C and 60 °C.

2.9.2 Primer titration

The effect of primer concentration was determined by preparing a primermix as outlined in Chapter 4, section 4.4.3. From the primermix, 1 µl, 1.5 µl, and 3.5 µl was taken and added to the reaction components

2.9.3 Cycle number

Amplification of cDNA samples were optimised at 28, 30, 33 and 35 cycles. These were carried out using the same thermal cycler.

2.9.4 Species specificity

For specie specificity study, extracted human DNA samples from blood, saliva and bone were collected from storage within the Forensic Genetics Group (FGG) in the university. Also, mammalian samples from horse, pig, wood mouse, rat, and rabbit were also collected with adequate permission and used for this study. All samples have been used previously for studies unrelated to specie identification.

2.9.5 Reproducibility study

Following preparation of primermix (See Chapter 4, section 4.4.3), a 350 µl aliquot was made and sent to another independent laboratory for use on their prepared mock casework samples. The samples were run on 3130 XL Genetic analyser as reported by the laboratory.

2.9.6 Mixture study

Mixture of all four mRNA body fluid samples were first prepared in equimolar concentrations, and then modified in order to obtain a near balanced peak height for the overall profile. Further experiments involved preparation of mRNA samples mixtures in duplex combinations. These included blood + saliva, semen + vaginal secretion, blood + semen and blood + vaginal secretion with mixture ratios of 1:2, 1:4, 1:9, 1:1, 9:1, 4:1 and 2:1. For each combination, 1 ng/µl was used, which represents one sample ratio.

2.9.7 Sensitivity study

Dilution series was carried out for the sensitivity study with a 5 µl (1ng/µl) starting mRNA concentration for each body fluids using the following series: 1:10, 1:50, 1:250, 1:500, 1:1000, 1:1500, and 1:3000. These were reverse transcribed and amplified in duplicate reactions.

2.9.8 Degradation experiments

The degradation experiment was carried out under two conditions viz controlled and uncontrolled degradation. For controlled degradation, three different experiments were designed.

2.9.8.1 Controlled degradation

First, blood samples were prepared on different substrates such as metal scalpel, hand gloves, glass slide and facial mask. For each substrate, a volume of 10 µL blood sample was spotted. These were left to dry at room temperature and then stored in a cool, dark cupboard for six weeks. Samples were taken with sterile and wet cotton swabs every two weeks for extraction and downstream analysis. Secondly, a 10 µL blood, semen and saliva samples were spotted on glass slides and incubated at 37 ^{0}C for six weeks. Samples were also taken every two weeks with wet and sterile cotton swabs for downstream analysis. The final controlled degradation study involved preparation of extracted mRNA samples from blood, saliva, vaginal secretion and semen, following degradation under UV crosslinker (X100 µL/cm^{2}) and incubator at 56 ^{0}C. Only the last degradation experiment with extracted mRNA samples was carried out in parallel between UV cross-linker and Incubator at 56 ^{0}C, others were carried out and analysed independently. For UV cross-linker and incubator at 56 ^{0}C, 10 µL of each sample was taken at intervals of 5, 10, 30, 60 and 90 mins for downstream analysis.

2.9.8.2 Uncontrolled degradation

Another member of FGG, UCLan (a former PhD student) in Saudi Arabia, prepared Blood, saliva and semen used for uncontrolled degradation study. These samples were the batches remaining following his degradation study. According to him, the samples were prepared in the summer of 2014 and left in a secure place outside, exposed to direct sunlight for a total of 51 days. For each body fluid sample, a 5 µl volume was pipetted on cloth (100% pure cotton), metal (a large stainless steel kitchen knife) and glass (microscopic slides). A metallic grid was placed on the samples to protect them from scavengers; otherwise, they were left exposed for the duration of the experiment. Samples were taken every 48-72 hours with swabs, the swabs heads were then cut out with a sterile scalpel and stored in 1.5 ml Eppendorf tubes at -20 ^{o}C for further use.

Environmental conditions reported during the sample preparation included maximum temperature of 49.5 ^{0}C, minimum temperature of 6.3 ^{0}C, maximum and minimum relative humidity of 95 %, respectively. Following ethical guidelines, the samples were requested and brought to the UK by the student in June 2016 on one of his visits to the University. These were packaged in a sealed plastic bag. It was picked up from the drop-off point after 3 days due to communication issues. The samples when opened were found in a poor state, considering they have been initially stored frozen and been in transit for at least 5 days. From the information that came with the samples, they were not in order of sampling as the student used most of the samples for his project. Based on the dates on each Eppendorf tube containing the swab heads, samples taken at days 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 51 were selected for this study.

2.10 RNA extraction

RNA was extracted from all body fluids using RNeasy Mini Plus kit (Qiagen Ltd) according to manufacturers' instructions. For every sample, 400 µL RLT Plus/βME mixture was added to a 1.5 ml microcentrifuge tube. The samples were then transferred (direct pipetting/swab head) into the microcentrifuge tube. These were vortexed and incubated for 10 min at room temperature. During incubation, the samples were vortexed for approximately 2 min, all the stains solubilized after 10 min. The samples were briefly centrifuged and the swab head/ cutting was transferred into a spin basket. This was later centrifuged at maximum speed for 5 min. The eluates were transferred into a genomic DNA (gDNA) eliminator spin column. Care was taken during pipetting to avoid air bubbles. Afterwards, the samples were centrifuged for 30 secs at 11, 000 rpm (11.2 x g), making sure no fluid remained in the spin column membrane. If any fluid is noticed on the membrane, the samples were re-centrifuged. The spin columns were discarded and 400 µL of 70% ethanol was added to the eluate and mixed well with pipette. While avoiding air bubbles, the eluate/ethanol was transferred to the RNeasy spin column and centrifuged for 15 secs at 11, 000 rpm (11.2 x g). The flow through was discarded and the spin column placed back in the collection tube. A volume of 700 µL RW1 buffer was added and centrifuged at 11, 000 rpm (11.2 x g) for 15 sec. Again, the flow through was discarded and the spin column placed back in the collection tube. Ethanol was added to the RPE buffer (following manufacturers' recommendation) and

500 µL RPE/ethanol was added to the spin column, centrifuged for 15 secs at 11, 000 rpm (11.2 x g), the flow-through was discarded and the spin column was placed back in the collection tube. A volume of 500 µL RPE buffer/ethanol mix was added again to the spin column and centrifuged for 2 min at 11, 000 rpm (11.2 x g). The flow-through and the used collection tubes were discarded. While ensuring that ethanol was not carried over into the RT-PCR, the spin column was centrifuged for 1 min at maximum speed and placed in a new 1.5 ml collection tube. Without touching the spin column membrane with the pipette tip, 30 µL RNase-free water was added directly to the membrane. This was incubated for 2 min at room temperature and centrifuged for 1 min at 11, 000 rpm (11.2 x g) to elute the RNA. For increased yield of RNA, the eluate was placed unto the membrane again, incubated for 5 min at room temperature and centrifuged for 1 min at 11, 000 rpm (11.2 x g) to elute the RNA (See Figure 2.2). Following final elusion, the eluate, which contain RNA, was then purified using Turbo DNA-free kit (Life Technologies Corp).

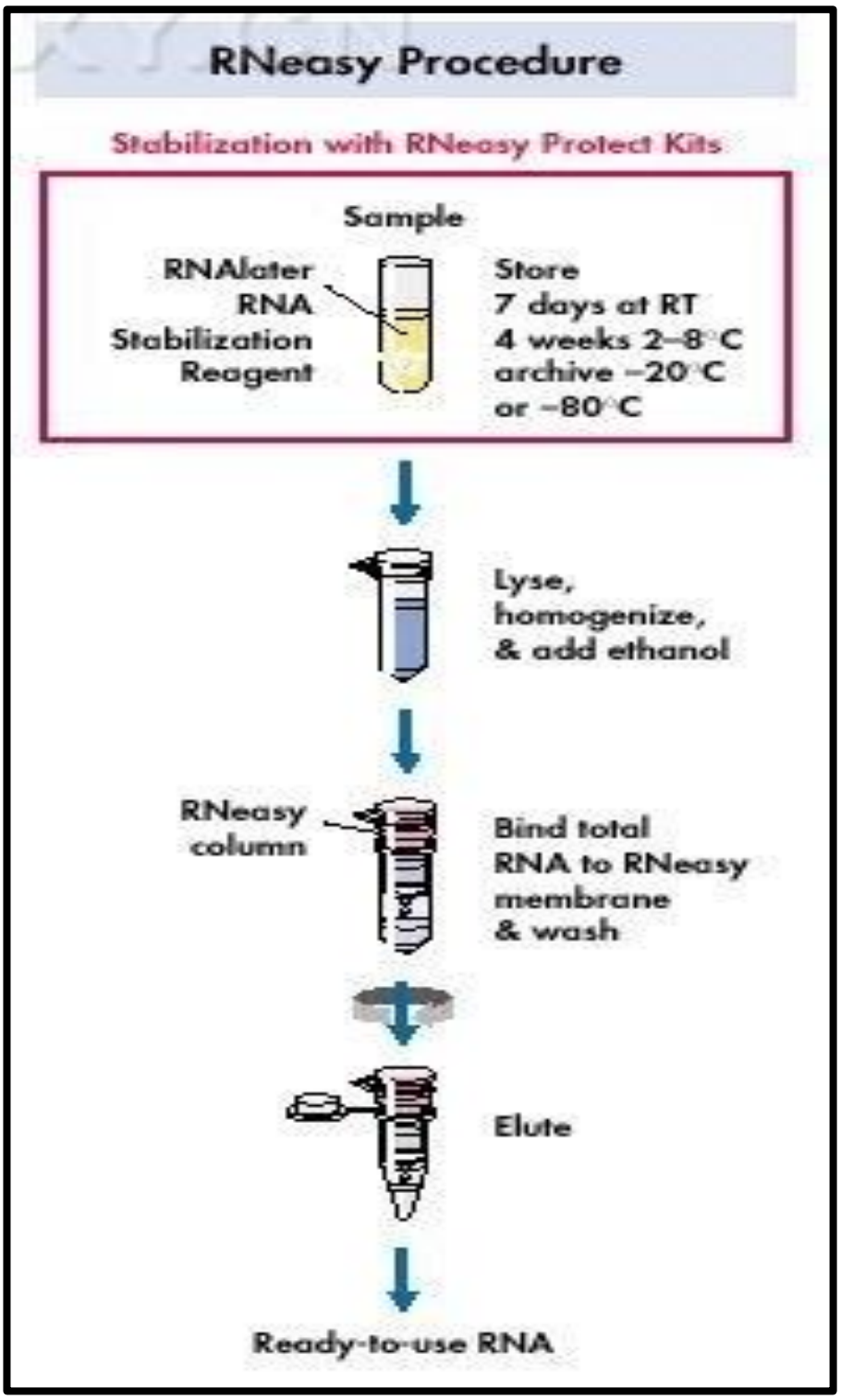

Figure 2.2: Schematic representation of RNeasy procedure of RNA isolation (Taken from www.qiagen.com on 21/02/2016).

2.11 Post RNA purification DNase treatment with TURBO DNA-free Kit™ (Life Technologies Corp.)

According to the manufacturer, this RNA purification method using DNase treatment allows for thorough removal of contaminating DNA compared to on-column DNase treatment. A volume of 30 µL RNA in the final elution stage was transferred into a 0.5 ml microcentrifuge tube. Similarly, a volume of 3 µL of 10X TURBO DNase buffer and 1 µL TURBO DNase was added to the sample and incubated for 30 min at 37 ^{o}C. Thereafter, a volume of 3.4 µL inactivation reagent was added and mixed well. This was incubated for 5 min at room temperature, mixing occasionally to re-disperse the inactivation reagent. The mixture was centrifuged at 10, 000 rpm (9.2 x g) for 1.5 min. The supernatant (about 30-32 µL) was then carefully transferred into a new 0.5 ml tube.

2.12 RNA Quantitation

Total RNA was quantified using NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE) featuring both micro volume pedestal and cuvette measurement options. Following manufacturer's instructions, all samples were measured using RNA-40 settings and wavelength monitored at 260 nm and 280 nm. Each purified sample was quantitated thrice and the average was taken as the sample concentration. A known concentration of standard RNA was quantitated at least twice with each batch of samples.

2.13 Total RNA quality

Only the samples with absorbance values greater than 1.8 at 260 nm/280 nm when quantitated using Nanodrop were selected as this indicated that they were relatively free of proteins.

2.14 cDNA synthesis

First strand cDNA was synthesized using SuperScript VILO MasterMix (Life Technologies (UK) Ltd)) in a total reaction volume of 20 µL. SuperScript VILO mastermix comprises SuperScript III reverse transcriptase, RNaseOUT recombinant Ribonuclease inhibitor, a proprietary helper protein, random primers, $MgCl_2$ and dNTPs (Life Technologies Ltd). Following manufacturer's instructions, for each sample, a mix of 4 µL SuperScript VILO

MasterMix and 6 µL nuclease free-water was prepared on ice. This was added to 10 µL (2 ng/µL) extracted RNA sample, gently mixed and incubated at 25 °C for 10 min. Thus, prepared sample was then incubated at 42 °C for 60 min and the reaction was terminated at 85 °C for 5 min. Negative controls (No Amplification Control (NAC)) were processed with the samples by first incubating a 4 µL SuperScript VILO MasterMix and 6 µL nuclease-free water at 65 °C for 10 min to denature the reverse transcriptase in the mastermix. This would detect any genomic DNA co-extracted with mRNA. Similarly, no template controls (NTCs) were processed by substituting RNA samples with nuclease free water. There was no amplification in both NACs and NTCs. All reverse transcribed samples were stored at -20 °C.

2.15 Real-time PCR cycling conditions

Real-time PCR was carried out on ABI 7500 system using two detection methods, which include SYBR Green and Taqman probes. The cycling parameters are listed in Table 2.7 and Table 2.8.

2.15.1 SYBR Green

Table 2.7: PCR cycling parameters used for SYBR Green experiments

Temperatures	Times	
50 °C	2 min	Stage 1
95 °C	2 min	Stage 2
95 °C	15 s	Stage 3
60 °C	1 min	**X 40 cycles**
95 °C	15 s	Stage 4 (melt curve)
60 °C	1 min	**X 1 cycle**
95 °C	15 sec	
60 °C	1 min	

2.15.2 Taqman probes

Table 2.8: PCR cycling parameters used for Taqman probes experiments

Temperatures	Times	
50 °C	2 min	Stage 1
95 °C	2 min	Stage 2
95 °C	15 s	Stage 3
60 °C	1 min	**X 40 cycles**

2.16 End-point PCR cycling conditions

Similar to real-time PCR, end-point PCR was carried out on the ABI veriti 96 thermal cycler (Life Technologies Ltd). Two different mastermix, Qiagen PCR multiplex mix and Platinum PCR mastermix were used. The cycling parameters are displayed in Table 2.9, for Qiagen multiplex kit and Table 2.10, for Platinum multiplex mix. The primer, mastermix and cDNA and water volumes were modified appropriately (See Table 2.11)

2.16.1 Qiagen PCR multiplex kit

Table 2.9: PCR cycling parameters used for Qiagen multiplex kit

PCR stages	Temperatures		Times
Initial denaturation	95 °C		15 min
Denaturation	94 °C		15 s
Annealing	58 °C	**33 cycles**	1 min
Extension	72 °C		1 min
Final extension	72 °C		20min
Final hold	4 °C		∞

2.16.2 Platinum PCR SuperMix Hifi Mastermix

Table 2.10: PCR cycling parameters used for Platinum PCR SuperMix

PCR stages	Temperatures		Times
Initial denaturation	94 °C		30 s
Denaturation	94 °C		30 s
Annealing	58 °C	**33 cycles**	30 s
Extension	68 °C		1 min
Final extension	68 °C		10min
Final hold	4 °C		∞

2.16.3 Reaction components for PCR

Table 2.11: PCR reaction components

Reagents	Reaction volumes (µL)	
Final volume	**10**	**16**
Mastermix	5	10
Primer mix	x	3.5
cDNA	x	x
Water	x	x

Primer mix, cDNA and water volumes were varied with each batch of reaction due to optimization. A final primer mix used for multiplex reaction was 3.5 µL.

2.17 Fragment analysis

The PCR amplicons using unlabelled primers were electrophoresed using AGE. PCR amplicons generated using fluorescently labelled primers were analyzed on 3500XL Prism Genetic Analyzer (Applied Biosystems Ltd).

2.17.1 Sample preparation for Agarose gel electrophoresis

PCR products amplified using unlabeled primers were electrophoresed using AGE. Unless otherwise stated, a 1.5% Agarose gel was used throughout the experiments and was prepared by adding 1,500 g of Agarose to 100 ml (1X) TAE buffer. This was swirled briefly, placed in the microwave and set on low heat until a clear solution was seen. The conical flask containing the gel was then left to stand till it was cool to touch. A volume of 2 µL safe-view was added to each 100 ml gel. The gel was then gently mixed avoiding bubbles and then cast in a gel tray. The gel was allowed to set and then transferred into a gel tank containing 1X TAE buffer. Sample were prepared for loading on the gel by adding 2 µL tracking dye and 3 µL water to 5 µL PCR product. A volume of 100 bp ladder (Thermofisher Scientific) was prepared by adding 3 µL of the ladder to 2 µL tracking dye and added to one well of the gel. The prepared samples were added to other designated wells. Gel electrophoresis was allowed to run at 100 V for approximately 25 min. The gels were viewed using BioDoc-It UVP trans-illuminator (UVP, LLC, CA).

2.17.2 Sample preparation for capillary electrophoresis

PCR samples amplified using fluorescent primers were analyzed using ABI 3500XL Prism Genetic Analyzer (Applied Biosystems Ltd). The fluorescent dyes are shown in Table 2.12. Each PCR sample was prepared by adding 1 µL PCR product to 8.5 µL Hi-Di Formamide (Life Technologies, UK) and 0.5 µL GeneScan 500 LIZ size standard (Life Technologies, UK). Samples were spun briefly then heated at 95 ^{o}C for 5 min and snap-cooled on ice for at least 10 min. Fragment analysis was carried out on ABI 3500 XL Prism Genetic Analyzer in a 50 cm long capillary and POP-6 polymer (Life Technologies, UK) using dye set G5 (Table 2.13).

Table 2.12: Florescent dye labels used for labelling the forward primer of various pairs of primers (Eurofins MWG Operon)

Fluorescent dyes	Colors
ATTO 565	Red
6-FAM	Blue
Yakima Yellow	Green
ATTO 550	Yellow

Table 2.13: 3500XL Prism Genetic Analyzer (Applied Biosystems Ltd) fragment analysis protocol (Fragment Analysis 50_POP6)
Dye set: G5

Parameters	Values
Oven temperature	60 ^{o}C
Run time	2600 s
Pre-run voltage	15.0 kV
Run voltage	15.0 kV
Pre-run time	180 s
Injection time	15 s
Injection voltage	3 kV
Data delay	600 s

2.18 Data Analysis (GeneMapper ID-X software)

ABI 3500 GeneMapper ID-X Software Version 1.2 (Thermofisher Scientific Ltd) was used to analyze raw data obtained from capillary electrophoresis (CE). The instrument default parameters were left unchanged throughout the course of the experiments (Table 2.14).

2.19 General statistical Analysis of data

Statistical analysis to determine primer and PCR efficiencies was carried out using Expression suite software (Thermofisher Scientific Ltd) and Microsoft Excel software. The expression suite software operates using geNORM analysis, which is the geometric mean of multiple carefully selected housekeeping genes validated as an accurate normalization factor (Vandesompele *et al.*, 2002). Student's t-test and Analysis of variance (ANOVA) were performed using R studio software (See page 72). R studio is an open source statistical analysis software that enables organization of data and maintenance of multiple projects using specific codes for various tests. Standard deviation was calculated to measure how data were scattered around mean values. Where applicable, RNA sample concentration, cycle threshold values (Ct) and peak heights (RFU) of samples were used for the statistical analysis. Further details on statistical calculation is discussed in relevant chapters. All data are presented as a single EPG, gel electrophoresis photos or amplification plot readings from 5-10 of such different experiments or as mean ± standard deviation (SD with n and p values where possible.

Table 2.14: Advanced Peak detection Algorithm used for each analysis of PCR fragments.

Parameters	Values
Size calling method	Local Southern
Analysis Range	Full Range
Baseline Window	51 points (pts)
Peak Detection	50 RFU
Polynomial Degrees	3 pts
Minimum Peak Half Width	2 pts
Peak Window Size	15 pts
Slope Threshold for peak start/end	0-0
Size Call Range	All sizes

CHAPTER THREE

OPTIMISATION OF REFERENCE GENES

3.0 Introduction

Reference genes, otherwise known as housekeeping genes, are constitutive genes that carry out the maintenance of basic metabolic functions in a cell. Irrespective of their patho-physiological conditions, they are expected to be uniformly expressed in the sample type they are found (Butte *et al.,* 2001; Pfaffl *et al.,* 2004). These unique characteristics make them ideal for use in calibration and normalization studies (Vandesompele *et al.,* 2002). Similarly, they are used as internal controls for some experimental studies (Robinson and Oshlack, 2010; Dheda *et al.,* 2004; Rubie *et al.,* 2005), most importantly, in mRNA profiling, which has been reported to yield good results (Wang *et al.,* 2013; Park *et al.,* 2013). Bustin, (2002) reported that validation of input RNA required for a reaction without inhibition is time consuming when RNA samples are to be compared by controlling the total input RNA. Correspondingly, comparison of RNA samples requires precise quantification. In forensic RNA studies, endogenous controls, usually housekeeping genes, are required for better normalisation of sample-to-sample variation as well as experimentally induced variations (Vandesompele *et al.,* 2002). This is achieved by reporting the amount of a target gene in reference to a reference gene (Tricarico *et al.,* 2002). Previously, traditional method of RNA analysis based on immunological assays such as Northern blots or RNase protection assays were used to determine varying amounts of RNA in samples (Thellin *et al.,* 1999). Recently, RNA measurement using real time PCR technology as well as microarray analysis is able to estimate slight variation in relative quantities for larger number of genes due to their high sensitivity (Yuen *et al.,* 2002). These variations are highly significant in any experimental study; therefore, to prevent ambiguity and spurious results; such variations must be quantified and minimised whenever possible. Equally, the choice of a reference gene for any study should not be based on their traditional use in other studies but on adequate validation of their stability in the intended experiment (Meller *et al.,* 2005). Although, it is possible for a single reference gene to be capable of possessing all the required characteristics, many authors however have reported that no single gene is universally stable. Hence, multiple reference genes have been proposed for use in normalization data analysis studies (Vandesompele *et al.,* 2002; Pohjanvirta *et al.,* 2006). For the purpose of this study, endogenous control genes

was empirically evaluated for stability and uniformity in expression between many types and states of body fluid samples.

3.1 Reference genes selection

In this study, 10 reference genes were picked from relevant published literature (Table 3.1). A thorough literature search showed these reference genes as commonly used in Forensic body fluid identification experiments. *Chapter 2*, Table 2.1 displayed the primer sequences that were used to amplify all target loci in this study. All primers were ordered from Life Technologies (UK) Ltd and no modification was made to the primer sequences.

3.2 Aim and objectives

The aim of this chapter was to evaluate and optimize a total of 10 reference genes commonly used in forensic body fluid identification studies for fitness of purpose.

The chapter has the following objectives:

To select a total of 10 reference genes commonly used in body fluid identification

To optimize the reference genes using SYBR Green detection chemistry and establish their efficiencies.

To select the genes with 90-110% efficiency and a slope of -3.33±10% and design Taqman probes for them.

To incorporate the robust reference genes into the multiplex assay that will be developed in Chapter 4.

3.3 Methods

Sample preparation for the reference genes study was reported in Chapter 2, sections 2.5 – 2.7.

3.4 Results

This section discussed the results obtained from SYBR Green and Taqman probes experiments.

3.4.1 qPCR efficiency

A total of 10 reference genes (UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and S15) were selected from relevant published literatures at the start of the study (Table 2.1). Human control RNA (Qiagen) with known RNA concentration was used as the sample source.

The results show that a few of the samples failed amplification using a 1:10 serial dilution with varying primer concentrations of 0.1-0.5 µM. This was modified by setting up further experiments with a 1:5 serial dilutions, keeping the primer concentrations constant. Primer efficiency of 90-110% was tested by generating a standard curve from the serial dilutions.

3.5. SYBR Green

Cycle threshold (Ct value) is the cycle number at which the florescent signal crosses the threshold. Amplification plot was generated, together with the cycle threshold (Ct) values. In Microsoft Excel spreadsheet, reaction efficiencies and slope were determined by plotting a graph of Ct values against the logarithm of dilutions. All these were in accordance to MIQE guidelines. From the data generated, five makers; ACTB, B2M, UCE, TEF and RPS29 were found to be 90-110% efficient, having a slope of -3.33 ± 10% (Figures 3.1-3.5).

3.6 Taqman probes

Similar to SYBR Green experiments, Taqman probe assay using the real-time PCR was used to evaluate the threshold cycle value (Ct: cycle number at which the fluorescence passes the threshold) should the sample extraction and reverse transcription succeed.

Real time PCR amplification curves of different amounts of total input RNA (25 pg– 2 ng) extracted from blood, saliva, menstrual blood, semen and vaginal secretion was obtained. Furthermore, calibration curve was constructed from the Ct values obtained in reference to the different RNA concentrations present in each sample. To compare the expression levels and normalisation of the target genes among body fluid samples, the delta Ct (dCt) value was calculated automatically using the ExpressionSuite Software v1.1 for Microsoft® Windows® 7, XP, 10 (Thermofisher Scientific Ltd). The expression suite software operates using geNORM analysis, which is the geometric mean of multiple carefully selected housekeeping genes validated as an accurate normalization factor (Vandesompele *et al.,* 2002). The data obtained from expression suite software were used to generate a histogram plot using the Ct values and the samples. This showed the frequency of distribution of the data generated (Figures 3.11, 3.12 and 3.13).

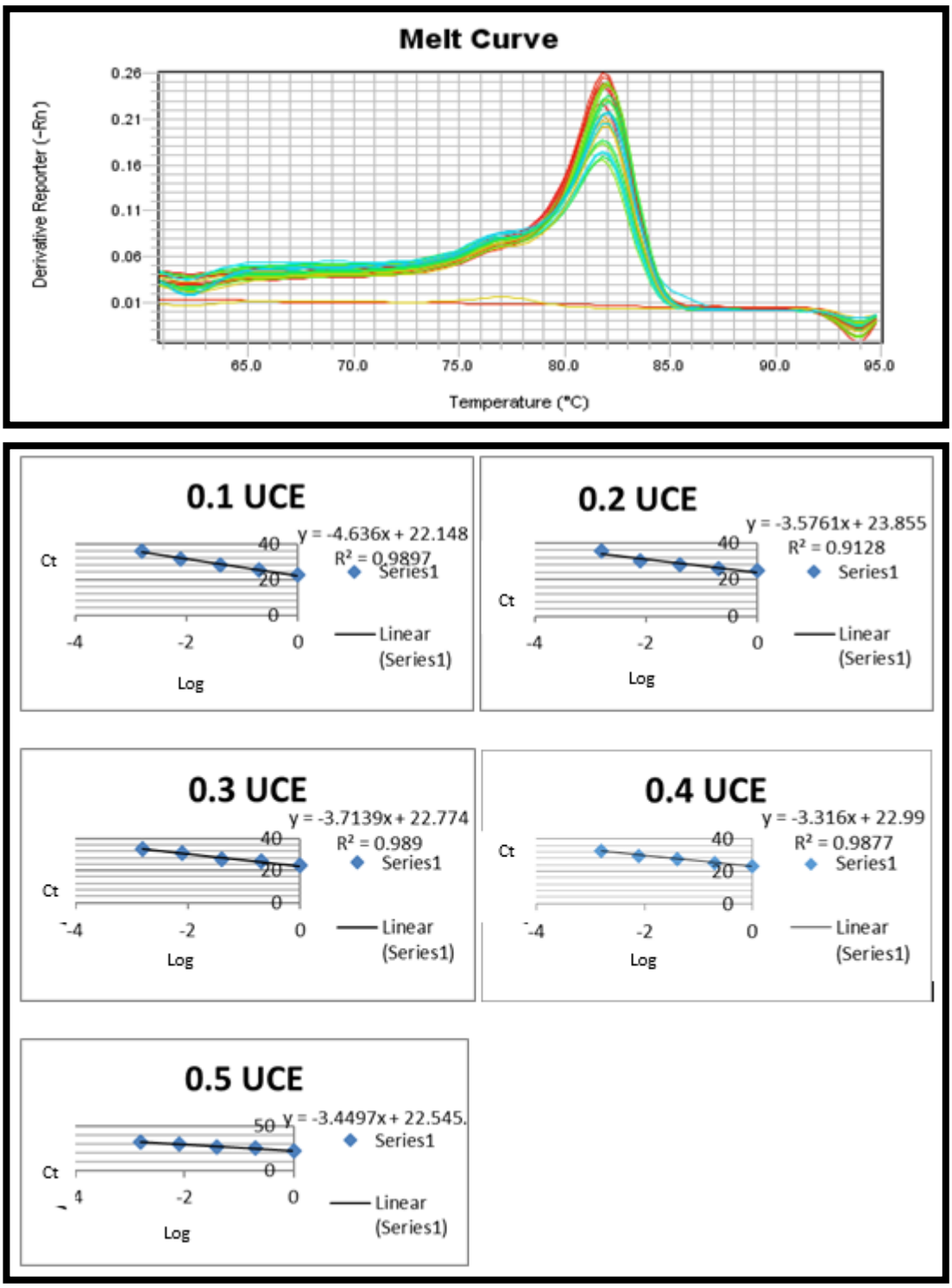

Figure 3.1: Melt curve plot of UCE. 0.1-0.5 µM represents UCE primer concentrations/volumes optimized. The slope of 0.2, 0.4 and 0.5 µM UCE showed 100% efficiency of -3.33 ± 10%. 0.1 and 0.3 displayed less than 100% qPCR efficiency; n=5, where n equals the number of repeats of experiment.

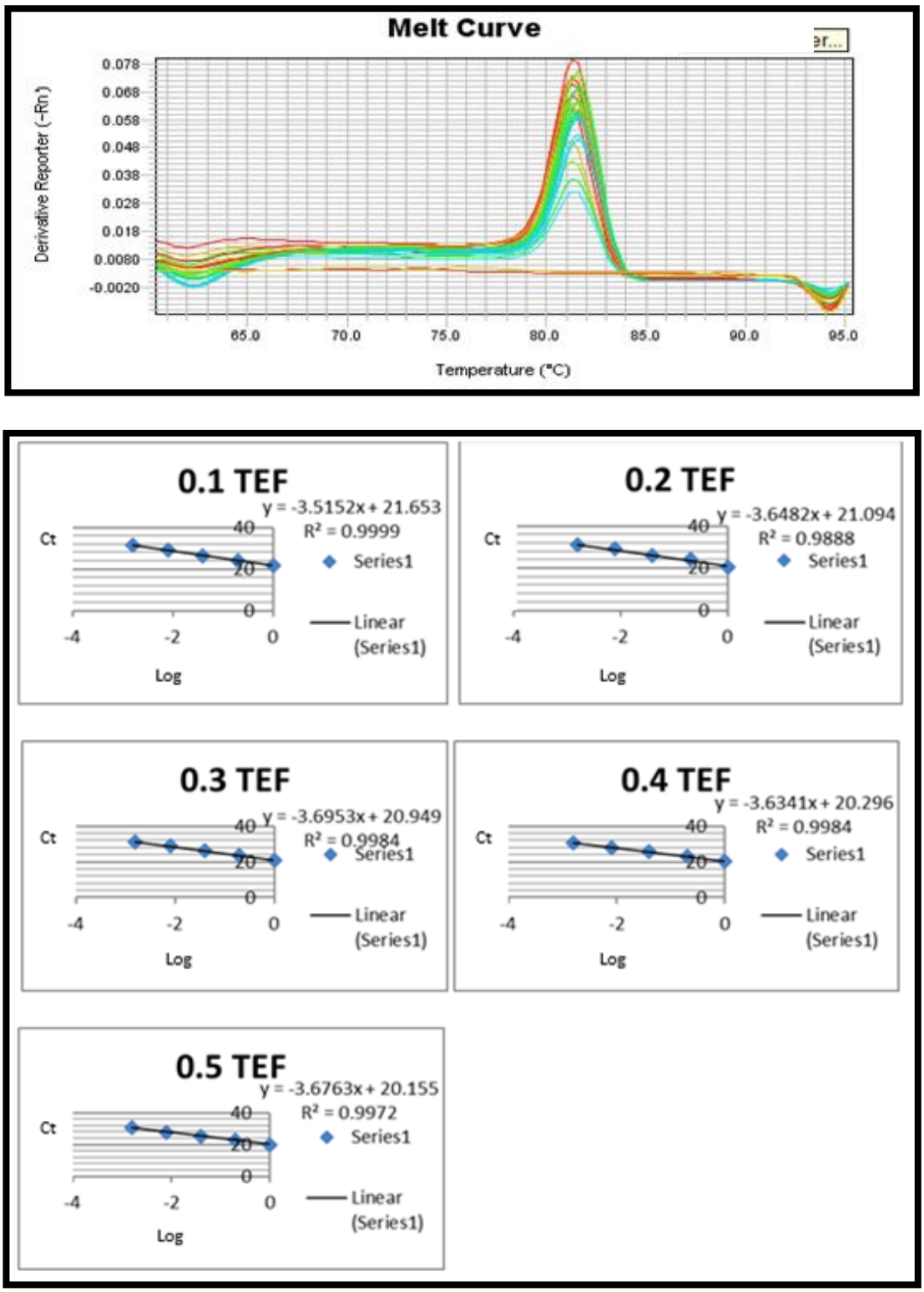

Figure 3.2: Melt curve plot of TEF. 0.1-0.5 µM represents TEF primer concentrations/volumes optimized. The slope of 0.1 TEF shows 100% efficiency of -3.33 ± 10%. Other concentrations/ slopes did not display 100% qPCR efficiency; n=5, where n equals the number of repeats of experiment.

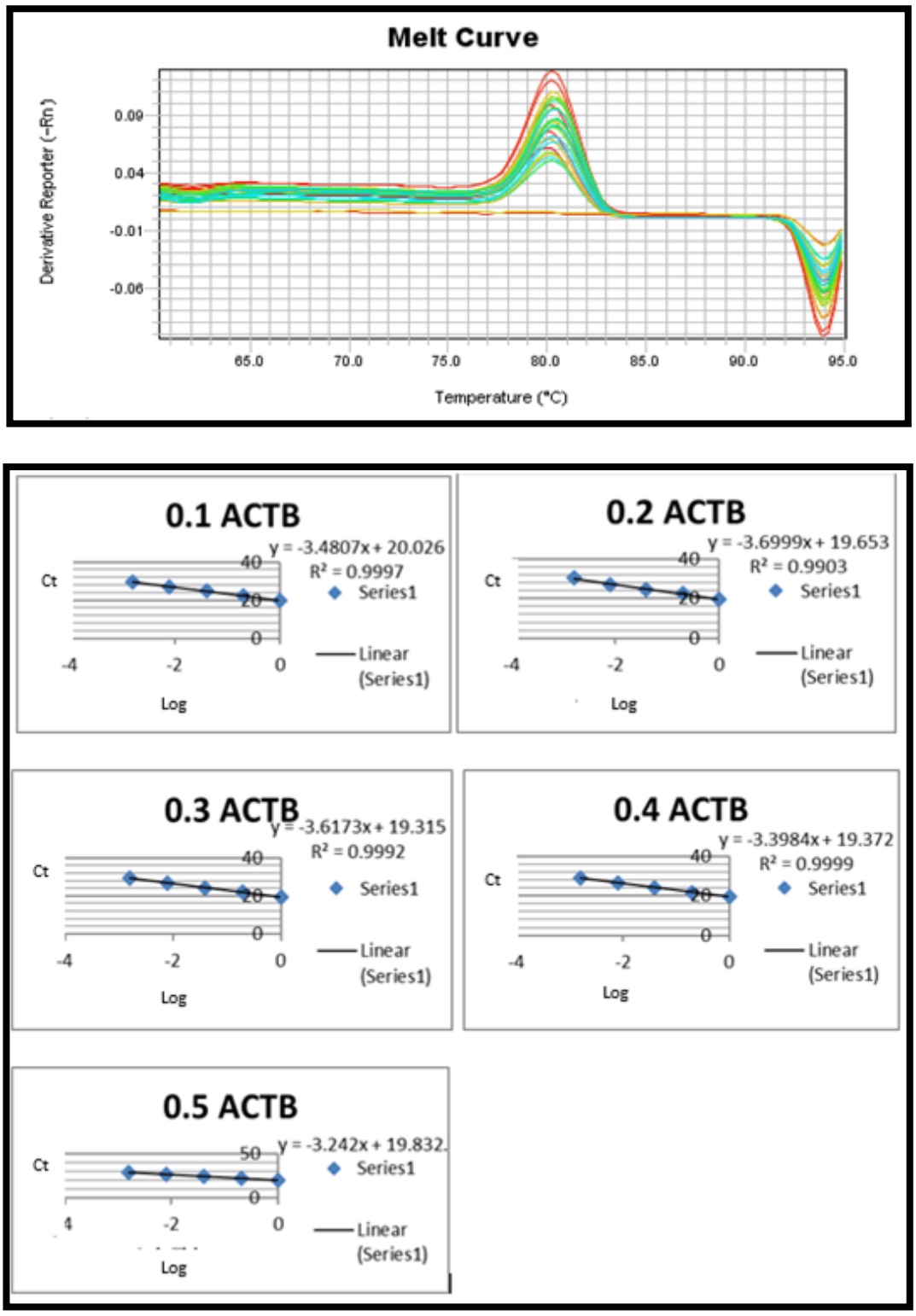

Figure 3.3: Melt curve plot of ACTB. 0.1-0.5 µM represents ACTB primer concentrations/volumes optimized. The slope of 0.1, 0.4 µM and 0.5 µM B2M shows 100% efficiency of -3.33 ± 10%. Other slopes showed less than 100% qPCR efficiency; n=5, where n equals the number of repeats of experiment.

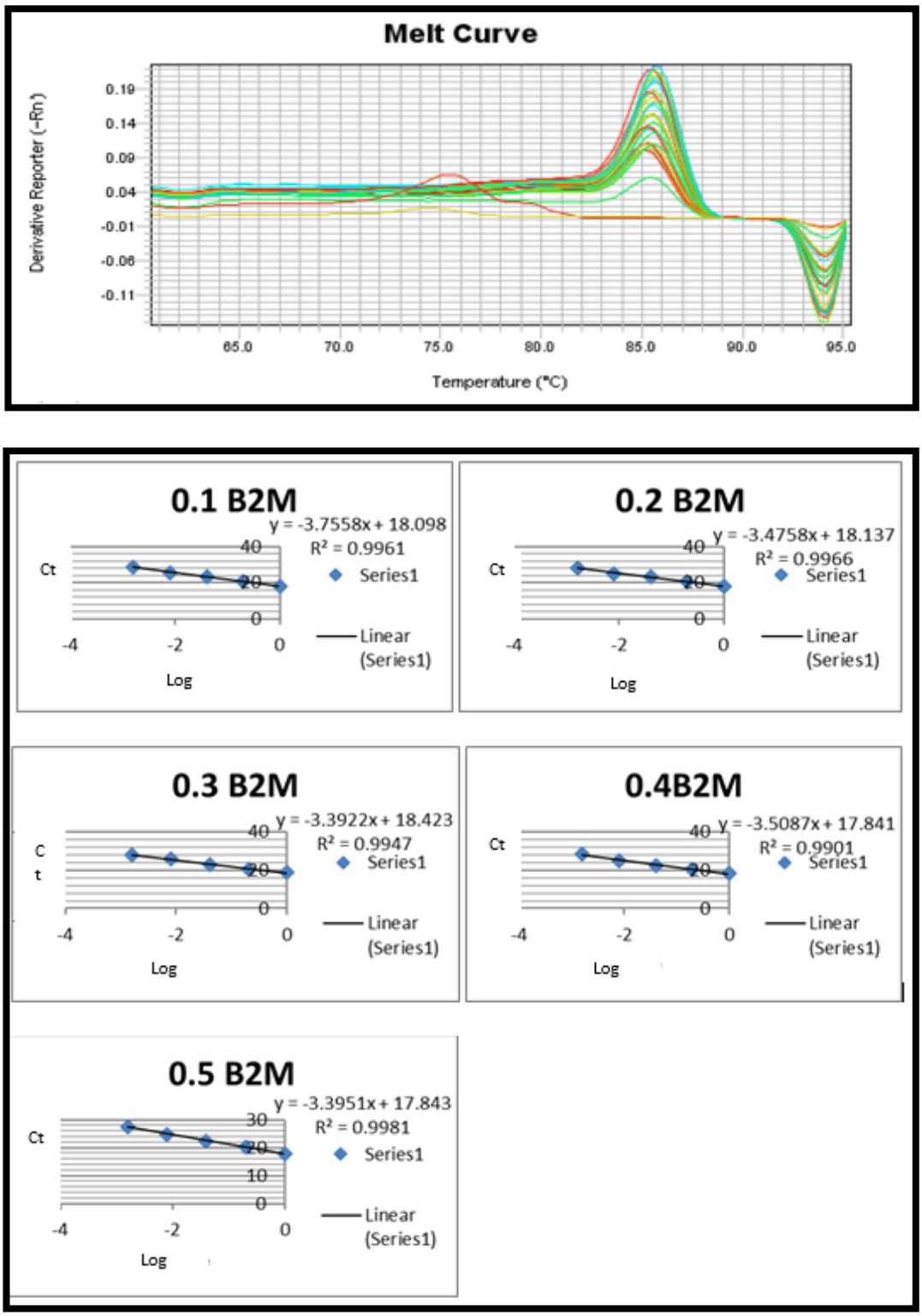

Figure 3.4: Melt curve plot of B2M. 0.1-0.5 µM represents B2M primer concentrations/volumes optimized. The slope of 0.2-0.5 µM B2M shows 100% efficiency of -3.33 ± 10%. 0.1 µM did not display 100% qPCR efficiency; n=5, where n equals the number of repeats of experiment.

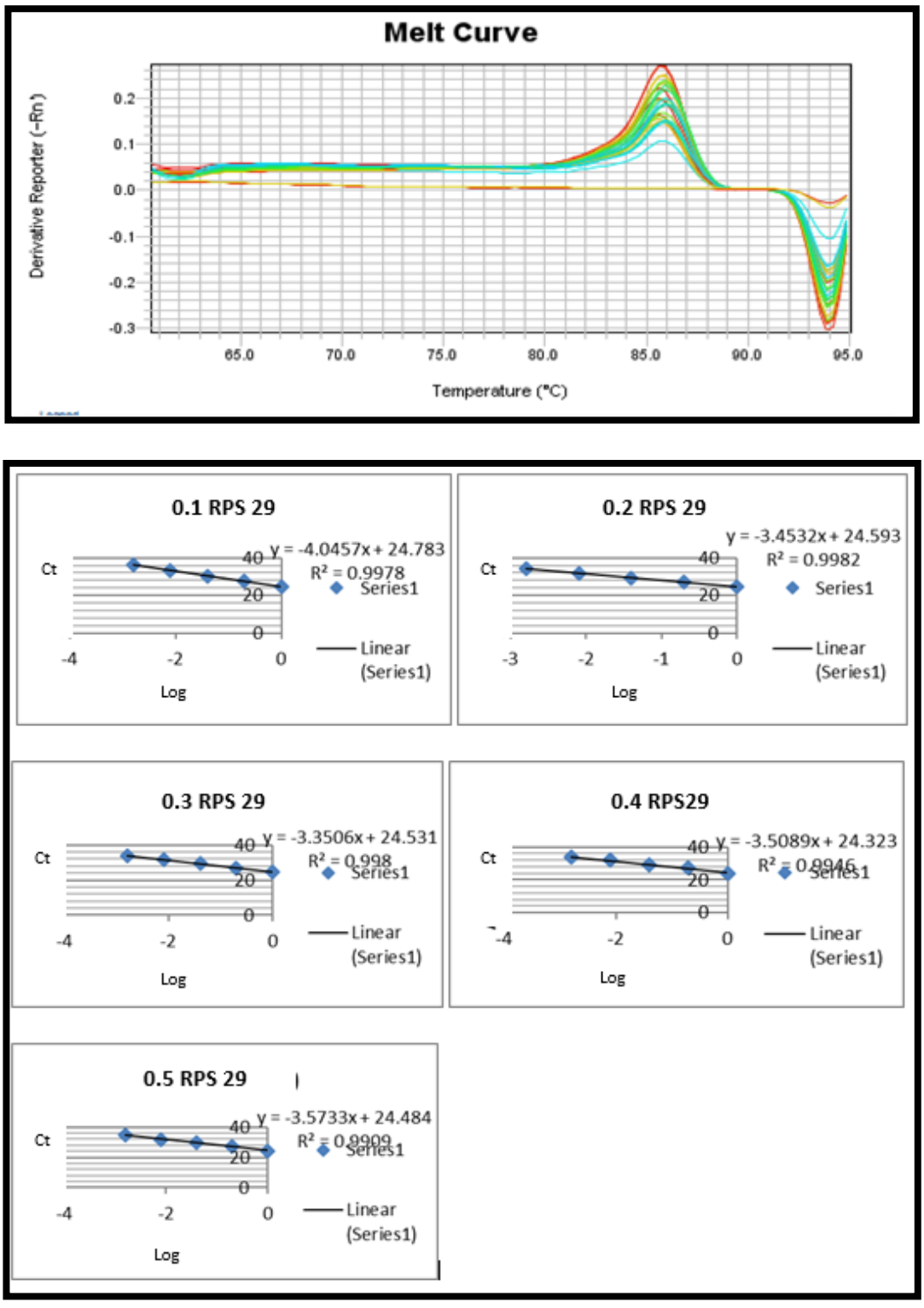

Figure 3.5: Melt curve plot of RPS29. 0.1-0.5 µM represents RPS20 primer concentrations/volumes optimized. 0.2-0.5 µM primer volumes showed 100% efficiency of -3.33 ± 10%. 0.1 µM primer volume displayed less than 100% efficiency; n=5, where n equals the number of repeats of experiment.

3.7 Primer selection for Taqman probes

The five markers listed (Table 2.4) attained the set efficiency threshold of 90-110% with a slope of -3.33±10%. Based on these criteria, they were then selected for Taqman probe design and further experiments.

3.7.1 qRT-PCR efficiency

Although the manufacturer (Thermofisher Scientific Ltd) suggested that there was no need to optimize the taqman probes as these were already carried out before shipment; to ascertain the qRT-PCR efficiency using the Taqman probes, a trial experiment was set up with UCE using 25 pg-2 ng of positive control sample (Human XpressRef universal total RNA (Qiagen Ltd). The standard curve data generated revealed that all the samples were amplified and detected down to 25 pg.

3.7.2 Sensitivity test/ Limit of detection

Limit of detection/ sensitivity test was carried out on UCE, TEF, RPS29, ACTB and B2M using the designed taqman probes. Fresh samples of blood, saliva, vaginal secretion, semen and menstrual blood were analysed. The results indicated that all the body fluids were detected up to 0.025 ng input RNA for all the makers except TEF. For semen sample, TEF was detected from 0.1 pg while menstrual blood and blood were both detected from 0.5 ng.

3.7.3 Taqman Probes Based Assays

A fluorescent TaqMan MGB probe (Thermofisher Scientific Ltd) with a non-fluorescent quencher at 3' end was used for attaining a higher specificity. The probes were designed to bind the sequences of the mRNA transcripts at exon/exon junctions to avoid the detection of DNA contamination. All probes were FAM labelled (as reporter dye) at the 5' end and a non-florescent quencher at the 3' end. The selected genes included: (B2M, Hs00187842_m1, 120bp), (ACTB, Hs03023943_g1, 75bp), (UCE, Hs00366152_m1, 241bp), (TEF, Hs01896387_g1, 206bp), (RPS29, Hs03004310_g1, 213bp). Both positive control (Human XpressRef Universal RNA (Qiagen Ltd) and negative controls were included in each batch. cDNA was amplified using Taqman probes (Thermofisher Scientific Ltd) and Taqman Universal PCR mastermix in a total volume of 20 µL, with 2 µl

input cDNA. For these experiments a range of mRNA were reverse transcribed (25 pg; 50 pg, 0.1 ng, 0.2 ng, 0.5 ng, 1 ng and 2 ng), to test the sensitivity and the limit of detection of the assay. All reactions were prepared in duplicates. Thermal cycling conditions were: 2 min at 50 ^{o}C; initial step of hold, 10 min at 95 ^{o}C followed by 40 cycles of denaturation at 95 ^{o}C for 15 sec and final annealing/ extension at 60 ^{o}C for 1 min. The amplification products were detected on a 7500 sequence detection system (Thermofisher Scientific Ltd).

3.8 Data analysis

The expression of reference genes was evaluated for use as endogenous control. The level of expression of each reference gene was assessed by analyzing the cycle threshold value (Ct: the cycle number at which fluorescence is detected). The limit of detection was assessed in fresh body fluid samples. Amplification curves of different amount of input RNA (25 pg- 2 ng) extracted from 30 µl fresh semen, blood, saliva, menstrual blood and vaginal secretion samples were generated. The Ct values were determined in the range 19-39.

The reference gene expression data for samples stored for 6 months was analyzed in a similar manner. A default instrument threshold of 0.133 was used for the analysis. Student's t test was performed for comparing the gene expression levels for the fresh and 6 month old samples. The level of significance was set at 5%.

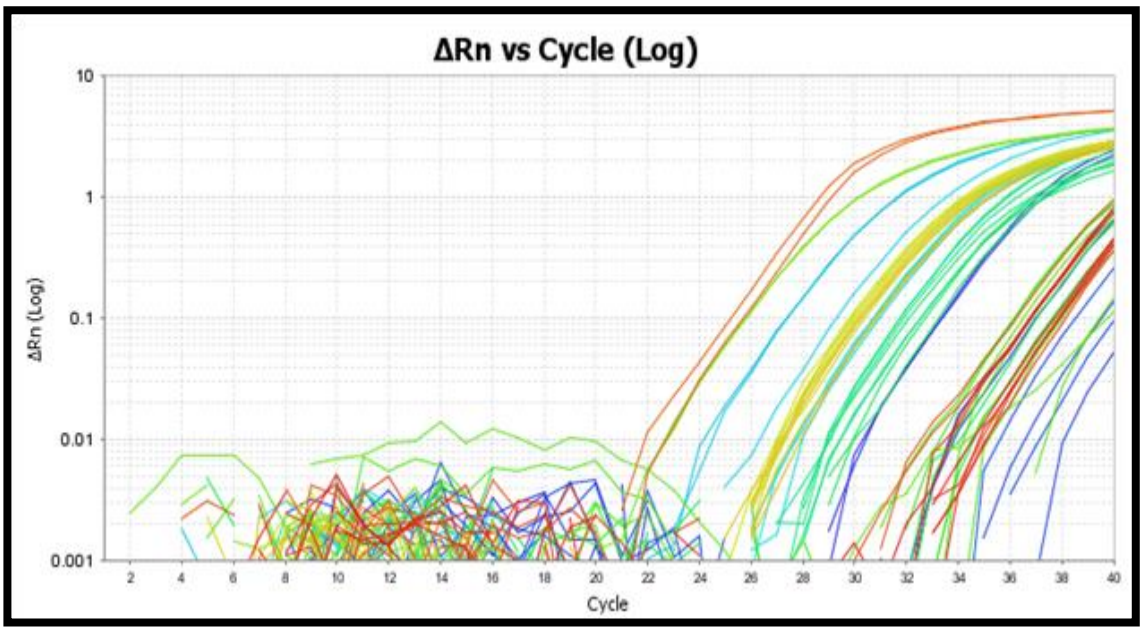

Figure 3.6: Pictorial illustration of Real-time PCR amplification plot of reference genes on semen sample; n=5, where n equals the number of repeats of experiment.

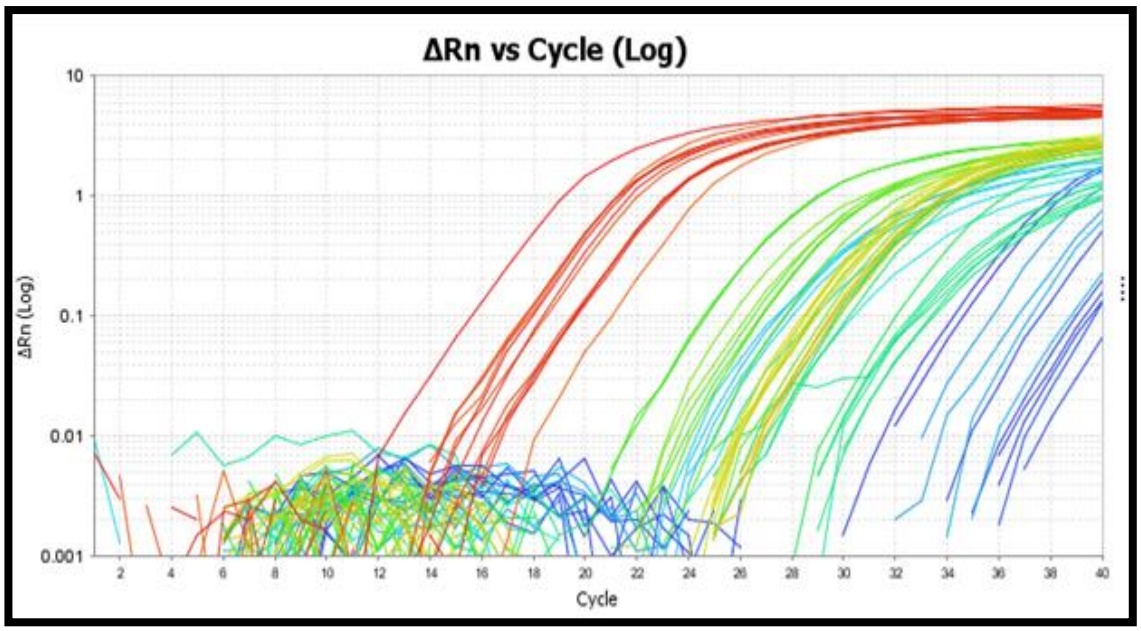

Figure 3.7: Pictorial representation of Real-time PCR amplification plot of reference genes on saliva sample; n=5, where n equals the number of repeats of experiment.

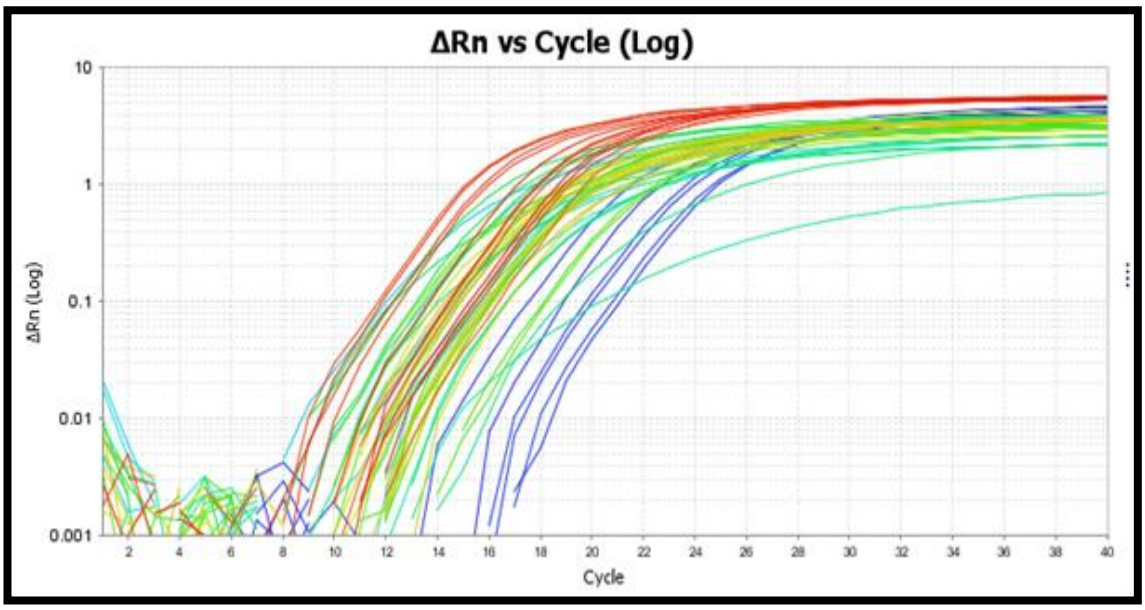

Figure 3.8: Pictorial representation of Real-time PCR amplification plot of reference genes on menstrual blood sample; n=5, where n equals the number of repeats of experiment.

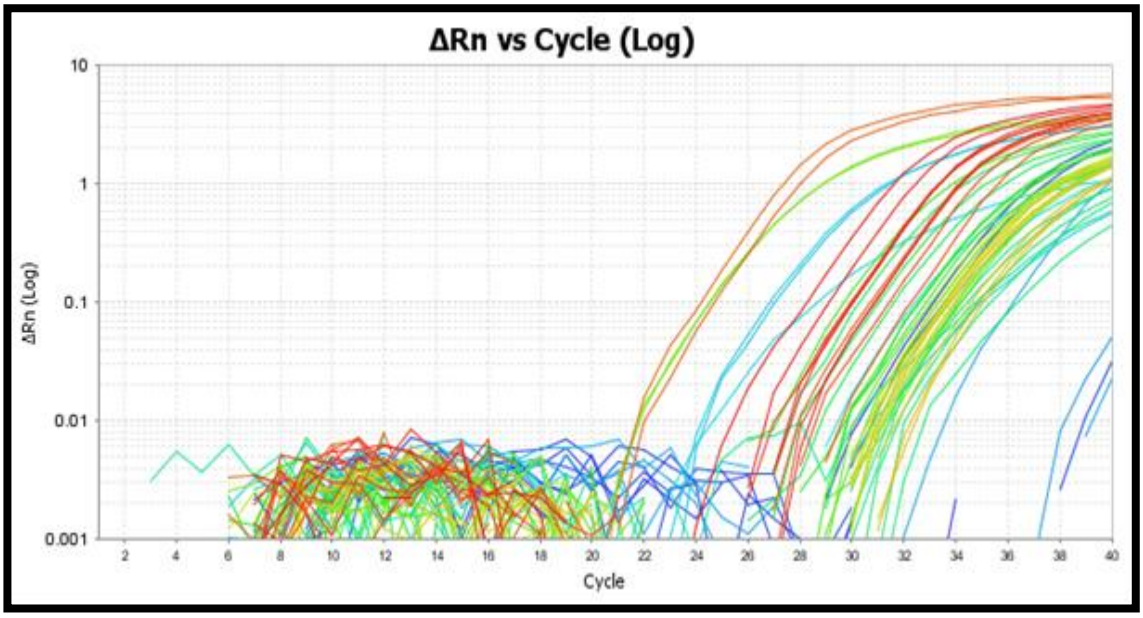

Figure 3.9: Pictorial representation of Real-time PCR amplification plot of reference genes on blood sample; n=5, where n equals the number of repeats of experiment.

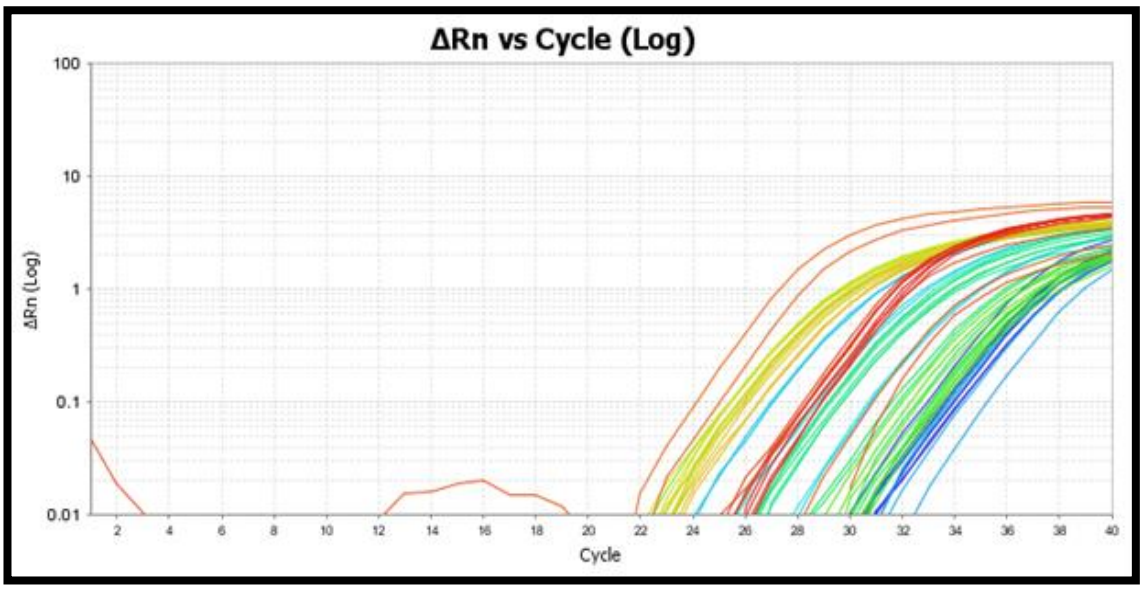

Figure 3.10: Pictorial representation of Real-time PCR amplification plot of reference genes on vaginal secretion sample; n=5, where n equals the number of repeats of experiment.

Key:

3.9 Six-month old samples

Samples stored at room temperature over six months were analysed for RNA sensitivity and stability. The results are given below.

3.9.1 Sensitivity

Four reference genes (UCE, RPS 29, ACTB, B2M) in different body fluids were detected down to 25 pg except TEF. TEF was detected at 0.1 ng in semen, and 0.5 ng in blood and menstrual blood.

3.9.2 Stability of RNA in stains over time

Since most forensic stains are not often recovered in a fresh state, the stability of UCE, ACTB, B2M, RPS29 and TEF were examined in samples stored at room temperature for

over 6 months. The Ct values of the reference genes were determined. All the markers were expressed in at least 4 of 5 different individual samples for each body fluid sample except TEF that was amplified in a total 2 individual samples across the 5 body fluids (Tables 3.1a, 3.1b). The stability of RPS29 was found to be higher across all the body fluids, followed by UCE, ACTB and B2M. TEF was either not expressed (saliva, vaginal secretion) or was selectively expressed in blood, semen and menstrual blood. Furthermore, the result of Anova test, using the Ct values indicated that the expression of each of the reference genes was not significantly different across the five body fluid samples ($P>0.05$)

Table 3.1a: Ct values for ACTB, UCE, TEF, RPS29 and B2M in blood, semen, vaginal secretion, menstrual blood and saliva samples stored at room temperature for 6 months, n=10.

Number of samples	**ACTB**	**UCE**	**TEF**	**RPS29**	**B2M**
Blood					
1	28.4428	24.8423	33.333	23.5240	30.0167
2	33.9818	34.0654	ND	36.3675	20.1324
3	29.4415	29.9992	ND	33.3829	20.0917
4	36.6418	30.9527	ND	33.9107	31.3060
5	32.6277	28.5818	ND	36.2352	32.7220
6	21.1790	27.5849	ND	30.7258	25.1986
7	27.5197	29.6871	ND	29.1299	25.0684
8	30.0445	26.9511	ND	31.3572	26.9798
9	31.7106	20.0092	ND	30.0906	25.2114
10	32.1233	27.0358	ND	19.0231	26.7152
Semen					
1	32.0857	37.1960	39.9977	32.4851	33.6951
2	32.7943	37.2625	39.8370	32.5104	33.8875
3	32.0727	37.0357	39.8261	33.1687	32.9396
4	31.9274	38.0549	ND	33.1324	34.0133
5	31.4121	22.2766	ND	33.5893	34.7402
6	37.9590	37.7419	ND	36.2108	36.3522
7	35.8241	37.6910	ND	35.2683	22.6165
8	39.2314	38.5445	ND	32.5896	34.0974
9	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND
Saliva					
1	24.9746	32.4266	ND	28.4760	21.9949
2	24.6462	32.2880	ND	28.4719	22.0539
3	24.5889	32.1213	ND	28.1391	21.7412
4	25.2094	34.2092	ND	29.1876	30.1004
5	25.0916	32.3652	ND	28.6078	21.7285
6	25.4542	36.3847	ND	28.8360	22.2883
7	25.1313	38.7327	ND	33.3331	28.7191
8	25.2543	39.4030	ND	37.3658	29.0209
9	25.2673	ND	ND	28.8023	22.1194
10	25.0442	ND	ND	28.4509	21.9907

Table 3.1b: Ct values for ACTB, UCE, TEF, RPS29 and B2M in blood, semen, vaginal secretion, menstrual blood and saliva samples stored at room temperature for 6months, n=10.

	ACTB	UCE	TEF	RPS29	B2M
Menstrual blood					
1	29.3250	34.2857	38.0689	33.1929	27.4627
2	29.2418	35.8603	38.1524	32.5346	27.1493
3	34.6060	28.8214	34.1209	35.5241	32.5487
4	38.7505	32.4830	ND	35.4180	32.9574
5	29.8688	28.3690	ND	31.6119	28.8910
6	30.6219	36.8748	ND	32.0296	29.6302
7	32.7020	33.7253	ND	32.1600	27.7370
8	18.5726	32.1010	ND	36.4235	29.8221
9	ND	38.9209	ND	ND	33.0698
10	ND	ND	ND	ND	32.5494
Vaginal secretion					
1	31.7349	35.5805	ND	39.3131	31.1240
2	30.2438	32.5852	ND	37.0601	31.7799
3	37.6809	34.6150	ND	34.6686	32.3642
4	37.9121	30.8288	ND	34.4291	27.9864
5	36.1747	35.4459	ND	36.7781	28.2896
6	34.0958	34.5916	ND	38.0804	34.9704
7	ND	28.0194	ND	34.5791	31.9286
8	ND	33.3759	ND	34.8006	29.6732
9	ND	33.7360	ND	ND	28.0460
10	ND	36.1619	ND	ND	29.7224

ND: Not detected.

Comparing the Ct values generated from fresh and six-month old samples, it was observed that there was no significance difference in the expression of the five markers on all the body fluids (See Tables 3.1a and 3.1b).

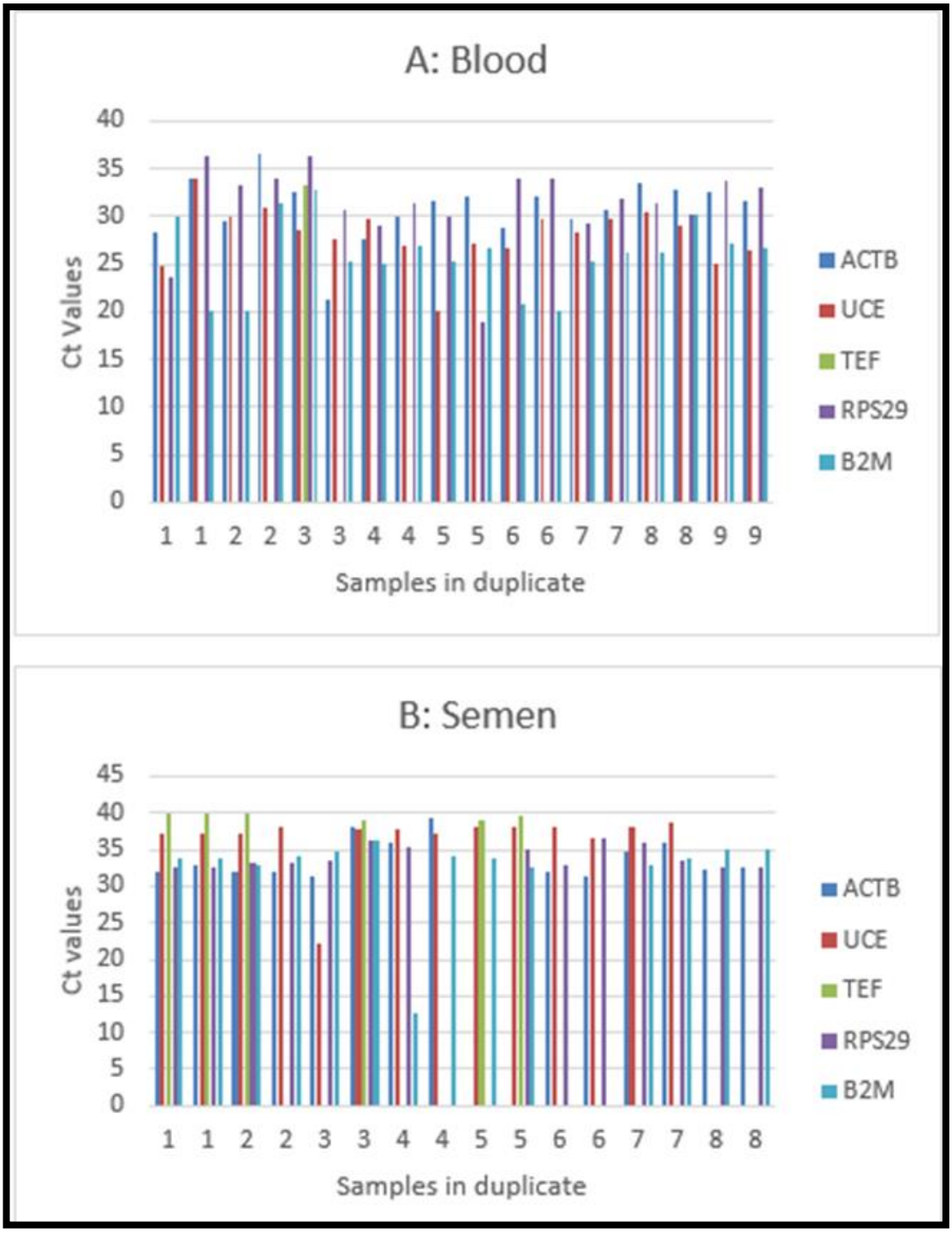

Figure.3.11: Histogram plot of Ct values generated from amplification of six-month old body fluid samples in duplicate. a) Blood, b) Semen. Data are represented as mean ±SD, n=10, P>0.05.

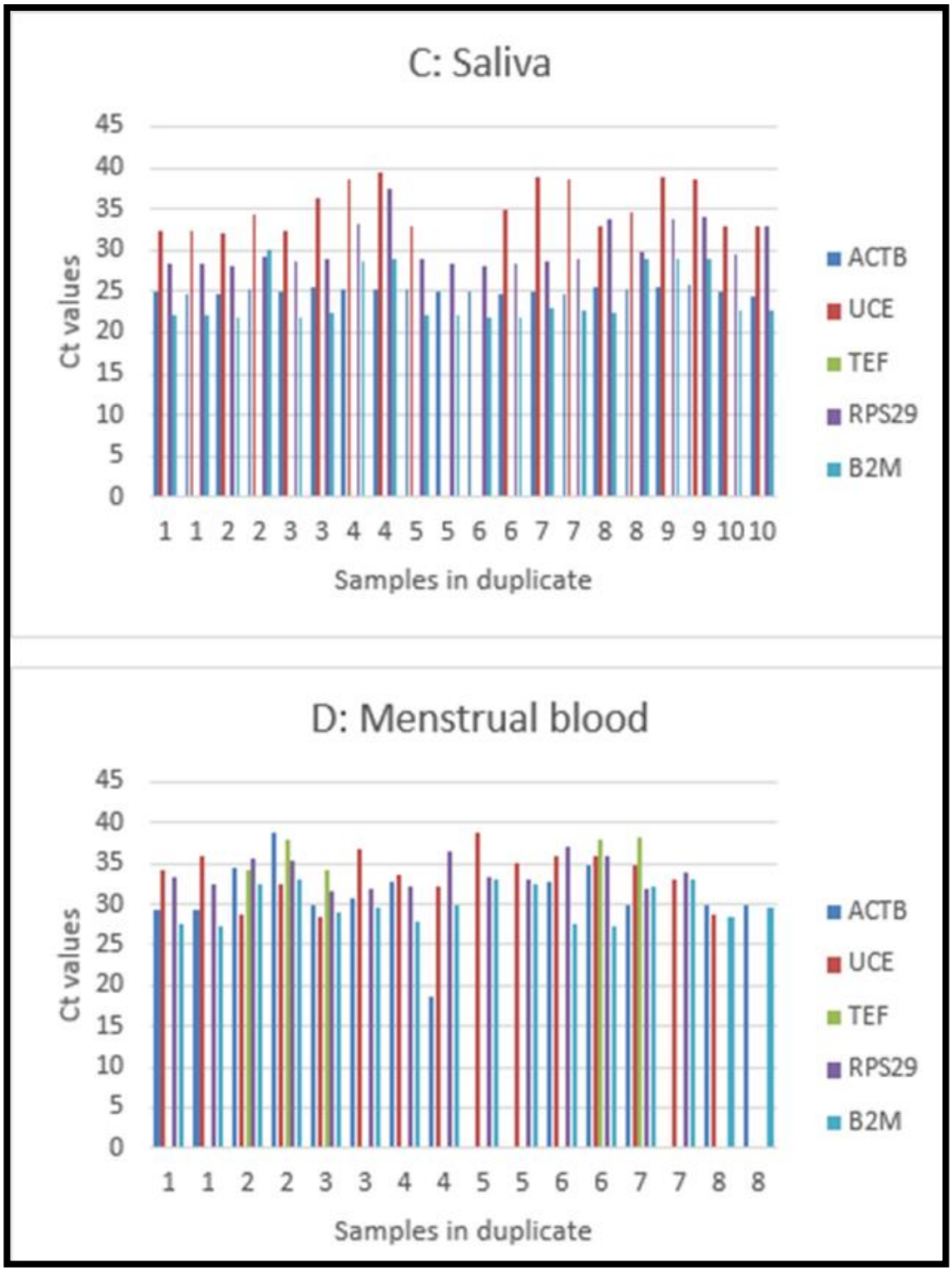

Figure 3.12: Histogram plot of Ct values generated from amplification of six-month old body fluid samples in duplicate. c) Saliva, d) menstrual blood, Data are mean ±SD, n=10, P>0.05.

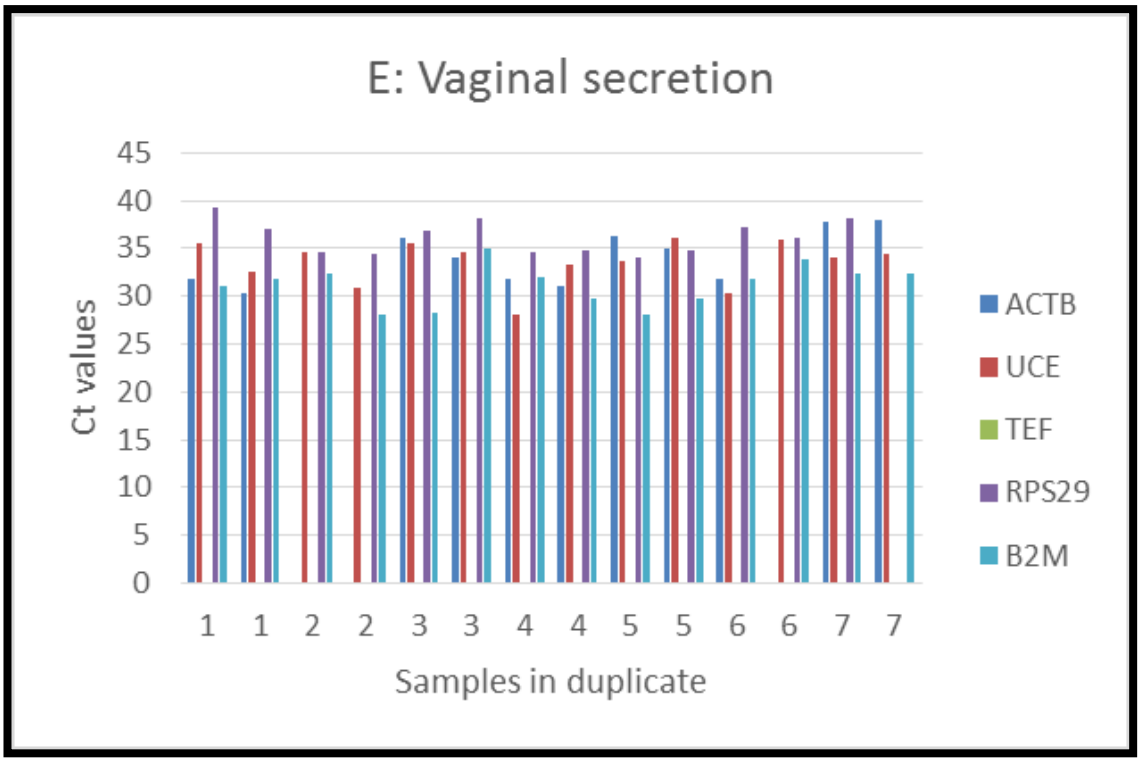

Figure 3.13: Histogram plot of Ct values generated from amplification of six-month old body fluid samples in duplicate. e) Vaginal secretion. Data are mean ±SD, n=10, P>0.05.

3.9.3 Result of negative controls

Although adequate precautions were taken through the whole workflow of each experiment to prevent contaminations, both positive (Figure 3.14) and negative controls were also prepared and processed along with the main samples to further check contamination. Reagent control, reverse transcription negative control (-RT) as well as negative control for the extraction, cDNA synthesis and qRT-PCR amplification respectively were prepared and processed along the target samples. All negative controls were analyzed and the result showed no amplifications (See Figure 3.15)

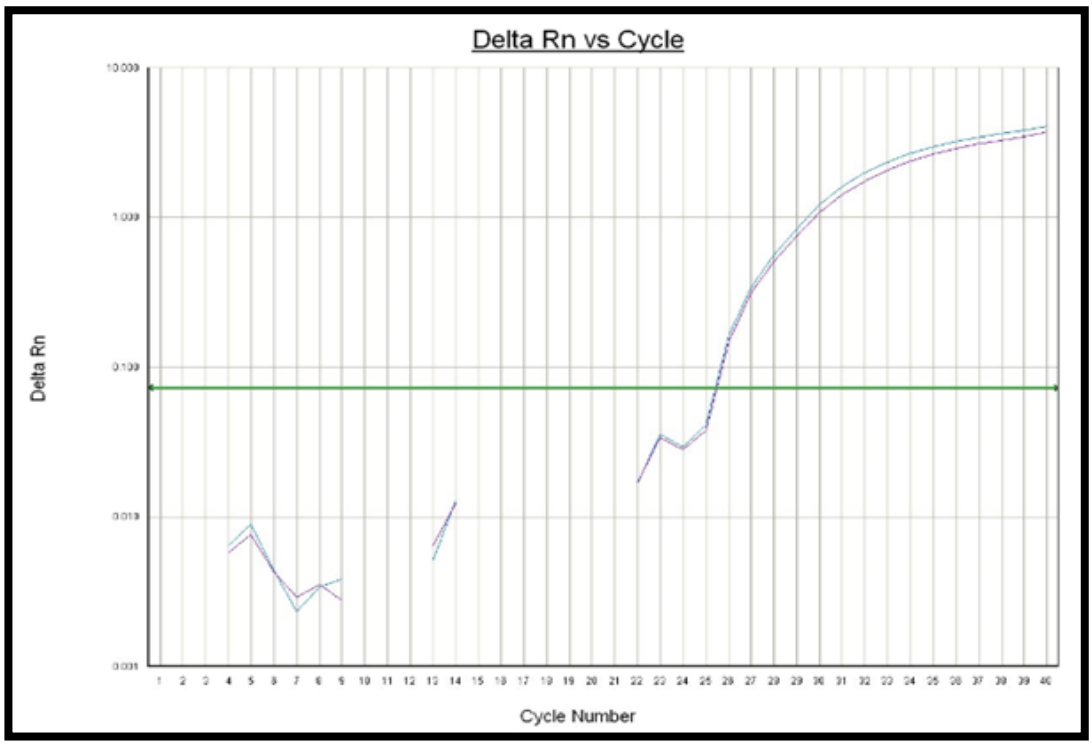

Figure 3.14: Pictorial representation of amplification plot of Human XpressRef positive control sample (Qiagen Ltd). 1 ng/µL of positive control sample was used in the reaction; n=10, where n represents the number of repeats of experiment.

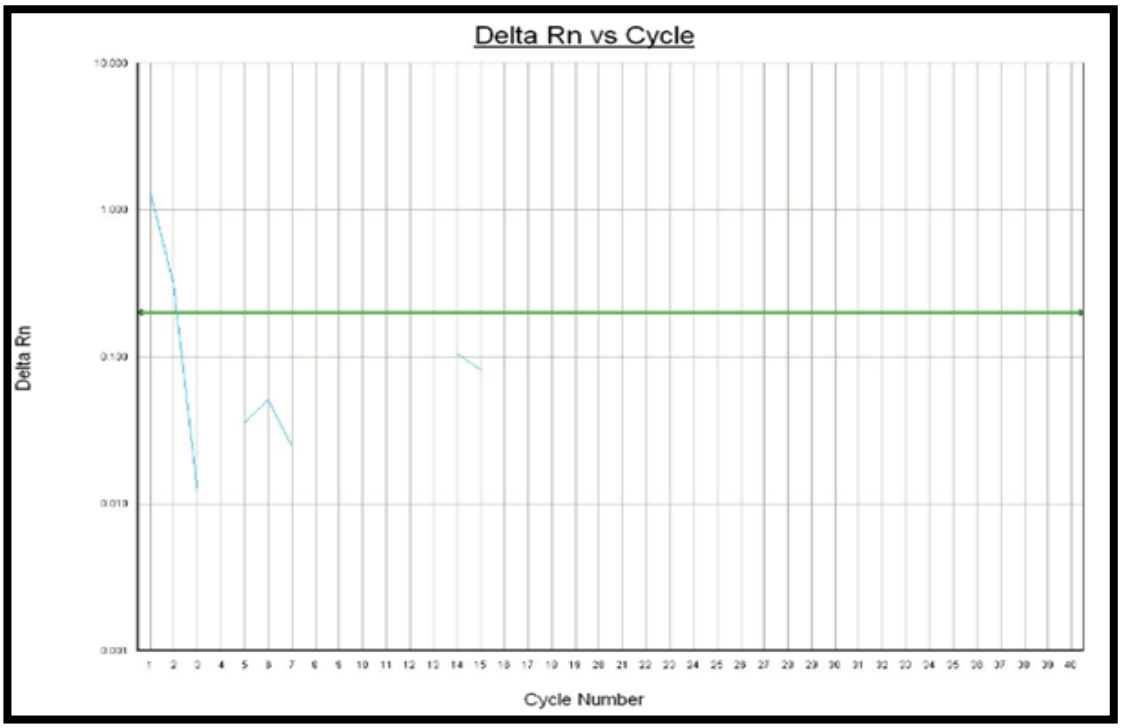

Figure 3.15: Pictorial representation of amplification plot of negative control sample (1 µL water was used in place of body fluid sample); n=10, where n represents the number of repeats of experiment.

3.10 Discussion

The data generated in this study were compared to the results reported in similar studies by other authors. This is discussed in the *Sections 3.10.1 – 3.11.*

3.10.1 Marker selection and analyses

The study in this chapter has demonstrated that it is possible to recover RNA from body fluids in sufficient quantity and quality required for forensic investigations. Using a number of relevant literature and database search, ten housekeeping genes were chosen and used for normalization in forensic body fluid identification analyses (Table 2.1). Total RNA, which comprise mRNA, ribosomal RNA and small RNA, was recovered from the body fluids in varying quantities. Following the extraction of total RNA, it was further determined whether mRNA of interest could be detected in the body fluids as it is known that mRNA comprises about 1-3% of total RNA. All the reference genes were detected in all the analysed body fluids using SYBR Green and Taqman probe detection systems (Figures 3.1-3.10).

SYBR Green chemistry detection method is less expensive and easy to use. However, it is not capable of multiplexing and fluoresces when it binds to any available dsDNA in the reaction leading to overestimation of the target amount. Conversely, Taqman probes however are more expensive, but with increased specificity, have the ability to multiplex and are highly specific (Jelena *et al.,* 2013).

The use of appropriate controls is important in any experiment. Here, the use of control was to confirm that absence of a particular amplification product was not the result of sample degradation, but because of absence of that gene. Warrington *et al.,* (2000) described reference genes as normally expressed to maintain various cell functions. As mentioned earlier, any reference gene should be validated before use for any forensic purposes. Among other characteristics, it should be expressed uniformly in all body fluids and tissues irrespective of the environmental or experimental conditions; it should be free of any known form of pseudogenes and it must be highly sensitive. In qRT-PCR experiments, reference genes are used for data normalization. This is to prevent variations that may arise from biological and environmentally induced sources (Vandesompele *et al.,* 2002; Dheda *et al.,* 2004).

A few of the selected reference genes are known to have pseudogenes, which have identical properties as real DNA segments. Their difference from real genes arise from accumulation of multiple mutations result in them losing some key characteristics that their real gene counterparts normally have (Raff *et al.,* 1997). The resulting effect of this is that it causes PCR bias leading to reduced reaction efficiency whereby the pseudogene might be preferentially expressed rather than the target gene. With this in mind, the reference genes were still selected based on other desirable characteristics and reports obtained from their use in published literature (Table 2.3). Five markers, namely GAPDH, 18S rRNA, β-Actin, OAZ1 and S15 failed the qRT-PCR were efficiency tested using SYBR Green detection chemistry. While most studies reported the use of these as reference genes, it is essential to confirm that the reference genes are adequately validated to ensure their suitability for the study type (Ghani *et al.,* 2013). For some reference genes, their expression level is affected by a number of factors such as hypoxia (Roberts *et al.,* 2003); and their involvement in functions other than their basic function of cell metabolism (Ishitani *et al.*, 1996). Due to these reasons, the use of reference genes could result in false positive amplification products in both negative, positive control and target makers if not adequately validated (Huggett *et al.,* 2005).

This study tested reference genes and as a result, there were no internal control genes to compare them. Microsoft excel statistical software was used to calculate the reaction efficiency using the generated Ct values. The premise for the study was because if the markers could pass qPCR efficiency using SYBR Green, which is a less specific, sensitive method, the use of taqman probes with increased sensitivity and specificity will further establish the efficacy of the markers.

For the SYBR Green experiment, GAPDH, 18S rRNA, β-Actin, OAZ1 and S15 failed the PCR efficiency test. Though these markers have been reportedly used as internal controls in forensic body fluid identification experiments, 100% efficiency was obtained for them during this study. It is envisaged that the markers were binding to all available double stranded molecules in the reaction, which is typical of SYBR Green detection system. 18S rRNA has the same chemical structure with mRNA. However, they are not in correlation as rRNA, a major component of 18S rRNA is not translated into protein like mRNA (Meyer *et al.*, 2010) which might be the reason for the efficiency test failure. Contrarily,

Lindenbergh *et al.,* (2012), Fleming and Harbison, (2010), Haas *et al.,* 2009, and Bas *et al.,* (2004) reportedly utilized 18S- rRNA in their experiment and recommended it for use in further studies. S15, b-actin and GAPDH were also reported by Juusola and Ballantyne, (2003) to yield positive result in their experiment. This, however, is not in accord with the results obtained in this study. The present conclusion for GAPDH failing the efficiency test irrespective of the results reported by several authors could be because of segmental duplications – a phenomenon whereby a near identical sequence of the target is also amplified (Ghani *et al.,* 2013). As reported, segmental duplication covers up to 60% of the entire GAPDH length. Thus, the primers will be seen amplifying a near identical sequence together with the target sequence. In turn, this can in effect lead to a reduction in their efficiency. ACTB was also reported by Ghani *et al.,* (2013) to possess segmental duplication characteristic property. However, it showed 100% efficiency in our study and this made us select it amongst others for taqman probe design. Meller *et al.,* (2005) reported OAZ1 and RPS 29 to be novel and promising candidate markers to be used in qPCR experiments. While RPS 29 passed the efficiency test, OAZ1 failed. These are still worthy of further research as they have not been validated for forensic body fluid identification and casework samples.

Likewise, for the taqman probe detection system, the five candidate markers - ACTB, B2M, RPS29, TEF and UCE that passed the efficiency test using SYBR Green detection system were selected for taqman probe design. Sensitivity test was carried out on them and all the markers were detected down to 25 pg of total input RNA with the exception of TEF which was detected from 100 pg in semen; and 500 pg in menstrual blood and venous blood, respectively. This result proves the markers as gold standard in qRT-PCR normalization studies, especially in a scenario where the perpetrator of a crime tries to conceal the evidential material by washing or cleaning up stains that can be recovered and used as evidence. As mentioned earlier, there was no internal control to compare the results since the study was centred on reference genes. Nonetheless, expression suite software (Life Technologies) was used for statistical analyses. This software makes use of geNORM algorithm as recommended by Vandesompele *et al.,* (2002). A histogram plot was generated for all the five reference genes on each body fluid (Figures 3.11-3.13). This reveals the spread of the data set from the minimum to maximum RNA

concentrations that was added to the reaction system. Since each RNA concentration was added in duplicates, the histogram plot showed the mean of each duplicate as well as the median. From the range of the data set obtained, the reference genes were seen to be expressed evenly across each body fluid except for TEF that showed a slight variation.

3.10.2 Six-month old samples

RNA was seen to be stable in the six-month old body fluid samples analysed. With samples from five different individual for each body fluid, expressions were detected in at least four individuals for each body fluid with ACTB, RPS29 and UCE being fairly stable across the five body fluids, (Figure 3.1). However, TEF was detected in a total of 2 individuals across the five body fluids. This result supports reports from other studies that no single reference gene is universally stable (Vandesompele *et al.,* 2002; Pohjanvirta *et al.,* 2006); hence, multiple reference genes have been proposed for use in normalization data analysis studies.

3.11 Conclusion

There have been several reports of reference genes being used as normalizers in body fluid identification experiments (Table 2.3). This study has demonstrated a novel approach to establish the efficiency of reference genes for use in forensic body fluid identification on five different body fluids – semen, saliva, vaginal secretion, blood and menstrual blood on the real time PCR using SYBR Green and Taqman probe detection systems. The genes selected include GAPDH, 18S rRNA, β-Actin, OAZ1, S15, ACTB, B2M, RPS29, TEF and UCE. The results showed that ACTB, B2M, RPS29, TEF and UCE passed the reaction efficiency test using SYBR Green and were further detectable in all the body fluids up to 25 pg using Taqman probes (Afolabi *et al.,* 2015). However, as shown in the present study, TEF did not demonstrate high sensitivity as other markers, especially on semen, blood and menstrual blood. So far, the high sensitivity of any marker is of high significance, particularly when stains with minimal amount of nucleated cells are encountered on crime scenes, which could be due to the perpetrator of a crime trying to conceal the evidentiary materials. Though TEF amongst the five markers tested showed no high sensitivity with semen, blood and menstrual blood, it will not be

discarded at this time. Meller *et al.,* (2005) and Pohjanvirta *et al.,* (2006) reported that since no single reference gene is universally stable, the use of more than one is highly recommended to be validated for any normalization experiment.

In summary, the results obtained from the study carried out in this chapter showed that SYBR Green chemistry is cost effective and are ideal to detect efficiencies of reference genes. In addition, all five-reference genes that passed the efficiency test and labelled with Taqman probes were all expressed in the six-month old samples tested. The approach outlined in this chapter is of high relevance as it established the suitability of the evaluated reference genes in both fresh and aged samples for forensic body fluid identification. More experiments would be carried out on the body fluids using TEF to further test its sensitivity.

CHAPTER FOUR

DESIGN, DEVELOPMENT AND OPTIMIZATION OF A MULTIPLEX PCR-BASED mRNA ASSAY FOR BODY FLUID IDENTIFICATION

4.1 Introduction

Currently, there are no mRNA assay kits commercially available for unambiguous identification of forensic body fluids. Each published multiplex assay kit has been developed in-house by research groups (Juusola and Ballantyne, 2005; Lindenbergh *et al*., 2012; Roeder and Haas, 2013). Although many authors have reported successful development of multiplex assays for forensic body fluid identification using various techniques, a large number of markers in these assays are reported to display cross reactivity with non-target body fluids (Song *et al.,* 2015; Roeder and Haas, 2013). Most forensic samples are usually recovered from the crime scene in a compromised state; all these factors necessitated the development of a multiplex PCR-based assay, which can unambiguously identify forensic body fluids in this study. Different marker sizes have been reported by several authors for the same mRNA PCR amplicons; an example of which could be seen in MUC4, a vaginal mucosal marker that is 141 bp (Lindenbergh *et al.,* 2012) and 235 bp (Roeder and Haas, 2013). mRNA markers used in this study were selected from relevant publications (See *Chapter 2*, Tables 2.1, 2.2).

4.2 Aim and objectives of the study

The aim of this study was to select and evaluate body fluid specific markers from relevant literature, employing two approaches, which include minimal cross-reactivity with non-target body fluids and smaller amplicon sizes as most forensic samples are commonly recovered in environmentally compromised form

The objectives were:

- To select body fluid specific markers with small amplicon sizes from relevant publications.
- To optimise the selected primer pairs and PCR conditions using non-labelled and florescent-labelled primers.
- To develop a specific multiplex for each of the five body fluids including blood, saliva, semen, vaginal fluid and menstrual blood.
- To combine the five multiplexes into a single multiplex assay, capable of identifying all the five body fluids.

4.3 Methods

Sample preparation and reaction conditions for assay development was highlighted in Chapter 2, section 2.8.

4.4 Results

This section shows the results generated in this study using gel electrophoresis and capillary electrophoresis. A comprehensive discussion of each result detailed in *Section 4.5*.

4.4.1 Gel electrophoresis

The following results showed gel electrophoresis of body fluid samples amplified using Platinum PCR multiplex kit and Qiagen PCR multiplex kit

4.4.1.1 PCR optimizations using Platinum PCR multiplex kit

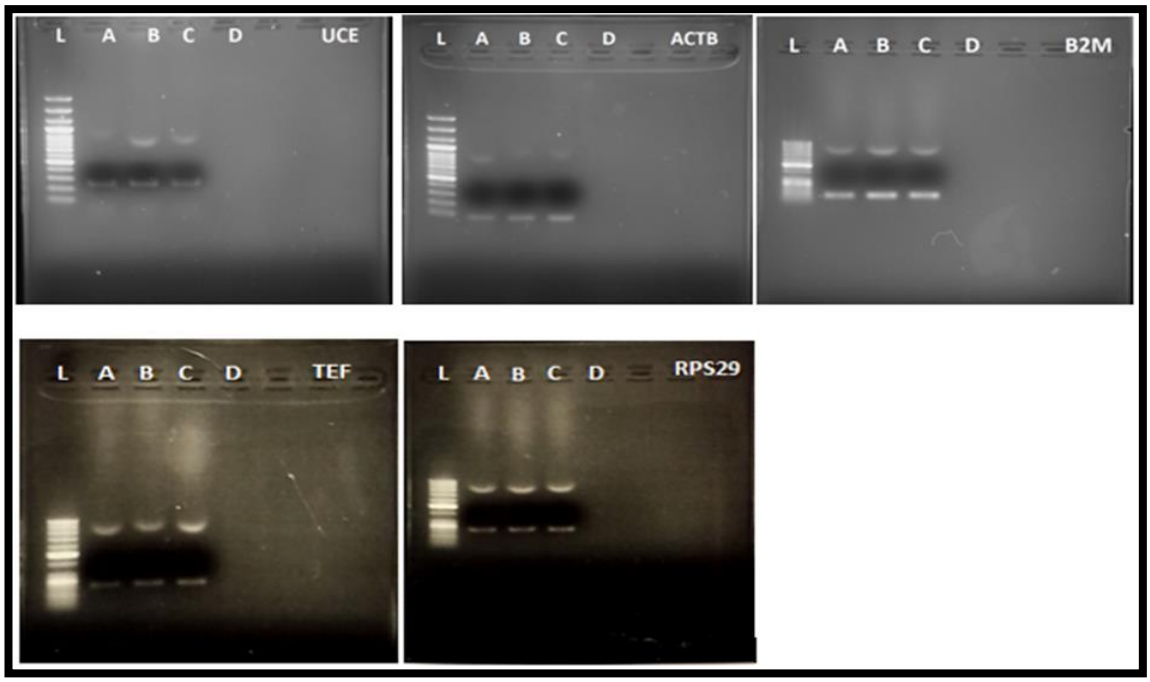

Figure.4.1: A pictorial representation of gel electrophoresis of positive control PCR product and reference genes stained with safeview. UCE – 241bp; ACTB – 75 bp; B2M – 120 bp; TEF – 206 bp; RPS29 – 213 bp; L: 100 bp ladder, A: 54 ^{0}C, B: 56 ^{0}C, C: 58 ^{0}C, D: Negative control sample; Number of cycles: 35, n=5; where n is the number of repeats of experiment.

Positive control RNA samples were amplified using Platinum PCR mastermix. All five reference genes (UCE, B2M, RPS 29, TEF and ACTB) were expressed with expected sizes (Figure 4.1). Although, non-specific amplification was noticed, this was further optimised using Qiagen PCR mastermix.

4.4.1.2 PCR optimization using Qiagen PCR multiplex kit

PCR amplification using Platinum PCR multiplex kit displayed non-specific amplification across all tested samples. Further optimization was carried out using modified cycling parameters, primer concentrations and a different mastermix (Qiagen multiplex PCR mastermix). The results show that all reference genes were fully expressed in vaginal secretion samples and reference genes (Figure 4.2).

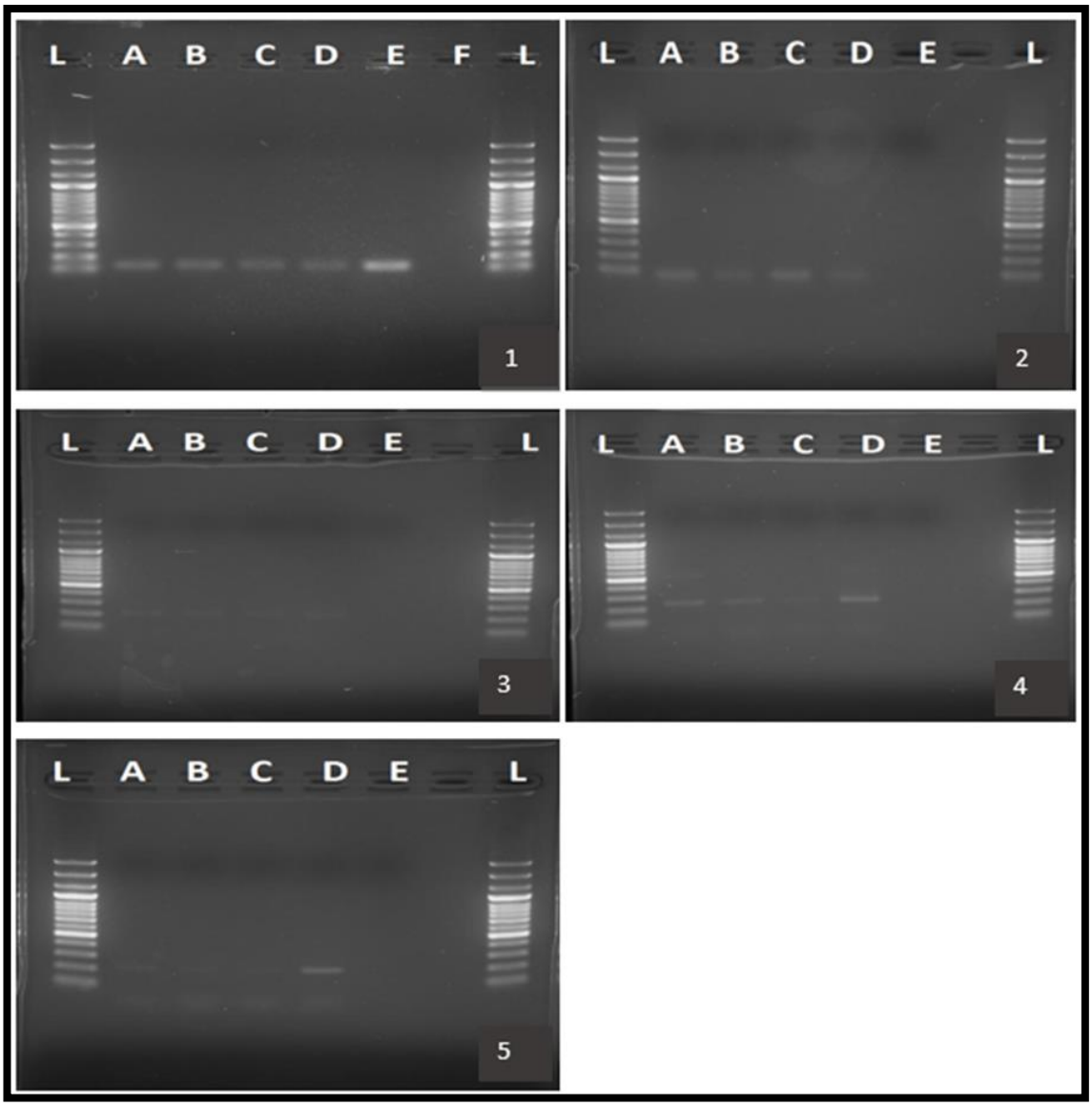

Figure 4.2: Pictorial representation of gel electrophoresis of vaginal secretion PCR product and reference genes stained with safeview. 1: B2M – 120 bp, 2: ACTB – 75 bp, 3: TEF – 206 bp; 4: UCE – 241 bp, 5: RPS29 – 231 bp. L: 100 bp ladder, A: 58 ^{0}C, B: 60 ^{0}C, C: 62 ^{0}C, D: Positive control, E: Negative control; Number of cycles: 33. B2M: A: 54 ^{0}C, B: 56 ^{0}C, C: 58 ^{0}C, D: 60 ^{o}C, E: Positive control, F: Negative control. n=5; where n is the number of repeats of experiment.

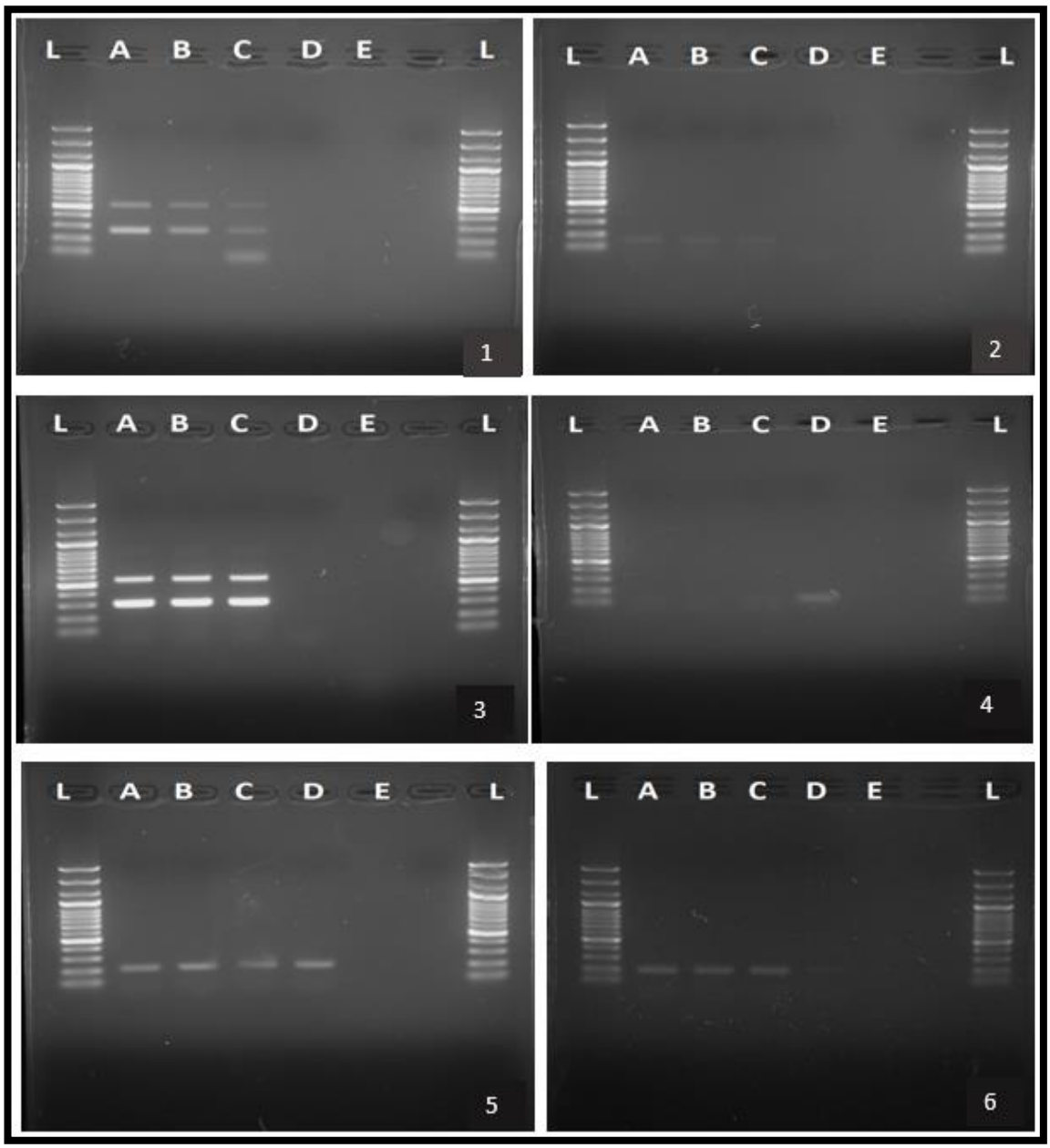

Figure 4.3: Pictorial representation of gel electrophoresis of vaginal secretion PCR product stained with safeview. 1: *L. gasseri* – 273 bp; 2: MUC4 – 141 bp; 3: *L. crispatus* – 267, 305 bp; 4: HBD1 – 101 bp; 5: CRYP2B7P1 – 198 bp, 6: SFTA2 – 183 bp. L: 100 bp ladder, A: 56 ^{0}C, B: 58 ^{0}C, C: 60 ^{0}C, D: Positive control, E: Negative control; Number of cycles: 33. n=5; where n is the number of repeats of experiment.

Figure 4.3 shows amplifications and gel electrophoresis of all vaginal secretion body fluid specific markers, positive and negative control samples at 56 ^{0}C – 60 ^{0}C. All markers were expressed at the three annealing temperatures optimised except for HBD1 which showed amplification with positive control sample only. There was no amplification in the negative control samples.

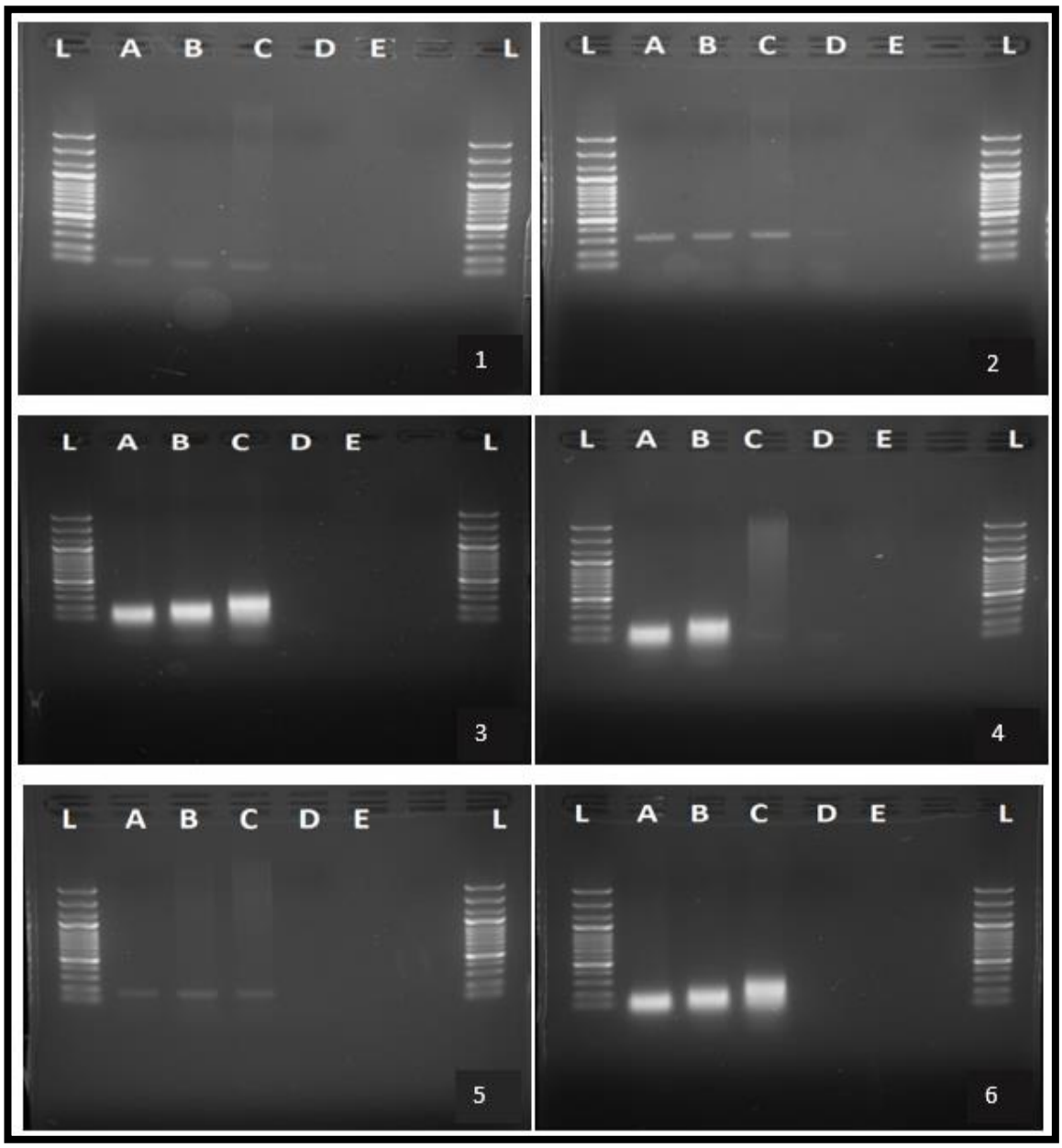

Figure.4.4: Pictorial representation of gel electrophoresis of semen PCR product stained with safeview. 1: PRM1 – 91 bp, 2: PRM2 – 294 bp, 3: TGM4 – 215 bp; 4: MSMB – 105 bp, 5: NKX3-1 – 144 bp, 6: SEMG – 183 bp L: 100 bp ladder, A: 56 ^{0}C, B: 58 ^{0}C, C: 60 ^{0}C, D: Positive control, E: Negative control; Number of cycles: 33. n=5; where n is the number of repeats of experiment.

Semen markers were all expressed in semen samples tested. PRM1, PRM2 and NKX3-1 displayed a good amplification at all the three annealing temperatures optimised. However, TGM4, MSMB and SEMG showed amplification with smeary bands. The smeary bands were more noticeable in MSMB at 60 ^{0}C (Figure 4.4)

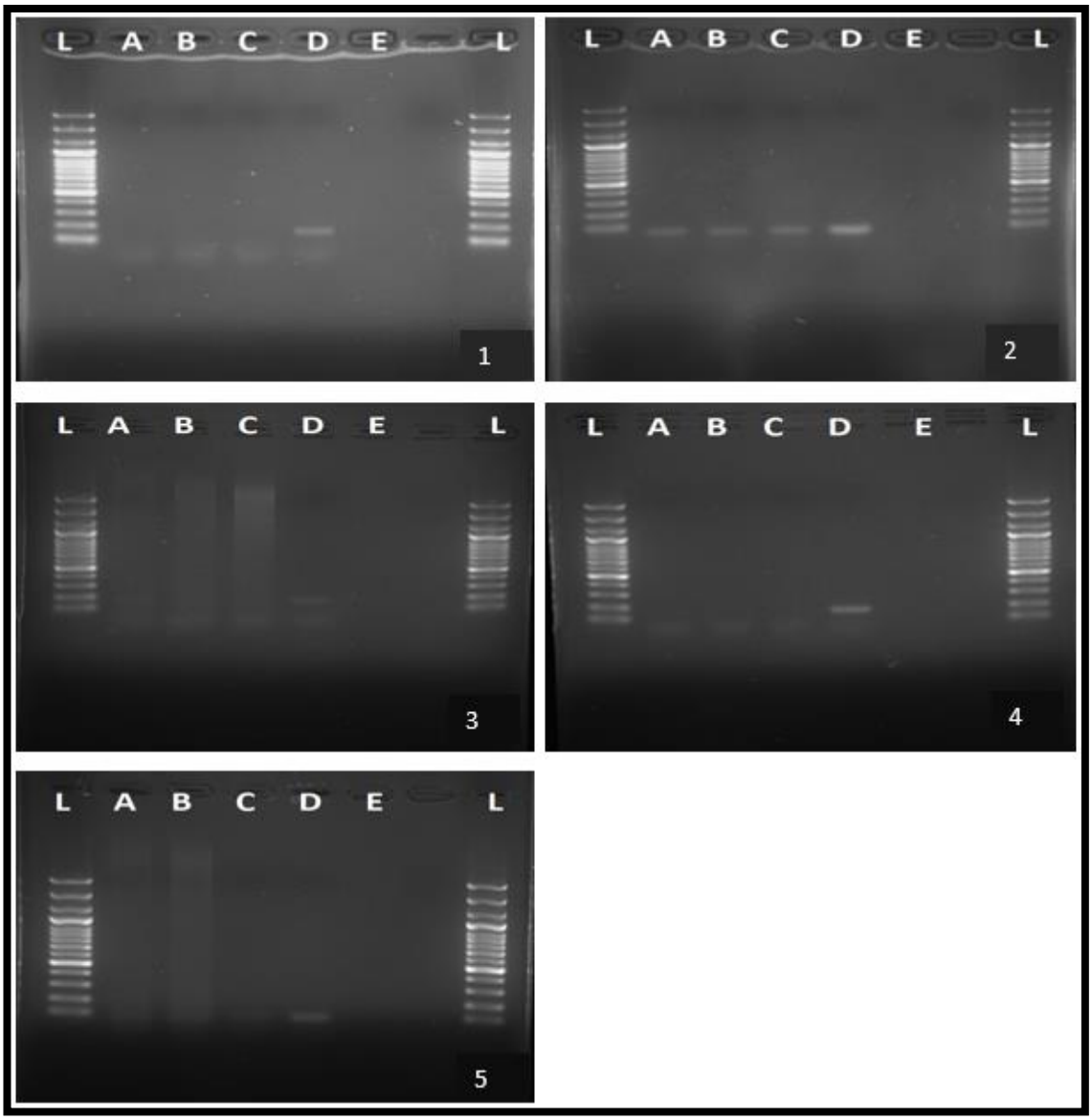

Figure 4.5: Pictorial representation of gel electrophoresis of blood PCR product stained with safeview. 1: CCL5 – 134 bp; 2: HBB1 – 61 bp; 3: β-SPECTRIN – 150 bp; 4: PPBP – 146 bp; 5: ALAS2 – 81 bp; L: 100 bp ladder; A: 56 ^{0}C, B: 58 ^{0}C, C: 60 ^{0}C, D: Positive control, E: Negative control; Number of cycles: 35. n=5; where n is the number of repeats of experiment.

Figure 4.5 shows amplification of blood specific markers at 56 ^{0}C, 58 ^{0}C and 60 ^{0}C with smeary bands. HBB1 appeared to display optimal amplification at all annealing temperatures. Smeary bands were prominent in CCL5, β-Spectrin and ALAS2, and all markers were expressed in the positive control sample.

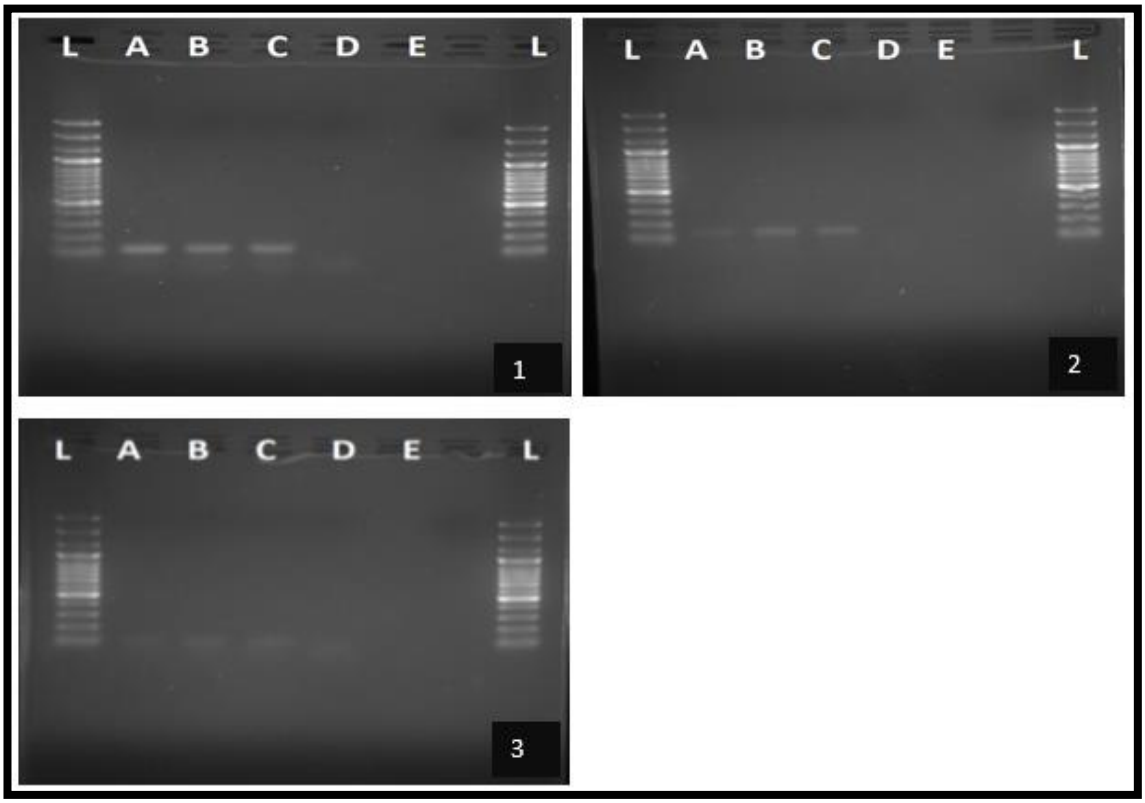

Figure.4.6: Pictorial representation of gel electrophoresis of saliva PCR product stained with safeview. 1: HTN3 – 134 bp, 2: FDCSP – 115 bp, 3: STATH – 93 bp; L: 100 bp ladder, A: 56 ^{0}C, B: 58 ^{0}C, C: 60 ^{0}C, D: Positive control, E: Negative control; Number of cycles: 35. n=5; where n is the number of repeats of experiment.

Three saliva markers were all expressed in saliva samples at the annealing temperatures. Brightest band was noticed in HTN3, which decreased across FDCSP and STATH respectively. None of the markers was expressed in the in the positive control samples (Figure 4.6).

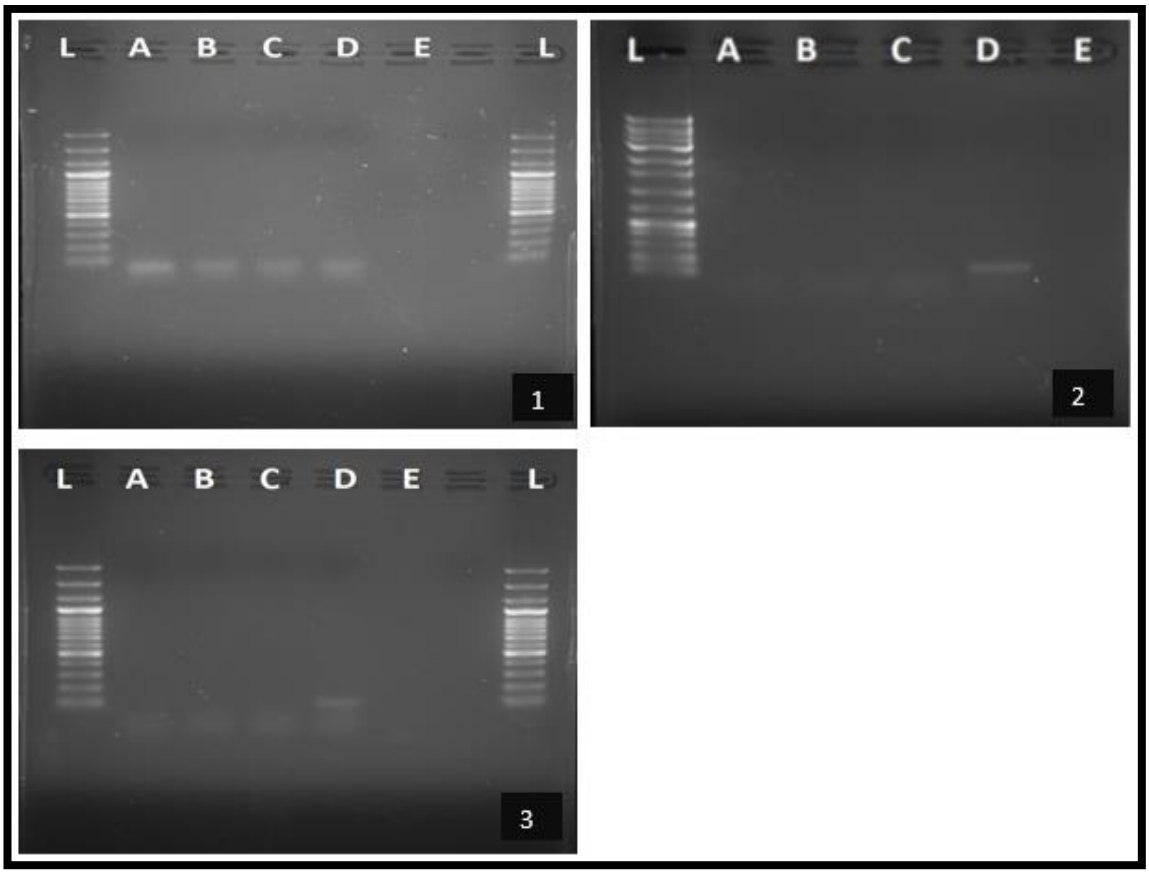

Figure.4.7: Pictorial representation of gel electrophoresis of menstrual blood PCR product stained with safeview. 1: MSX1 – 96 bp; 2: SFRP – 136 bp; 3: MMP11 – 96 bp; L: 100 bp ladder; A: 56 ^{0}C, B: 58 ^{0}C, C: 60 ^{0}C; D: Positive control; E: Negative control; Number of cycles: 35. n=5; where n is the number of repeats of experiment.

Figure 4.7 shows amplification and gel electrophoresis of menstrual blood markers. MSX1 was expressed in menstrual blood sample at all annealing temperatures while SFRP and MMP11 were only expressed in the positive control sample. There was no amplification noticed in the negative control samples.

4.4.2 Optimisation of Multiplex PCRs using Capillary Electrophoresis (CE)

Capillary electrophoresis was carried out on the ABI 3500 genetic analyser. The results are reported in the sections below.

4.4.2.1 Five-plex multiplex PCR

Capillary electrophoresis experiment resulted in the development of a five-plex multiplex assay which consist of saliva-plex, semen-plex, vaginal secretion-plex and blood plex. All the markers were fully expressed in their respective body fluids, which indicated specificity of the body fluid markers (Figures 4.9 – 4.13). This implied no expression of the specific body fluid markers in non-target body fluids. A schematic representation of all the five-plex combined is represented in Figure 4.8.

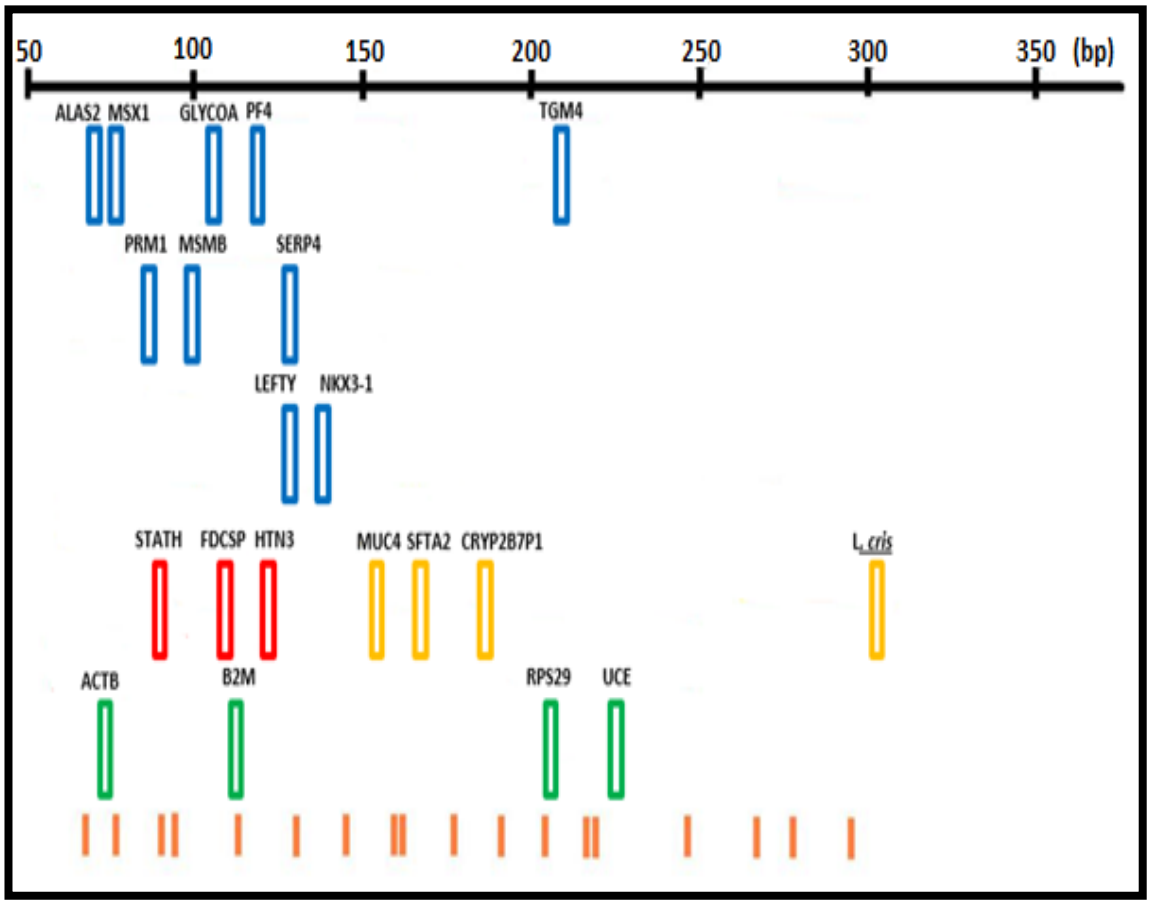

Figure 4.8: Schematic representation of developed and modified body fluid-specific multiplex assay. The following dye colours indicate markers for each body fluid: Blue: blood, menstrual blood and semen, Red: saliva, Yellow: vaginal secretion, Green: reference genes, Orange: Size standards.

Table 4.3: Final primer concentrations in the five-plex multiplex reactions. An initial equimolar concentration of 0.5 µM was used and modified based on the RFUs generated in the first rounds of multiplex optimisation.

Body fluid markers	Primer concentrations (F/R) µM
GLYCOA	2.0
ALAS2	0.11
PF4	0.11
SPTB	1.40
MSX-1	0.86
SERP4	1.44
LEFTY	1.44
PRM1	0.10
PRM2	0.35
TGM4	0.87
MSMB	0.10
NKX3-1	0.07
CRYP2B7P1	0.46
L. crispatus	0.03
SFTA2	0.08
MUC4	0.93
HTN3	0.18
FDCSP	1.22
STATH	0.09

4.4.2.1.1 Blood-plex

The EPG below shows a multiplex panel of all the blood markers. Three blood markers were successfully amplified out of four that were optimised (Figure 4.9). SPTB dropped out during amplification. An artefact was noticed just before ALAS2, with amplicon size of 70 bp. This would not be an issue in profile interpretation due to the sensitivity of the genetic analyser used – this is capable of detecting amplicon sizes of a single base-pair difference. In addition, ALAS2 is the marker with the smallest amplicon size in the overall multiplex panel; therefore, any artefact below this range could be disregarded.

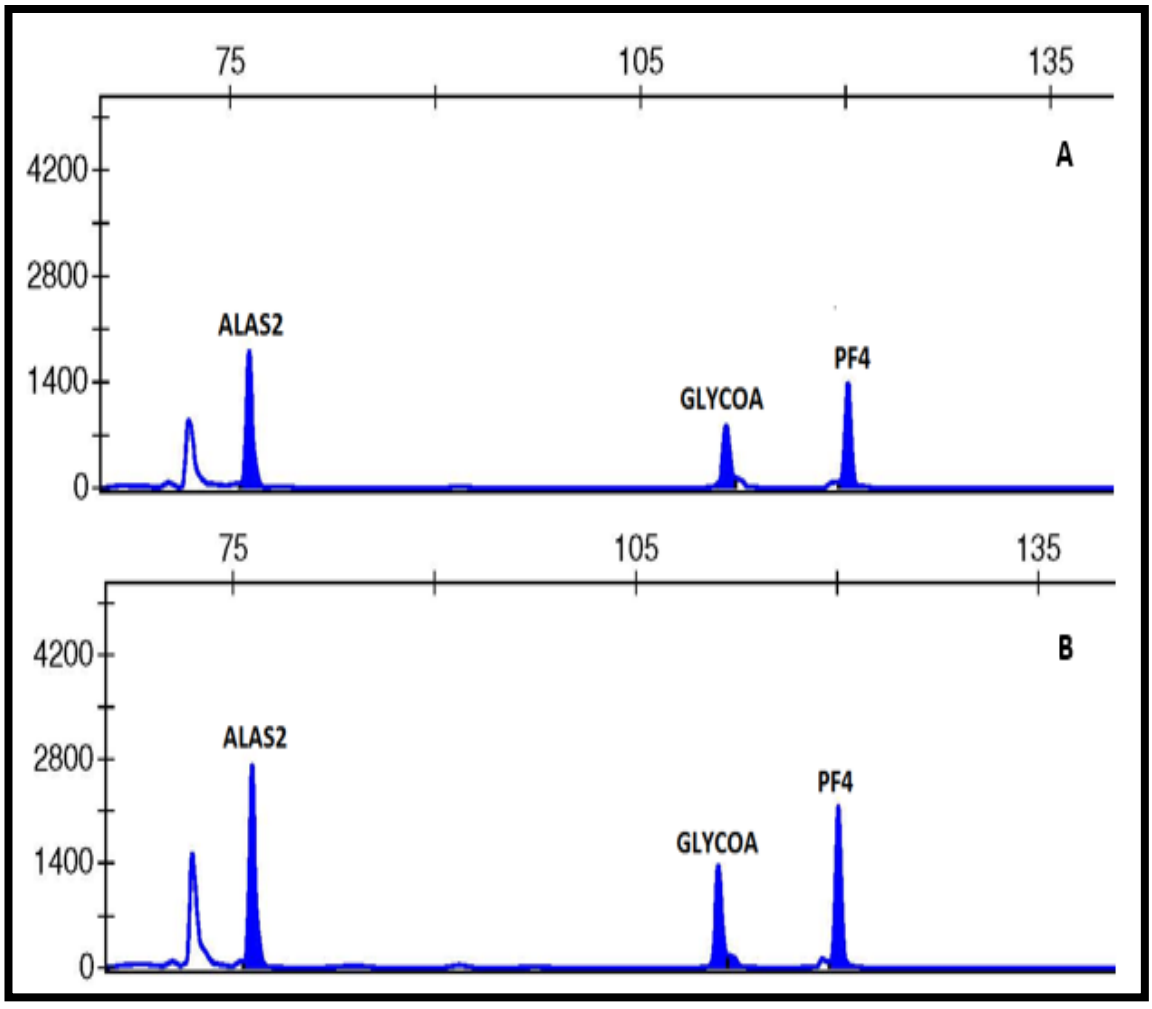

Figure 4.9: EPG showing multiplex panel of blood markers ALAS2: 76 bp; GLYCOA: 110 bp; PF4: 119 bp. (A) and (B) indicates duplicate PCR of the same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.2 Saliva-plex

All three saliva markers were successfully expressed in the saliva-plex assay. Unlike blood-plex that displayed an artefact, none was observed in the saliva-plex. Also as expected, the three markers were expressed at different proportions (Figure 4.10).

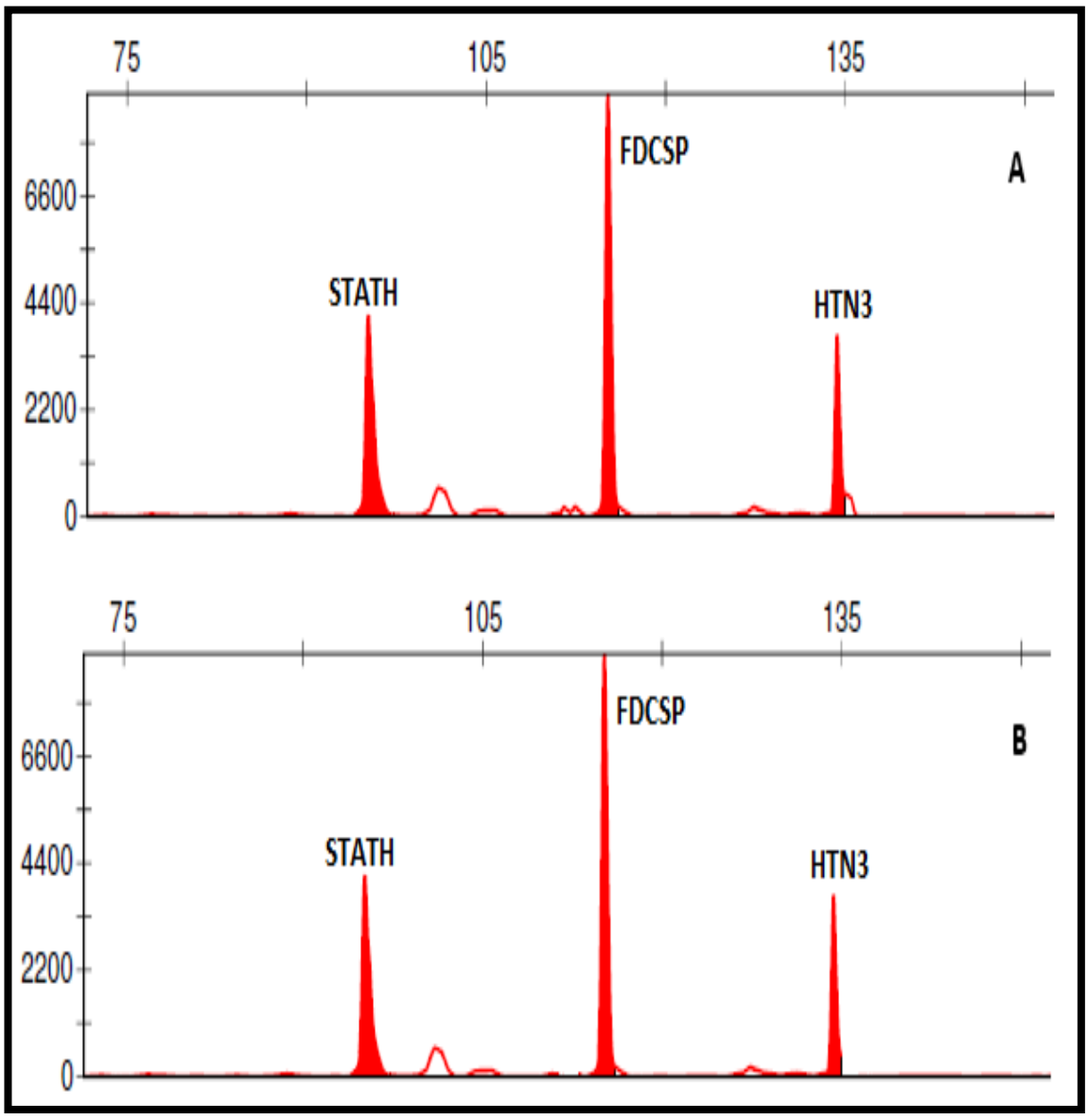

Figure 4.10: EPG showing multiplex panel of saliva markers STATH: 95bp; FDCSP: 115 bp; HTN3: 134 bp. (A) and (B) indicates duplicate PCR of the same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.3 Semen-plex

Four markers out of five was expressed in the semen-plex (Figure 4.11). PRM2 dropped out in the multiplex. Further modifications were made to the primer concentration, which yielded no positive results.

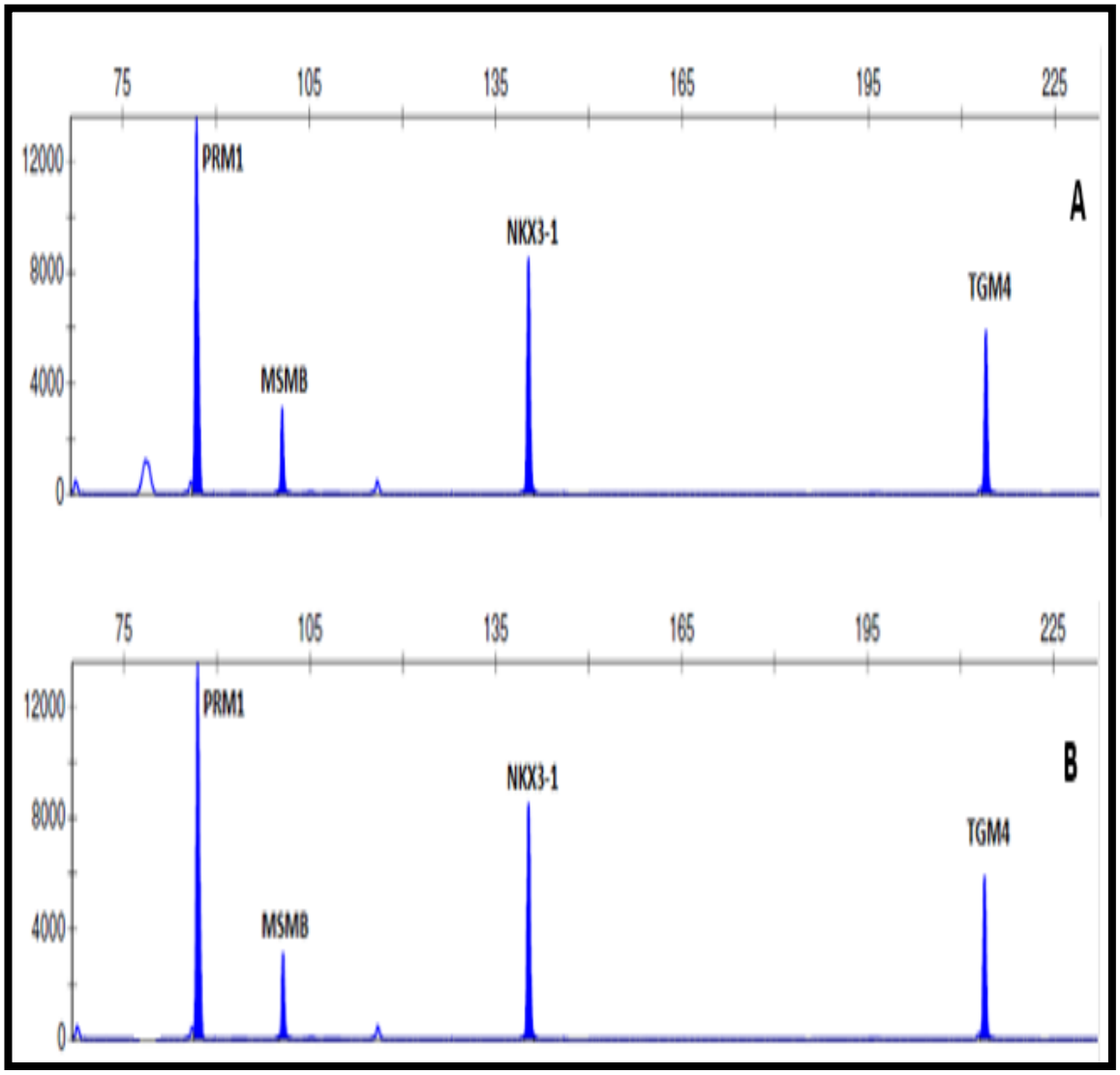

Figure 4.11: EPG showing multiplex panel of semen markers PRM1: 86 bp; MSMB: 100 bp; NKX3-1: 140 bp; TGM4: 213 bp. (A) and (B) indicates duplicate PCR of same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.4 Vaginal secretion-plex

Four markers were optimised for vaginal secretion detection; however, three markers were amplified with MUC4 dropping out of the multiplex. Several attempts made to modify the primer concentration yielded no result (See Figure 4.12).

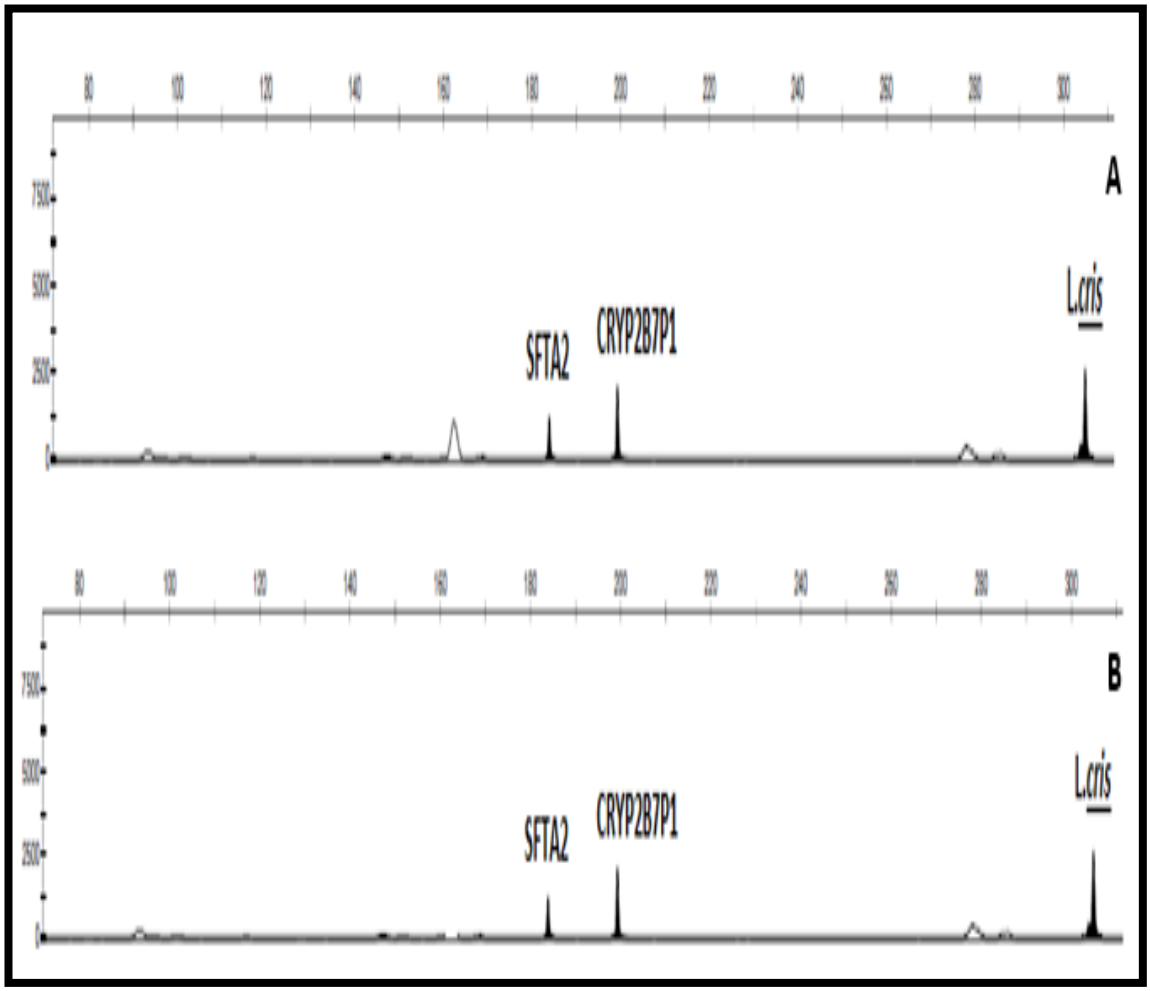

Figure 4.12: EPG showing multiplex panel of vaginal secretion markers SFTA2: 183 bp; CRYP2B7P1: 199 bp; *L. cris:* 304 bp. (A) and (B) indicates duplicate PCR of the same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.5 Menstrual blood-plex

Three markers were initially optimised for menstrual blood detection. SFRP4 and MSX-1 were expressed with LEFTY dropping out of the multiplex. Further increase in LEFTY primer concentration yielded no result (Figure 4.13)

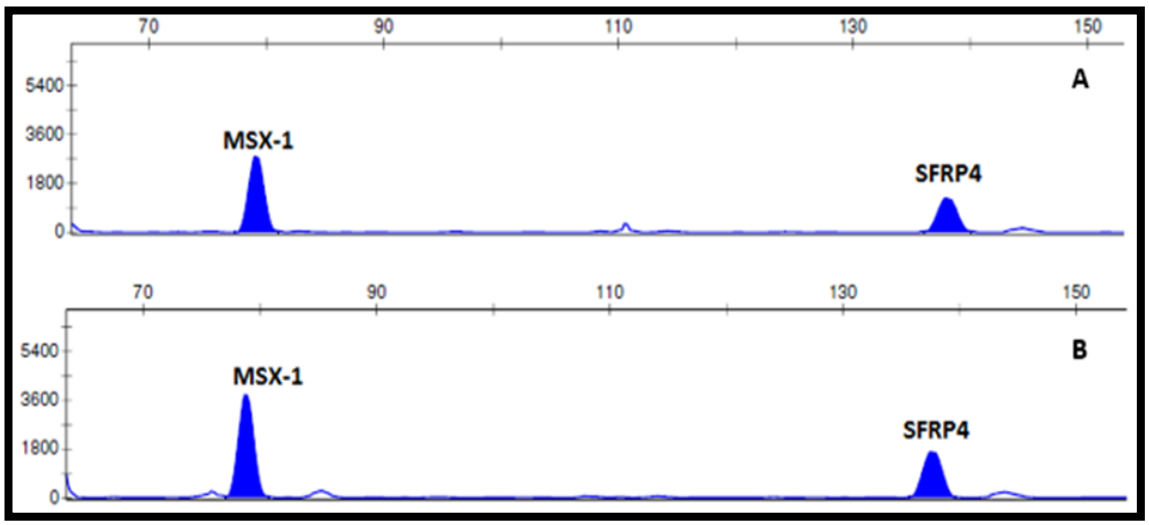

Figure 4.13: EPG showing multiplex panel of menstrual blood markers MSX-1: 79bp; SFRP4: 134 bp. (A) and (B) indicates duplicate PCR of the same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.6 Reference genes-plex

Human XpressRef Universal mRNA sample was used initially to optimise the five reference genes. Donated body fluids were then used in the combined multiplex. In the EPG (Figure 4.14), TEF and B2M dropped out of the multiplex.

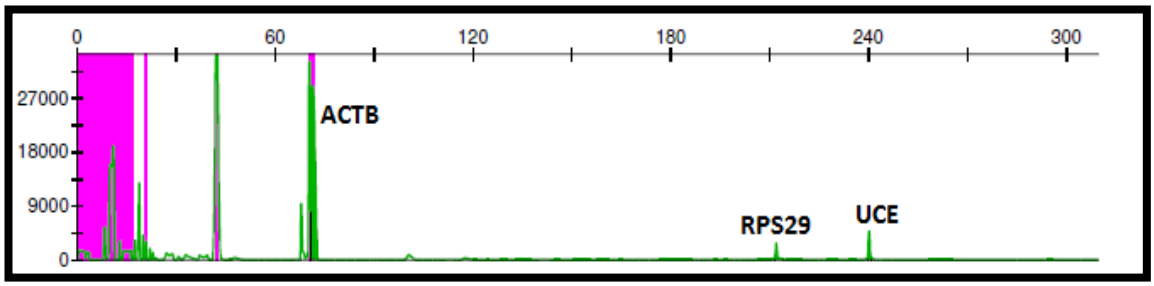

Figure 4.14: EPG showing multiplex panel of reference gene markers ACTB: 75 bp; UCE: 241 bp; RPS29: 213 bp for positive control sample (Human XpressRef universal RNA). (A) and (B) indicates duplicate PCR of the same sample, n=5; where n is the number of repeats of experiment.

4.4.2.1.7 Negative controls

Figure 4.15 shows EPG of negative control samples. These were cDNA prepared from original negative control with water. No amplification was seen in any of the four panels. This infers there was no form of contamination in any of the reactions.

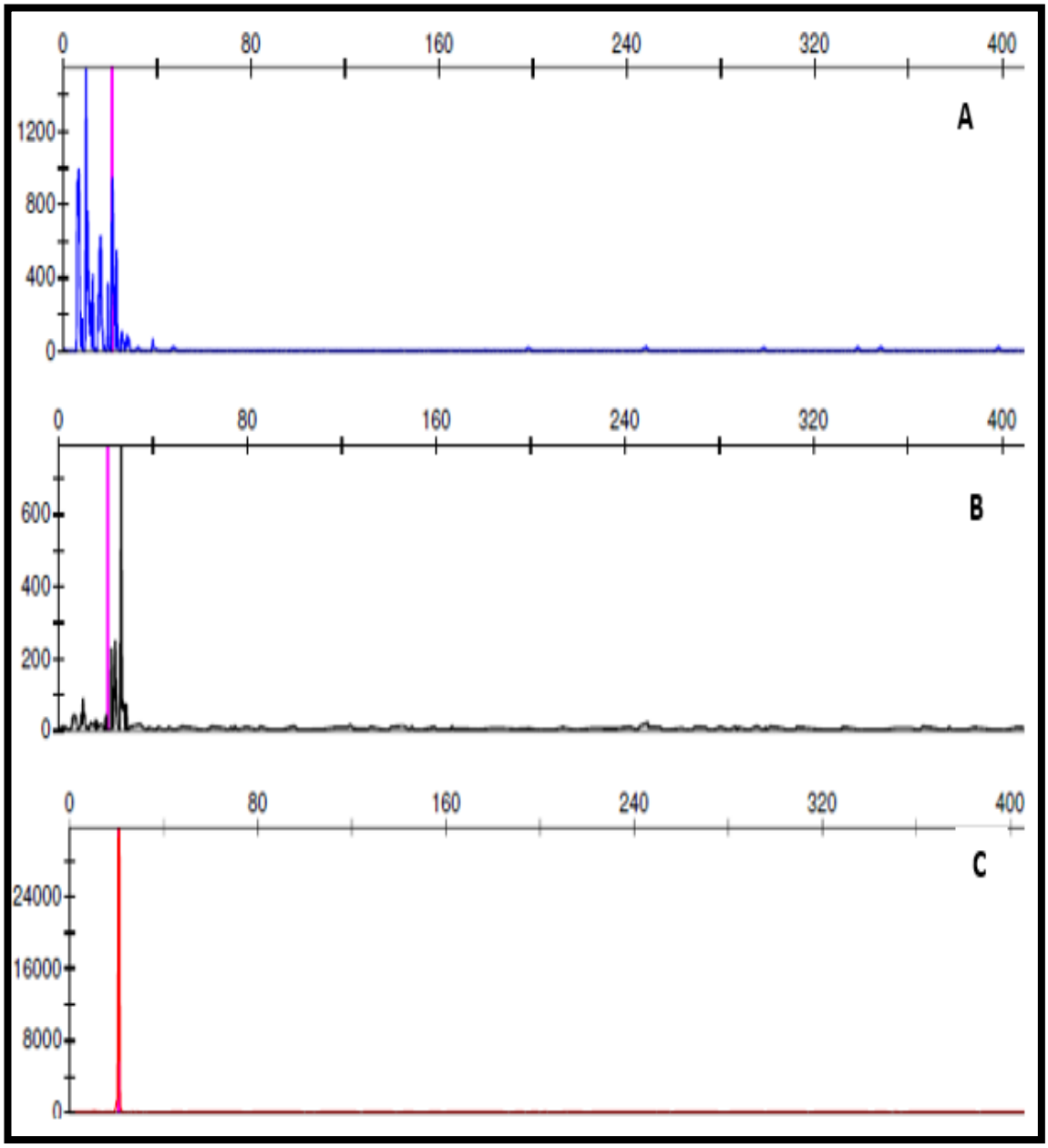

Figure.4.15: EPG showing negative controls of all the panels utilised in the multiplex reactions of each body fluid. (A), (B) and (C) represents blood, menstrual blood and semen panels; vaginal secretion panel; and saliva panel respectively, n=5; where n is the number of repeats of experiment.

4.4.3 Combination of five-plex assay into a single multiplex

Final primer concentrations used in the combined 5-plex are shown Table 4.4. The results displayed expression of all the body fluid markers in their target body fluids with no cross-reaction with non-target body fluids (See Figure 4.16).

Table.4.4: Final primer concentration in the combined multiplex reaction. This include primers for blood, saliva, semen, vaginal secretion and reference genes.

Body fluid specific markers	Final primer concentration (F/R) * µM
ALAS2	0.26
PF4	0.92
HTN3	0.19
STATH	0.19
PRM1	0.16
TGM4	0.46
MSMB	0.28
NKX3-1	0.18
UCE	0.92
ACTB	0.16
L. *crispatus*	0.19
CRYP2B7P1	0.92
MUC4	0.92
SFTA2	0.92

F/R* indicates forward and reverse primer

The result in Figure 4.16 shows the EPG generated from cDNA mixture of all body fluids. These were amplified using equimolar concentration of primers (0.5 µL). Menstrual blood markers dropped out of the combined multiplex. In addition, two blood markers, four semen markers, two saliva markers, two vaginal secretion markers and 2 reference gene markers were expressed. There was a pull-up noticed in both blue and red panels with ACTB. This remained persistent despite reducing ACTB primer concentration. As shown in Figure 4.16, all body fluid markers were displayed varied expression.

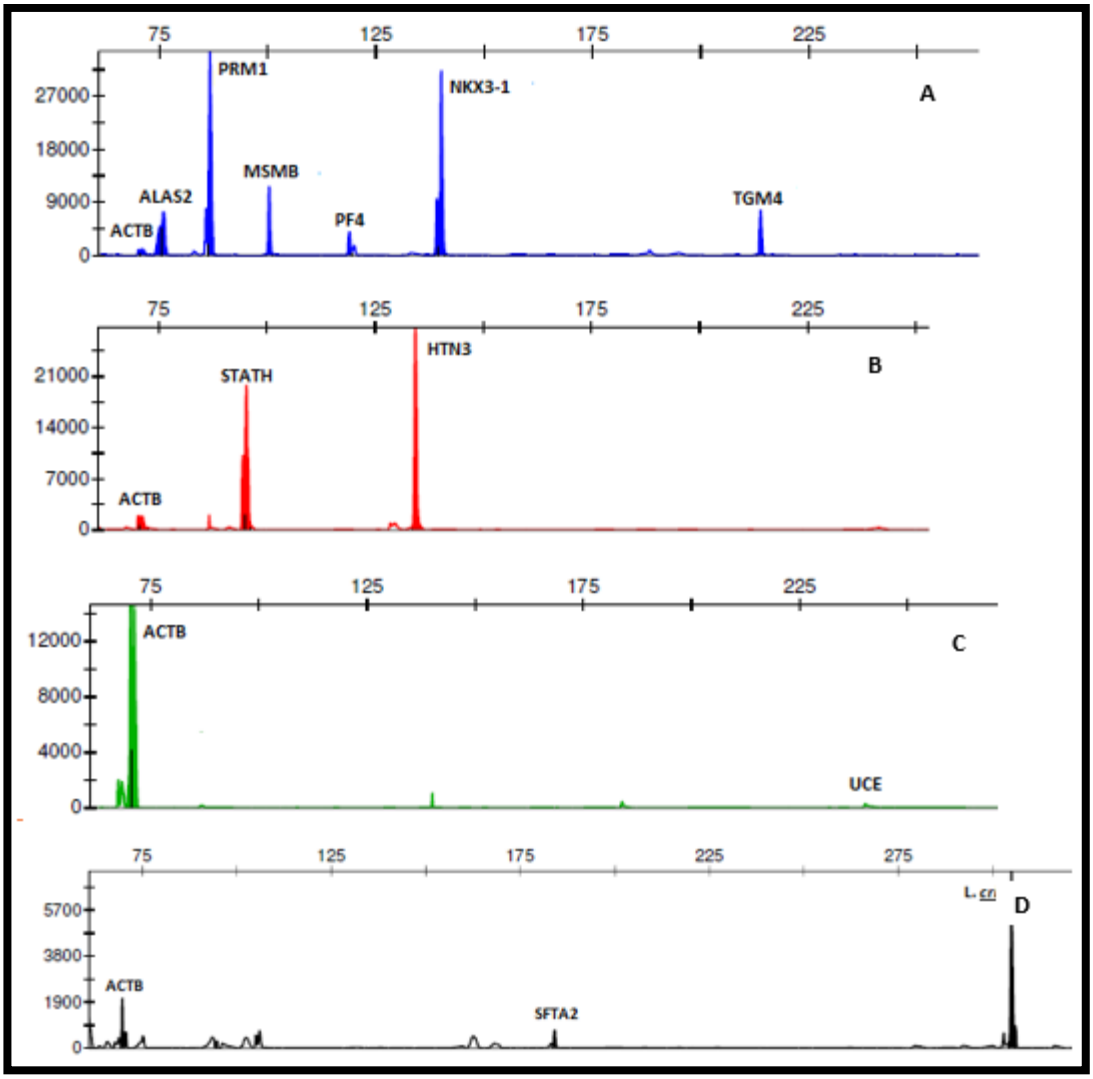

Figure 4.16: EPG showing the combined 5-plex into a single multiplex panel using **equimolar** primer concentration. A: Blue panel displays blood and semen markers; B: red panel displays saliva markers; C: Green panel displays reference gene markers; D: Yellow panel displays vaginal secretion markers. Reference gene ACTB is expressed in all the body fluids while UCE was expressed in only the reference genes panel, n=5; where n is the number of repeats of experiment.

A modified primer concentration was used to generate the EPG in Figure 4.17. A better EPG was obtained and the samples did not display over-amplification as noticed in Figure 4.16. CRYP2B7PI vaginal secretion marker was expressed this time. The primer concentration of UCE was increased due to low expression noticed in Figure 4.16. However, this increase did not seem to make any significant difference in the markers expression. In addition, pull-ups were still noticed for ACTB in both blue and red panels. Most importantly, all markers displayed high specificity with no record of non-specific amplification to non-target body fluids noticed.

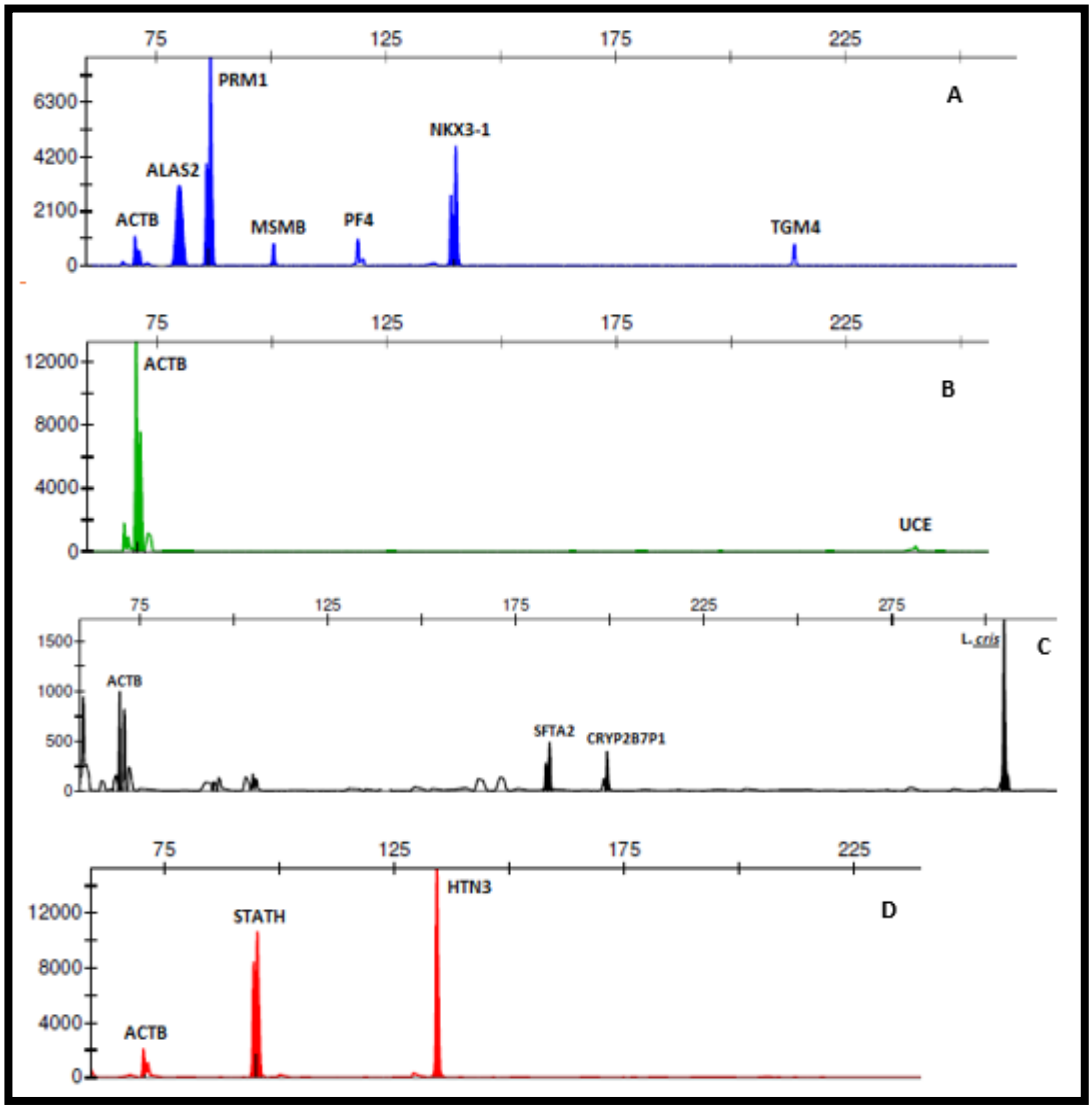

Figure 4.17: EPG showing the combined 5-plex into a single multiplex panel using **modified primer concentrations** in Fig. 4.4 above. A: Blue panel displays blood and semen markers; B: Green panel displays reference gene markers; Yellow panel displays vaginal secretion markers. D: red panel displays saliva markers; D: Reference gene ACTB was expressed in all the body fluids while UCE was expressed in only the reference genes assay, n=5; where n is the number of repeats of experiment.

4.5 Discussion

Multiplex PCR, which offers the flexibility to combine two or more primer pairs in a single reaction, has remained an indispensable tool in the forensic science field. Since its first description by Chamberlain *et al.,* (1988.), its application has witnessed a significant increase across various biochemical fields of study, especially in areas of forensic DNA study. It is specifically important in forensic area of body fluid identification as most body fluid stains comprise of heterogeneous mixture of cellular materials and are mostly recovered with other body fluids (Juusola and Ballantyne, 2003). Multiplex PCR is made up of critical parameters, which must be adequately optimised in order to establish its success. These include primer concentration, cycling parameters, and role of various reagents involved in the reaction (Saiki, 1989).

In this study, a mRNA multiplex assay for forensic body fluid identification was developed and adequately optimised for unambiguous identification of human body fluids (Figures 4.1-4.17). Unlike various human identification multiplex assays that are commercially available (Applied Biosystems Ltd, Promega Corporation, and Qiagen Ltd), there is no known commercial body fluid identification assay till to date. All reported different research groups have developed body fluid identification assays in-house, which is intended for use in their respective laboratories.

Initially, 32 markers were selected from relevant literature for the mRNA assay development. These include six markers each for vaginal secretion and semen respectively, five markers for blood, three markers each for saliva and menstrual blood, and five markers for reference genes (Table 2.2). The first phase of the study evaluated the reference genes that were initially screened in chapter 3, but in this case, they were fluorescently labelled and analysed with body fluid specific markers on the genetic analyser. This was optimised using Platinum PCR supermix (Life Technologies Ltd) and Qiagen PCR mastermix (Qiagen Ltd) using manufacturers recommended protocols. Upon amplification with Platinum PCR supermix, Agarose gel electrophoresis displayed the expected sizes for each marker, however, with non-specific amplifications (Figure 4.1). Although, the use of Platinum PCR mastermix has not been reported in any body fluid identification study, it was considered and used in this project because of its reported higher specificity and fidelity (Life Technologies Ltd). Further optimization was

carried out to detect the source of the non-specific binding (See Tables 4.1, 4.2 for modified cycling parameters). This included optimization of reaction cycles (20–35), annealing temperatures (54 ^{o}C – 64 ^{o}C) and most importantly, the cDNA (Kapley *et al.*, 2000). In addition, since the non-specific amplifications were noticed above the expected amplicon sizes (See Fig. 4.1), attempts were made to reduce the final extension time from 10 min to 8 min, 5 min, and 2 min, respectively. The reduction in final extension time was targeted at terminating the PCR reaction just shortly after the target product has been amplified. These modifications did not yield significant change in amplification products obtained as all the reactions produced similar result with the presence of non-specific amplifications. Having established this, Qiagen PCR master mix was then used for further optimisation with both positive control and vaginal secretion samples. This was done as a blind test to check if there would be any difference in cDNA amplification using Qiagen PCR multiplex mix, compared to the results obtained using Platinum PCR multiplex mix. The amplification produced similar results with Platinum mastermix; however, there were no non-specific amplifications (See Figure 4.2). Similar studies have reported amplification success using Qiagen PCR mastermix (Lindenbergh *et al.*, 2012; Roeder and Haas, 2013; Jakubowska *et al.,* 2013).

Figure 4.3 shows the result obtained from optimization of all vaginal secretion markers. Optimal amplification was recorded at 33 cycles for all markers except HBD1. All markers displayed expected sizes as reported in the literature (See *Chapter 2,* Table 2.2; Figure 4.3). Positive control sample was only expressed in CRYP2B7P1 marker. HBD1 was not expressed in vaginal secretion sample other than in the positive control. This was found to be in agreement with a study by Lindenbergh *et al.,* (2012). These authors reported no expression of this marker in their developed 19-plex assay. In addition, cross-reaction of the marker with saliva was reported by Roeder and Haas, (2013) and Jakubowska *et al.* (2013); and menstrual blood (Juusola and Ballantyne, 2005). This result coupled with other authors' reports could infer the marker might not be ideal in unambiguous identification of vaginal secretion.

PCR optimisation optimization strategy proposed by Chamberlain *et al.,* (1988) and Saiki, (1989) was adopted in this project. This include basic PCR cycle (Tables 4.1 and 4.2), and annealing temperatures were first tested. Optimised annealing temperature was 58 ^{o}C

for all samples. This was found to be consistent with the reports of Lindenbergh *et al.,* (2012). However, for semen and vaginal secretion specific markers, 33 cycles appeared to be optimal whereas 35 cycles were optimal for blood, saliva and menstrual blood markers. The 35 cycles for menstrual blood amplification was again consistent with the reports of Roeder and Haas, (2013). The variations in PCR primer concentrations appeared to have a limited effect after the initial optimisation so these were kept constant.

Semen specific markers displayed optimal amplification and sizes at 33 cycles using semen samples. However, positive control samples were not expressed in any of the markers (Figure 4.4). Unlike, vaginal secretion and semen markers, blood markers were optimally expressed at 35 cycles (Figure 4.5). HBB1 was expressed at all optimized annealing temperatures. A smeary band was observed in β-Spectrin and ALAS2, and positive control was constantly expressed in all the markers. Although CCL5 and PPBP was reported by Park, *et al*., (2013) to be a robust candidate marker for the identification of blood, both markers displayed no expression in this study. Most of the discrepancies noticed in the results in comparison to other authors' reports could be due to using different equipment, amplification platforms and reagents (Roeder and Haas, 2013; Lindenbergh *et al.,* 2012).

The three saliva markers displayed expected size expression at 35 cycles (Figure 4.6) which further validates the reports of Lindenbergh *et al.,* (2012) and Park *et al.*, (2013). All menstrual blood markers were expressed in positive control samples, however, only MSX1 was expressed in menstrual blood sample (Figure 4.7). SFRP4 and MMP11 were both reported to display specificity for menstrual blood by Roeder and Haas, (2013) and Lindenbergh *et al.* (2012), respectively; however, these were not expressed in this study. The reason for this could be because of the nature of sample used. Menstrual blood is made up of complex composition (Divall and Ismail, 1983), and expression of its markers are dependent on the time of the month the sample was taken. As no specific time of menstrual flow was given to the participants regarding the sample collection, this could be a main reason some of the markers were not expressed.

The results of PCR optimization obtained using the non-florescent primers was used for fluorescent labelling of primers with high specificity. Four different florescent dyes were

selected and these include ATT0 565(Red), 6-FAM (Blue), Yakima Yellow (Green) and ATT0 550 (Yellow) (Table 2.8). The use of four different florescent dyes was necessary due to some markers having similar sizes as well as to prevent overlap of sizes (Figure 4.8). The florescent markers were optimised in singleplex PCR to establish their sizes using capillary electrophoresis, a platform with vastly increased sensitivity compared to gel electrophoresis.

Further attempts made to develop a 5-plex multiplex panel for all the five body fluids resulted in three markers each for blood and saliva, four markers each for semen and vaginal secretion and two markers for menstrual blood (See Table 4.3). An initial approach was to use equimolar primer concentrations for all the primers in each multiplex as recommended by Chamberlain *et al.,* (1988) and Saiki, (1989). These were then modified based on the result obtained (RFU) on the electropherogram (EPG). Primer pairs with low RFU were increased while those with very high RFUs were reduced in order to obtain a nearly balanced peak height (Henegariu *et al.*, 1997). A Tm of 58 ^{0}C and 33 cycles produced better amplification (Lindenbergh *et al.,* 2012). Magnesium Chloride ($MgCl_2$) and dNTP concentrations as recommended by Henegariu *et al.*, (1997) did not alter the results as a ready-made multiplex mix (Qiagen PCR multiplex mix) was used.

In this study, blood-plex assay displayed three markers from a total of four (Figure 4.9), excluding SPTB. This was expected as many studies that focused on blood identification have reported this marker as either not specific or not optimal for mRNA profiling of blood (Kohlmeier and Schneider, 2012). MUC4 expression for vaginal secretion in this study displayed a very low RFU of 183 and could not be shown on the vaginal secretion-plex (Figure 4.12). Mara *et al*., (2012); Nussbaumer *et al.*, (2006); Haas *et al*., (2009); and Cossu *et al.*, (2009), have all reported this marker to exhibit cross reactivity with saliva. The results obtained from saliva-plex (Figure 4.10) and semen-plex (Figure 4.11) were consistent with all the studies where these markers were used (Lindenbergh *et al.*, 2012; Roeder and Haas, 2013; Park *et al*.,2013; Mara *et al*., 2012). LEFTY2 was not detected in menstrual blood-plex in this study (Figure 4.13). This was less of a concern as Roeder and Haas, (2013) reported this marker coupled with other menstrual blood specific markers to be non-specific as they were all detected in the non-target body fluids.

Reference genes-plex displayed three markers out of five in the multiplex assay (Figure 4.14). Although B2M was one of the markers that was expressed across all body fluids in previous experiments (Chapter 3), it was not detected in this study. While the primer concentration and other cycling parameters were modified to enhance this with no positive result, a less concern was placed on the non-expression of TEF as this displayed the least expression profile in the previous experiments (Chapter 3). This contrasted with the reports of Roeder and Haas, (2013) as the authors reported full expression of this marker in their developed assay. In order to keep in check the presence of contamination in this study, negative control samples were prepared with each batch of reactions. No amplification was observed in any of the negative control samples (Figure 4.15).

The developed five-plex multiplex assay displayed unambiguous identification of the target body fluids with increase in time and reagents used for the experiments. In addition, cross contamination may not be ruled out in the preparation of separate multiplex if the analyst does not take adequate care (Ghani *et al*., 2013). This necessitated the need to attempt a single multiplex assay, which will incorporate all the markers expressed in the five-plex multiplex assay and reference genes.

A few modifications in the reaction components were made at the initial stage of the multiplex development. A total of 10 µL of mastermix was used in each reaction instead of the initial 5 µL in the five-plex reactions. This increased the total reaction volume to 17 µL as against 10 µL in the five-plex. All the markers that were expressed in the five-plex reactions were included in the multiplex development. Starting with equimolar primer concentrations (Table 4.4), ALAS2 and PF4 (blood), STATH and HTN3 (saliva), SFTA2 and *L. crispatus* (vaginal secretion), PRM1, TGM4, MSMB, NKX3-1 (semen), ACTB and UCE (reference genes) were expressed leaving GLYCOA (blood), FDCSP (saliva), MUC4, CRYP2B7P1 (vaginal secretion), MSX-1, SFRP4 (menstrual blood) and RPS29, B2M (reference genes) unexpressed in the final assay. A minimum of two markers were expressed for each target body fluids other than menstrual blood markers that were not expressed at all (Figure 4.16).

Further attempts were made to balance out the peak heights of the markers in the final multiplex assay. Markers that displayed relatively high RFUs were decreased and those

that were unexpressed or displayed relatively low RFUs were increased (Table 4.4). An improved assay was developed with these further optimisations (Figure 4.17). The final multiplex assay comprises the following markers: ALAS2 and PF4 (blood), STATH and HTN3 (saliva), PRM1, TGM4, MSMB, NKX3-1 (semen), ACTB and UCE (reference genes), CRYP2B7P1, SFTA2, and *L. crispatus* (vaginal secretion). Menstrual blood markers were not expressed in the final multiplex assay despite increase in primer and cDNA concentrations. This led to the exclusion of these markers in the final multiplex assay. In addition, ACTB was consistently expressed in all the four panels representing the body fluids while UCE was only expressed in the reference genes panel (Figure 4.17b). Despite all optimisations made to UCE, it was either consistently low in expression or not expressed at all in the assay. The consistent expression of ACTB across all body fluid panels further validates the marker as a robust reference gene marker for forensic body fluid identification (Lindenbergh *et al*., 2012; Ghani *et al*., 2013).

In conclusion, body fluid specific markers have been adequately optimised using both gel and capillary electrophoresis technique. The results led to the development of a 14-marker multiplex assay, which was used for the identification of semen, saliva, vaginal secretion and blood. For each tested sample, there was no expression of non-target body fluid by the assay. Validation experiments were carried out (See Chapter 5) using degraded samples, mixtures, mock casework and aged samples. Sensitivity experiments were also undertaken in order to establish the overall efficacy of the 14-plex assay.

CHAPTER FIVE

DEVELOPMENTAL VALIDATION OF A 14-mRNA MARKER MULTIPLEX PCR-BASED ASSAY FOR BODY FLUID IDENTIFICATION

5.1 Introduction

The drive to unambiguously identify body fluids deposited at the crime scene has been a major concern for forensic investigators. Use of RNA markers for body fluid identification has been researched in recent years. The multiplexes based on RNA markers follow the same principles as the commercial STR based multiplex kits for human identification.

Validation of methods in Forensic field is a key factor that cannot be overemphasized. It is used to establish 'fitness of purpose' for methods involved in conducting specific examinations. In 2015, the Scientific Working Group on DNA Analysis Methods (SWGDAM) published guidelines for Collection and Serological Examination of Biological Evidence. The document highlights key rules to follow by Forensic DNA laboratories in order to evaluate reliability, sensitivity, specificity, stability, and reproducibility of their methods. Unlike mRNA profiling, many validation experiments have been carried out on DNA marker based multiplex assay (Ensenberger *et al.*, 2010; Oostdik *et al.*, 2013; Oostdik *et al.*, 2014). The authors have reported specifically validation of DNA multiplex kits with addition of more loci to the 13 core CODIS loci, enhanced buffer system with hot-start *Taq* DNA polymerase, sensitivity, concordance, inhibition tolerance amongst other criteria, following SWGDAM developmental validation guidelines; all these have made DNA typing more efficient and less time consuming. In addition, the guidelines have enhanced the ease of human STR typing and its standardization between laboratories.

In this study, the developmental validation of a 14-RNA marker multiplex PCR assay was carried out. The study was designed to satisfy SWGDAM, as there are currently no validation guidelines published on mRNA analysis.

5.2 Aim and objectives

The main aim of this section of the study was to carry out developmental validation of the mRNA-based multiplex PCR assay.

Specific objectives of this chapter were:

- To carry out sensitivity study using the assay on four different body fluids – blood, saliva, vaginal secretion and semen.
- To carry out degradation of the body fluid sample and study assay performance using such samples.
- To conduct other validation experiments including mixture, reaction volume, different cycle number and annealing temperatures, cross reactivity, species specificity studies.
- To establish the use of most appropriate master mix by testing different PCR mastermix for the assay.
- To establish the robustness of the assay using mock casework samples.

5.3 Methods

The methods used for the validation experiment was reported in Chapter 2, section 9.

5.4 Results

All results and experimental conditions are stated below each electropherogram generated. Unless otherwise stated, electrophoresis conditions were outlined in Chapter 2, sections 2.17-2.18.

5.4.1 Sensitivity study

All samples were diluted using the dilution series in *Chapter 2, Section 2.9.7.* The results are discussed in the sections below.

5.4.1.1 Blood

The results in the Figures 5.1i-5.1iv display a gradual decrease in peak heights with increase in dilution ratio of body fluid samples. Both blood markers (ALAS2 and PF4) were fully expressed at 1:1500 dilution. The relative sensitivities of the assay on blood sample is shown in Figures 5:1a-h. There was a significant decrease in peak heights of both markers with increase in dilution ratio. PF4 dropped out at 1:3000th dilution.

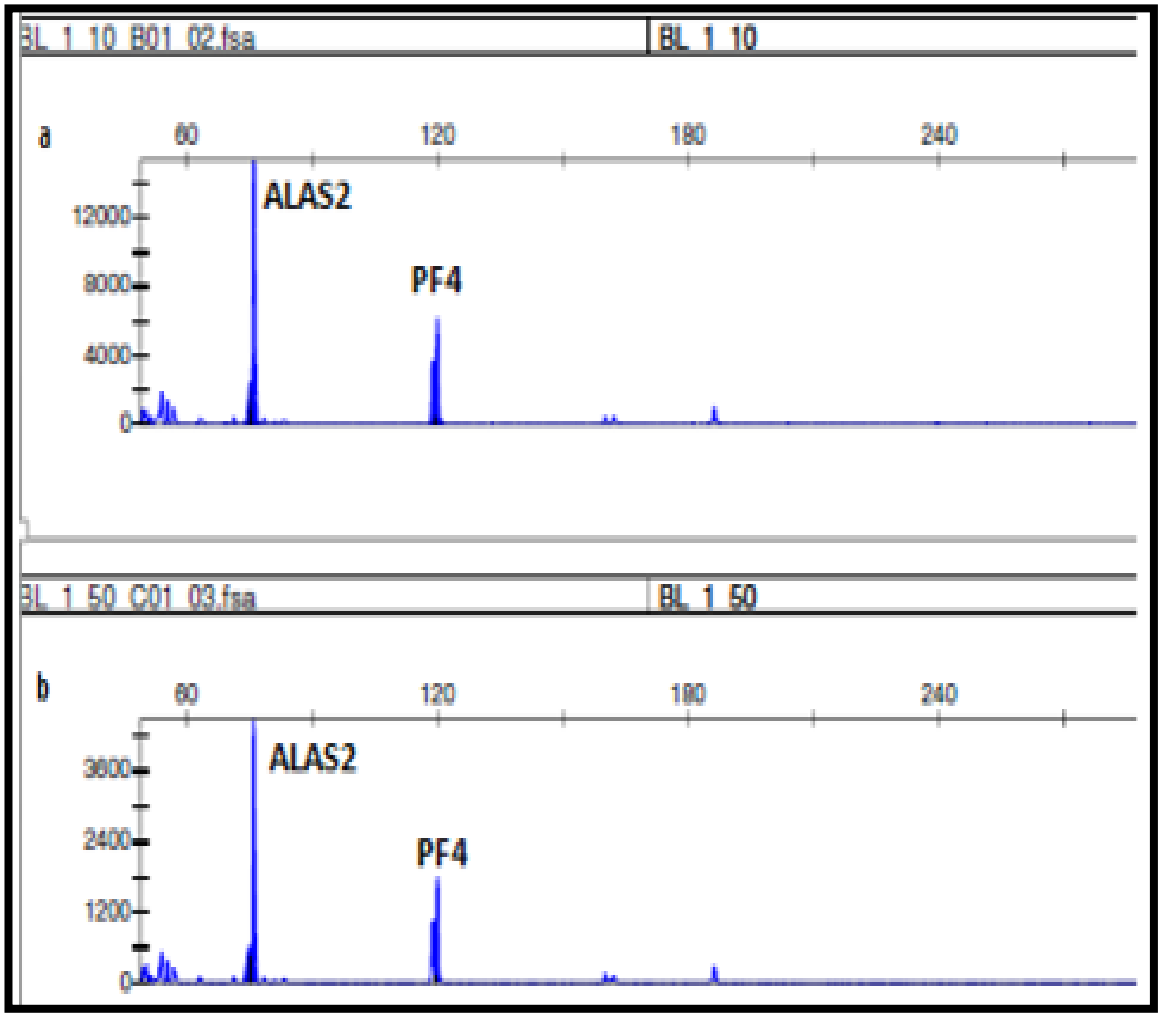

Figure 5.1i: EPG showing (a) 1:10, (b) 1:50 of 5 ng/µl input mRNA of blood sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

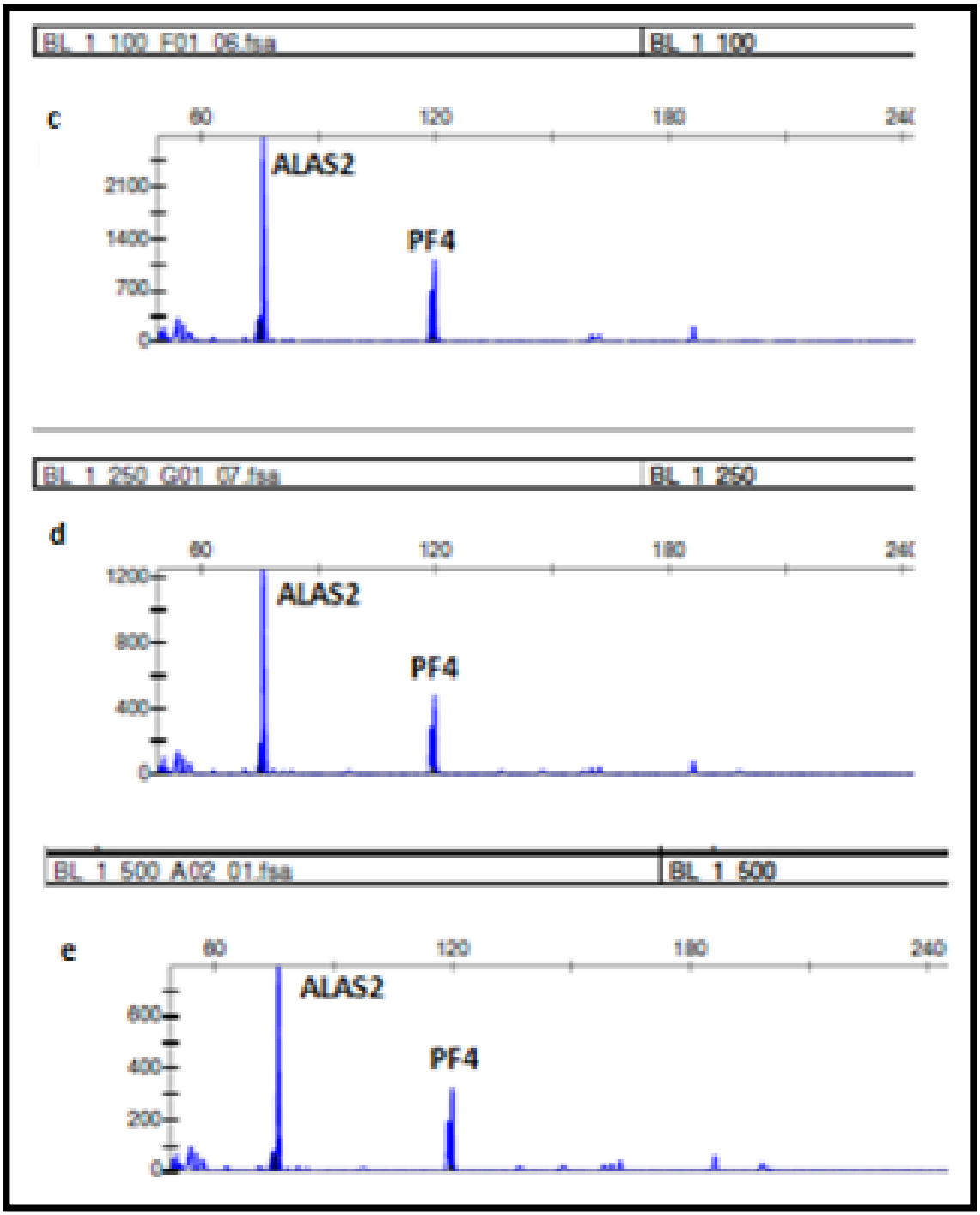

Figure 5.1ii: EPG showing (c) 1:100, (d) 1:250 and (e) 1:500 dilutions of 5 ng/µl mRNA of blood sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment

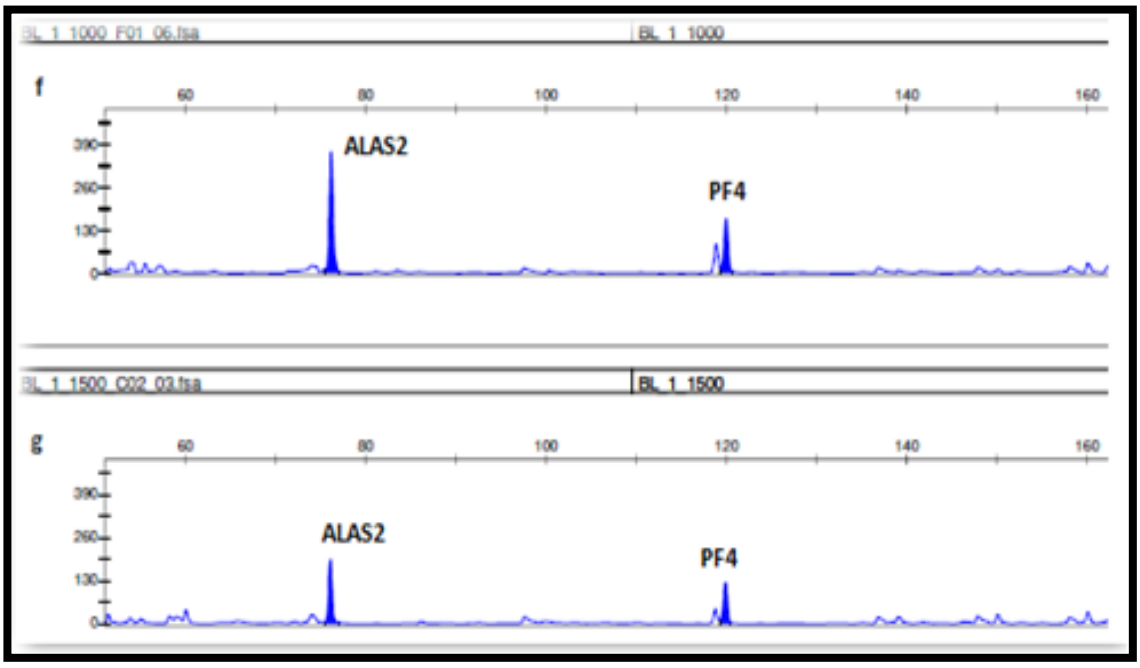

Figure 5.1iii: EPG showing (f) 1:1000, (g) 1:1500 dilutions of 5 ng/µl mRNA of blood sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

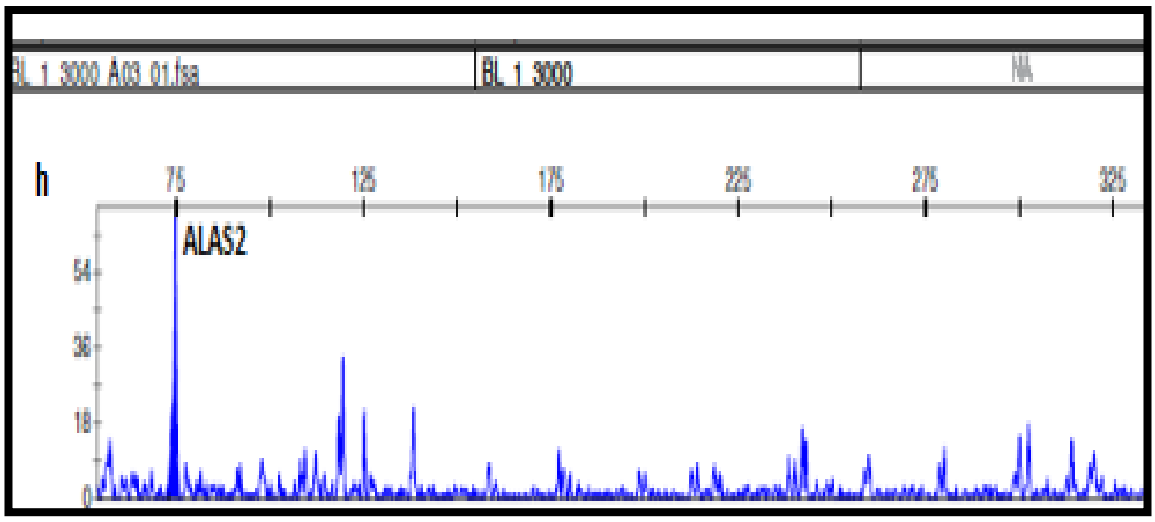

Figure 5.1iv: EPG showing (h) 1:3000 dilution of 5 ng/µl mRNA of blood sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.1.2 Semen

Four markers were incorporated into the 14-plex assay for semen sample identification; these included PRM1, MSMB, NKX3-1, and TGM4. All the four markers were well expressed throughout the dilution series. MSMB displayed the highest expression amongst the four, with the remaining three having a near balanced expression (Figures 5.2i-iii). All the four markers were still fully expressed at 1:3000 dilution.

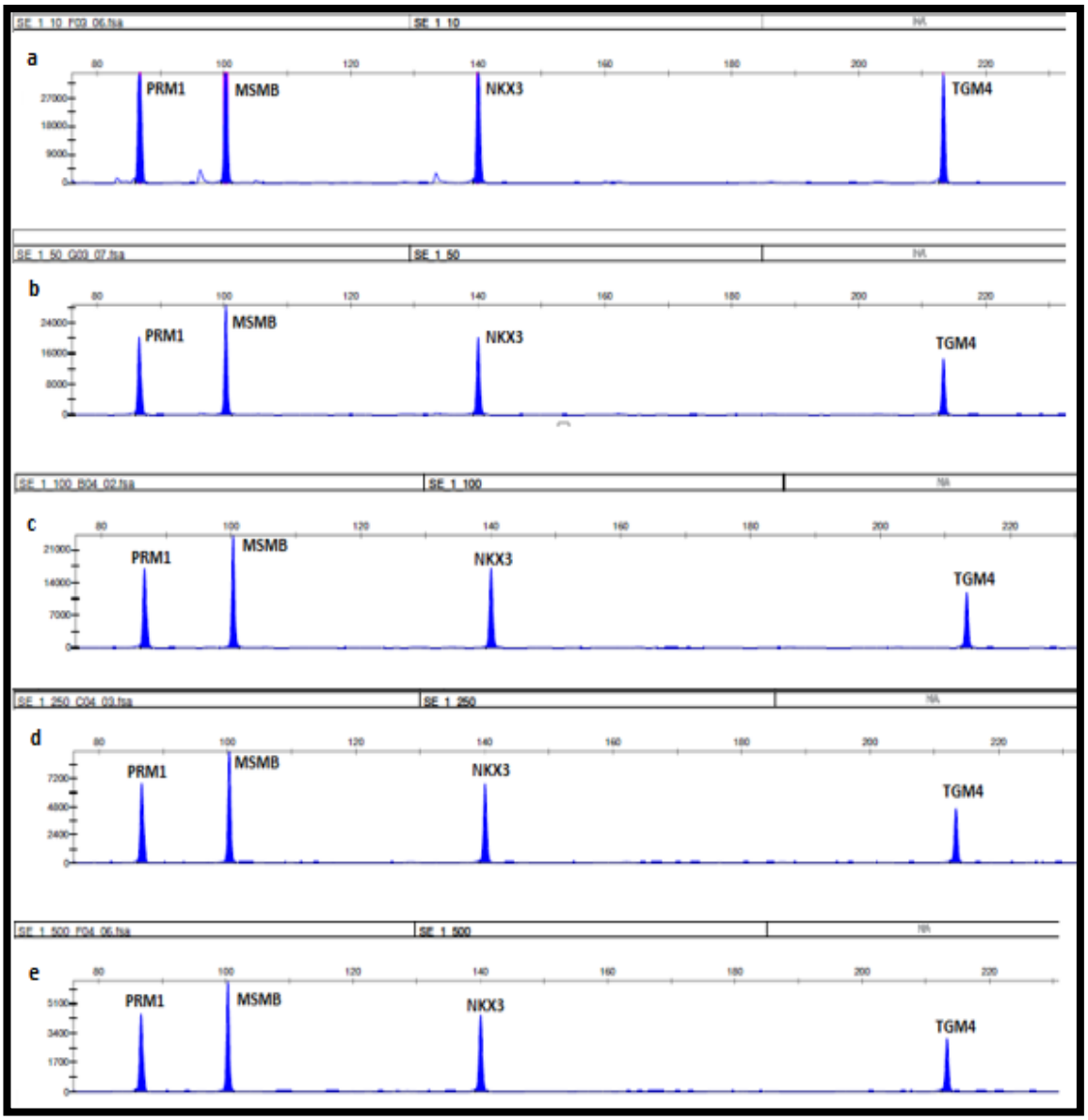

Figure 5.2i: EPG showing (a) 1:10, (b) 1:50, (c) 1:100, (d) 1:250, (e) 1:500 dilutions of 5 ng/ µl mRNA of semen sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

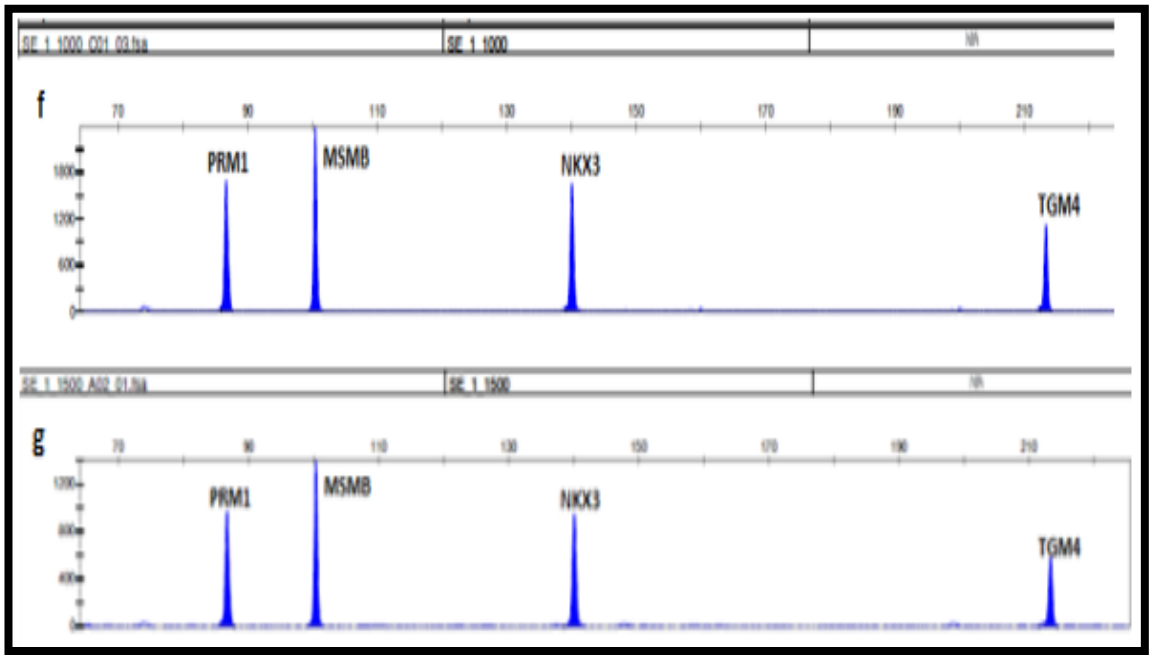

Figure 5.2ii: EPG showing (f) 1:1000, (g) 1:1500 dilutions of 5 ng/ µl mRNA of semen sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

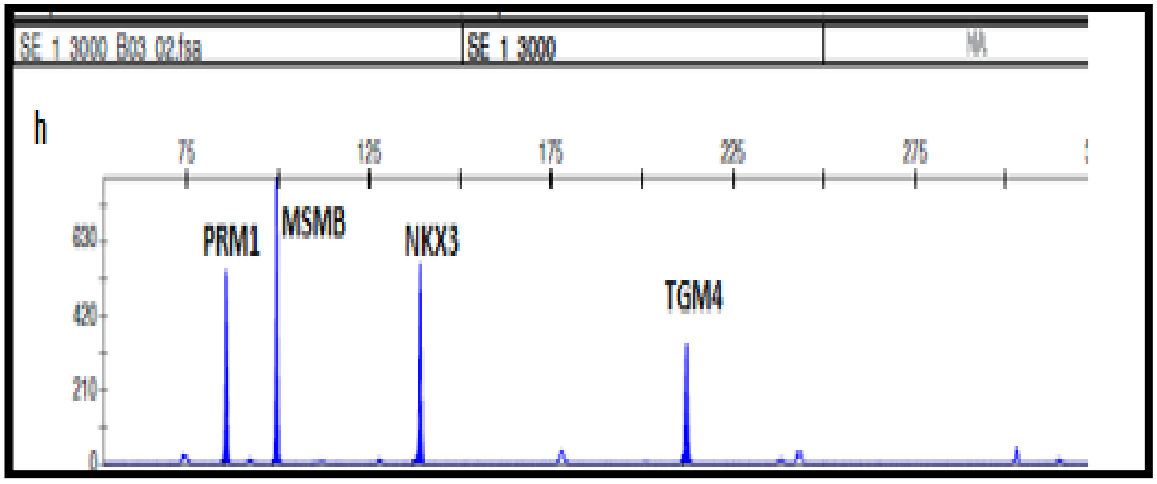

Figure 5.2iii: EPG showing (h) 1:3000 dilution of 5 ng/ µl mRNA of semen sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.1.3 Saliva

Saliva markers (STATH and HTN3) were both expressed in the assay all through the dilution series and represented in the red panel (Figures 5.3i-iii). Also in this panel are the reference genes ACTB and UCE. Despite several attempts at increasing the concentration of ACTB, it was not expressed at all in the assay. However, UCE was expressed in every amplification. An increase in dilution of saliva sample resulted in a significant decrease in expression of UCE, the expression of which was lost at 1:1000, 1:1500 and 1:3000 dilutions.

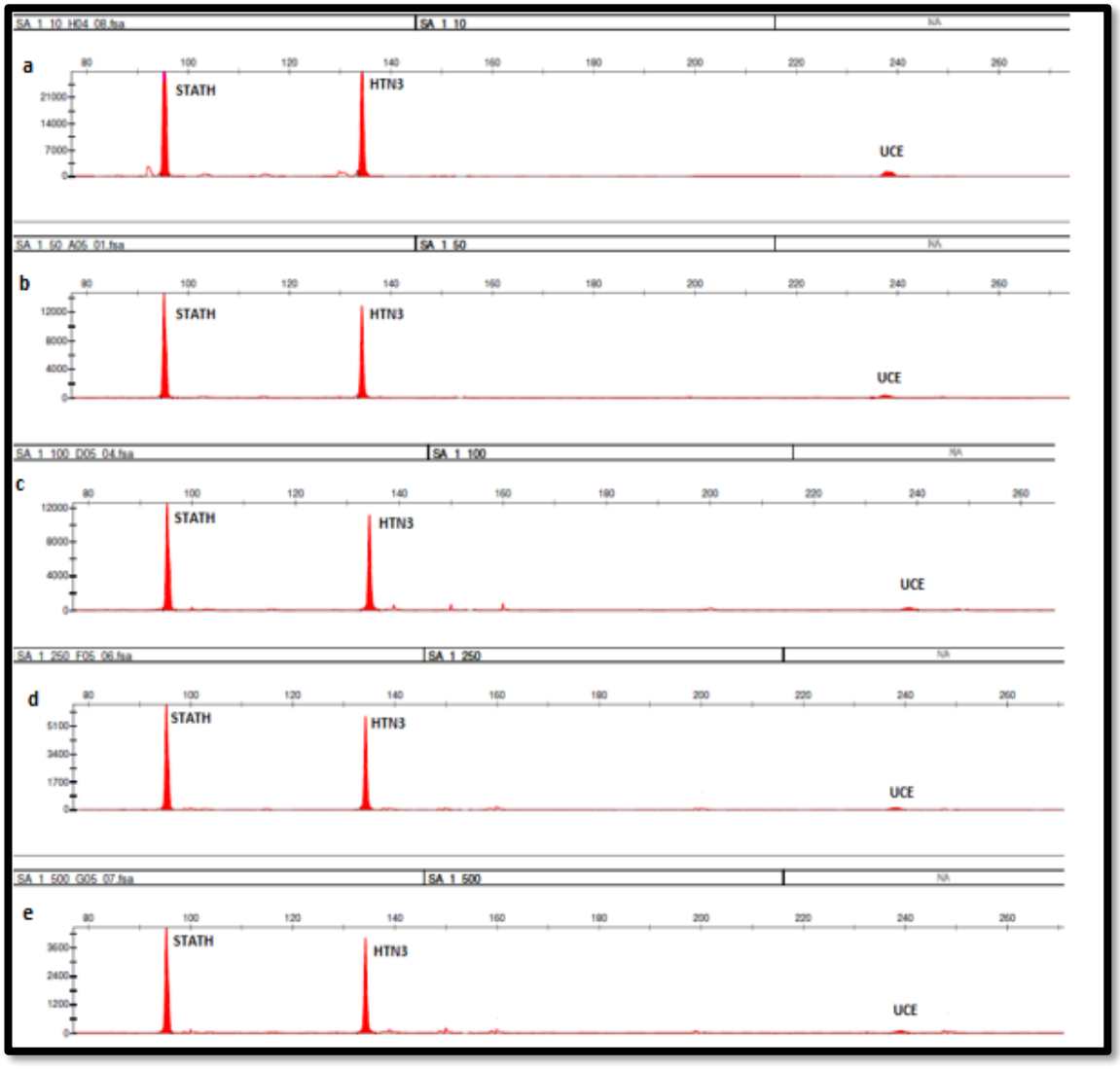

Figure 5.3i: EPG showing (a) 1:10, (b) 1:50, (c) 1:100, (d) 1:250, (e) 1:500 dilutions of 5 ng/ µl mRNA of saliva sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

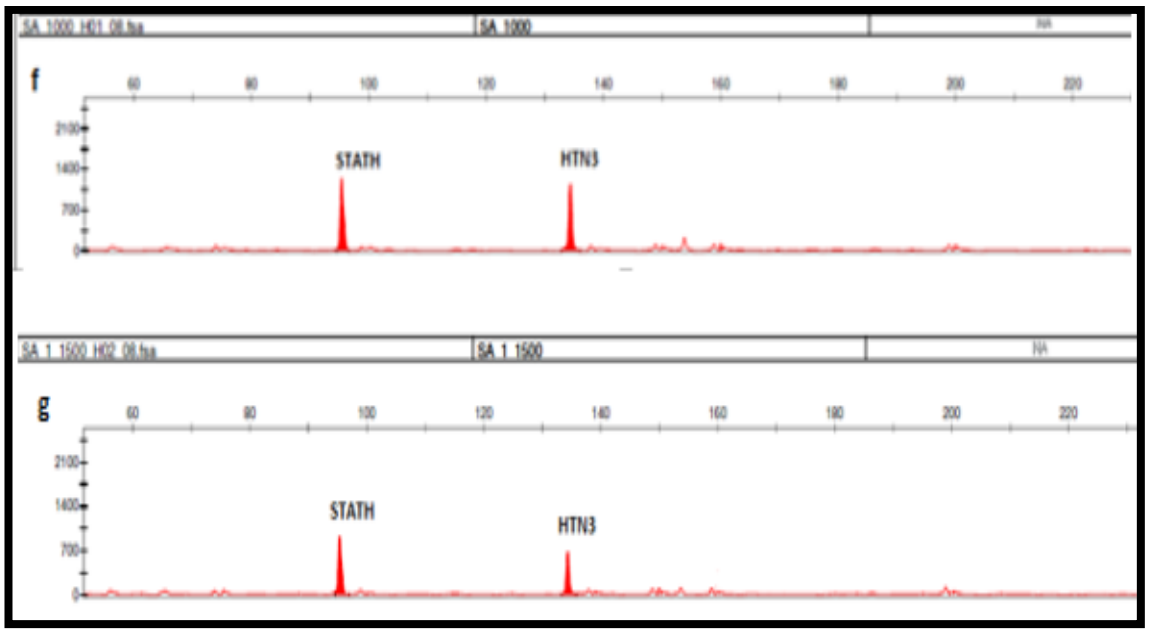

Figure 5.3ii: EPG showing (f) 1:1000, (g) 1:1500 dilutions of 5 ng/ µl mRNA of saliva sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

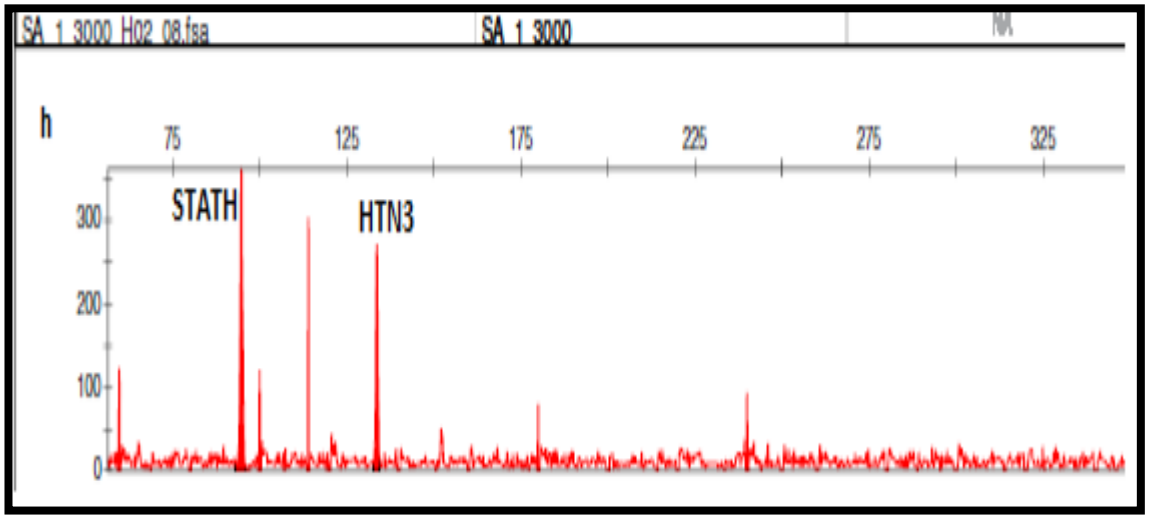

Figure 5.3iii: EPG showing (h) 1:3000 dilution of 5 ng/ µl mRNA of saliva sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.1.4 Vaginal secretion

Vaginal secretion markers (MUC4, CRYP2B7PI, SFTA2 and *L. crispatus*) were optimised for specificity on vaginal secretion samples. While the four markers displayed specific expression for vaginal secretion in the five-plex assay (Chapter 4), SFTA2 and *L. cris* were the only markers expressed in the validation study. They both displayed a high expression till 1:1500th dilution series (Figures 5.4i/ii). However, both markers dropped out at 1:3000th dilution (Figures 5.4iii).

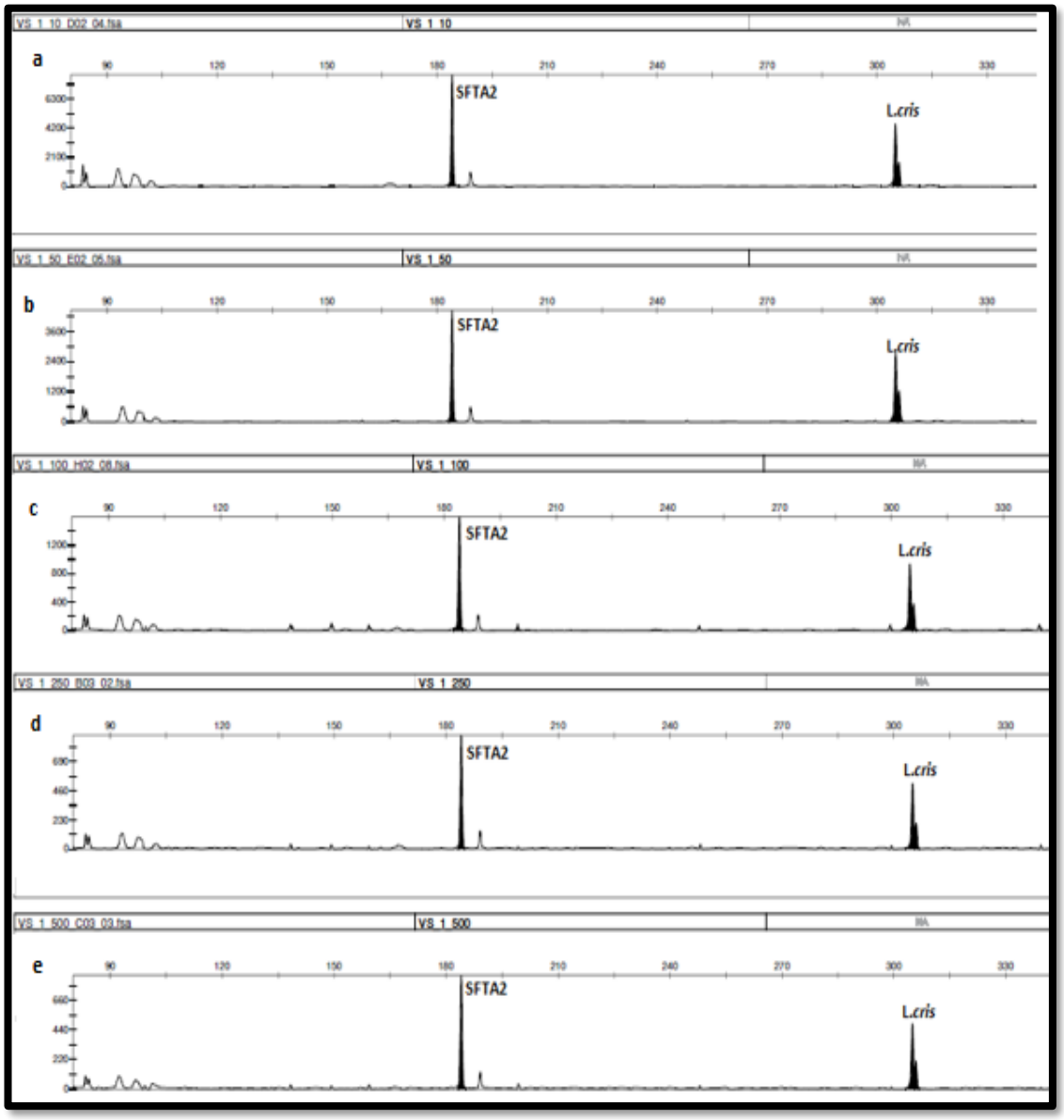

Figure 5.4i: EPG showing (a) 1:10, (b) 1:50, (c) 1:100, (d) 1:250, (e) 1:500 dilutions of 5 ng/ µl mRNA of vaginal secretion sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

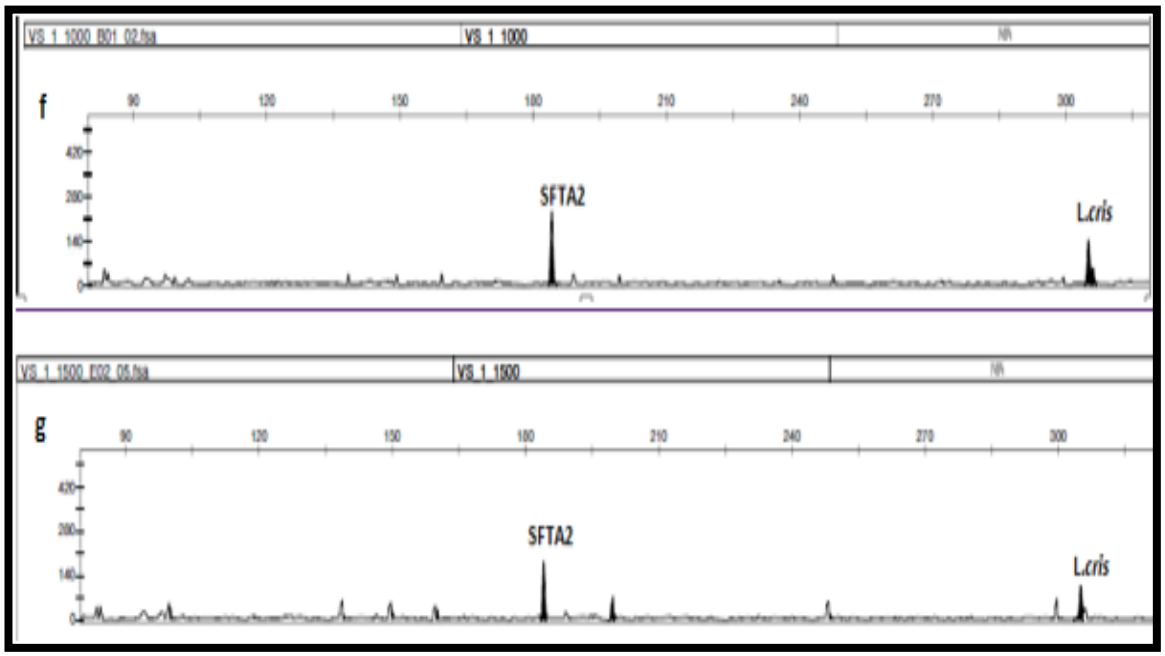

Figure 5.4ii: EPG showing (f) 1:1000 and (g) 1: 1500 dilutions of 5 ng/ µl mRNA of vaginal secretion sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles, n=5; where n is the number of repeats of experiment.

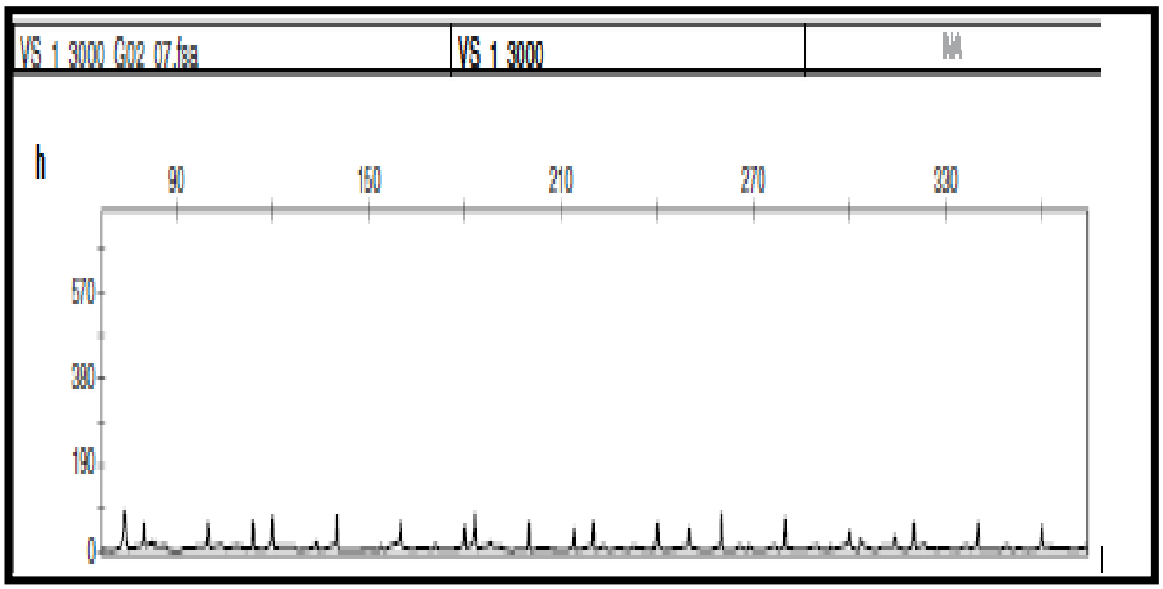

Figure 5.4iii: EPG showing (h) 1: 3000 dilutions of 5 ng/ µl mRNA of vaginal secretion sample. A volume of 1 µl of the dilution was used for sample amplification at 58 ^{0}C, 33 cycles.

Table 5.1: Amplification success levels of mRNA multiplex assay using samples at various dilution ratios of blood, saliva, semen, vaginal secretion and the reference genes.

Dilution factor	Blood	Saliva	Semen	Vaginal secretion	Reference genes
1:10	*+++*	*+++*	*+++*	*+++*	*++*
1:50	*+++*	*+++*	*+++*	*+++*	*++*
1:100	*+++*	*+++*	*+++*	*+++*	*++*
1:250	*+++*	*+++*	*+++*	*+++*	*++*
1:500	*+++*	*+++*	*+++*	*+++*	*++*
1:1000	*+++*	*+++*	*+++*	*+++*	*++*
1:1500	*++*	*+++*	*+++*	*+++*	*++*
1:3000	*+*	*++*	*++*	-	*+*

+++ = strong expression; ***++*** = weak expression; ***+*** = very weak expression; **-** = negative/no expression.

The scale was developed based on the use of number of markers expressed in the study. For semen with four markers, the expression was classified as strong when all four or three are amplified, weak with two markers, very weak with one marker and no expression with no marker amplified (See Table 5.1)

5.4.2 Reproducibility study

The independent laboratory where the primermix was sent for reproducibility study generated similar results with experiments carried out at UCLan laboratory as all the markers were expressed for the target body fluids. However, over-amplification of samples was generated and this was reported to be because of unquantified and sample overload (Appendix 5).

5.4.3 Mixture study

Mixture of all four body fluids were first prepared in equimolar concentrations, and then modified in order to obtain a near balanced peak height for the overall profile (see Figures 5.26iii-iv). In practise, occurrence of mixture of all four body fluids at any specific

time is rarely possible; this prompted subsequent mixture experiments to be carried out in duplex reactions.

5.4.3.1 Semen + Vaginal secretion

Mixture of semen and vaginal secretion samples in equimolar and varying proportions is shown in Figures 5.5i-iv. All four semen markers displayed high expression for all mixture ratios. However, three out of four vaginal secretion markers were expressed in all mixture ratios. Equimolar mixture of semen and vaginal secretion showed a decrease in peak heights of vaginal secretion markers (Figures 5.5ii (di-ii)). The same was noticed to decrease further with increase in proportion of semen sample (Figures 5.5iii-iv). Overall, all four semen markers and three out of four vaginal secretion markers were expressed through the dilution series.

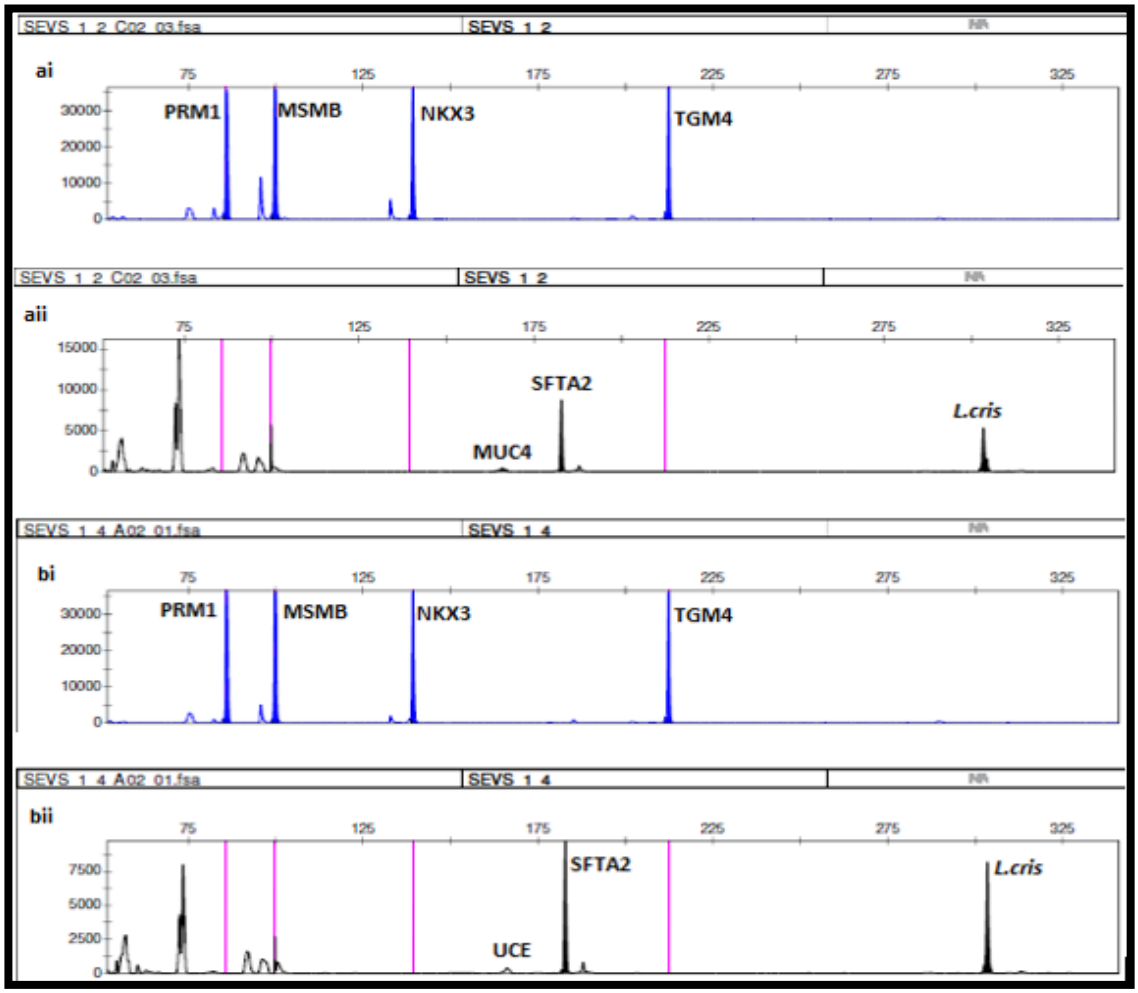

Figure 5.5i: EPG showing mixture of semen and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ai/aii) 1:2, (bi/bii) 1:4. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

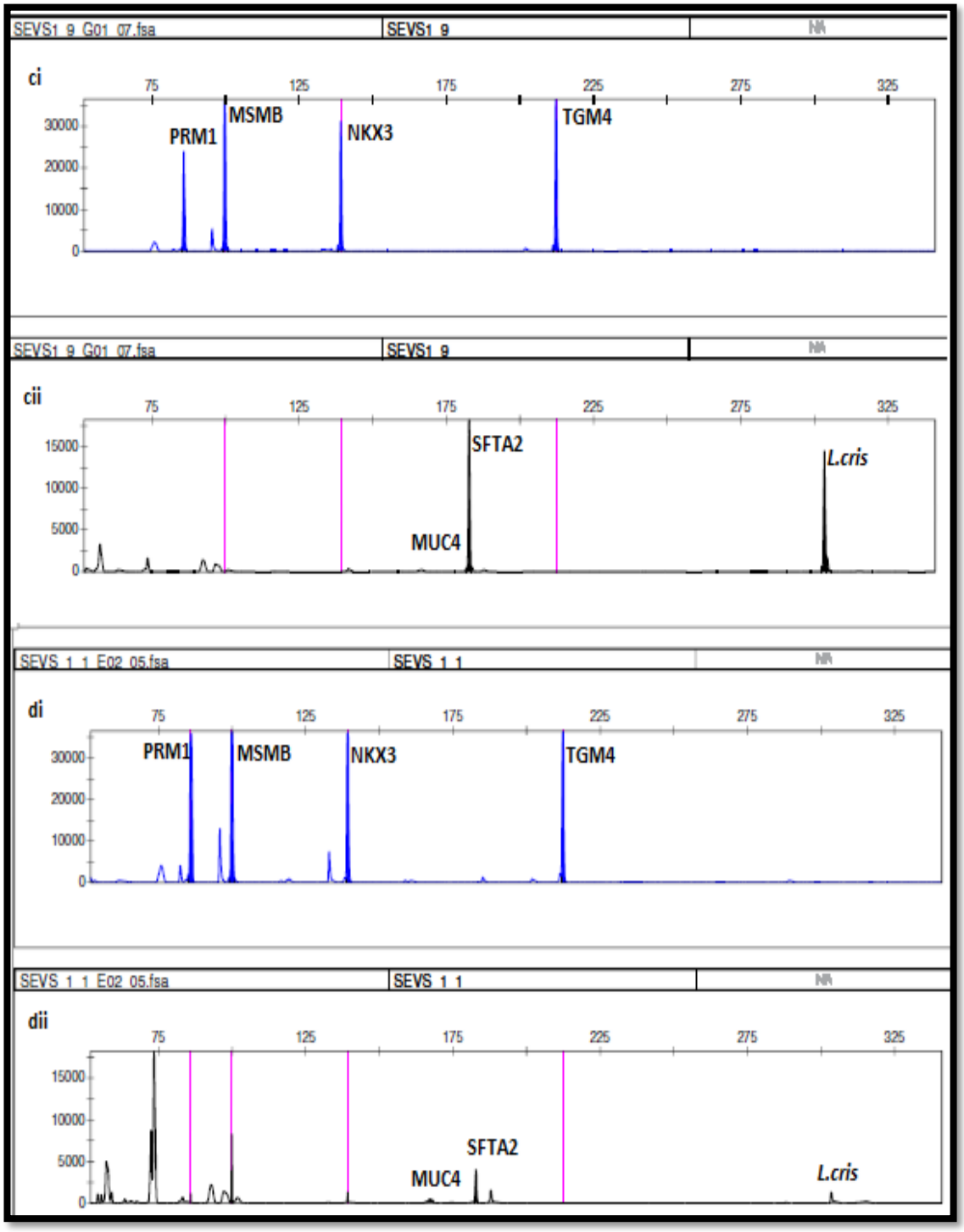

Figure 5.5ii: EPG showing mixture of semen and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ci/cii) 1:9, (di/dii) 1:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

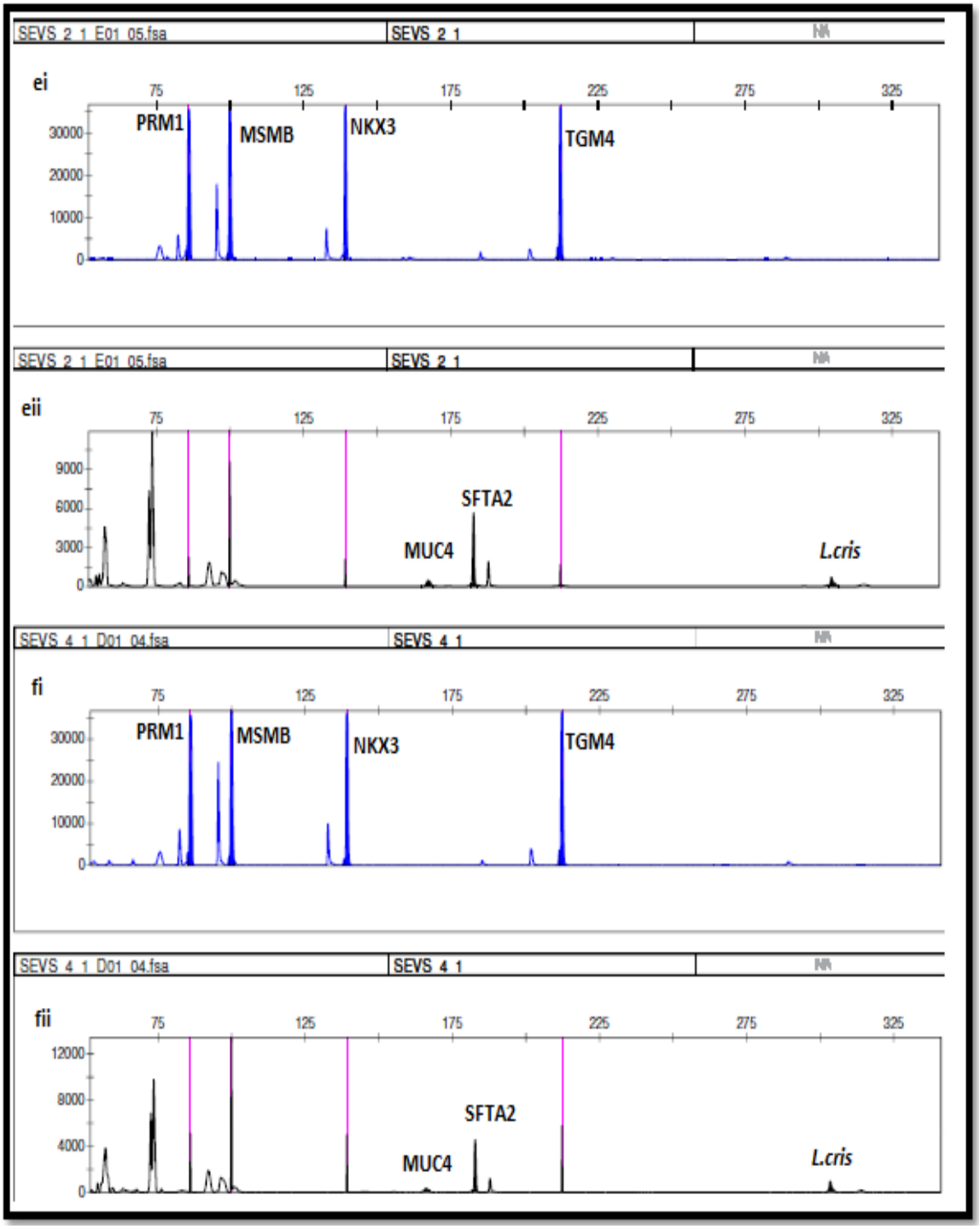

Figure 5.5iii: EPG showing mixture of semen and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ei/eii) 2:1, (fi/fii) 4:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

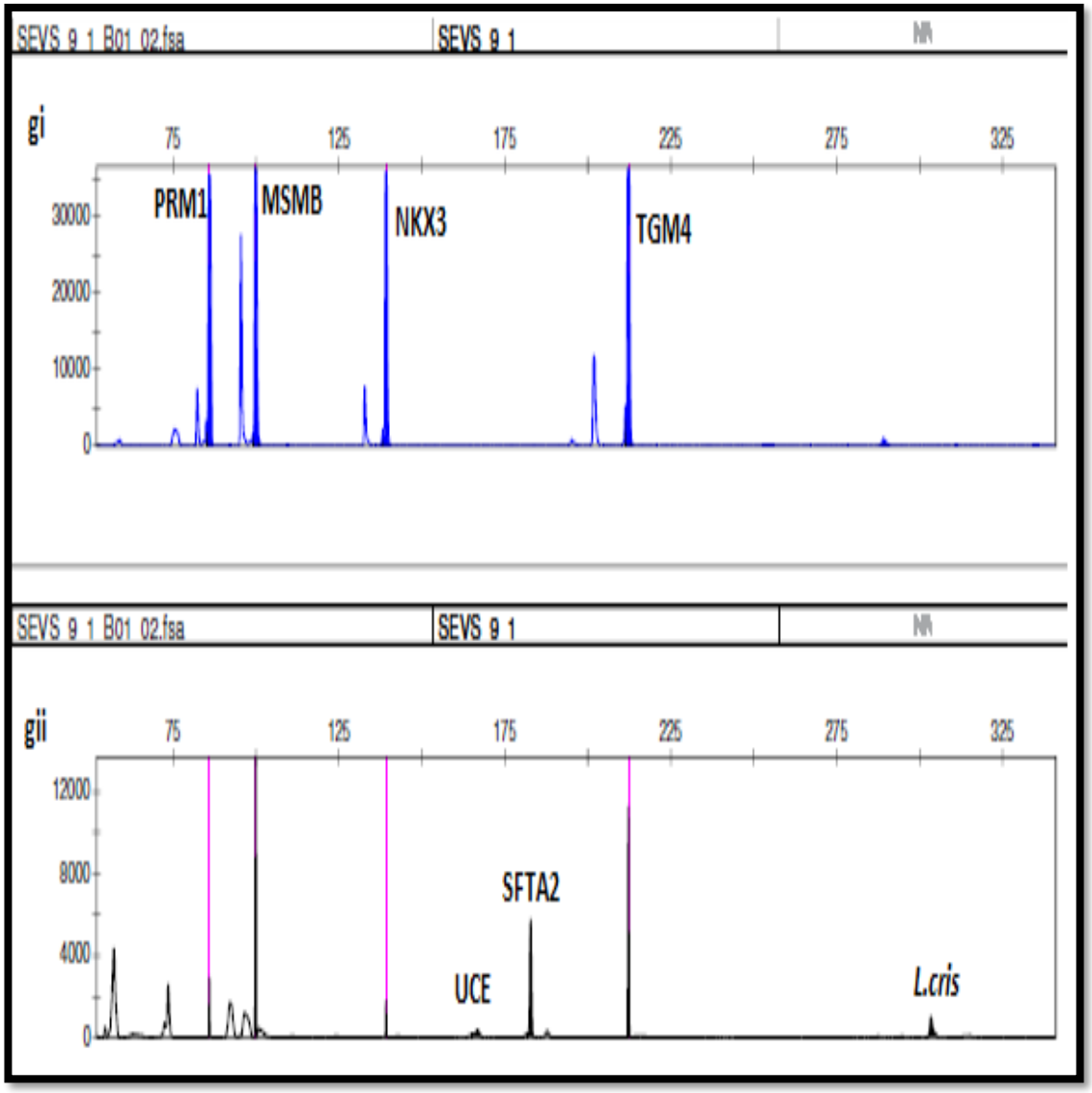

Figure 5.5iv: EPG showing mixture of semen and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (gi/gii) 9:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

Several artefacts were noticed in the EPGs. These were because of overload of a particular cDNA sample at any given mixture ratio.

5.4.3.2 Blood + Vaginal secretion

Two blood and three vaginal secretion markers, respectively were expressed upon mixture in both equimolar and varied proportions. There was a decrease in the expression of vaginal secretion markers upon increase in proportion of blood samples. All markers for both body fluids were expressed in all proportions (Figures 5.6 i-iv).

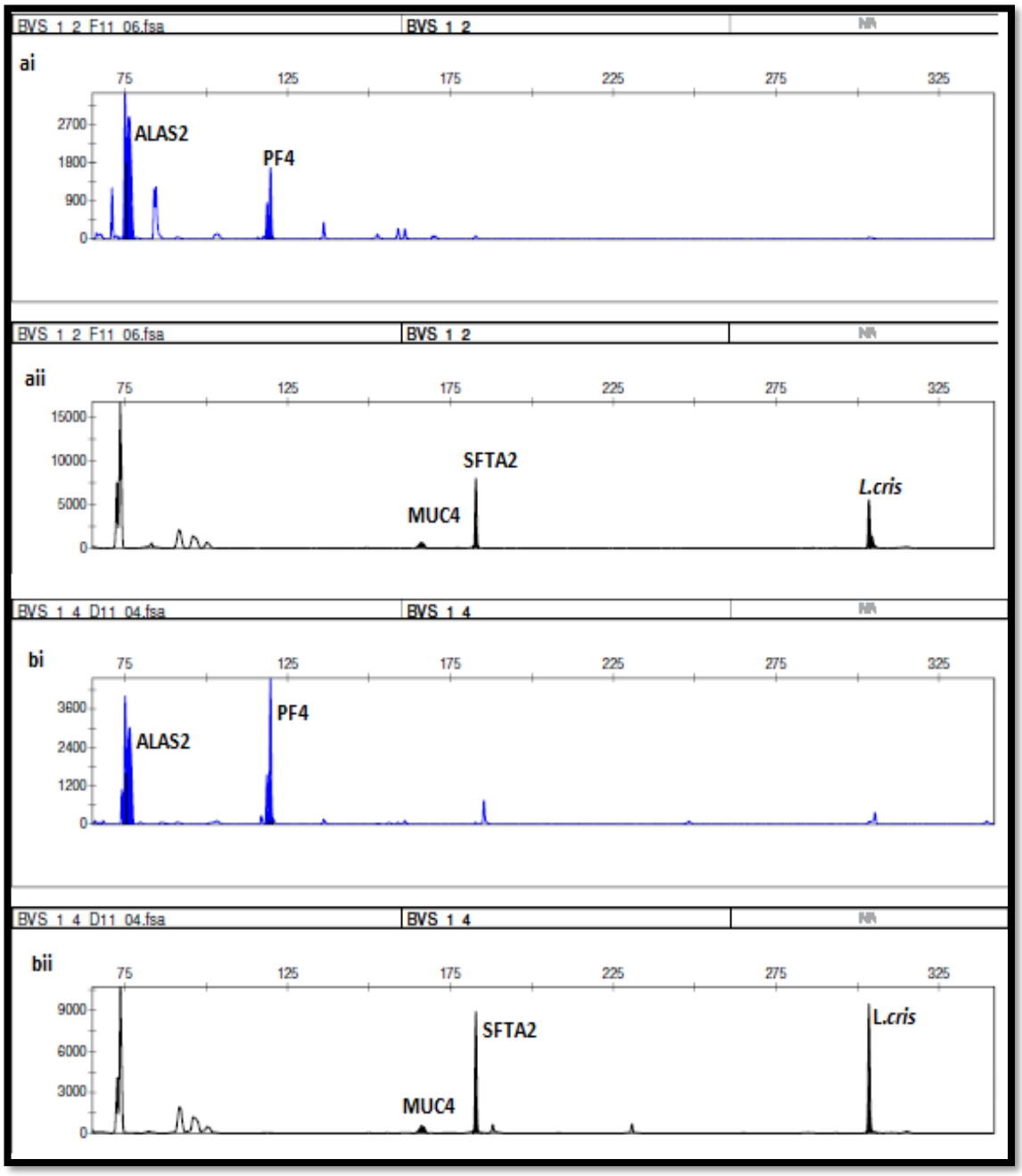

Figure 5.6i: EPG showing mixture of blood and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ai/aii) 1:2, (bi/bii) 1:4. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

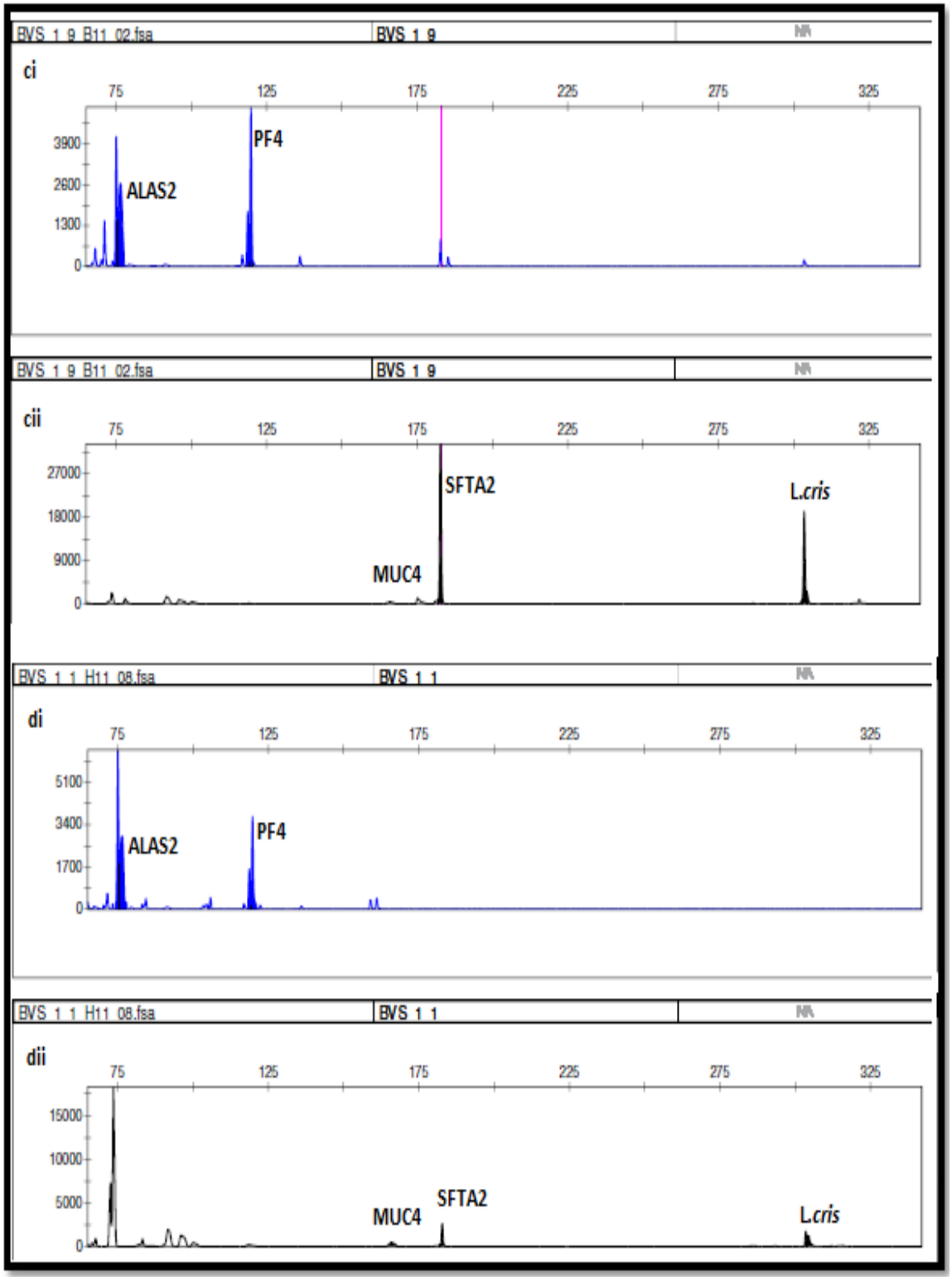

Figure 5.6ii: EPG showing mixture of blood and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ci/cii) 1:9, (di/dii) 1:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

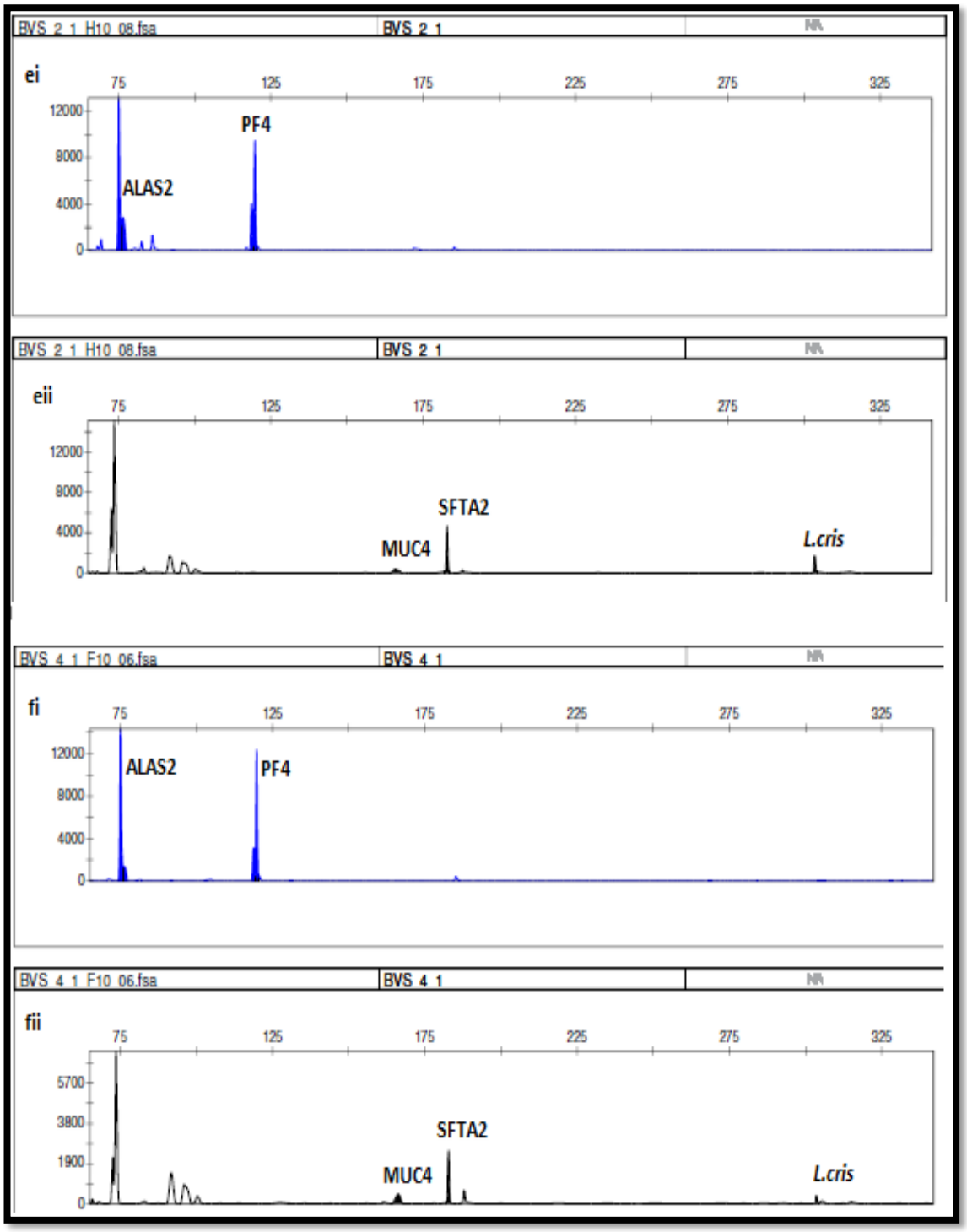

Figure 5.6iii: EPG showing mixture of blood and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ei/eii) 2:1, (fi/fii) 4:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

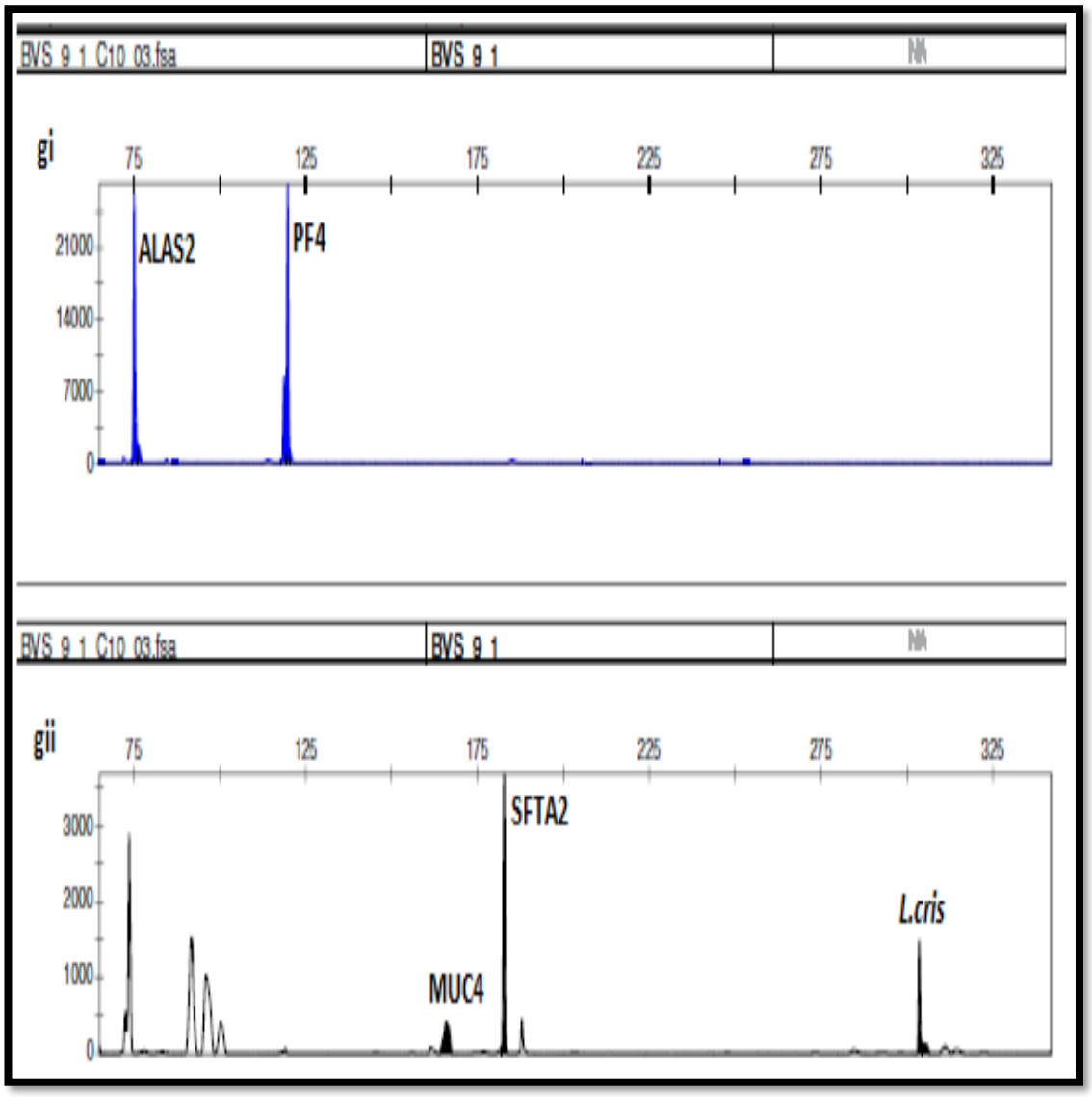

Figure 5.6iv: EPG showing mixture of blood and vaginal secretion in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (gi/gii) 9:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.3.3 Blood + Semen

Semen markers were seen to display a stable and high expression through all the dilution series. A 1:1 dilution showed a low expression of blood markers, which only were increased at 4:1 mixture of blood and semen. Increase in the mixture proportion of blood markers did not have an effect the expression of semen markers (Figures 5.7i-ii).

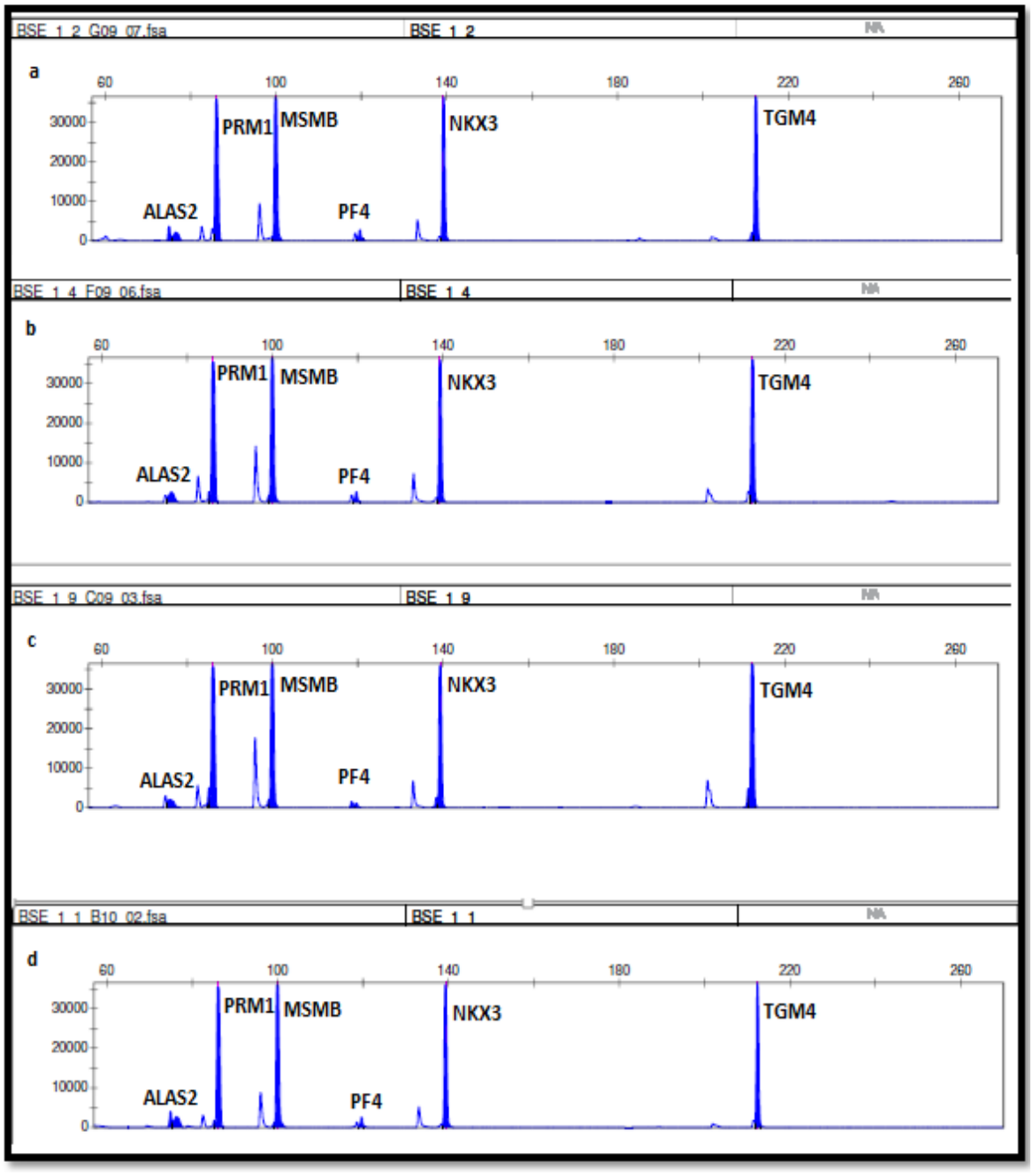

Figure 5.7i: EPG showing mixture of blood and semen in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (a) 1:2, (b) 1:4, (c) 1:9, (d) 1:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

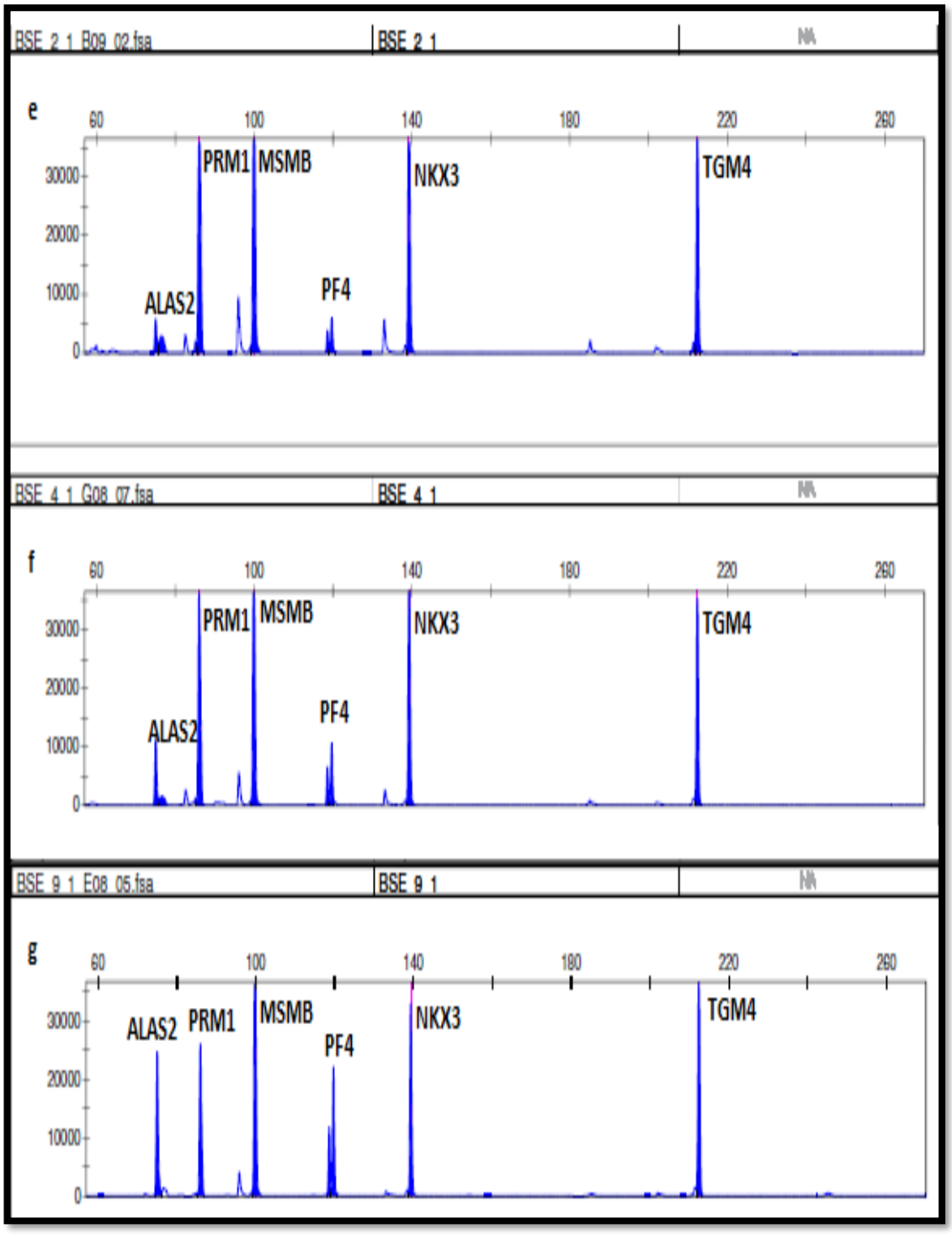

Figure 5.7ii*:* EPG showing mixture of blood and semen in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (e) 2:1, (f) 4:1, (g) 9:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.3.4 Blood + Saliva

An increase in mixture proportion of saliva had no major effect on expression of blood markers ALAS2 and PF4. Both markers were stably expressed in all dilution series, with increase in RFU generated at 4:1 and 9:1 blood and saliva (Figures 5.8iii (fi-gi)). Saliva markers as well as reference gene marker, UCE also displayed a stable expression throughout the dilution series (Figures 5.8i-iv).

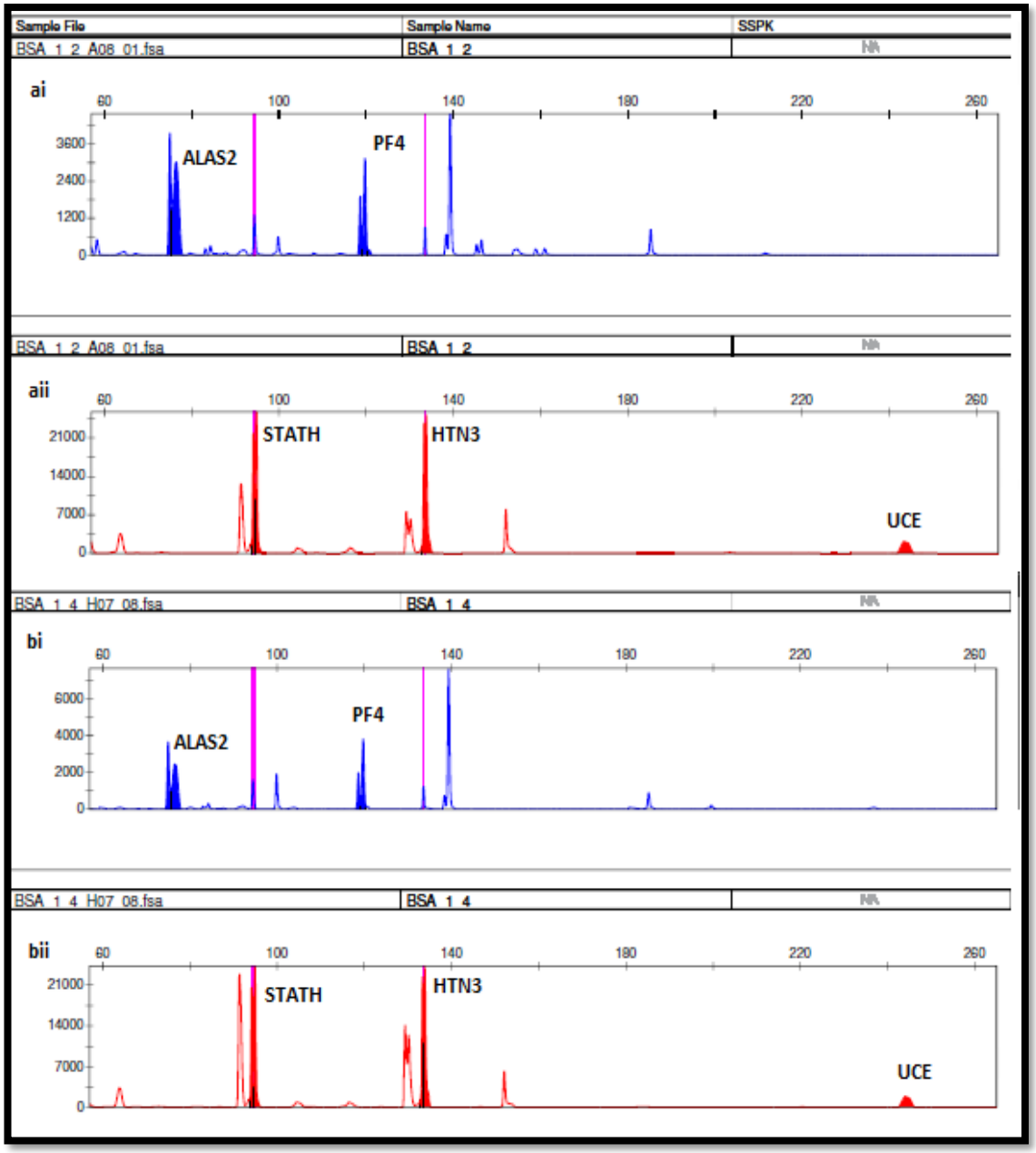

Figure 5.8i: EPG showing mixture of blood and saliva in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ai/aii) 1:2, (bi/bii) 1:4. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

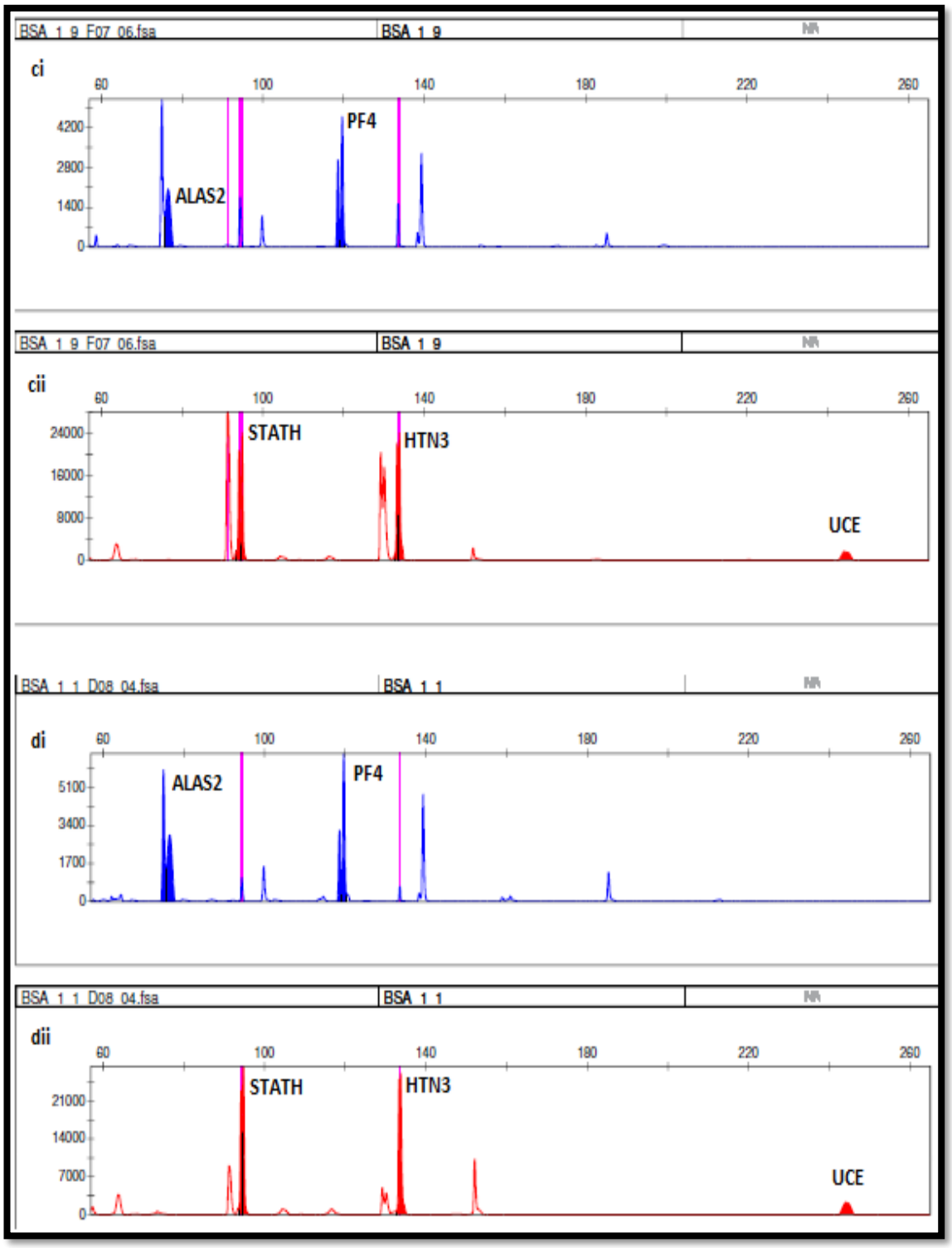

Figure 5.8ii: EPG showing mixture of blood and saliva in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ci/cii) 1:9, (di/dii) 1:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

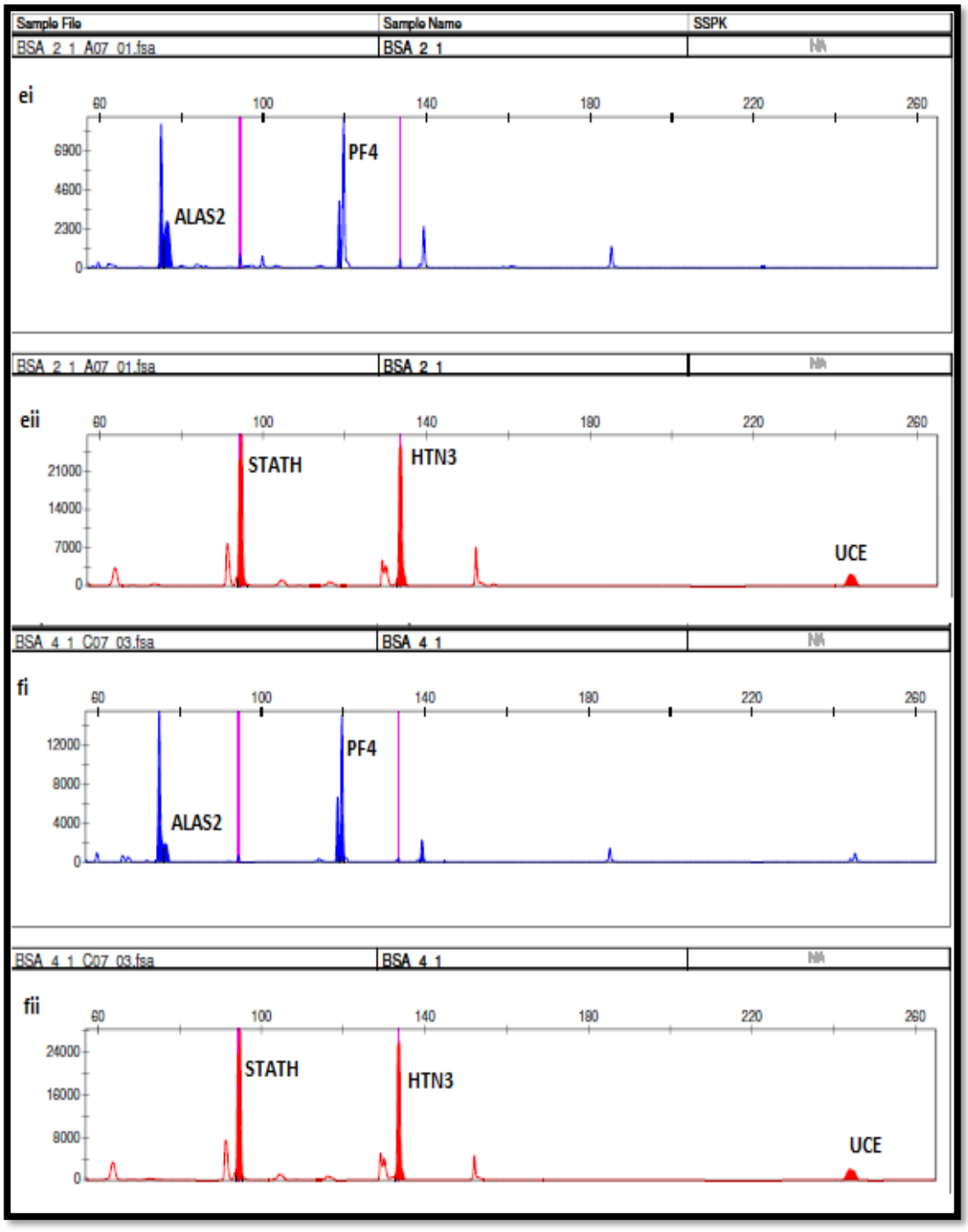

Figure 5.8iii: EPG showing mixture of blood and saliva in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ei/eii) 2:1, (fi/fii) 4:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

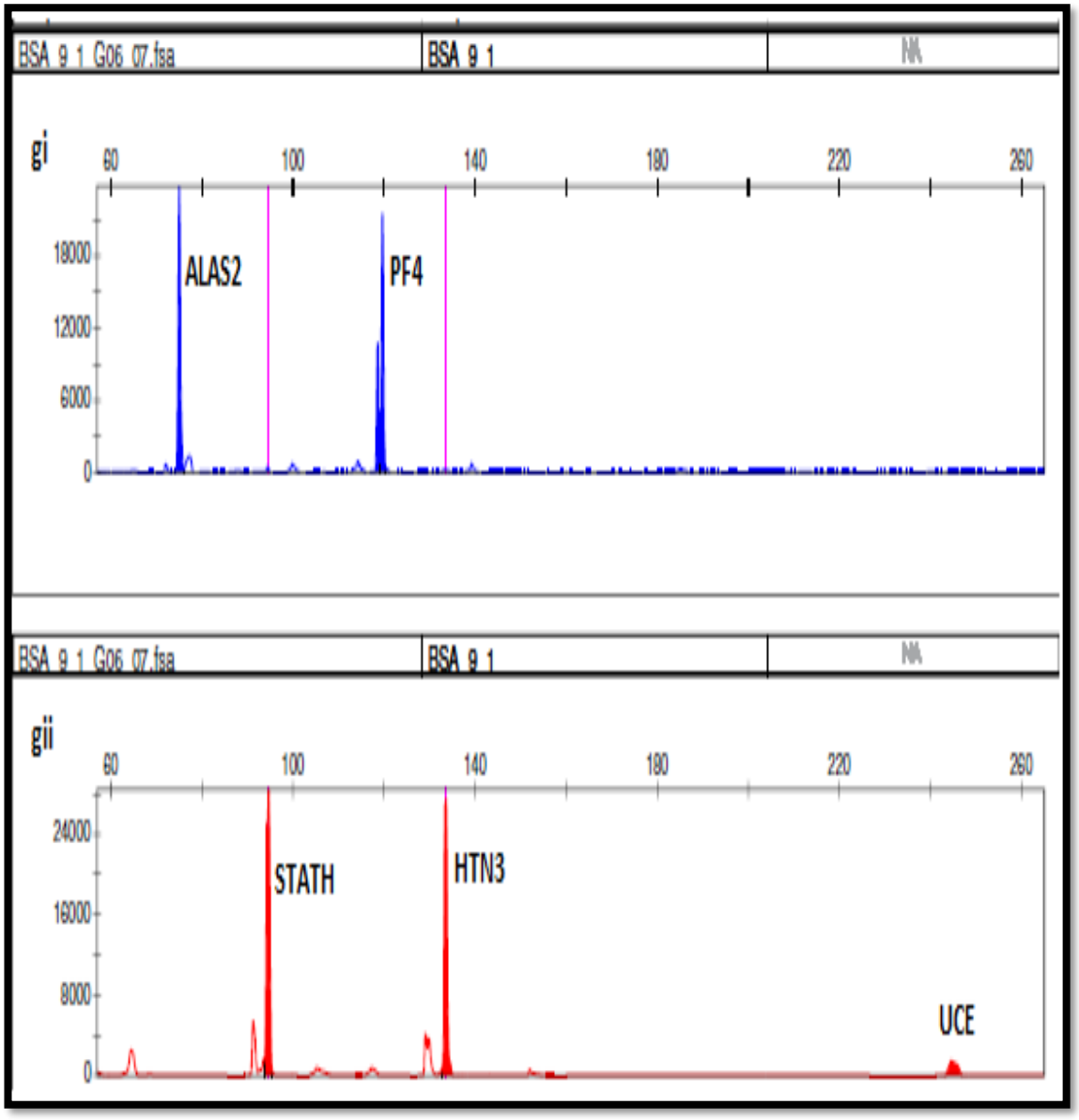

Figure 5.8iv: EPG showing mixture of blood and saliva in proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (gi/gii) 9:1. These were amplified at 58 ^{0}C and 33 cycles, n=5; where n is the number of repeats of experiment.

5.4.4 Controlled degradation

Controlled degradation experiment was carried out for all body fluid samples at room temperature, 37 ^{o}C, 56 ^{o}C and under UV crosslinker. The results are shown in sections 5.4.4.1 -5.4.4.4.9.

5.4.4.1 Blood: Room temperature

The blood samples that were prepared on microscopic slides showed no apparent effect of degradation in the first four weeks of the experiment (Figures 5.9a, b). However, at the end of the sixth week, there was a noticeable change as the amount of mRNA recovered was almost halved compared to what was recovered at week 0 (Figure 5.9c). See appendix 4d-h for EPGs showing results of blood sample prepared on glass slide, can of coke, plastic bag, facemasks, and scalpels.

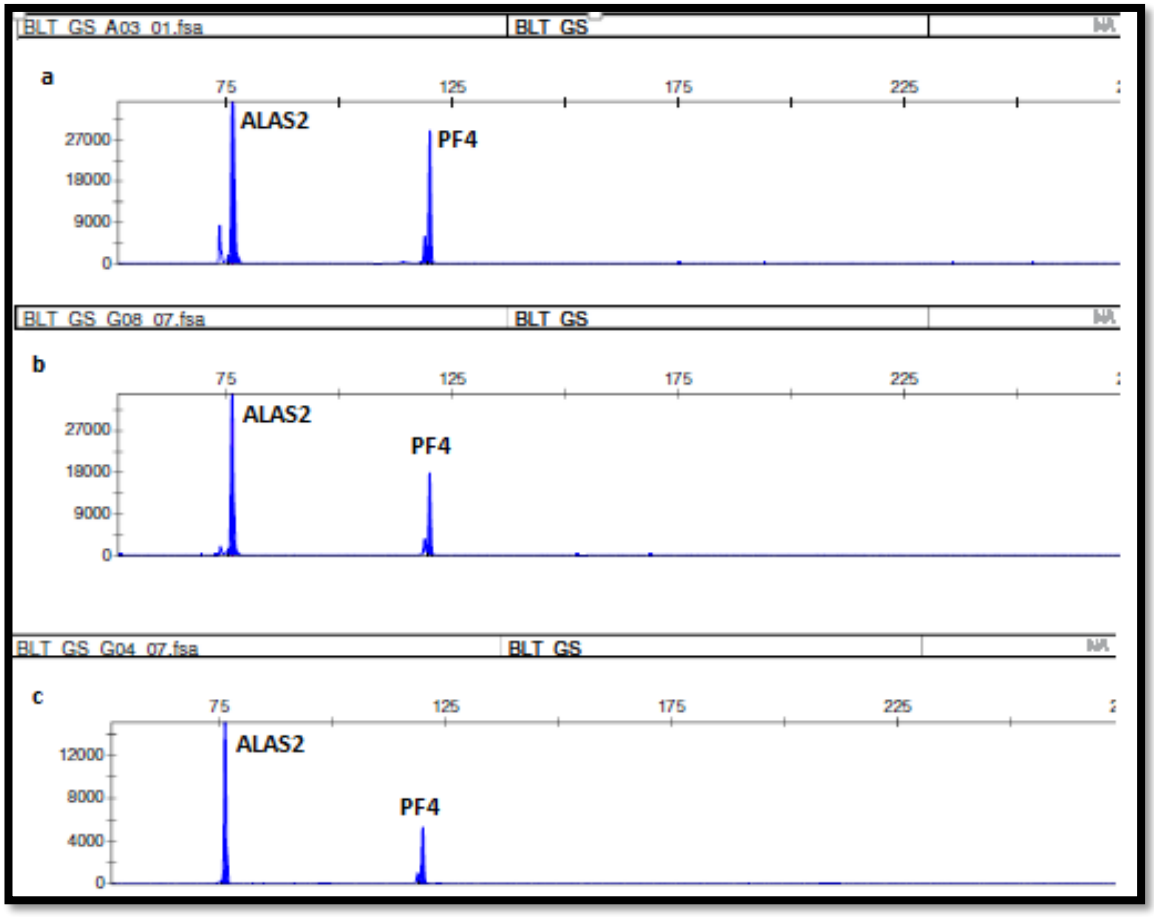

Figure 5.9: EPG showing degradation of blood sample in a dark, cool cupboard at room temperature over a period of six weeks. (a) week 0, (b) week 2 (c) week 6. A volume of 1 µl amplified sample was used for CE, 56 ^{o}C, n=5; where n is the number of repeats of experiment.

5.4.4.2 Semen

Low RFUs were generated for expression of three semen markers –MSMB, NKX3-1 and TGM4, which was consistent over the six weeks' period. Also, there was a noticeable difference in amount of mRNA sample recovered at the end of six weeks compared to the results at weeks zero and four (Figure 5.10).

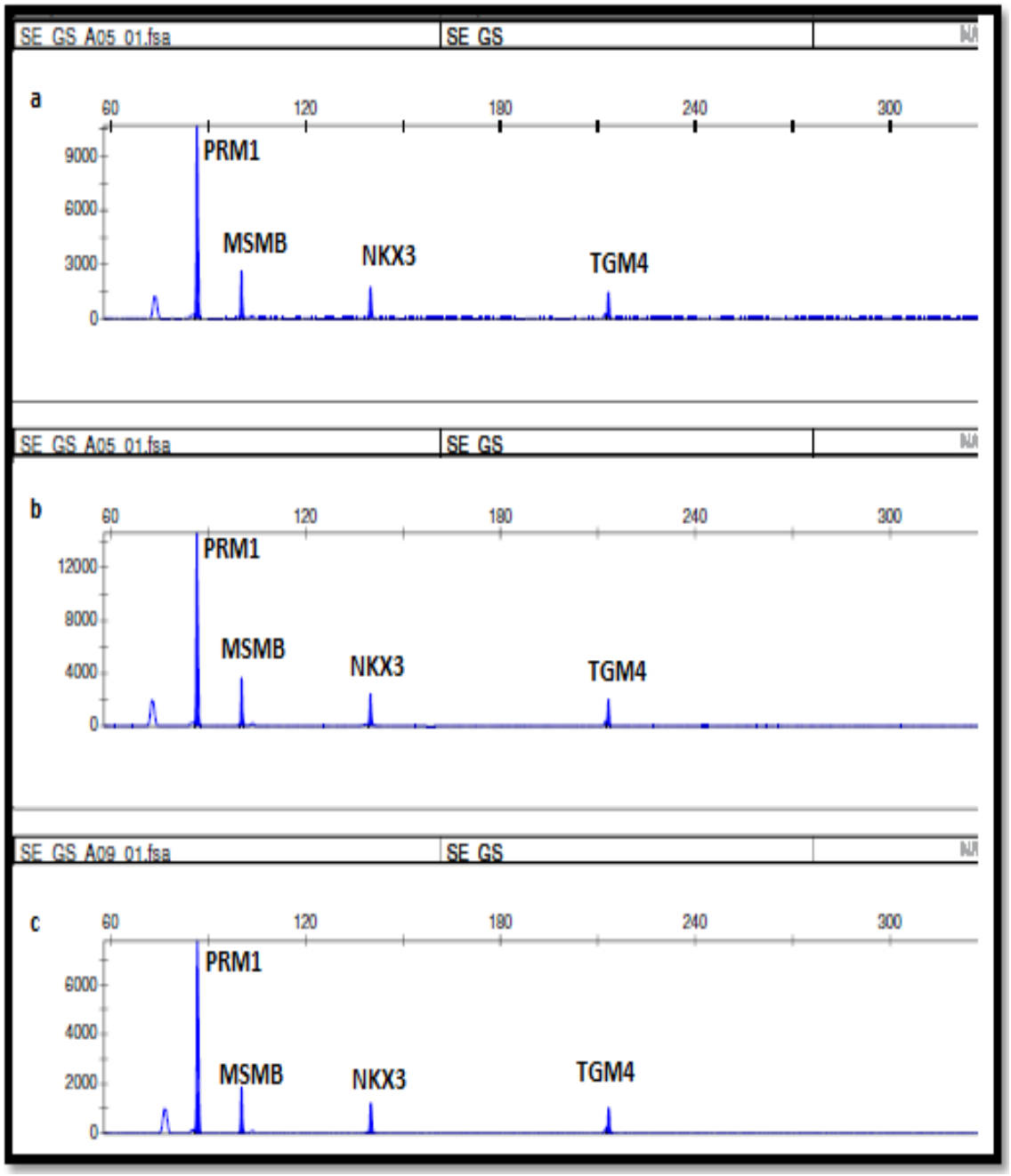

Figure 5.10: EPG showing degradation of semen sample in a dark, cool cupboard at room temperature over a period of six weeks. (a) week 0, (b) week 2 (c) week 6. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.3 Saliva

Unlike semen and blood samples, saliva displayed a notable degradation rate over the six weeks' period. The amount of mRNA recovered and amplified was almost halved at the end of week 6, compared to week 1. This was established by the RFU generated in Figure 5.11.

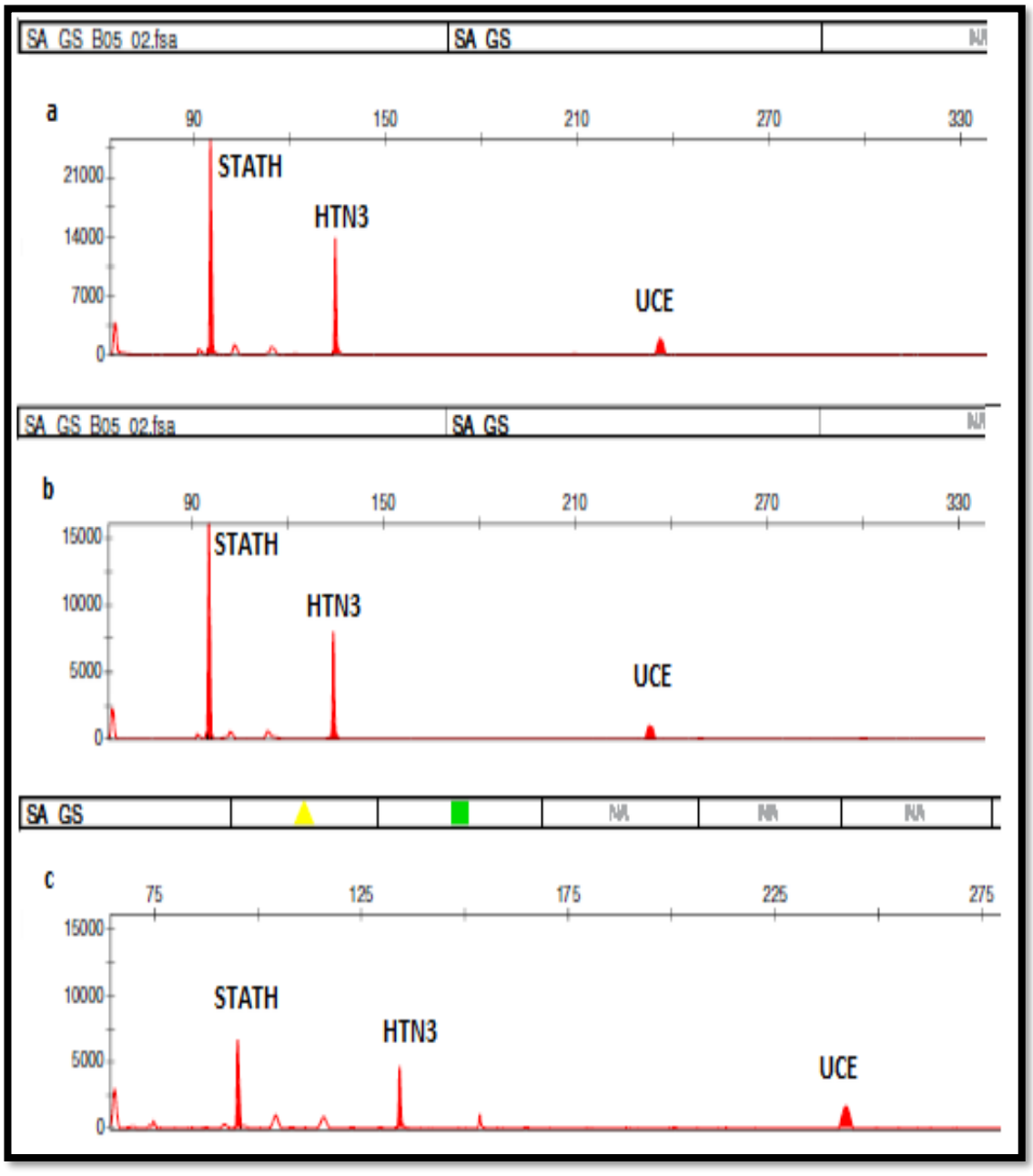

Figure 5.11: EPG showing degradation of saliva sample in a cool, dark cupboard at room temperature over a period of six weeks. (a) week 0, (b) week 2 (c) week 6. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.2 Blood: Incubator

From the result obtained in Figure 5.12, the high temperature appeared to have a significant effect on the blood mRNA sample as the expression of both blood markers decreased gradually with the most reduced effect noticeable at the end of 90 mins (Figure 5.12e).

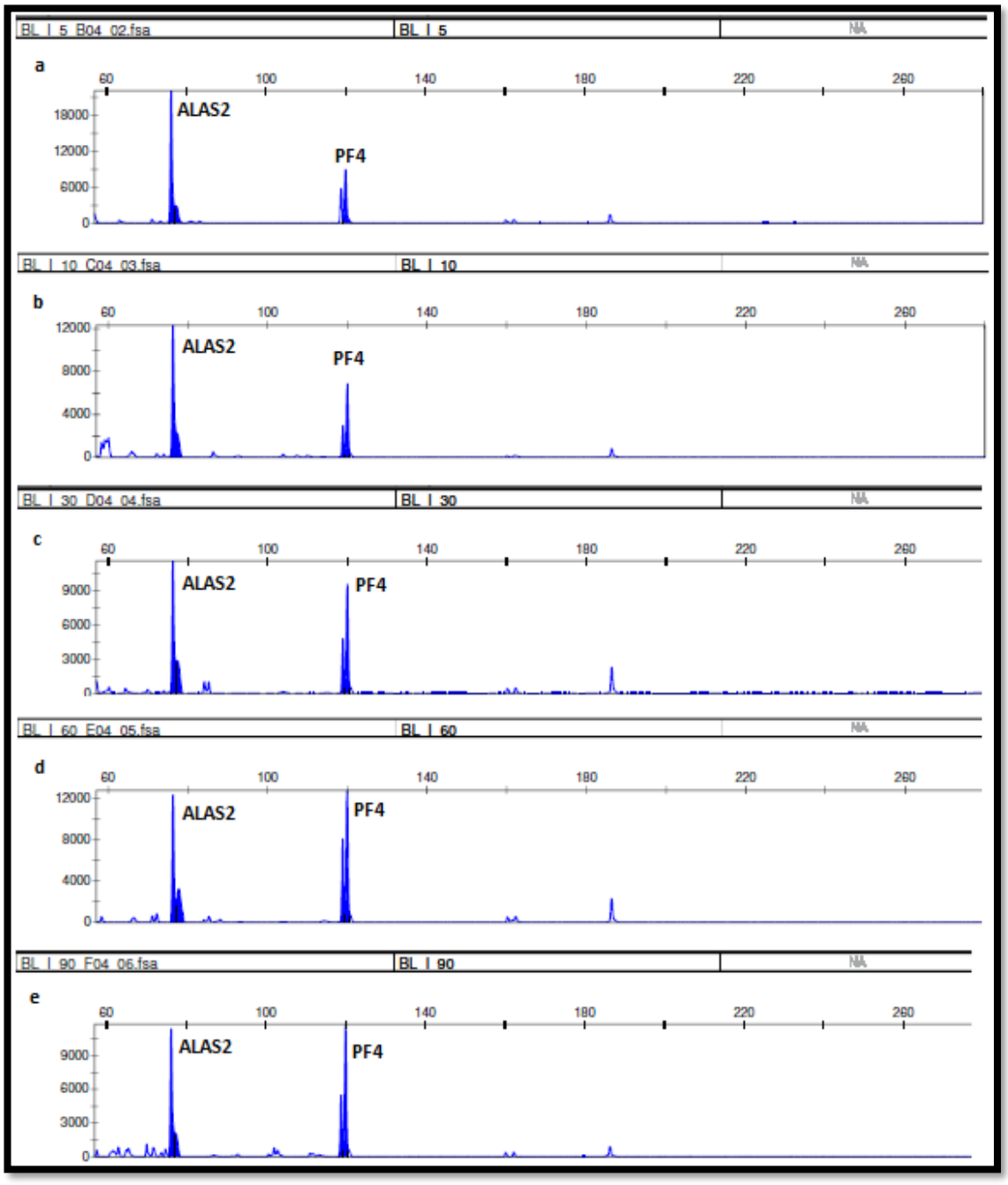

Figure 5.12: EPG showing degradation of blood sample in an incubator at 56 °C over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.3 Blood: Ultraviolet cross-linker

The effect of UV light on mRNA blood sample was distinct after 5 mins of exposure. While the expression of both blood markers were considerably reduced after 5 min, PF4 dropped out after 10 mins of exposure to UV (Figure 5.13).

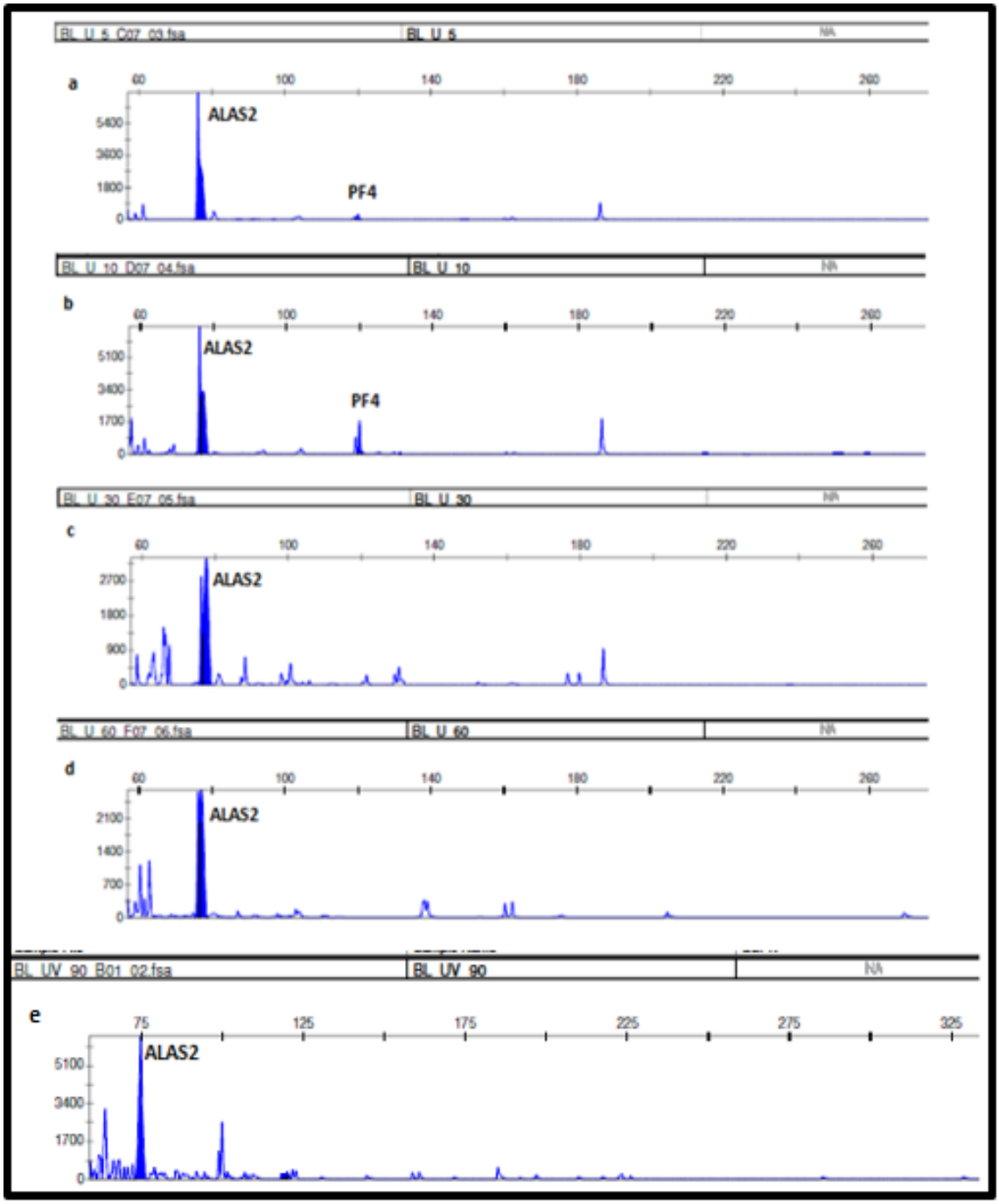

Figure 5.13: EPG showing degradation of blood sample in ultraviolet (UV) cross linker (X100 µJ/cm^2) over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.4 Saliva: Incubator

Saliva markers appeared stably expressed post incubation as there was no noticeable change in the RFU generated all through the incubation period (Figure 5.14).

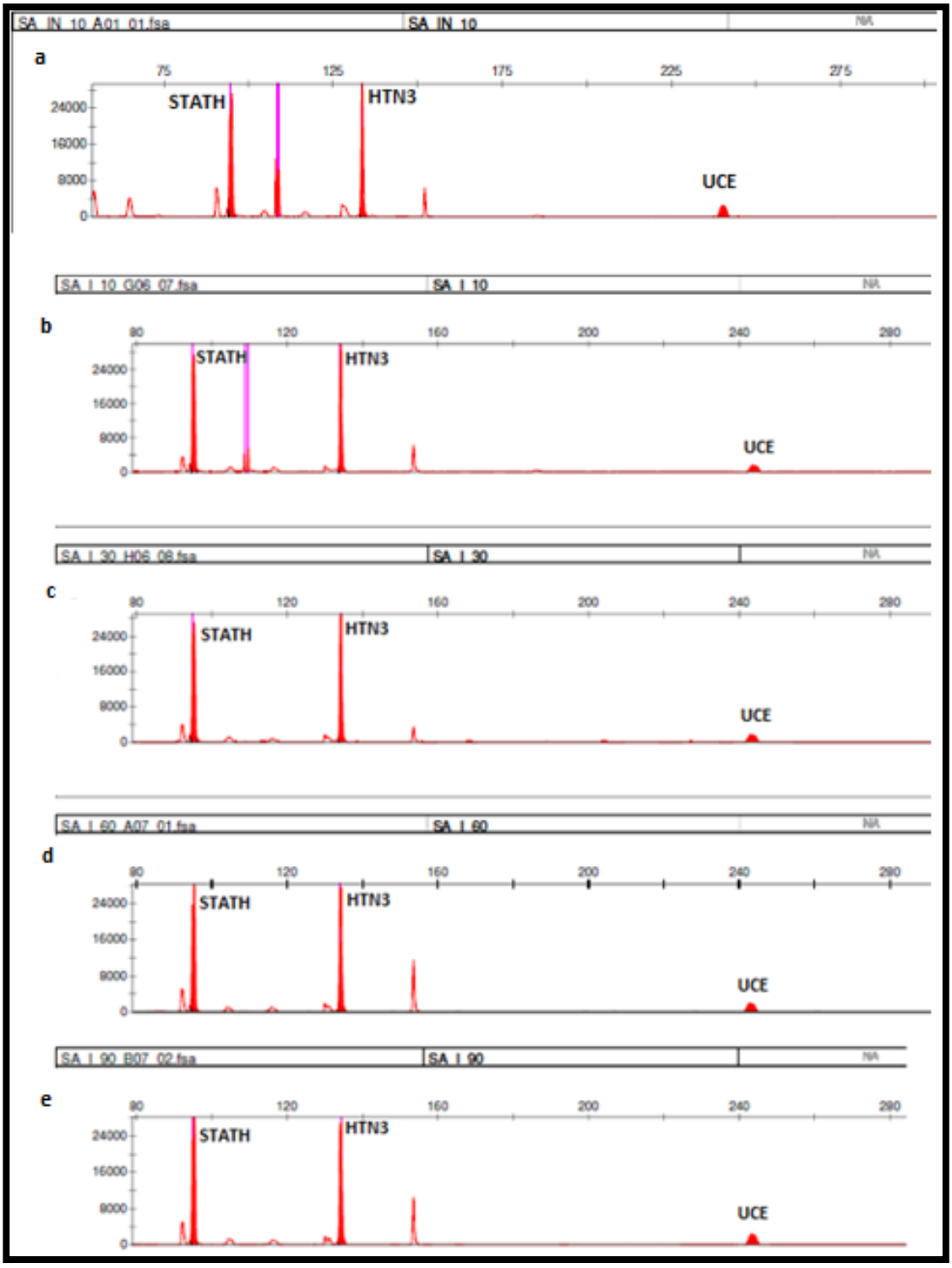

Figure 5.14: EPG showing degradation of saliva sample in an incubator at 56 ^{o}C over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.5 Saliva: Ultraviolet cross-linker

In contrast to the result obtained from samples degraded in the incubator, UV had a significant effect on saliva mRNA samples. The RFU generated reduced with increasing time of degradation. After 60th minute, the expression of STATH dropped out, leaving HTN3, which eventually dropped out at before the 90th minute. The expression of UCE was seen to be stable and not affected by the UV light (Figure 5.15).

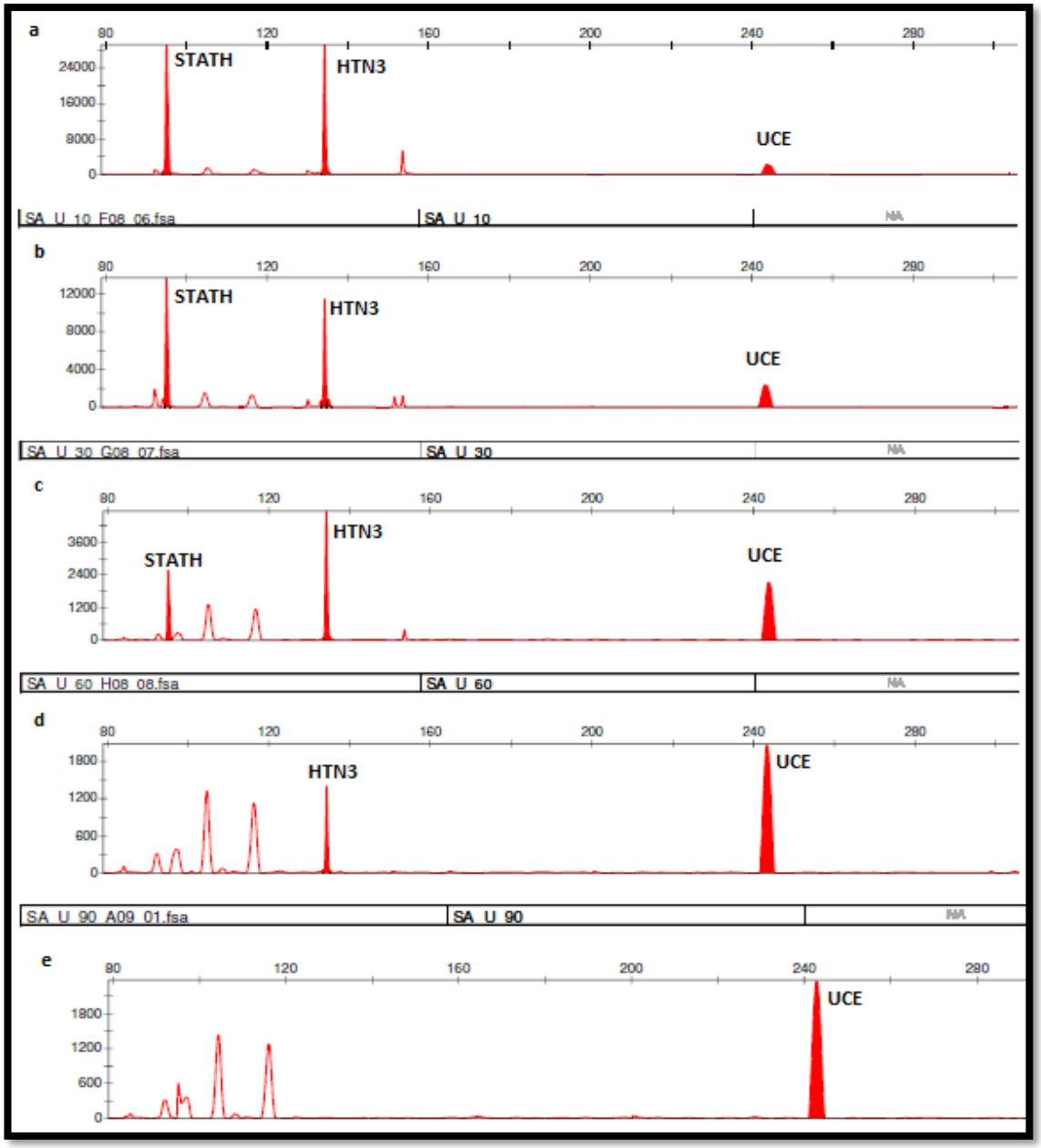

Figure 5.15: EPG showing degradation of saliva sample in ultraviolet (UV) cross linker (X100 µJ/cm^2) over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.6 Semen: Ultraviolet light

The results obtained from exposure of semen mRNA samples to UV light showed all four markers with high expression. After 10 min of exposure, TGM4 reduced gradually reaching an RFU of less than 1000 at the end of 90 min (Figure 5.16).

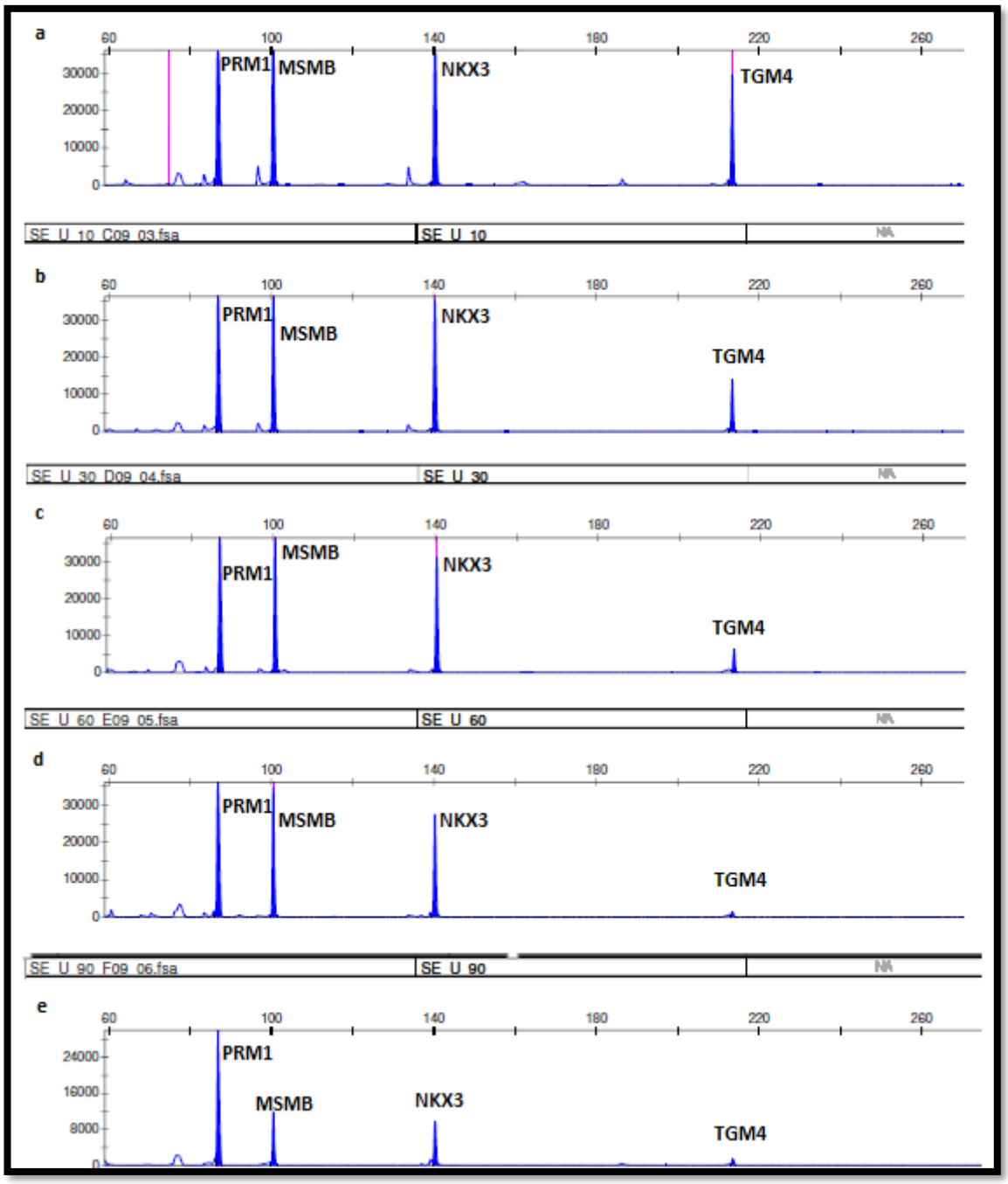

Figure 5.16: EPG showing degradation of semen sample in ultraviolet (UV) cross linker (X100 µJ/cm^2) over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.7 Semen: Incubator

Incubation of semen samples at 56 ^{o}C did not appear to have to have much effect on its degradation. All the four markers displayed high expression which remained stable all through the incubation times (Figure 5.17).

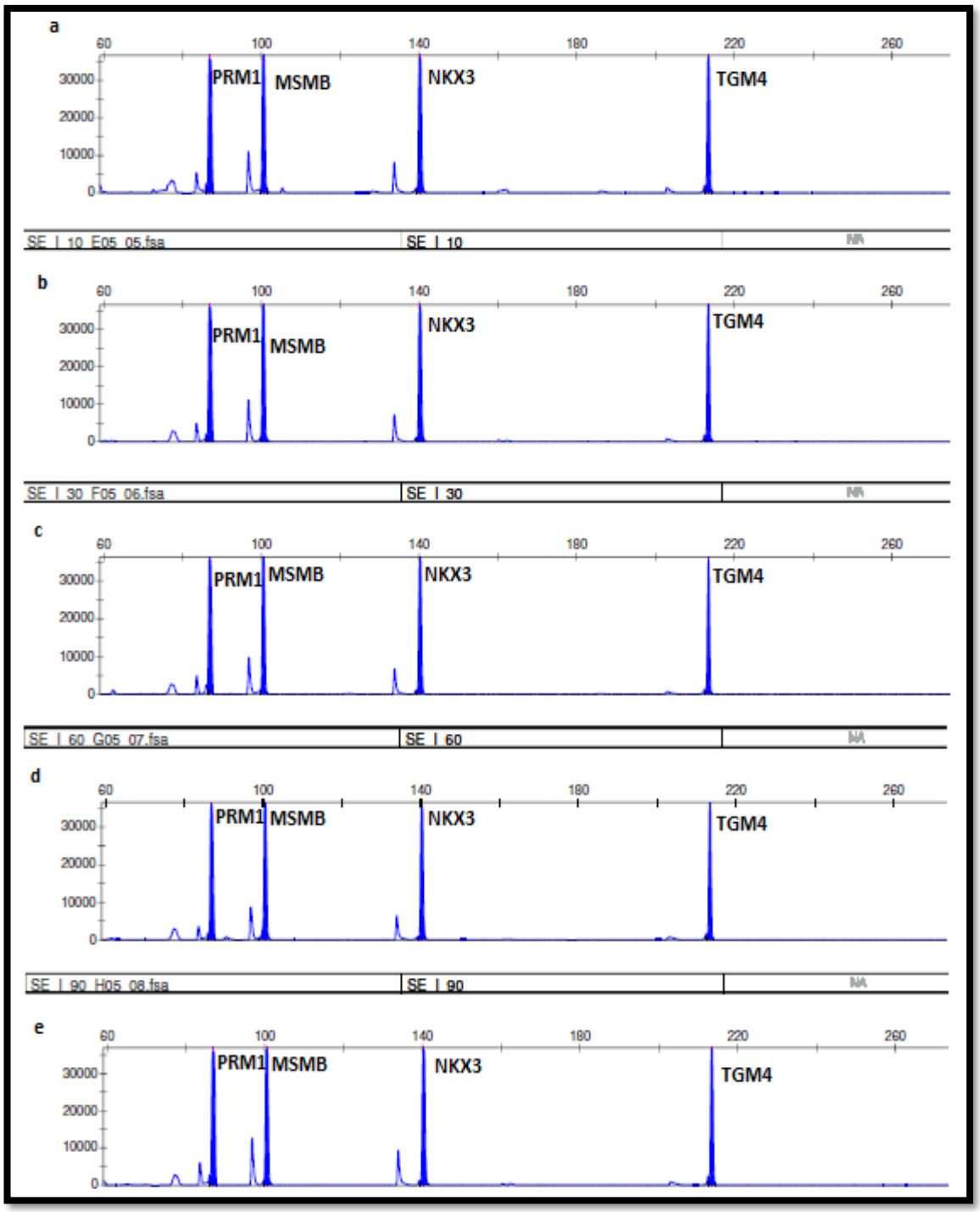

Figure 5.17: EPG showing degradation of semen sample in an incubator at 56 ^{o}C over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.8 Vaginal secretion: Ultraviolet light

Three out of four vaginal secretion markers were expressed after exposure to UV light. MUC4 and vaginal bacterial marker *L. crispatus* both showed a decrease in RFU through the 90 minutes of exposure. However, SFTA2 dropped out at 30 and 90 minutes (Figure 5.18).

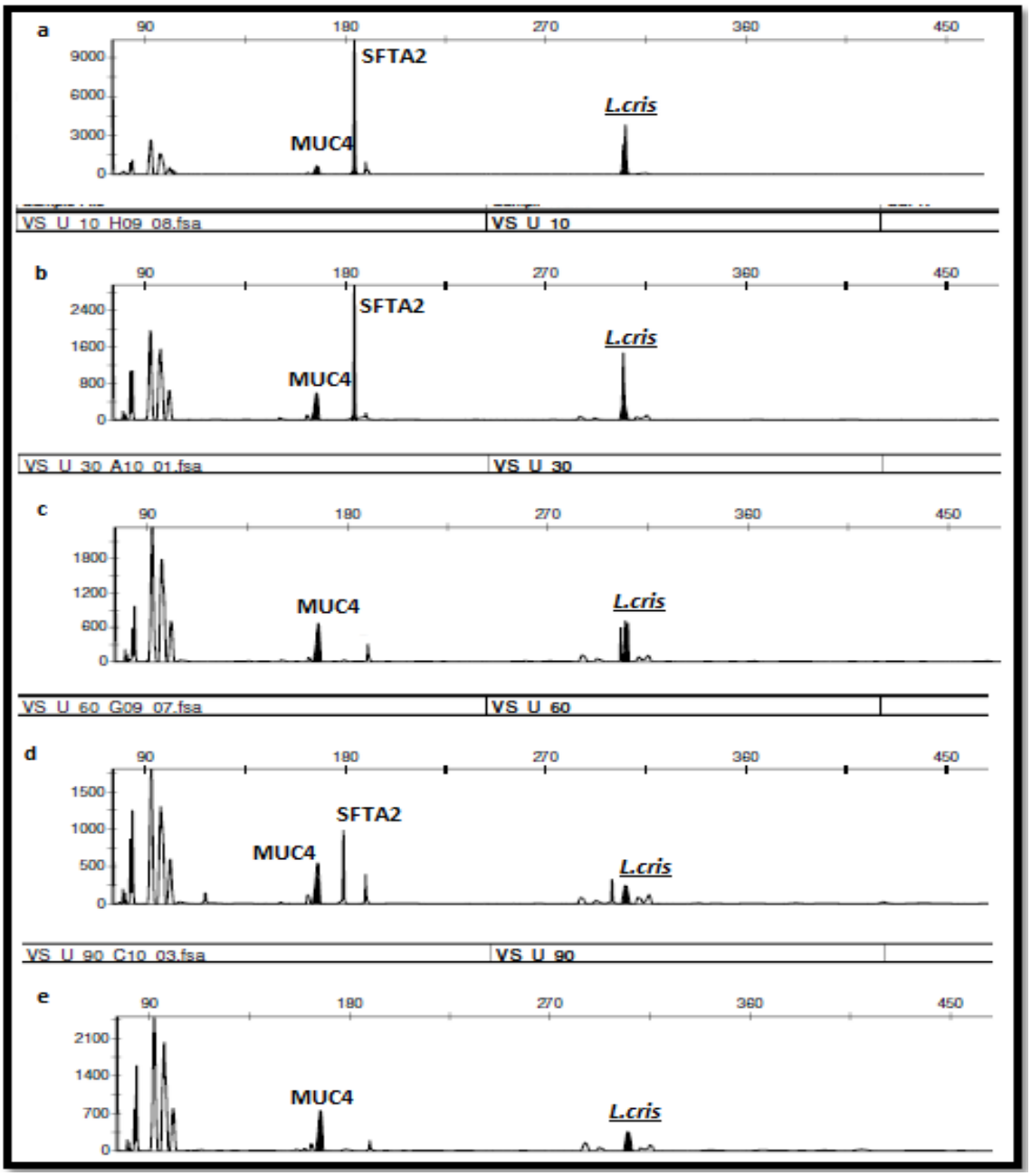

Figure 5.18: EPG showing degradation of vaginal secretion sample in ultraviolet (UV) cross linker (X100 µJ/cm^2) over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.4.4.9 Vaginal secretion: Incubator

Incubation at 56 °C appeared not have a noticeable effect on the expression of MUC4, SFTA2 and *L. cris* as RFU generated remained the same (Figure 5.19).

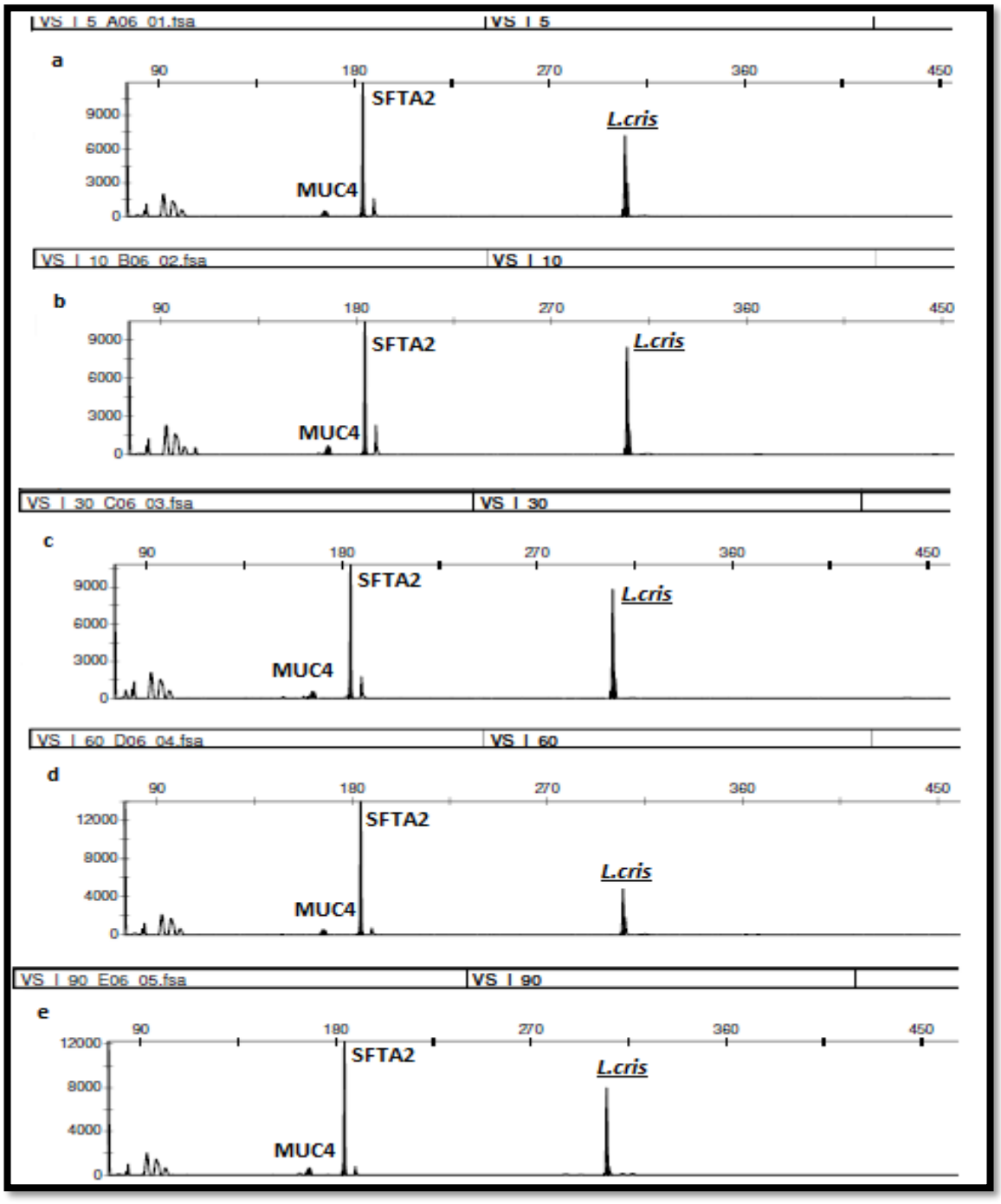

Figure 5.19: EPG showing degradation of vaginal secretion sample in an incubator at 56 °C over a period of 90 minutes. (a) 5 min, (b) 10min (c) 30 min. (d) 60 min, (e) 90 min. A volume of 1 µl amplified sample was used for CE, n=5; where n is the number of repeats of experiment.

5.4.5 Non-controlled degradation

Non-controlled degradation was carried out as outline in Chapter 2, section 2.9.8.2. The result is reported in sections 5.4.5.1 – 5.4.5.3.

5.4.5.1 Blood sample

The high temperature and humidity did not affect the expression of the two blood markers ALAS2 and PF4. They both displayed a stable but reduced expression throughout the period of the experiment. Figure 5.20 displays EPG obtained from samples prepared on glass slide and recovered on days zero (day sample was prepared), day 25 and day 50.

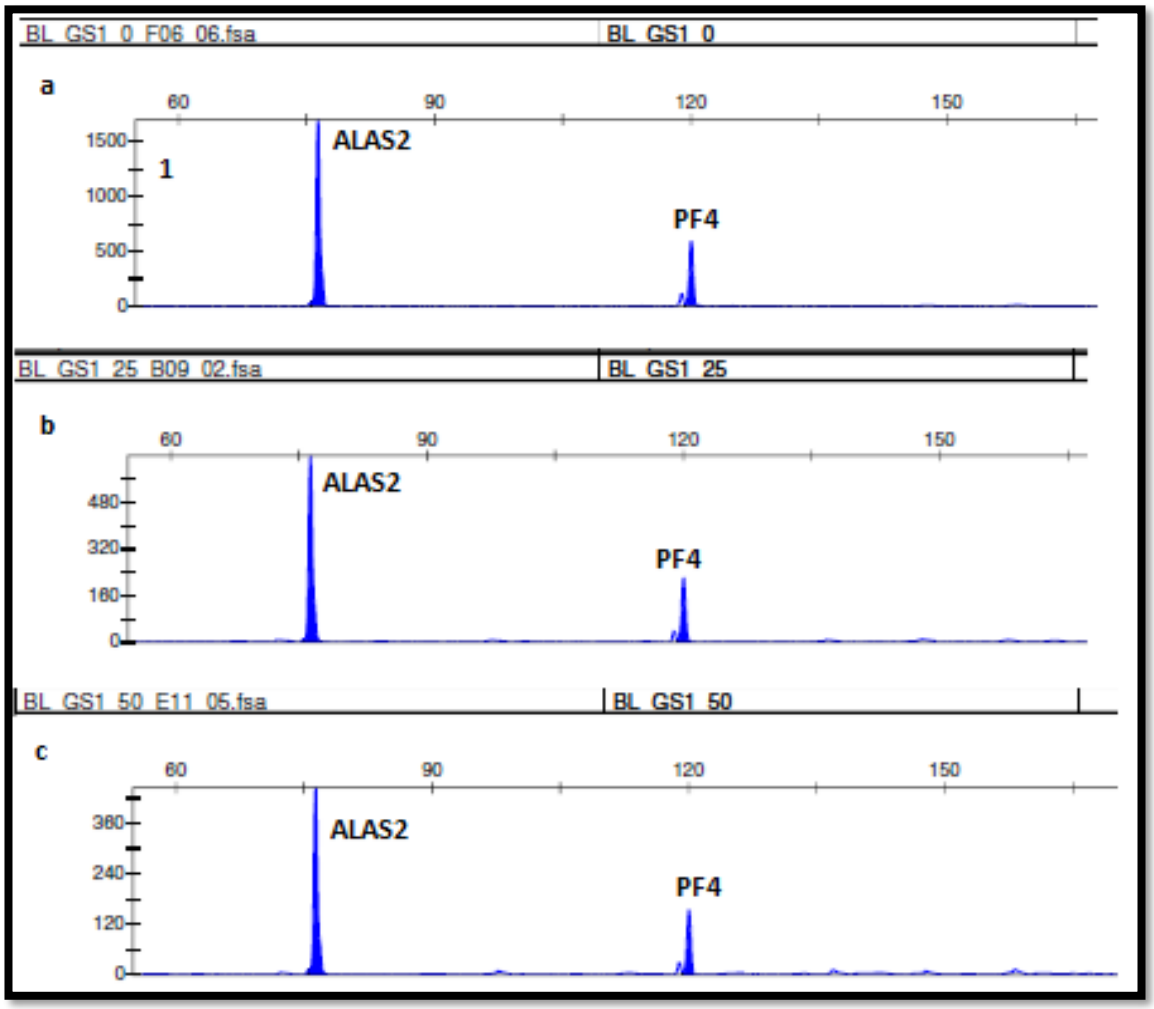

Figure 5.20: EPG showing uncontrolled degradation of blood sample stained on glass slide over a period of 51 days in Saudi Arabia, in the summer of 2014. Samples were taken every 48-72 hrs under 49.5 ^{o}C, 6.3 ^{o}C maximum and minimum temperatures respectively, 95% maximum and minimum relative humidity. (a) day 1, (b) day 25 and (c) day 50, n=5; where n is the number of repeats of experiment.

5.4.5.2 Saliva

Unlike blood samples, the environmental conditions had an adverse effect on saliva samples as both saliva markers and reference gene were only expressed up till day 25 of the experiment. The markers dropped out after day 25 (Figure 5.21).

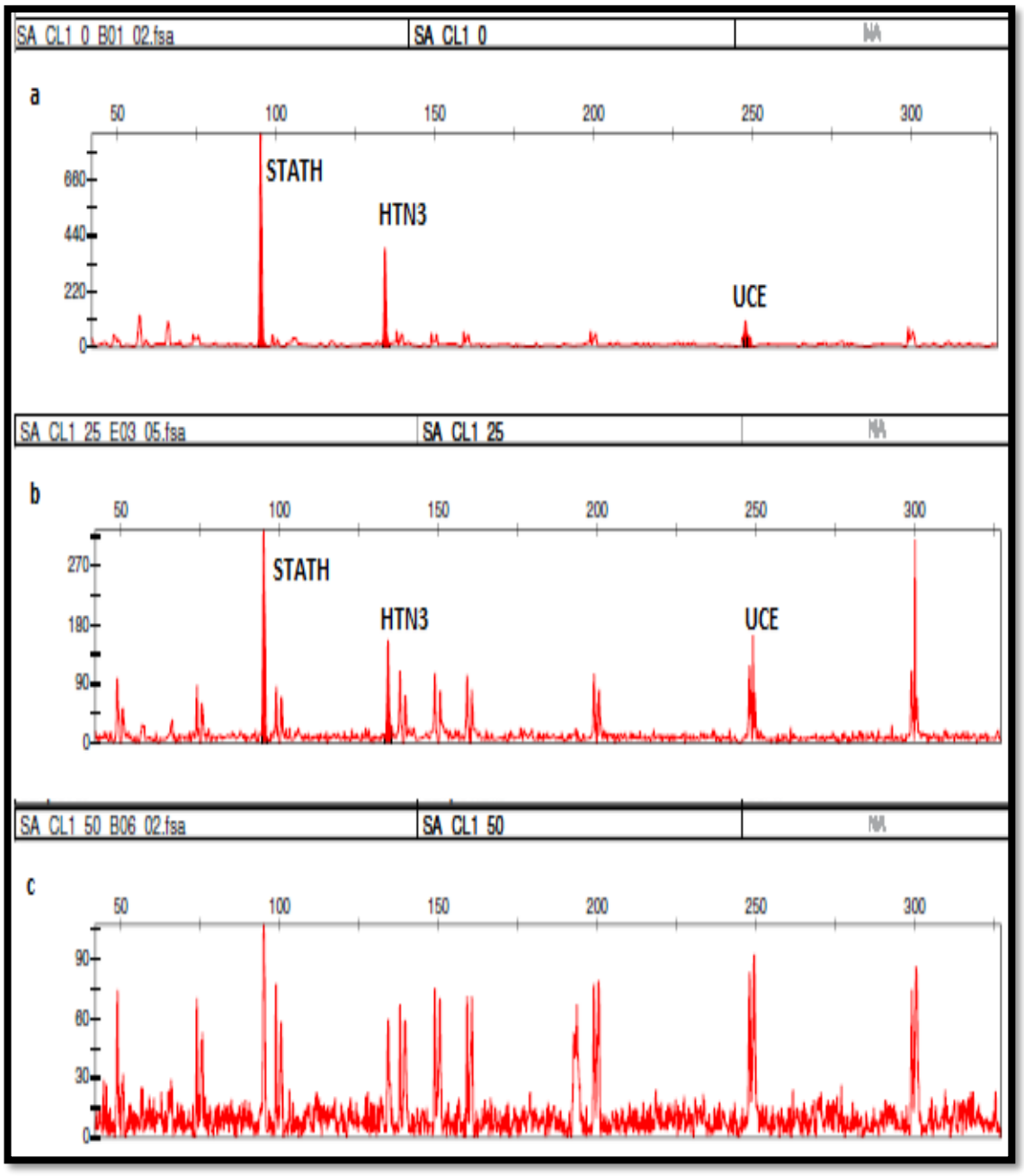

Figure 5.21: EPG showing uncontrolled degradation of saliva sample stained on cotton cloth over a period of 51 days in Saudi Arabia, in the summer of 2014. Samples were taken every 48-72 hrs under 49.5 ^{o}C, 6.3 ^{o}C maximum and minimum temperatures respectively, 95% maximum and minimum relative humidity. (a) day 1, (b) day 25 and (c) day 50, n=5; where n is the number of repeats of experiment.

5.4.5.3 Semen

Semen samples prepared and recovered from glass slides, cotton cloth and metal all displayed expression up until day 30. All four markers dropped out after day 30 on samples recovered from metal and cotton cloth while MSMB and NKX3-1 were the only markers expressed at day 30 on samples recovered from glass slides (Figure 5.22).

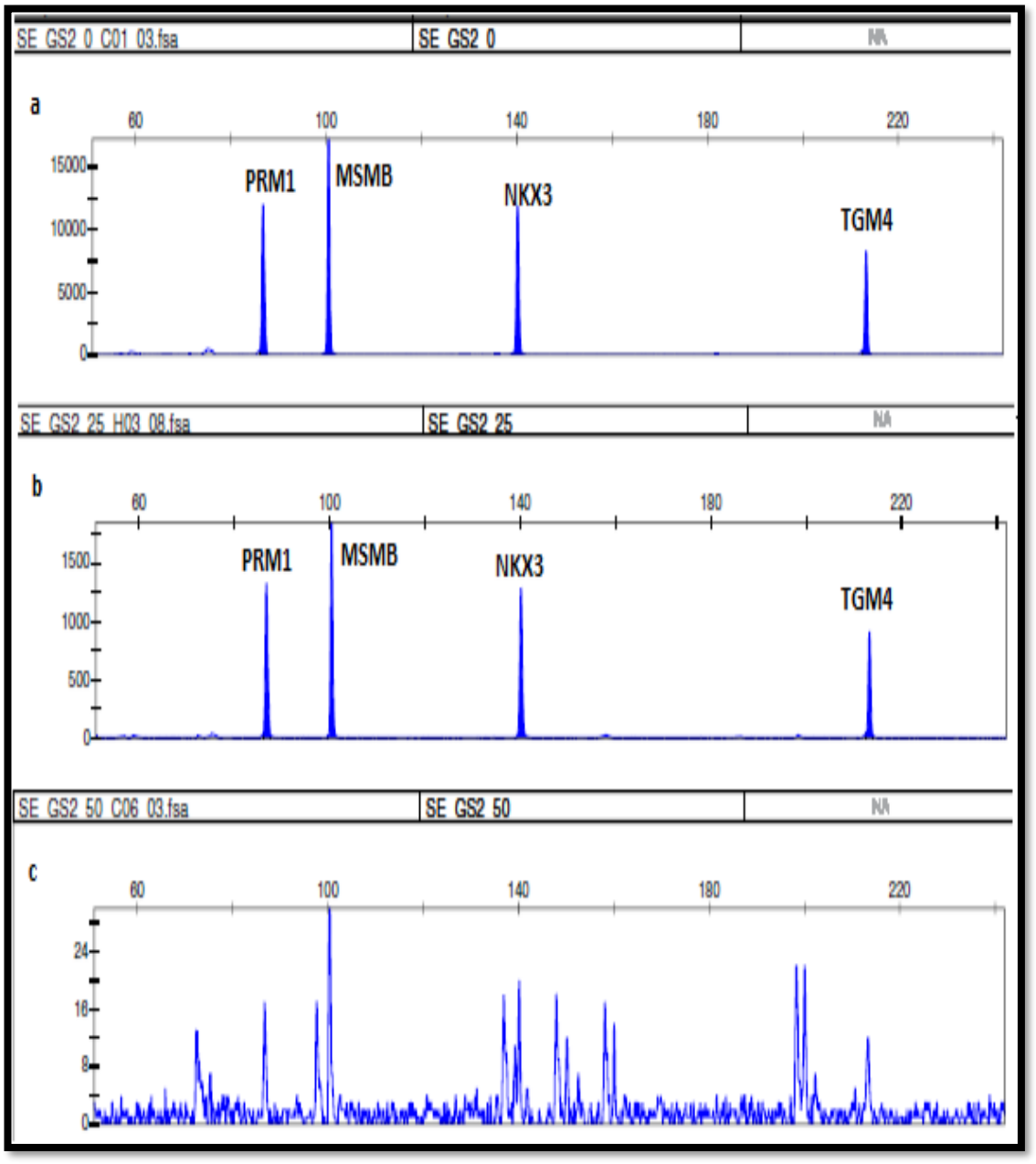

Figure 5.22: EPG showing uncontrolled degradation of semen sample stained on glass slide over a period of 51 days in Saudi Arabia, in the summer of 2014. Samples were taken every 48-72 hrs under 49.5 °C, 6.3 °C maximum and minimum temperatures respectively, 95% maximum and minimum relative humidity. (a) day 1, (b) day 25 and (c) day 50.

Table.5.2: Expression of body fluid markers in environmentally degraded samples over 51 days.

	Sample								
	Blood			Saliva			Semen		
Day	C	M	G	C	M	G	C	M	G
1	*+++*	*+++*	*+++*	*+++*	*+++*	*ns*	*+++*	*+++*	*+++*
5	*+++*	*+++*	*+++*	*+++*	*++*	*ns*	*+++*	*+++*	*+++*
10	*+++*	*+++*	*+++*	*+++*	*++*	*ns*	*+++*	*+++*	*+++*
15	*+++*	*+++*	*+++*	*++*	*++*	*ns*	*+++*	*+++*	*+++*
20	*+++*	*+++*	*+++*	*++*	*+*	*ns*	*+++*	*+++*	*+++*
25	*+++*	*+++*	*+++*	*++*	*+*	*ns*	*++*	*+++*	*+++*
30	*+++*	*+++*	*+++*	*+*	*+*	*ns*	*++*	*+*	*++*
35	*+++*	*+++*	*++*	-	*+*	*ns*	*++*	*+*	*+*
40	*+++*	*+++*	*++*	-	-	*ns*	-	-	-
45	*+++*	*++*	*++*	-	-	*ns*	-	-	-
50	*+++*	*++*	*++*	-	-	*ns*	-	-	-

+++ = very strong expression; **++** = weak expression; **+** = very weak expression; **-** = no expression, **ns**= not supplied.

C= cotton cloth, **G**= glass slide, **M**= metal/knife.

5.4.6 Equimolar mixture of all body fluid sample

All body fluid samples; blood, semen, saliva and vaginal secretion were mixed in 1:1 proportion and 1µl of the mixture was amplified. All blood, saliva, semen and reference gene markers were highly expressed (Figures 5.23ai, aiii). However, only SFTA2 was expressed for vaginal secretion (Figures 5.23ii).

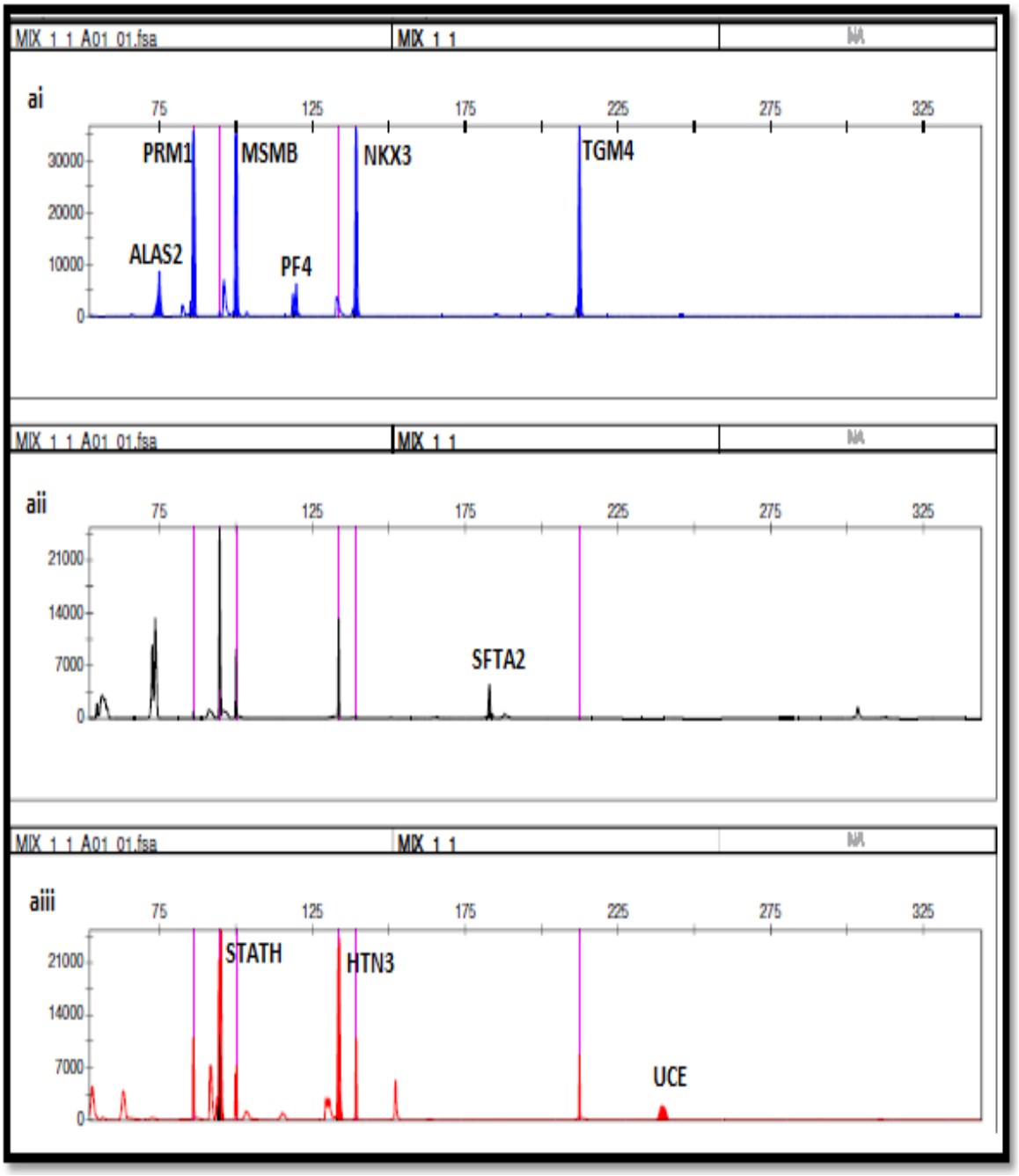

Figure 5.23: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in equal proportions. A volume of 1 µl of each sample which indicates 1 proportion was used. (ai/aii/aiii) 1:1, n=5; where n is the number of repeats of experiment.

5.4.7 Primer titration

Primer concentration plays a key role in amplification reaction. The effect of primer concentration was determined by optimising 1 µl, 1.5 µl, and 3.5 µl primer mix added to the reaction components. As primer concentrations increase, there was an increase in peak heights of all the markers (Figures 5.24i-vi). With an optimal primer concentration being 3.5 µl, better profile was generated using Qiagen PCR mastermix (See Figures 5.24iv and 5.24vi).

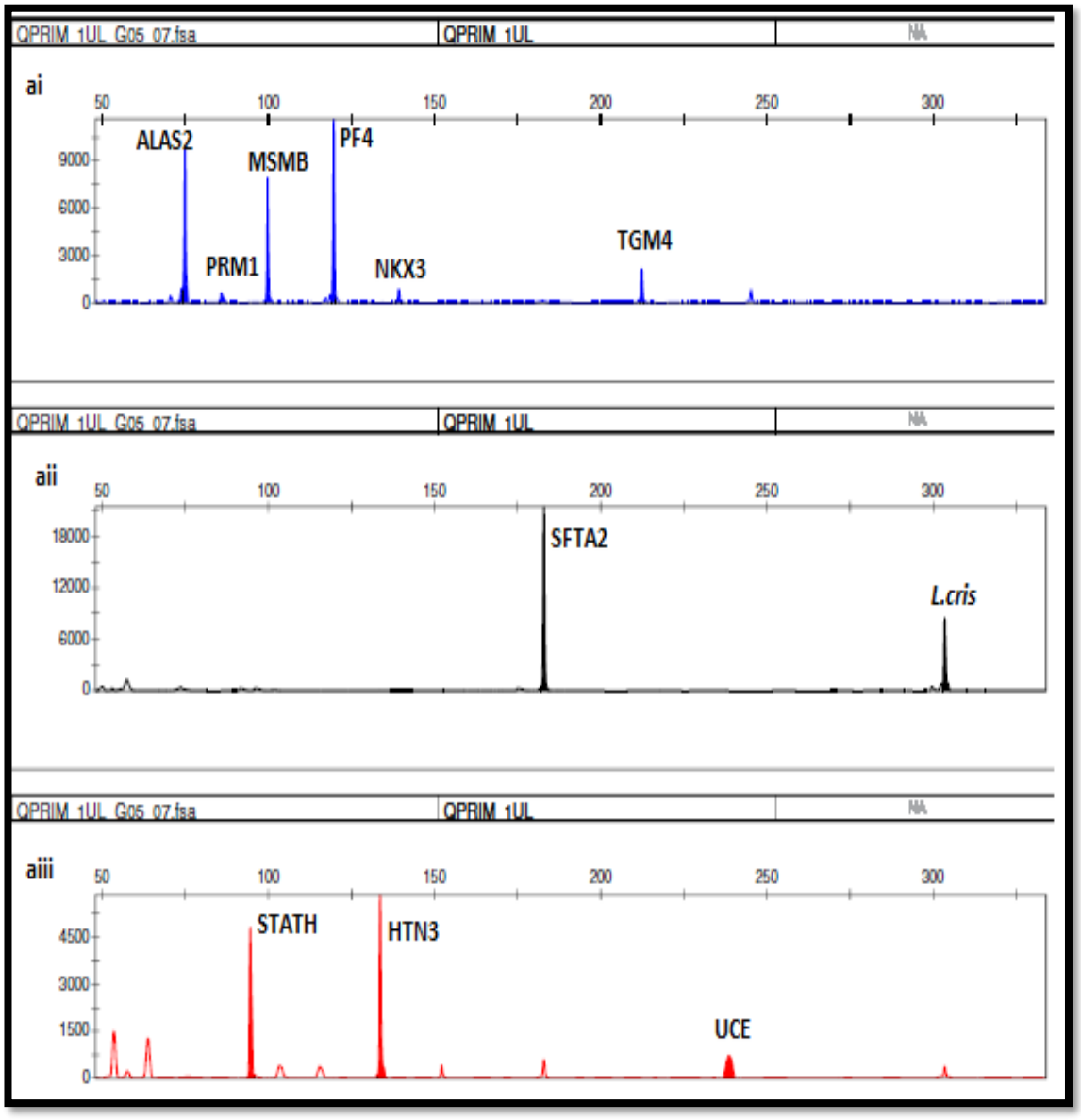

Figure 5.24i: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and a volume of 1 µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

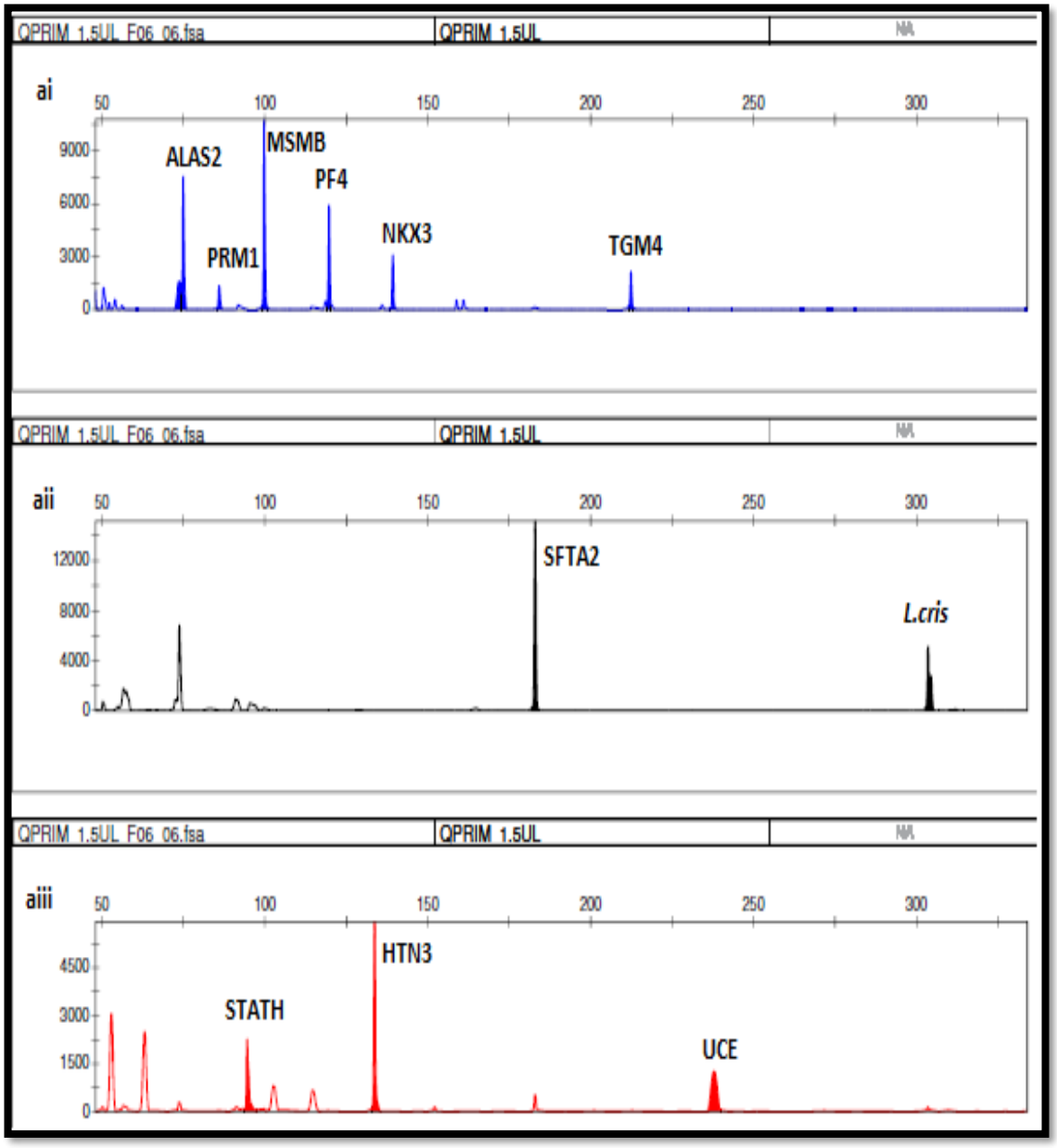

Figure 5.24ii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and a volume of **1.5** µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

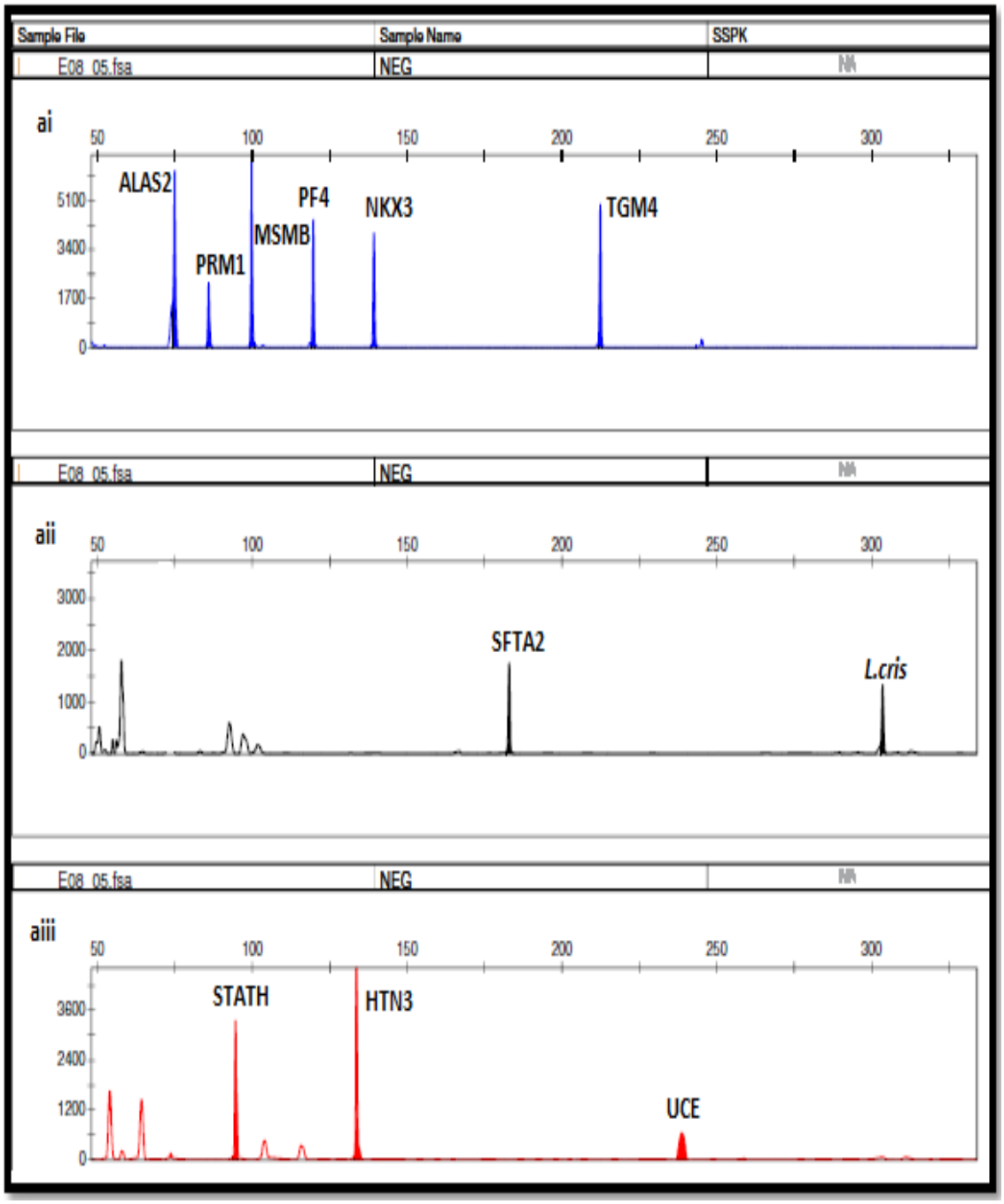

Figure 5.24iii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and a volume of **3.5** µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

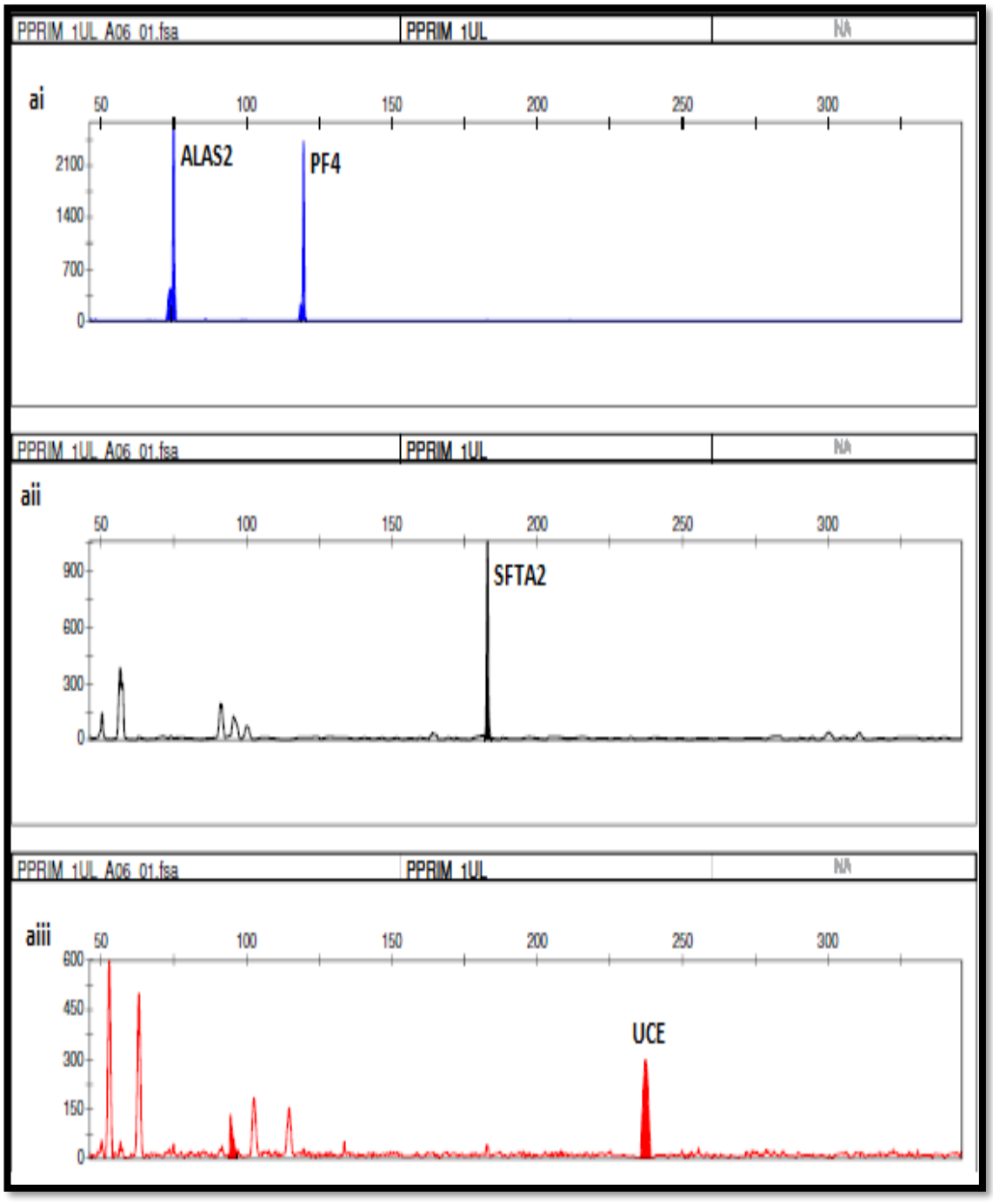

Figure 5.24iv: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

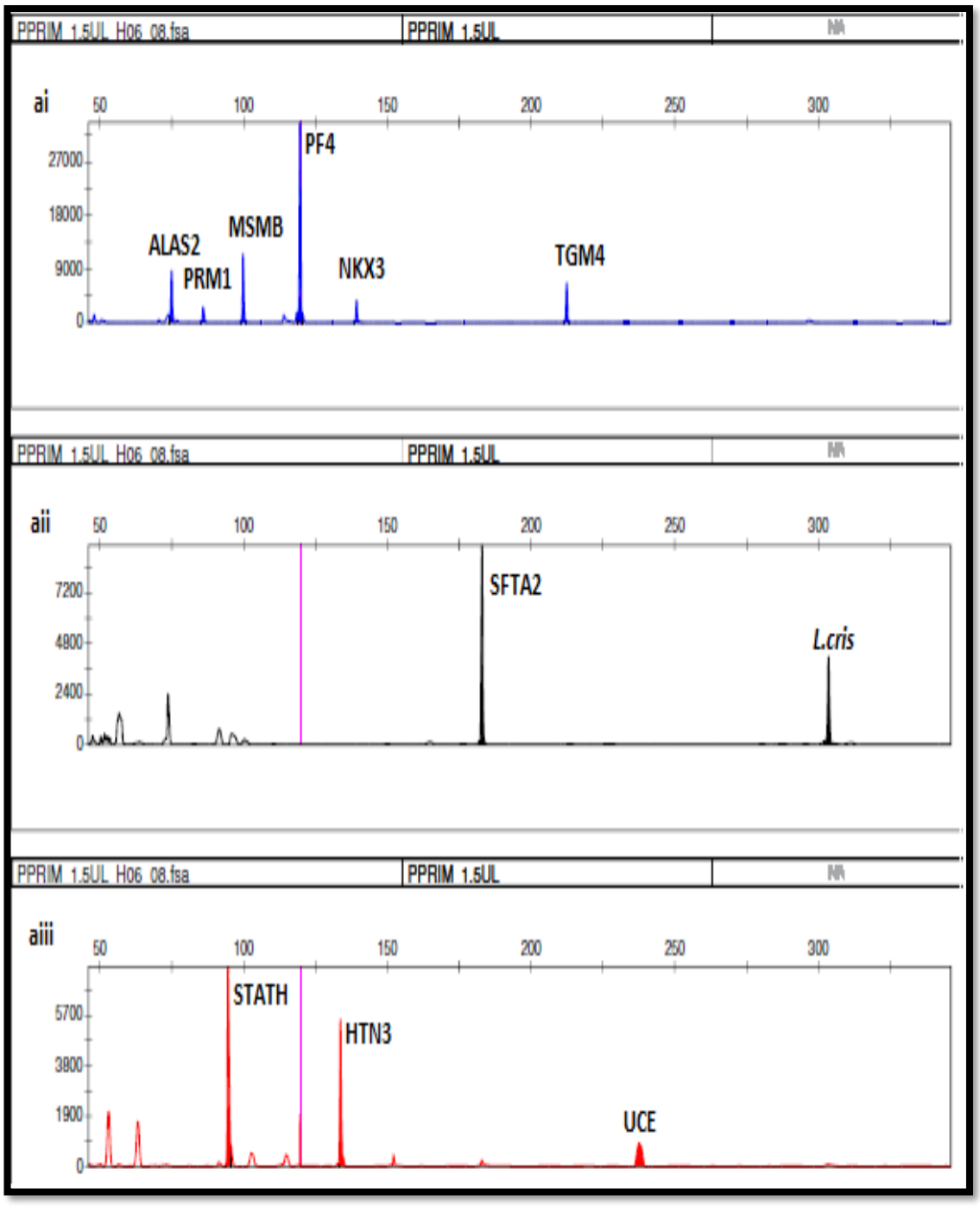

Figure 5.24v: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **1.5** µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

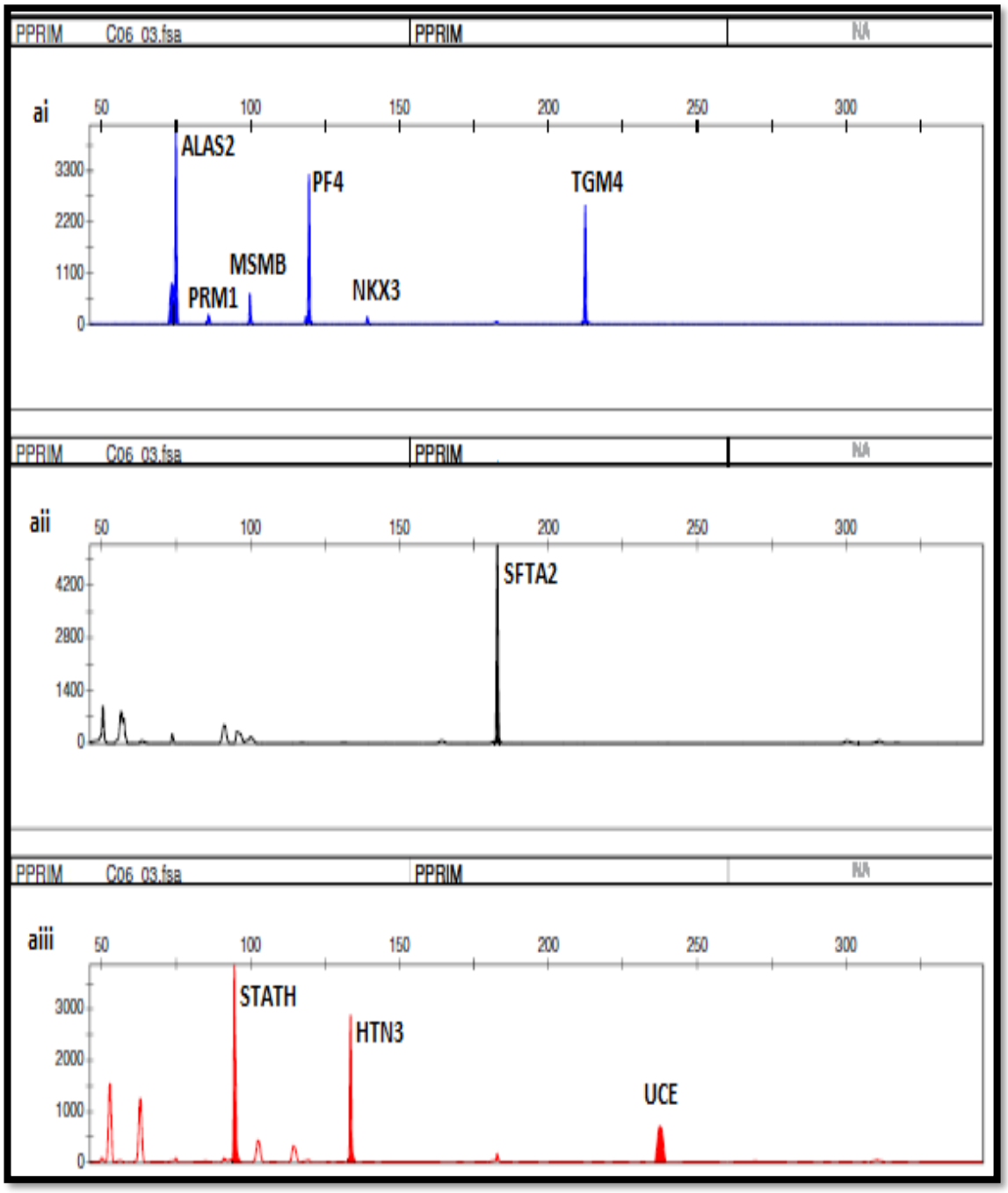

Figure 5.24vi: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

5.4.8 Reaction components and thermal cycling conditions

Reaction components need to be optimised as a slight departure from optimal annealing temperature, cycle number or master mix could lead to generation of artefacts. For consistent and robust results, two different mastermix, Qiagen PCR multiplex mix (Qiagen Ltd) and Platinum supermix (Life technologies Ltd) were optimised at 54 ^{o}C, 56 ^{o}C, 58 ^{o}C and 60 ^{o}C with 58 ^{o}C being the optimal annealing temperature (see Figures 4:1-7; 5.25i-iv).

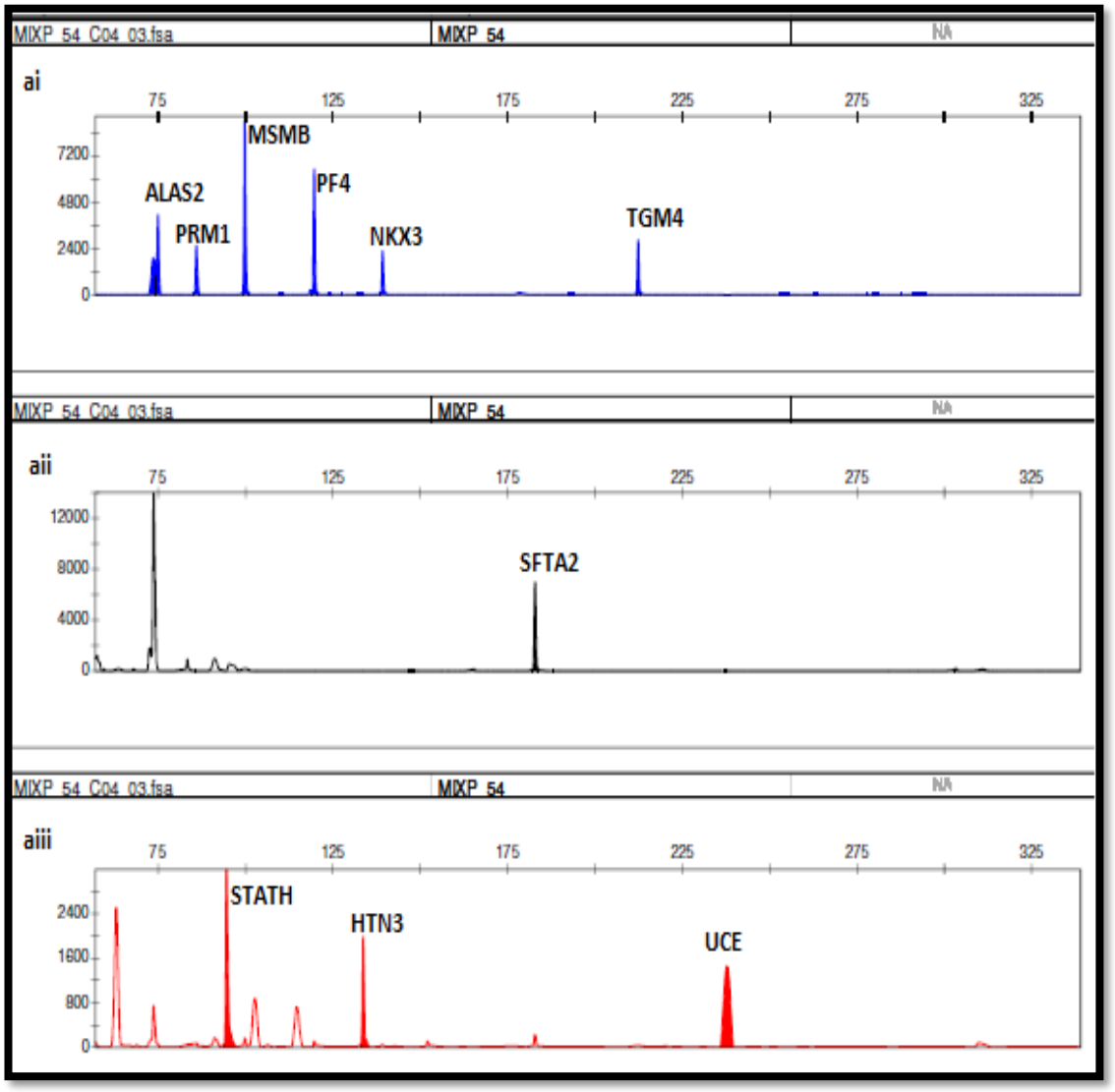

Figure 5.25i: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix at **54 °C Tm**. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

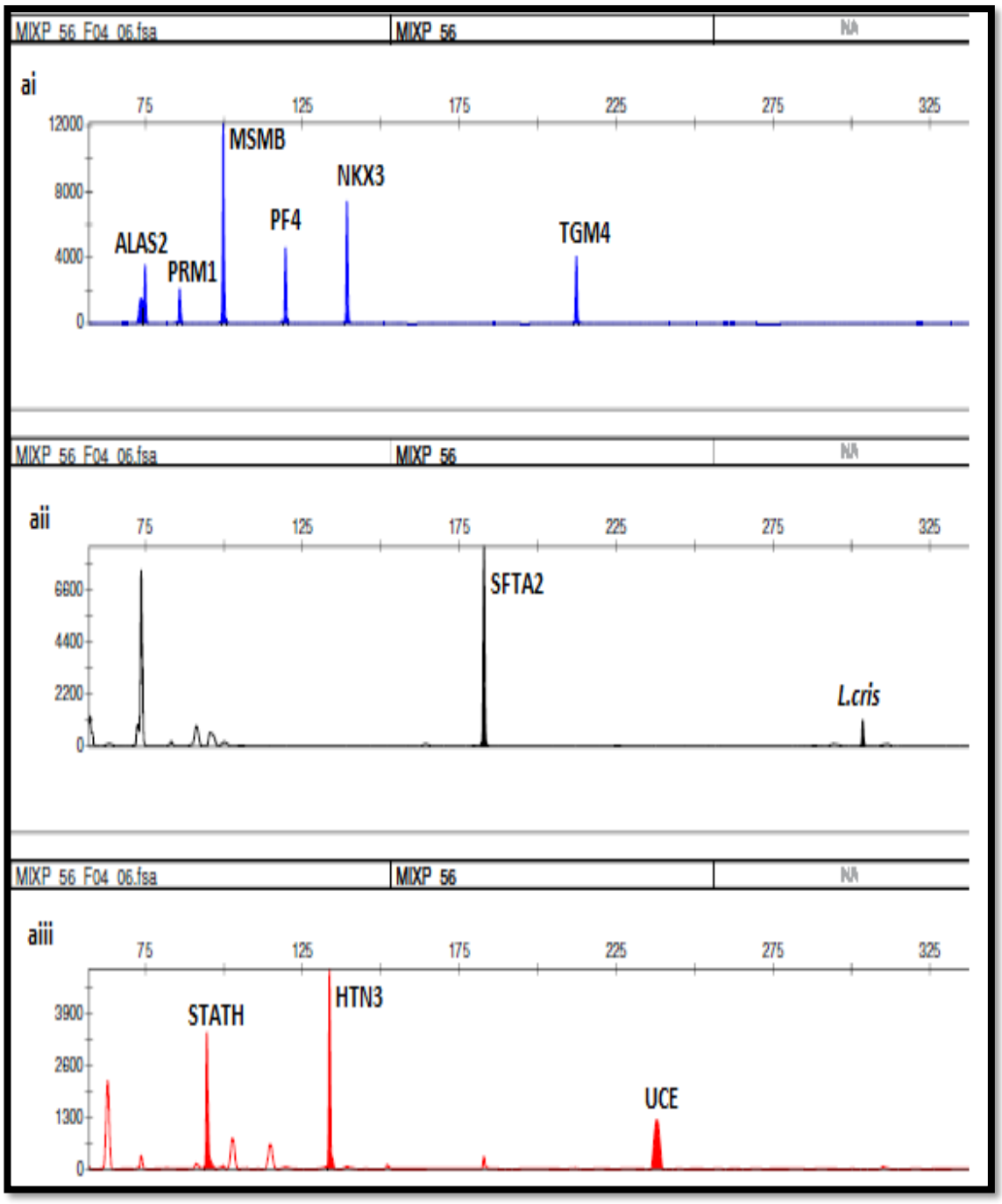

Figure 5.25ii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix at **56 ºC Tm**. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

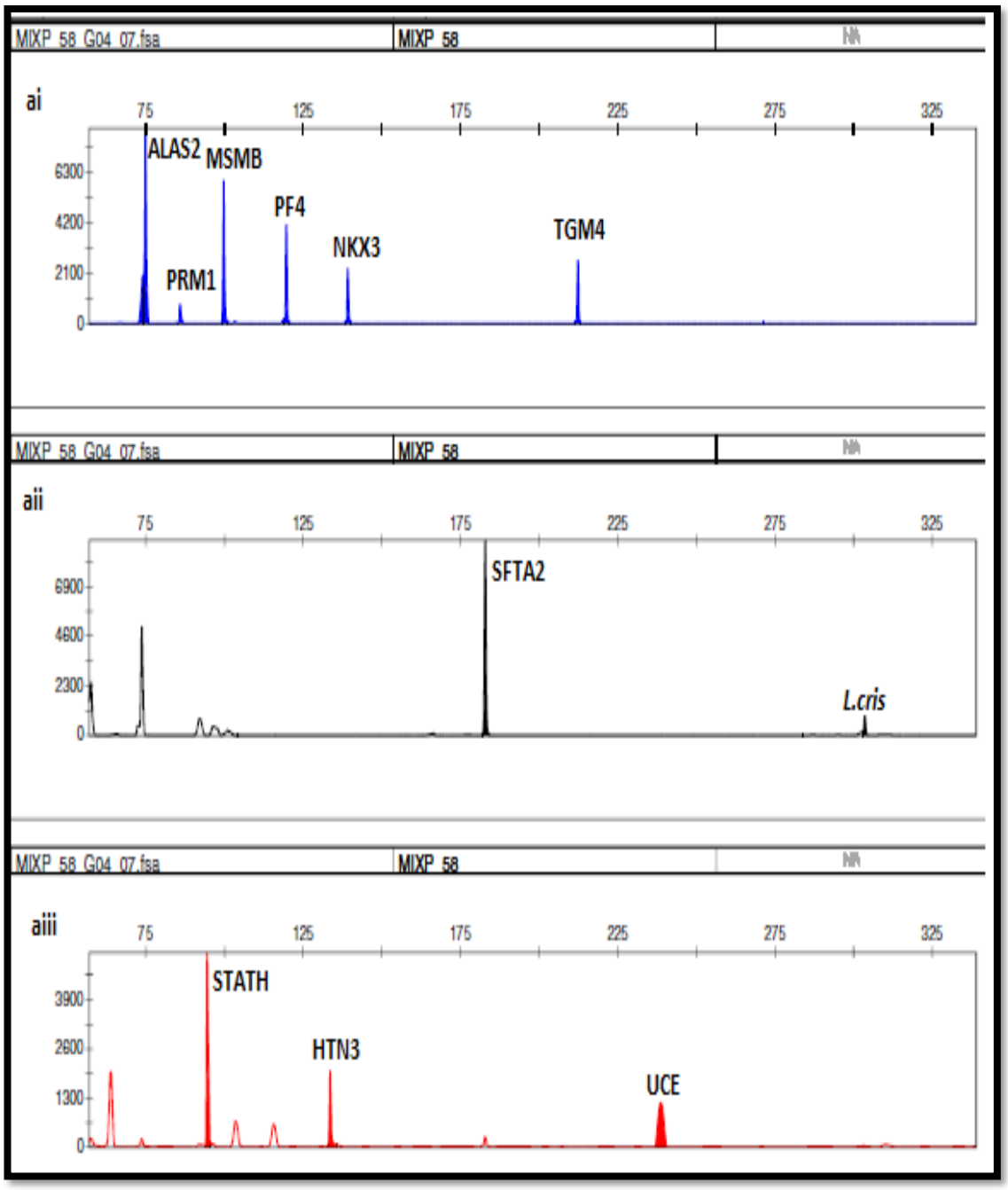

Figure 5.25iii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix at **58 °C Tm**. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

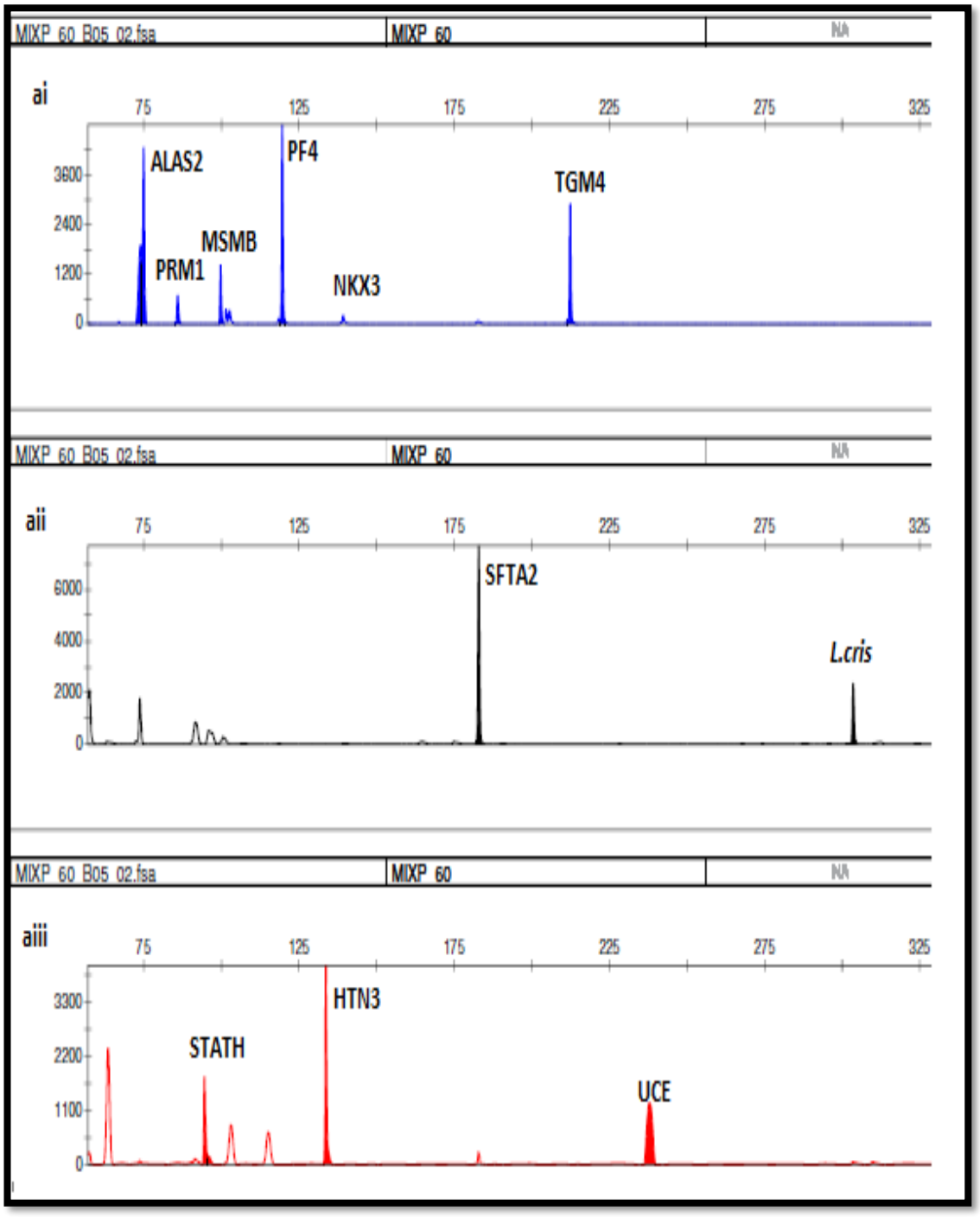

Figure 5.25iv: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Platinum PCR mastermix (Life Technologies Ltd) and a volume of **3.5** µl primermix at **60 °C Tm**. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

5.4.9 Reduced reaction volume

Many forensic laboratories tend to reduce the reaction volume for their experiments as a cost saving measure. In this study, final reaction volume was modified by reducing the volume of mastermix in the reaction while other reaction components were kept constant. Final reaction volumes of 12.2 µl and 17.2 µl were tested. The result in Figures 5.26i shows expression of all the body fluid markers, although in varied proportions and a low efficiency in amplification using 5 µl Qiagen PCR mastermix (Figure 5.26i). A similar profile was obtained using 5µl Platinum PCR mastermix but with low expression of vaginal secretion markers (Figure 5.26ii). The use of 10 µl Platinum PCR and Qiagen mastermix (Figures 5.26iii and 5.26iv respectively) generated an improved profile with expression of all the body fluid markers.

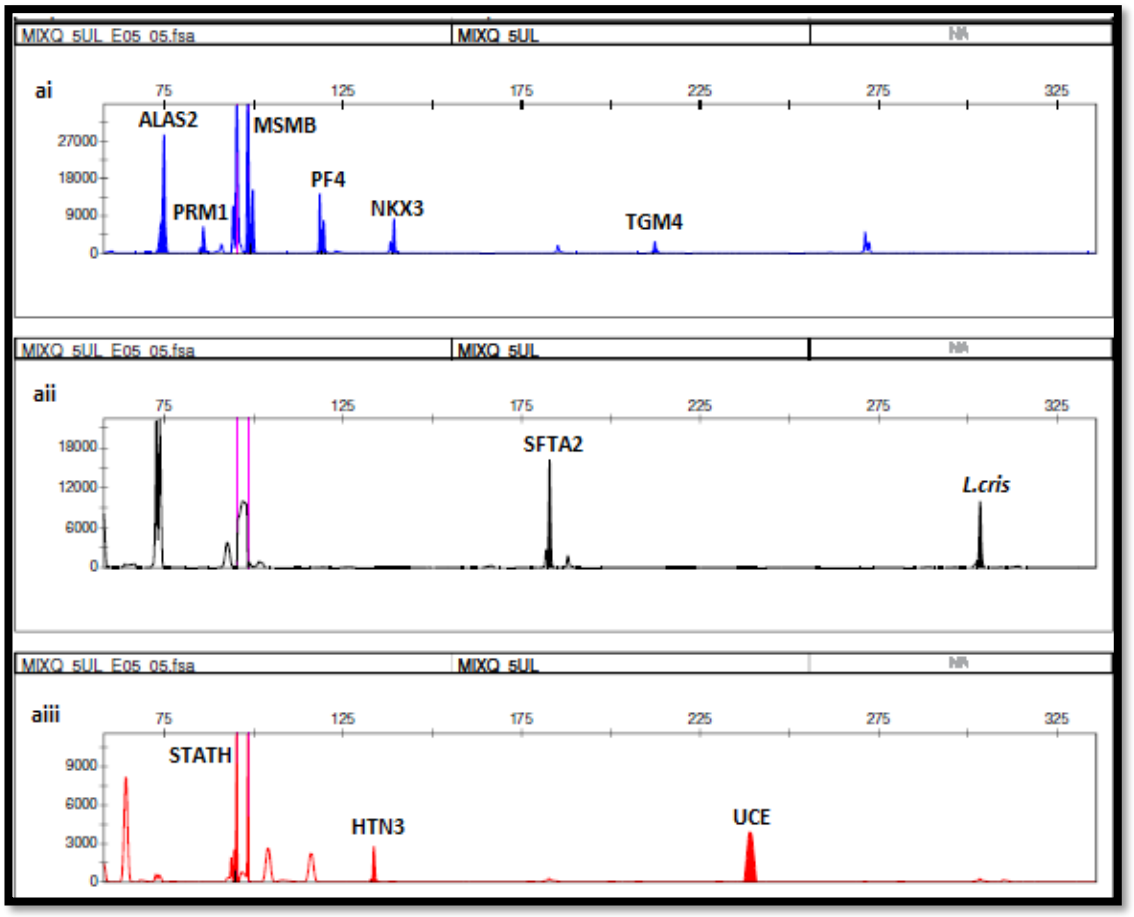

Figure 5.26i: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in proportions with a reduced volume **(5** µl**)** Qiagen PCR mastermix (Qiagen Ltd). Varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl proportion was used. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene. Final reaction volume was 12.2 µl., n=5; where n is the number of repeats of experiment.

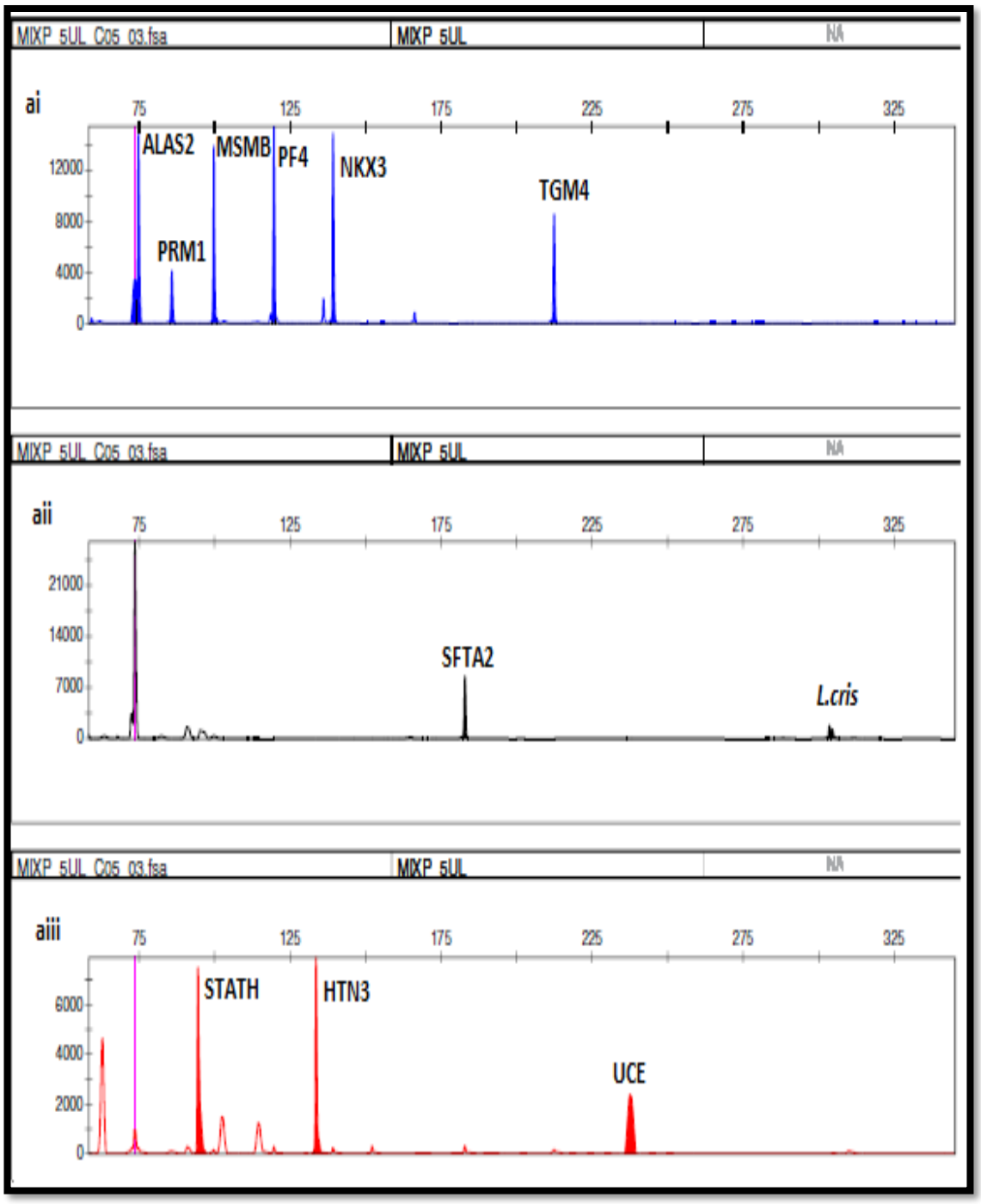

Figure 5.26ii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in proportions with a reduced volume **(5** µl**)** Platinum PCR mastermix (Life Technologies Ltd). Varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl proportion was used. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene. Final reaction volume was **12.2** µl, n=5; where n is the number of repeats of experiment.

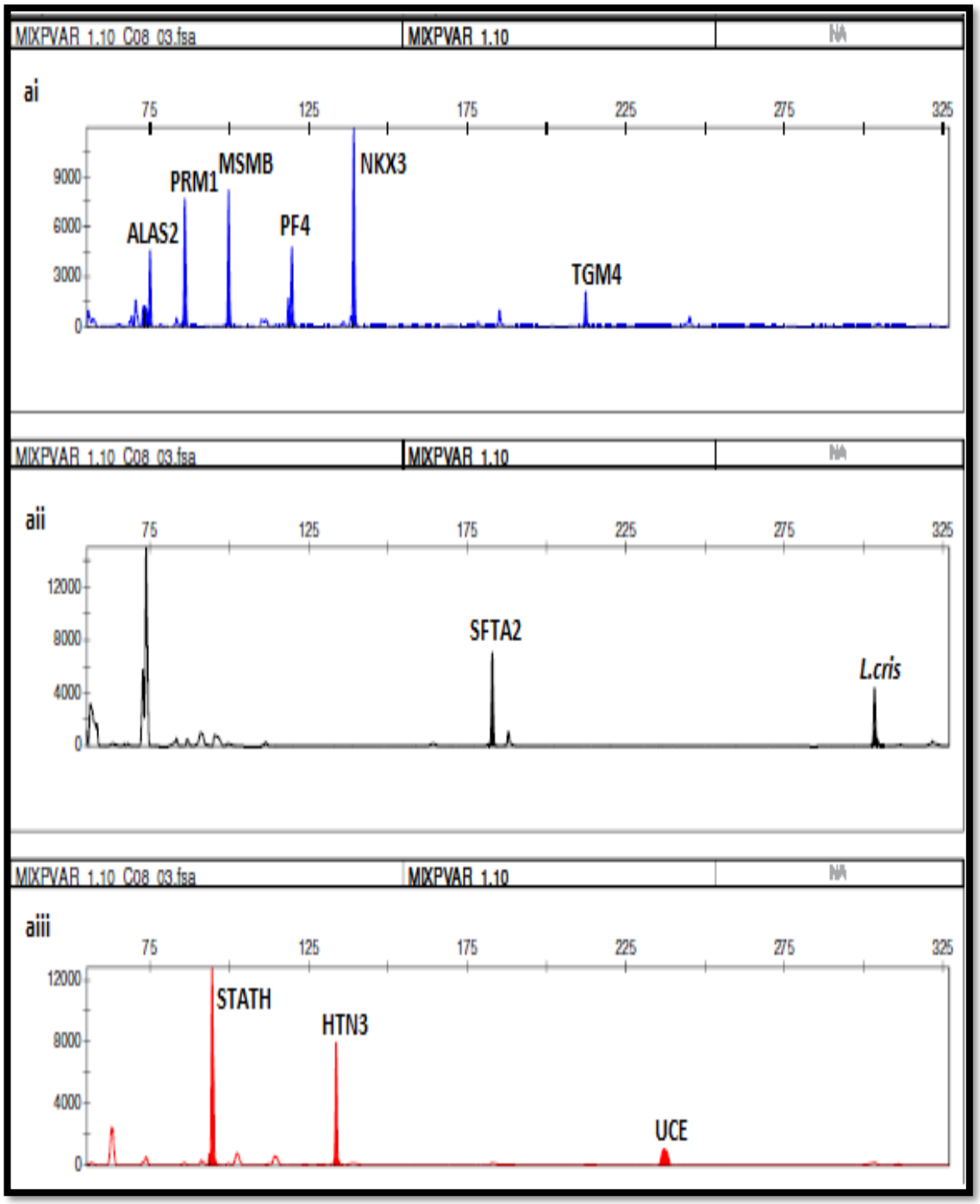

Figure 5.26iii: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in proportions with a reduced volume **(10** µl**)** Platinum PCR mastermix (Life Technologies Ltd). Varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl proportion was used. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene. Final reaction volume was **17.2** µl**,** n=5; where n is the number of repeats of experiment.

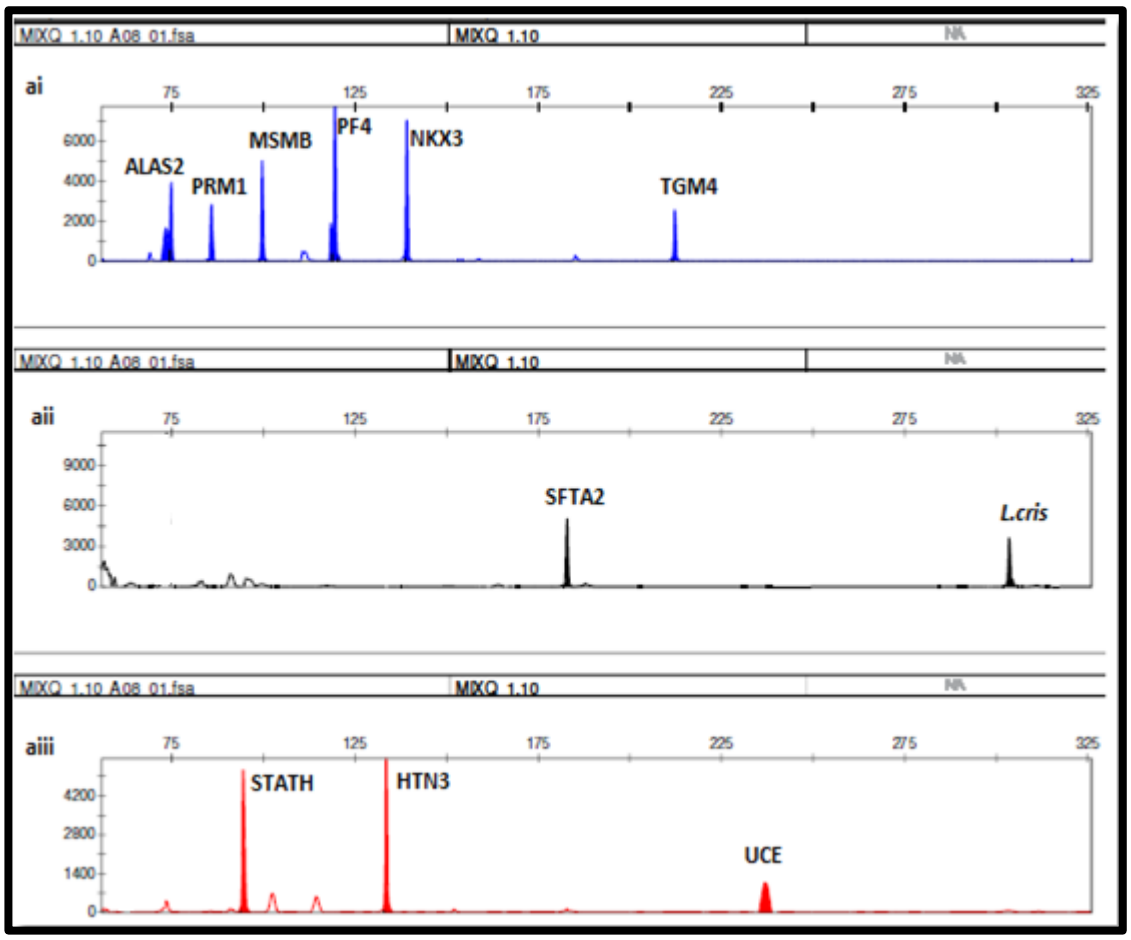

Figure 5.26iv: EPG showing mixture of all body fluids: blood, saliva, semen and vaginal secretion in proportions with a reduced volume **(10** µl**)** Qiagen PCR mastermix (Qiagen Ltd). Varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl proportion was used. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene. Final reaction volume was **17.2** µl**,** n=5; where n is the number of repeats of experiment.

5.4.9i Comparison of reduced and full reaction volume results

Figures 5.26i-iv display the results of full and reduced reaction volumes. As expected, both multiplex mix used (Qiagen PCR and Platinum PCR multiplexes) displayed varied results with each reaction volume. These effects were evident mostly in semen markers PRM1, NKX3-1, and TGM as low RFUs were generated with reduced reaction volume using Qiagen multiplex mix (Figure 5.26i). An improved EPG was generated with reduced and full volume Platinum multiplex mix, however, with artefacts (Figures 5.26ii, iii). A more improved result was generated with full volume Qiagen PCR mastermix with minimal artefacts (Figure 5.26iv).

5.4.10 Cycle number

Extracted mRNA was evaluated using 28, 30, 33 and 35 amplification cycles. Partial profile was generated for 28-cycle amplification as only one semen marker was expressed. Saliva markers had reduced peak heights and there was no expression for any of the vaginal secretion markers (Figure 5.27i). An improved profile was generated with 30 cycles of amplification (Figure 5.27ii) while many artefacts were observed at 35 cycles of amplification (Figure 5.27ii). Optimal amplification was recorded at 33 cycles with all semen, blood, saliva and two vaginal secretion markers expressed (Figure 5.27iii).

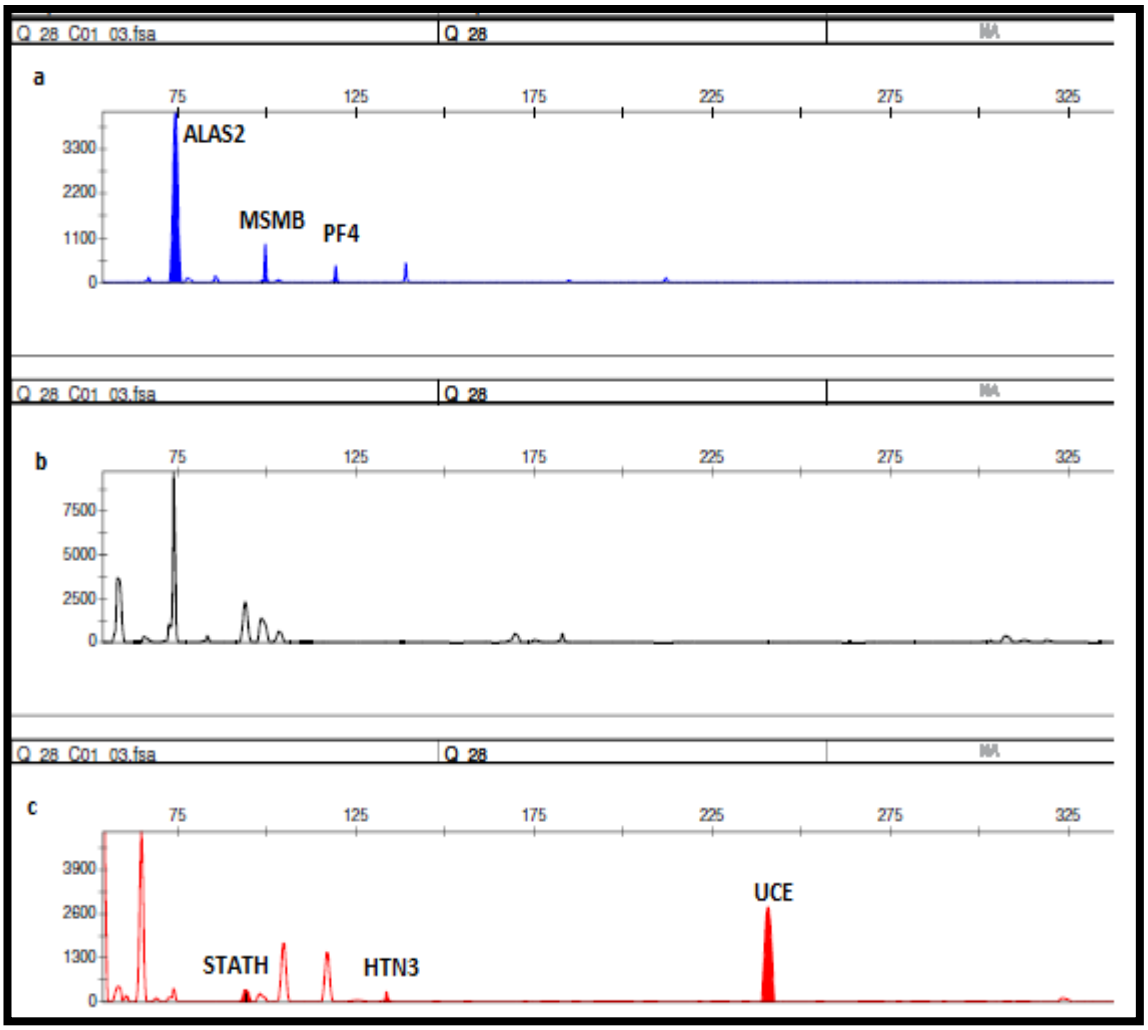

Figure 5.27i: EPG showing amplification of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and **3.5** µl primermix at **58 ºC Tm, 28** cycles. (a) blood and semen, (b) vaginal secretion, (c) saliva and reference gene, n=5; where n is the number of repeats of experiment.

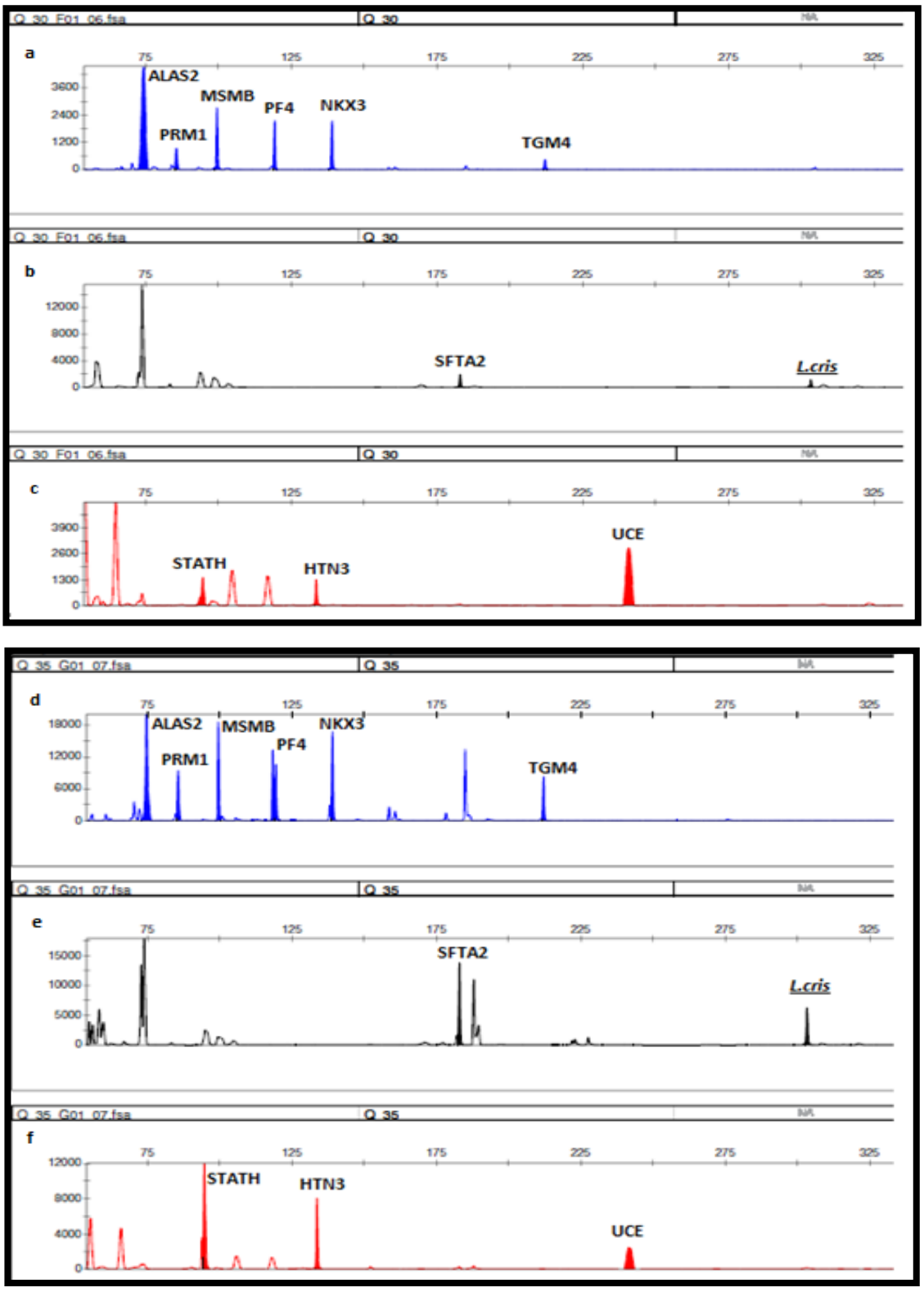

Figure 5.27ii: EPG showing amplification of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and a volume of **3.5** µl primermix at **58 °C Tm 30** (a-c) **and 35** (d-f) cycles. (a/d) blood and semen panel, (b/e) vaginal secretion panel, (c/f) saliva and reference gene panel, n=5; where n is the number of repeats of experiment.

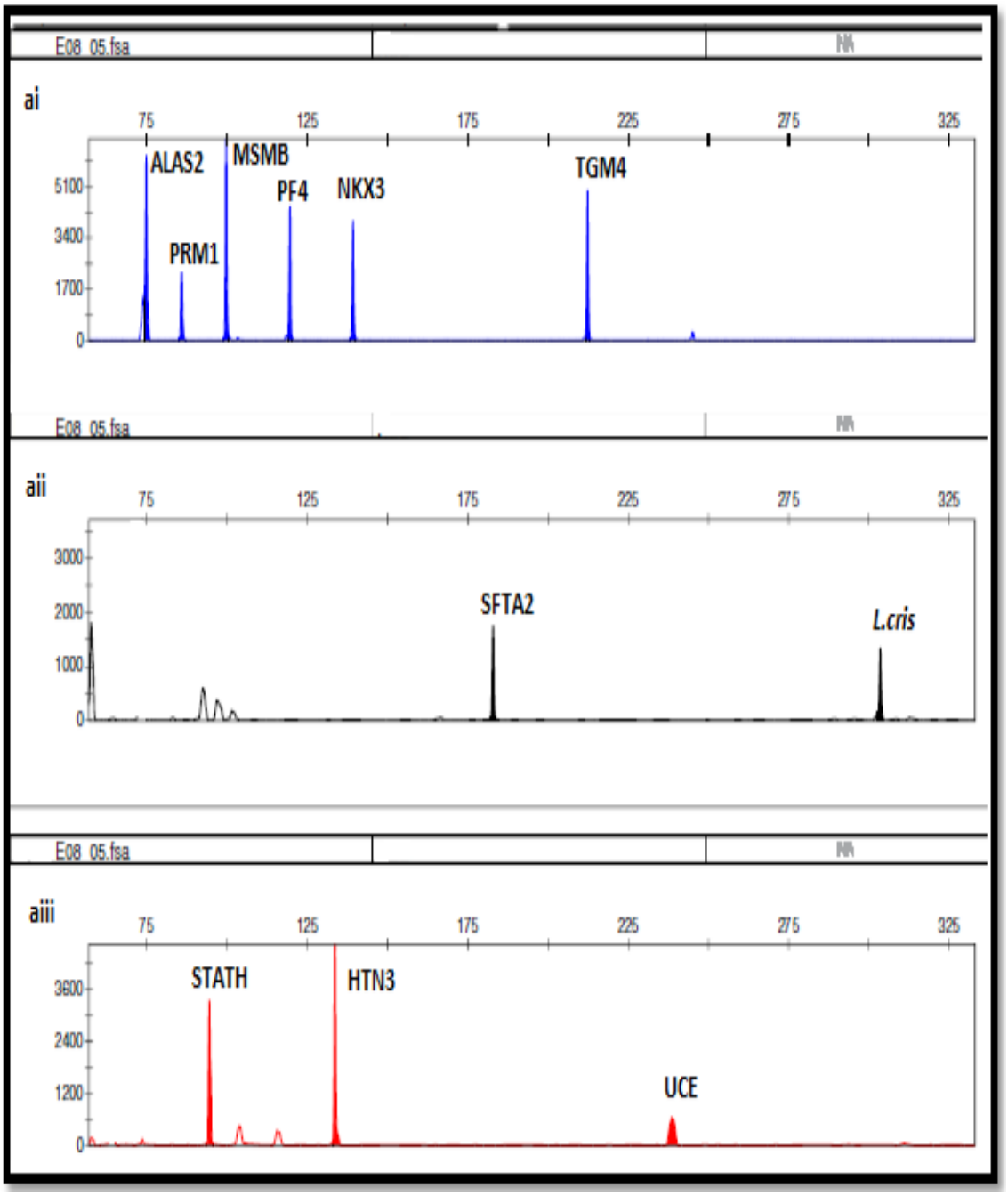

Figure 5.27iii: EPG showing amplification of all body fluids: blood, saliva, semen and vaginal secretion in varied concentration of each sample: semen: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl using **10** µl Qiagen PCR mastermix (Qiagen Ltd) and **3.5** µl primermix at **58 °C Tm, 33** cycles. (ai) blood and semen, (aii) vaginal secretion, (aiii) saliva and reference gene, n=5; where n is the number of repeats of experiment.

5.4.11 Species specificity

Human DNA samples from blood, saliva and bone were tested for specificity of the assay. A few peaks were observed but were not within the range of the body fluid specific markers. Furthermore, mammalian samples from horse, pig, wood mouse, rat, and rabbit was amplified with the assay. In addition, no peaks were observed within the range of body fluid specific markers.

5.4.12 Negative controls

Negative controls were prepared with every batch of sample amplified. There was no amplification in any of the negative controls (See Figure 5.28i).

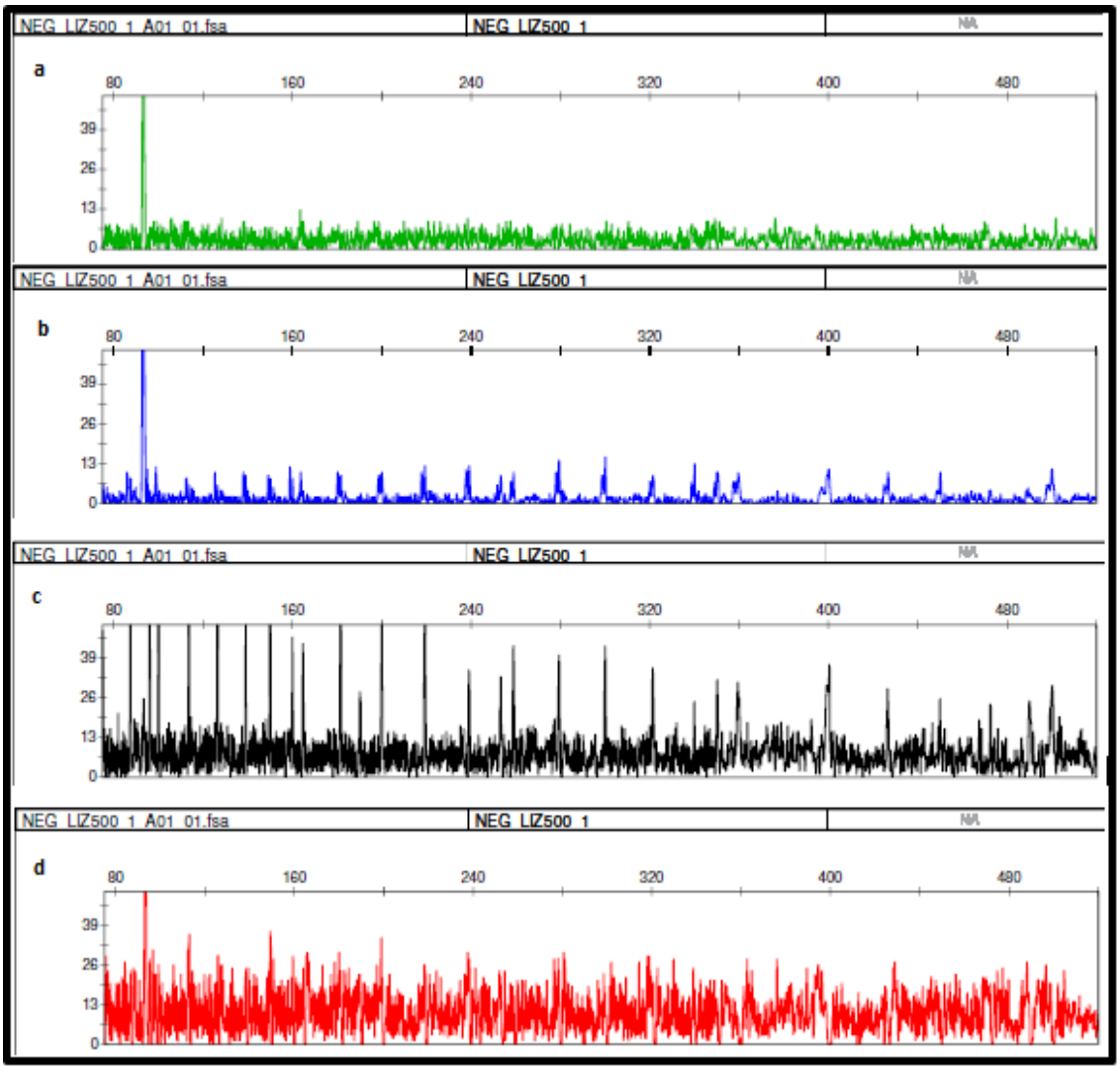

Figure 5.28i: EPG showing negative control and RFU's determined for each dye channel. (a) Yakima yellow, (b) FAM, (c) ATTO550, (d) ATTO565, n=5; where n is the number of repeats of experiment.

Table 5.3: Heat map of validation experiment and body fluid expression under different reaction conditions (Summary of validation study in Chapter 5)

Body fluid	Blood		Saliva		Semen				Vaginal secretion				Reference genes	
	ALAS2	PF4	STATH	HTN3	PRM1	MSMB	NKX3-1	TGM4	SFTA2	MUC4	*L. cris*	CRYP2B7P1	UCE	ACTB
Degradation														
Sensitivity														
Reproducibility														
Mock casework														
Reduced vol.														
Mixtures														
Separate multiplex														
Diff. mastermix														
Cycle number														
Spp. Specificity														
Reduced primer														

Key: Full expression/observed not observed Partial expression/sporadically observed

Furthermore, LIZ 500 and LIZ 600 size standards were used for marker size detection. LIZ 500 displayed the expected size for every marker; however, there was a maximum of one base pair difference in size of markers when LIZ 600 size standard was used.

Minimum and maximum peaks were noted for all the negative controls and used to establish the limit of detection for each panel. Two separate calculations were done as marker sizes were detection on ABI 3500 using LIZ 500 and LIZ 600 size standards.

5.5 Limits of detection (LOD) and quantitation (LOQ)

The lowest concentration of sample (LOD) that can be detected, but not necessarily quantified was calculated. In addition, limit of quantitation (LOQ) which is the lowest concentration of sample that can be determined with acceptable precision and accuracy was also calculated using the formula below:

LOD = (Average peak height + (3 x Standard deviation of the noise))

LOQ = (Average peak height + (10 x Standard deviation of the noise))

Table 5.4: Results obtained from 50 PCR negative controls analysed with LIZ500 at 1RFU across four dye channels.

Dye	Blue (FAM)	Yellow (ATTO550)	Green (YAKIMA YELLOW)	Red (ATTO565)
Minimum RFU	1	2	1	2
Maximum RFU	68	61	52	56
Average RFU	23.48	23.8	22.39	21.34
SD	5.09	5.15	4.01	4.05
Ave. RFU + 3SD	38.75	39.25	34.42	33.49
Ave. RFU + 10SD	74.38	75.3	62.49	61.84
2 x (Ymax – Ymin)	**134**	**118**	**102**	**108**

From table 5.4, the limits of detection for blue, yellow, green and red panels are **134 RFU, 118 RFU, 102 RFU and 108 RFU** respectively using LIZ 500 size standard.

Table 5.5: Results obtained from 30 PCR negative controls analysed with LIZ600 at 1RFU across four dye channels.

Dye	Blue (FAM)	Yellow (ATTO550)	Green (YAKIMA YELLOW)	Red (ATTO565)
Minimum RFU	1	1	1	1
Maximum RFU	62	54	45	48
Average RFU	20.01	18.03	16.52	16.91
SD	5.68	4.66	3.15	3.76
Ave. RFU + 3SD	37.05	32.28	25.97	28.19
Ave. RFU + 10SD	76.81	63.63	48.02	54.51
2 x (Ymax – Ymin)	**122**	**106**	**88**	**94**

Table 5.5 displays the limits of detection for blue, yellow, green and red panels, which are **122 RFU, 106 RFU, 88 RFU and 94 RFU** respectively using LIZ 600 size standard.

5.6 Statistical Analysis

Statistical analyses were carried out in R studio using 'R' statistical software. Blood, semen and saliva samples prepared on glass slides, cotton cloth and knives, and exposed to environmental insults were analysed using ANOVA (Analysis of variance). The model was used to test if there was any difference in means of sample quantities prepared and recovered from glass slides, cotton cloth and knives.

Given the null hypothesis:

$\mathbf{H_0}$ = "*There are no significant differences in the degradation rates of samples prepared on glass slides, cotton cloth and knives*", the null hypothesis is rejected if $p<0.05$ and accepted if $p>0.05$.

In order to determine the ideal type of model, the samples were tested for normal distribution using qq norm plot (see Figure 5.29a). The data appeared to be normally distributed.

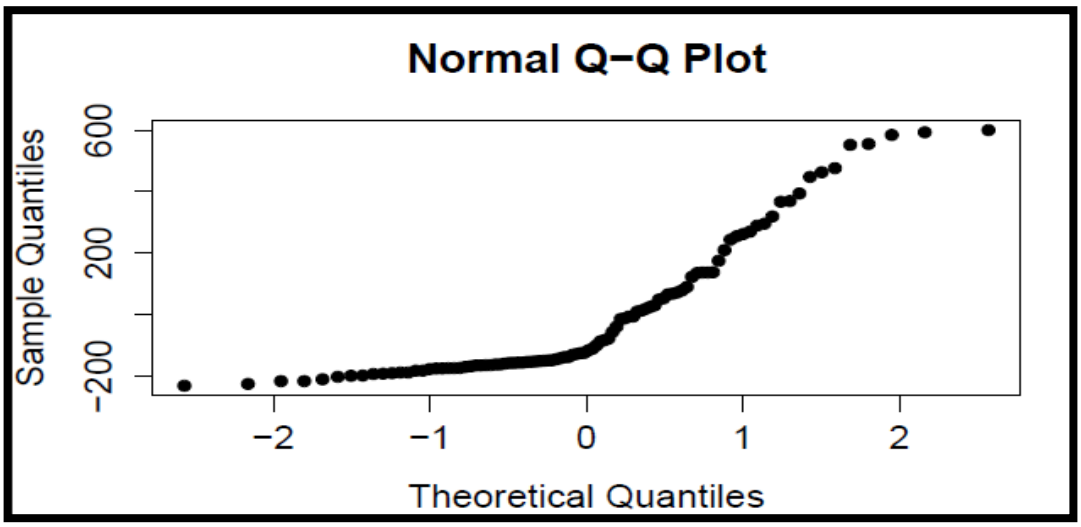

Figure 5.29a: qq-norm plot of saliva samples showing the normal distribution of data generated from the uncontrolled degradation of saliva and semen that were spotted on cotton cloth and knife, p>0.05, n = 33; where n equals the number of samples.

For saliva samples, there was no significant difference (p>0.05) between quantity of mRNA recovered from samples prepared on cotton cloth and knife ($F_{1,96}$ = 0.37, p =0.54). Saliva mRNA recovered from knife (mean = 284.75 RFU) were not significantly more than those recovered from cotton cloth (mean = 256.47; TukeyHSD: p=0.54), (See box plot, Figure 5.29b).

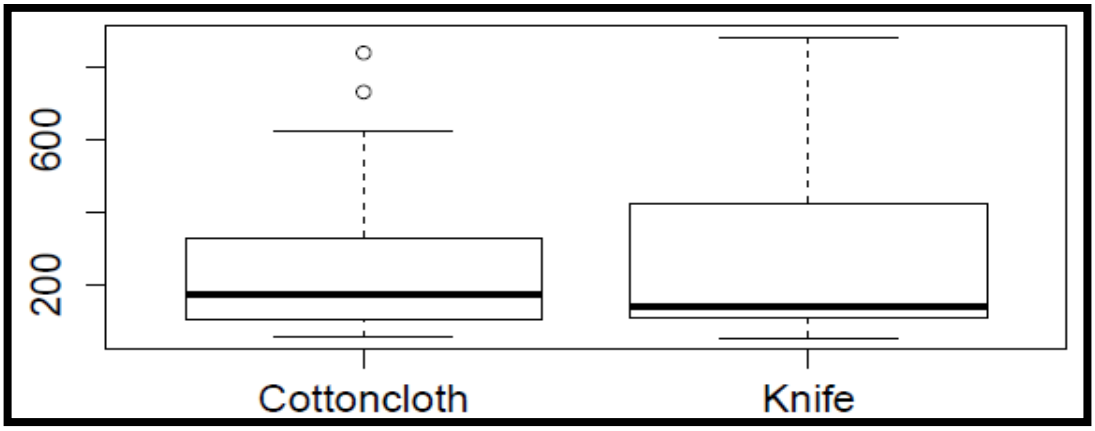

Figure 5.29b: Boxplot showing interactions between peak heights (RFU) and substrates (saliva samples prepared and recovered from cotton cloth and knife), n =5, where n is the number of repeats of experiments, p>0.05.

There was a significant difference (p<0.05) between quantity of semen mRNA recovered from samples prepared on cotton cloth, glass slide and knife ($F_{2.167}$ = 72.95, p <0.001).

Semen samples recovered from glass slide (mean = 7585.18 RFU) was significantly greater than cotton cloth samples (mean=941.43RFU). Knife samples were intermediate (mean =1265.52), but not significantly different from cotton (Tukey HSD: $p>0.05$). See Figure 5.30a,b below.

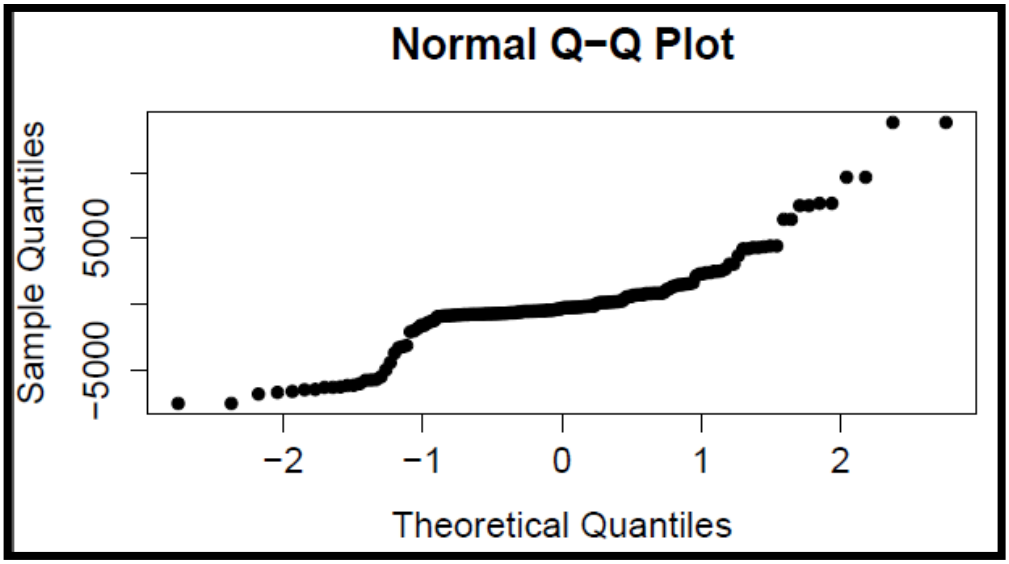

Figure 5.30a: qq-norm plot of semen samples showing the normal distribution of data generated from the uncontrolled degradation of saliva and semen spotted on cotton cloth, glass slide and knife, $p>0.05$, n = 33; where n equals the number of samples.

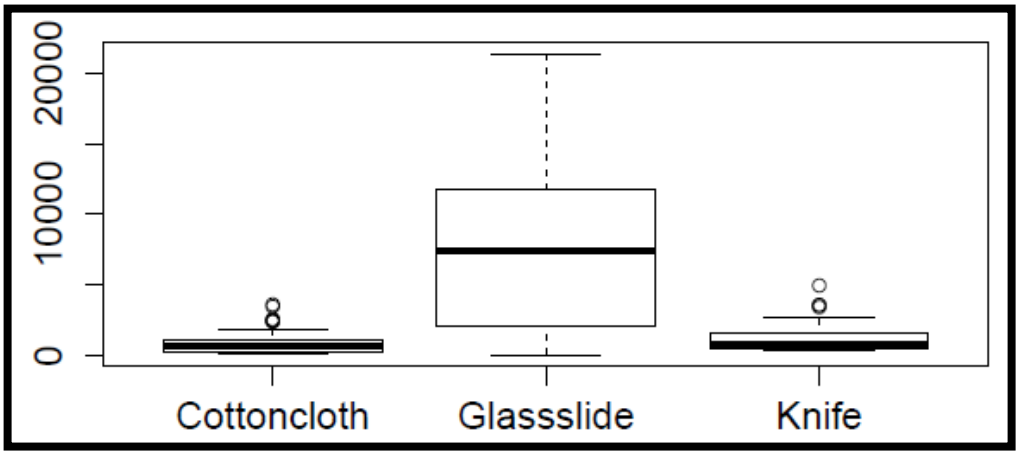

Figure 5.30b: Boxplot showing interactions between peak heights (RFU) and substrates (semen samples prepared and recovered from cotton cloth, glass slides and knife), n =5, where n is the number of repeats of experiments, $p<0.05$ for cotton and knife compared to glass slide.

In addition, there was a significant difference ($p<0.05$) between quantity of blood mRNA recovered from samples prepared on cotton cloth, glass slide and knife ($F_{2.119}$ = 0.37, p

<0.001). Blood mRNA recovered from cotton cloth (mean = 2371.89 RFU) being significantly more than glass (mean=541.09RFU) and knife being intermediate (mean =1373.14), but not significantly different from cotton (Tukey HSD: p=0.05). See Figures 5.31a and 5.31b.

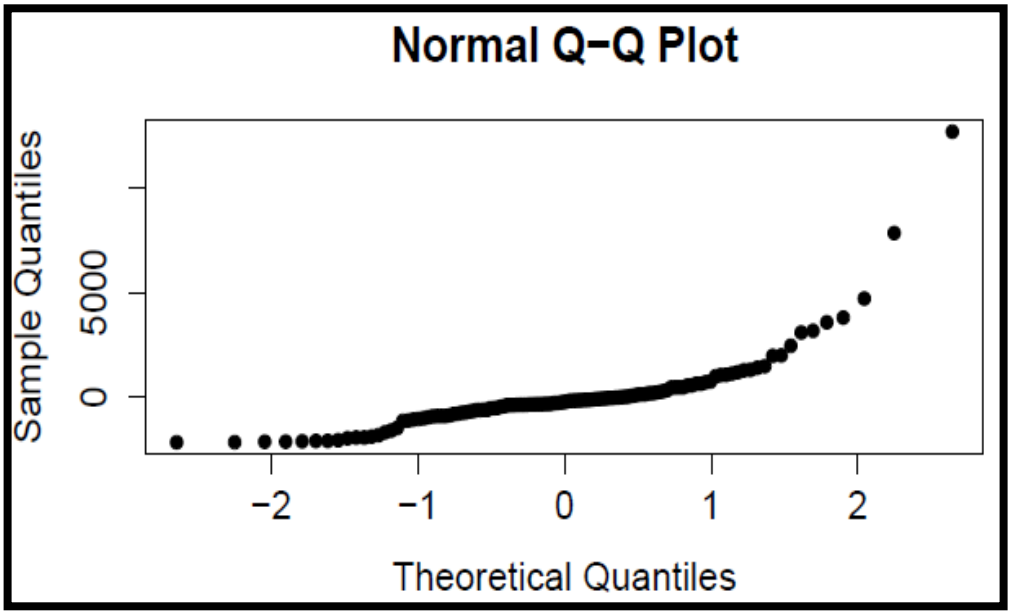

Figure 5.31a: qq-norm plot of blood samples showing the normal distribution of data generated from the uncontrolled degradation of saliva and semen spotted on cotton cloth, glass slide and knife, p=0.05, n = 33; where n equals the number of samples.

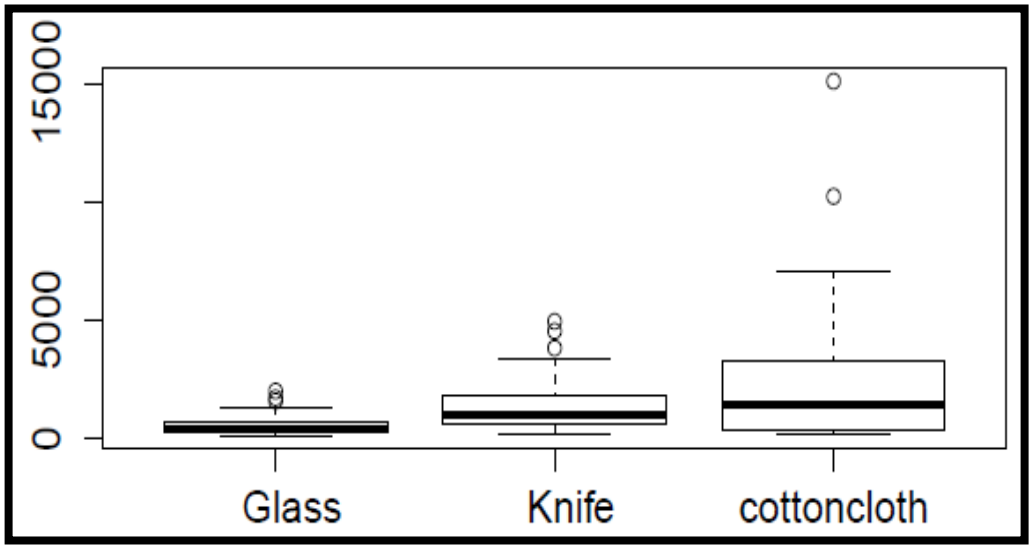

Figure 5.31b: Boxplot showing interactions between peak heights (RFU) and substrates (blood samples prepared and recovered from cotton cloth, glass slides and knife), p=0.5, n =5, where n is the number of repeats of experiments.

5.7 Final validated conditions for the developed assay

Upon adequate optimisation and validation, 11 markers were consistently expressed for their respective target body fluids. These include ALAS2 and PF4 (Blood), PRM1, MSMB, NKX3-1 and TGM4 (Semen), STATH and HTN3 (Saliva), SFTA2 and *L. cris* (Vaginal secretion) and UCE (Reference gene). ACTB reference gene, MUC4, and CRYP2B7P1 vaginal secretion markers displayed either low or no expression through the validation process.

Optimised reaction conditions include: 0.3 ng/µl, saliva: 0.3 ng/µl, blood: 1.3 ng/µl and vaginal secretion: 1.8 ng/µl, **10** µl Qiagen PCR mastermix (Qiagen Ltd) and **3.5** µl primermix at **58 ºC Tm** (See Figure 5.27c). Cycling parameters and CE conditions are highlighted in Chapter 2, Tables 2.10.1 and 2.9 respectively.

5.8 Discussion

This chapter describes the developmental validation of a 14-marker mRNA assay for forensic identification of human body fluids. A synopsis of the overall analyses is represented in Figure 5.27c and Table 5.3. As most forensic samples are recovered in a compromised state, with varying degree of degradation because of microbial and environmental impact, the developed assay was robust, enabling each of the body fluids to be detected without ambiguity and non-specificity.

Reference genes, which were selected as positive controls, displayed varied expressions throughout the course of the study. While UCE was uniformly expressed, ACTB was only expressed in the reproducibility and independent multiplex studies, which was inconsistent with the reports of Ghani and Rogaeva, (2013) and Lindenbergh, *et al.,* (2012). The expression of a minimum of one reference gene marker indicates optimal PCR conditions and that no single reference gene is ideal for body fluid identification (Vandesompele, *et al.,* 2002). While several authors (Lindenbergh, *et al.,* 2012; Juusola, and Ballantyne, 2005; Cossu *et al.,* 2009; Roeder and Haas, 2013) have reported cross reaction of their body fluid specific markers with non-target body fluids, the 14-markers in this study displayed high specificity with no cross reactivity noticed with the non-target body fluids. (Roeder and

Haas, 2013) reported a majority of the markers in their assay being detected at low frequencies and in non-target body fluids, hence, the need to develop a scoring system that was used to identify the body fluids. Similarly, sporadic expression of blood markers was determined in saliva by (Lindenbergh *et al.,* 2012) which the authors described to occur because of possible presence of blood in the saliva samples.

Both blood markers (ALAS2 and PF4) were expressed throughout the course of the experiment without cross reactivity with non-target body fluids. A similar result was seen with saliva markers. Although, an added transcript of STATH for saliva was reported by (Juusola and Ballantyne, 2005) which was explained to be a possible representation of a spliced Histatin isoform, this was not detected in this study; both saliva markers (STATH and HTN3) were expressed in their target body fluids. Only two out of four vaginal secretion markers (*L. crispatus*, SFTA2, MUC4 and CRYP2B7P1) were consistently expressed in this study. These were SFTA2 and vaginal bacterial marker *L. crispatus.* The composition of vaginal secretion is complex as it is mostly affected by the menstrual cycle and which in turn has an effect on the hormonal component of the sample donor (Gipson *et al.,* 1999; Fleming *et al.,* 2003). Although these factors were not considered during sample collection, it had no effect on the expression of vaginal secretion samples and no cross-reactivity occurred. A cross-reactivity was reported by Haas *et al.,* (2012) and Song *et al.,* (2015) with vaginal secretion markers and saliva samples; the authors described this to be due to both vaginal secretion and saliva originating from identical source –the epithelial cell. Vaginal bacterial marker, *L. cris.* was consistent in expression and did not cross react with non-target body fluids. This was expected theoretically, as the bacteria is known to be specific to vaginal. There has been reports of non-stability of its components during menstrual cycle due to variation in numbers of lactobacilli (Eschenbach *et al.,* 1989; Gupta *et al.,* 1998; Keane *et al.,* 1997). Although, expressed consistently, non-stability could not be determined with certainty, as menstrual cycle of participants was not put into consideration when samples were donated. All four semen specific markers were expressed consistently for semen samples only with no cross reaction with non-target body fluids; this is in concordance with Lindenbergh *et al.,* (2012) and Roeder and Haas, (2013).

Sensitivity test on the assay established all the body fluid markers to be highly sensitive (See Figures 5.1-5.4). A similar result was observed for the specificity test on the body fluids. Sample from human bone, pig tissue and DNA from mammals -horse, wood mouse, rabbit, dog and rat were tested. Reference gene UCE was expressed in all the samples. In addition, only bone samples displayed expression of four peaks, however, these were not in the range of any body fluid specific markers. Conversely, other authors have reported cross reactivity of their assay with primate samples, this was concluded to be as a result of very close evolutionary relatedness between the primates and humans (Haas *et al.,* 2011; Haas *et al.,* 2012).

In order to control sample variations that could occur, equimolar concentration of mRNA extracts from blood, semen, vaginal secretion and saliva was used for the controlled degradation in the laboratory. This was done over six weeks in incubator and under UV light. While mRNA samples degraded in incubator at 56 ^{o}C showed little or no significant level of degradation, samples degraded quicker and more significantly under UV light. This was found to be inconsistent with the speculation of Hall *et al.,* (2014), and Sirker *et al.,* (2016) that there are radiation-absorbing molecules in the cytoplasm, which could prolong samples subjected to UV-induced degradation. This speculation cannot be totally faulted as the authors used fresh samples and not mRNA extracts. The degradation study using the mRNA markers was novel. While most degradation studies were carried out in controlled environmental conditions, mostly at room temperature, therefore this was replicated and then an extended study on uncontrolled environmentally degraded samples was carried out. This tested the developed mRNA marker based assay using highly degraded samples. The results obtained from this further established the robustness of the assay. Table 5.2 summarises the expression of blood, saliva and semen that were prepared for uncontrolled degradation study over the period of 51 days.

The two blood markers were expressed up to the last day of the experiment while semen and saliva displayed only sporadic expression after day 30 (Table 5.2). In theory, it is known that RNAs degrades faster compared to DNA due to the presence and activities of ubiquitous RNases. However, mRNA could still be recovered from saliva and semen samples

until day 30 and blood throughout the course of the experiment. This was expected as mRNA expression in blood has been previously demonstrated in 23- year old bloodstain, although using a different marker HBB (Kohlmeier and Schneider, 2012).

Semen and saliva samples only displayed sporadic expression after day 30. Even though Sirker *et al.,* (2016) demonstrated the expression of semen samples throughout their entire study, this was carried out in a controlled environment. A similar result was reported by Haas *et al.,* (2009), Noreault-Conti, (2007), Setzer *et al.,* (2008) with semen samples stored in 1-2 years. Saliva markers were also sporadically observed after day 30. This was consistent with the results of Setzer *et al.,* (2008) and Sirker *et al.,* (2016) where the authors suggested that saliva markers were highly sensitive to hydrolytic damage. Fordyce *et al.,* (2013) further argued that when samples are dehydrated, RNases activities are inhibited, which in turn shields the nucleic acids against degradation. Also discussed was the presence of stronger N-glycosidic bonds and depyrimidation process in RNA molecules, enabling them to resist hydrolytic depurination and prevention of phosphodiester bond hydrolysis. These reasons could be valid as to why mRNA was still expressed in blood, semen and saliva after being exposed to such harsh environmental conditions.

Efforts were made to not to overload the PCR with cDNA as this could result in a major PCR failure. The results obtained from mixture studies showed that each body fluid was still detectable irrespective of the mixture ratios. Though there were a few artefacts –mainly pull-ups that were noticed in the EPGs generated, there was no cross-reaction with non-target body fluids. Several studies have reported cross-reactivity with non-target body fluids especially with an increased amount of template (Cossu *et al.,* 2009; Haas *et al.,* 2011; Fleming and Harbison, 2010), thereby leading to false positive and negative identification of body fluids. A similar result was reported by Moore *et al.,* (2016) where the authors described artefacts observed in PowerPlex Y23 kit associated with excess background quantities of female DNA.

Further attempt was made to balance the peak heights generated during the validation test. Despite optimising all possible parameters, with the main parameter being the template

quantity, it was nearly impossible to balance the peak heights. This could be due to differences in the expression of mRNA in cell types, individuals and pathological conditions (Bauer 2008; Carson *et al.,* 2002; Raj and van Oudenaarden, 2008; Whitehead, 2005).

In conclusion, methods that are more appropriate were adopted during the development of the mRNA assay during this study. Analyses in separate multiplexes were carried out first to study cross reactivity, which was reported for mRNA markers earlier (Lindenbergh *et al.,* 2012; Roeder and Haas, 2013; Song *et al.*, 2015). It had been determined in previous studies that no single mRNA marker is capable of identifying a particular body fluid; hence, the 14-plex mRNA assay was developed, with a minimum of two markers specific for a particular body fluid. The high sensitivity and specificity displayed by the assay during the degradation study further established its robustness in forensic body fluid identification. The developmental validation carried out on the developed 14 plex mRNA assay for body fluid identification has been carried out for the first time showing that the SWGDAM standards for STR kit validation are appropriate for mRNA marker based CE assays though further developments might also be required.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND SCOPE OF FUTURE WORK

6.1 General discussion

The main aim of this project was to evaluate genetic markers in order to identify five commonly encountered body fluids, which include blood, semen, saliva, vaginal secretion and menstrual blood. The outcomes of the study are assessed in this section with reference to the original aims (Section 1.11.2; *Chapter 1).*

A majority of crimes involve the deposition of body fluids at the scene. The identification of body fluid provides important clues in casework. Numerous studies have been reported recently on the significance of body fluid identification. Traditionally, techniques involved in forensic body fluid identification rely mainly on enzymatic reactions, which require high template, are prone to contamination with presumptive tests being carried out before a confirmatory test, are non-specific, and body fluid mixtures are difficult to analyse. New techniques have been reported which might enable body fluids to be identified with more robustness and limited ambiguity.

In this study, 10 commonly used reference genes were evaluated and analysed for specificity, sensitivity and efficiency. This was the first such study at the best of our knowledge. This was necessary before the start of the project as most authors reported the use of their reference genes to be because of reports from other studies but no in depth evaluation of the reference genes used in body fluid, identification was reported. Ghani *et al.,* (2013) had recommended adequate evaluation of reference genes for fitness of purpose before being used. For this study, the markers included 18S-rRNA, ACTB, UCE, B2M, OAZ1, RPS29, S-15, β-Actin, TEF and GAPDH. Initially, marker efficiency was tested using SYBR Green chemistry. Although this has major disadvantages of no multiplexing ability and fluorescence on binding to any available dsDNA which overall lowers the efficiency of the system, it was still considered as it is less expensive and still a robust enough method for initial evaluation. The hypothesis was that if the markers were able to attain 95-100% efficiency with SYBR Green chemistry, better efficiency would be expected for Taqman probes. Efficiency test using SYBR Green showed five markers with less than 95-100% efficiency and these were GAPDH, 18S rRNA, β-Actin, OAZ1 and S15. Contrary to this finding,

these were previously reported by several authors to be ideal reference markers for forensic body fluid identification; however, they failed the efficiency test. The reasons for the markers not attaining the set threshold were discussed earlier (Section 3.10.1; *Chapter 3).* ACTB, UCE, RPS29, B2M and TEF passed the efficiency test and were selected for evaluation using Taqman probe detection method. Taqman probes were designed and real time PCR assays were optimized for each reference gene marker. The sensitivity test using Taqman probes displayed detection down to 25-picogram input RNA with the exception of TEF, which was detected at 100 picogram of input RNA. Furthermore, these markers were tested on 6-month old body fluid samples stored at room temperature. Ten samples were used from each of the body fluids for this purpose, from five volunteers.

ACTB, B2M, UCE and RPS29 were stably expressed across the whole samples, with TEF had the least expression (Table 3.1a/b). This result with TEF was contrary to the findings of Fleming and Harbison, (2010) where the authors' reported a high expression for the marker. The variable expression of all the reference genes across the body fluids tested further established the reports of Meller *et al.,* (2005), Pohjanvirta *et al.,* (2006), and Vandesompele *et al.,* (2002) that no single reference gene is universally stable. Hence, the use of multiple reference genes in an assay had been recommended. This study empirically showed the value of using more than one reference gene in an assay.

Chapter four of this study highlighted the design and optimization of the developed mRNA assay. Initially, 32 markers were selected from relevant literature. These comprised; seven markers (Glyco-A, ALAS2, PF4, HBB, CCL5, PPBP and SPTB) for blood; four markers (MSX-1, SFRP, MMP11 and LEFTY2) for menstrual blood; six markers (PRM1, PRM2, TGM4, MSMB, and NKX3-1, SEMG) for semen; six markers (SFTA2, MUC4, *L. crispatus, L. gasseri,* CRYP2B7P1, and HBD1) for vaginal secretion; and three markers (HTN3, FDCSP and STATH) for saliva; and five markers (B2M, ACTB, RPS29, TEF and UCE) for reference genes. Optimization was carried out first using non-florescent primers and results analysed on agarose gel. Two different multiplex mix were also used, Platinum PCR mastermix (Life technologies Ltd) and Qiagen PCR mastermix (Qiagen Ltd). Upon optimization and agarose gel analysis, the markers that displayed clearly visible bands on the gel (of expected sizes)

were noted and the forward primers for these were then labelled with fluorescent dyes (Figure 4.8).

First phase of optimization resulted in 19 body fluid specific markers, which were expressed in their respective body fluids (Table 2.12, 4.3). Five separate multiplex were developed, one for each body fluid. This was required in order to establish the specificity of each marker to the body fluid of origin. For the vaginal secretion assays, all the markers except HBD1 were expressed in vaginal secretion at 33 cycles. However, CRYP2B7P1 and HBD1 were only expressed in positive control sample from Qiagen Ltd. The non-expression of HBD1 was in concordance with the results of Lindenbergh *et al.,* (2012) as the authors reported similar issue with expression of the marker. Also, Roeder and Haas, (2013) and Jakubowska *et al.,* (2013) reported the marker non-expression in saliva samples, and in menstrual blood samples (Juusola and Ballantyne, 2005). These could infer the markers non-specificity for vaginal secretion samples.

Blood and Saliva assays were optimised at 35 amplification cycles. CCL5 and PPBP were not expressed in blood, contrary to the reports of Park *et al.,* (2013). The three saliva markers were expressed in saliva, which further supported the report of Park *et al.,* (2013), Lindenbergh *et al.,* (2012), and Park *et al.,* (2013).

All semen markers were expressed in semen sample. Only MSX-1 was expressed in menstrual blood. MMP11 (Roeder and Haas, 2013) and SFRP4 (Lindenbergh *et al.,* (2012) were both reported specific for menstrual blood; however, they were not expressed in this study. Further optimization was carried out on the five-plex assay using fluorescently labelled dyes and the results analysed on the ABI 3500 genetic analyser. All blood markers were expressed apart from SPTB, which has been previously reported not optimal for mRNA profiling of blood (Kohlmeier and Schneider, 2012).

All vaginal secretion markers were expressed except MUC4, which displayed low expression. The marker had been reported to cross- react with saliva (Mara *et al.,* 2012; Nussbaumer *et al.,* 2006; Cossu *et al.,* 2009; Haas *et al.,* 2009). All saliva and semen markers expressed in saliva and semen respectively which was consistent with the reports of

Lindenbergh *et al.,* (2012), Roeder and Haas, (2013), Mara *et al.,* (2012), and Park *et al.,* (2014). LEFTY2 was not expressed in menstrual blood sample. Roeder and Haas, (2013) reported this marker to be detected in non-target body fluids as well. This was not an issue in this project as menstrual blood markers were not included in the developed assay.

In order to reduce sample processing time and cross-contamination between primers, all the five markers that displayed high expression in the individual five-plex assay were combined into a single multiplex assay. A 14-marker assay, comprising of ALAS2 and PF4 (blood), STATH and HTN3 (saliva), PRM1, TGM4, MSMB, NKX3-1 (semen), ACTB and UCE (reference genes), CRYP2B7P1, SFTA2, MUC4 and *L. crispatus* (vaginal secretion) was developed and optimised. Menstrual blood markers were excluded from the final assay, as they were not expressed despite all modifications to the concentration and cycling conditions. In addition, ACTB was highly expressed in all experiments carried out and led to pull up peaks in the blue, red and yellow panels of the electropherograms (Figure 4.17). The primer concentration was modified by reducing the working concentration; however, this did not make much difference in the high expression of ACTB. Overall, all markers were expressed in their target body fluids except for MUC4 in vaginal secretion that did not display expression.

A developmental validation was carried out for the 14-marker assay, this was essential in order to establish robustness of the developed assay. SWGDAM guidelines were used to conduct this validation. Because of pull-ups displayed by ACTB during assay optimisation both reference genes (ACTB & UCE) were modified by labelling the forward primers with ATT0565, which is a red florescent dye, therefore making the green panel free for possible incorporation of more markers. The validation experiments commenced with sensitivity study where all body fluid samples were diluted in different proportions, with the least being 1:10 and highest 1:3000 (Table 5.1). All body fluid samples were detected down to 1:3000 dilution except vaginal secretion that displayed expression up to 1:1500. In addition, specificity test was carried out using samples from bone, pig tissue, human DNA (blood), horse, wood mouse, rabbit, dog and rat. None of the body fluid specific markers was

expressed in any of these samples. However, reference genes were expressed which was a normal and expected occurrence.

For the degradation experiments, the main aim was to validate the robustness of the developed mRNA assay on samples that have undergone various stages of degradation and not to make comparisons between the rates of degradation that have occurred using different mechanisms. Degradation study was carried out in a controlled manner in the laboratory at room temperature, in the incubator and under the UV-light. In addition, samples that were exposed to outdoor environmental conditions (uncontrolled) were analysed. UV light was found to have a significant effect on sample degradation compared to temperature. Although Hall *et al.,* (2014) and Sirker *et al.,* (2016) speculated that UV would have a lower effect on degradation as there are radiation- absorbing molecules in the cytoplasm which could shield samples from UV rays, thereby prolonging their rate of degradation. These authors used fresh samples in their study whereas mRNA samples were used in this study. mRNA samples were used as these compared sample concentrations as against sample volume as used by other authors. By using sample concentrations and keeping it constant, it is possible to monitor the rate of degradation, which is not possible when volumes are used. For instance, when same aliquots of blood sample is extracted for mRNA, different concentrations would be obtained. This infers that when the authors mentioned prepared their mock casework samples, the rate of degradation would be monitored efficiency, knowing that each aliquot of body fluid sample prepared contained different amount of mRNA.

In the uncontrolled degradation experiment, body fluid samples were identified up to at least day 30 of 51. Interestingly, to current knowledge, this was the first attempt to carry out an extensive degradation study for evaluating the genetic markers for body fluid identification. There were only a few experiments that had been reported studying the stability of RNA over a longer period with the longest being detection of blood in 23-year old stains (Kohlmeier and Schneider, 2012), however, these studies were all carried out in controlled environment. This further established the robustness of the developed assay for body fluid identification as theoretically; RNA's are not stable and thus easily degraded

because of ubiquitous ribonucleases in the environment. Mixture study displayed no cross-reaction of the markers in non-target body fluids. In order to reduce the cost of reagents and time of analysis, different mastermix were optimised and the primer concentrations and volumes were reduced. While Platinum PCR mix also yielded an acceptable result, Qiagen mastermix (Qiagen Ltd) was found to be optimal for this developed assay.

Finally, attempts were made to obtain balanced peak heights for all the markers in the assay. This was not possible as other authors have reported it to be due to difference in mRNA expression in different cells, and because of pathological conditions (Bauer, 2008; Carson *et al.,* 2002; Raj *et al.,* 2008; Whitehead, 2005). However, as the expression of the markers in various body fluids can be ascertained through the presence or absence of a peak in a CE based system; the features of the peaks for individual loci were consistent and comparable across the samples and the assay led to identification of body fluids in different laboratories.

6.2 Conclusion

Initial multiplex design in this study targeted a total of 35 body fluid specific markers and reference genes (See Figure 6.1). However, after extensive validation process, a final assay, which comprise 14-markers, was developed (See Figure 6.2). In addition, all previously developed mRNA assays for body fluid identification have reported a minimum of one cross-reaction of one or more markers, which were detected in non-target body fluids. There were no cross reactions with non-target body fluids in the developed assay reported in this thesis. The reference gene marker study, together with extensive study of body fluids that were subjected to non-controlled environmental degradation, have sufficed for the novelty of this project. Thus, the developed novel assay was found to be robust and fit for purpose.

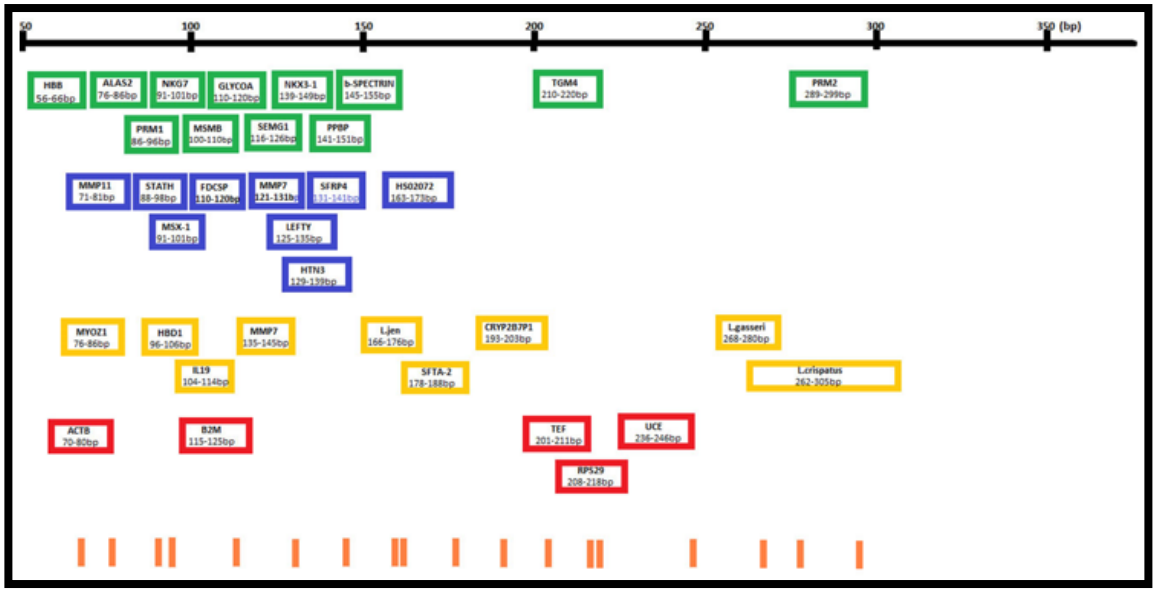

Figure 6.1: Initial schematic representation of the developed multiplex assay consisting of 35 body fluid specific marker and reference genes. Each marker was represented based on their expected amplicon size before optimisation (Drawn by hand).

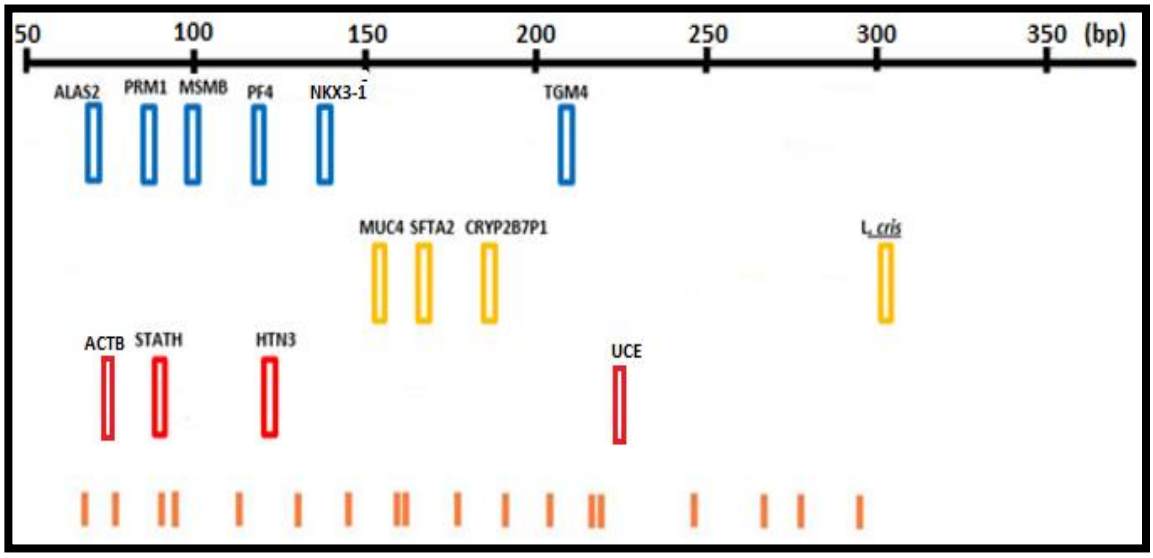

Figure 6.2: Final schematic representation of the developed multiplex mRNA assay. This consists of 14 mRNA markers that have been adequately optimised, validated, and reported fit for purpose. Each marker was represented based on the established amplicon size after optimisation. The blue panel represents blood and semen markers; yellow panel represents vaginal secretion markers; red panel represents saliva and reference genes (Drawn by hand).

6.3 Pitfalls and troubleshooting issues

1. The main limitation of the project was sample collection. Most potential donors were hesitant to participate in the study, especially with intimate samples such as semen, vaginal secretion and menstrual blood. This slowed down the project though a fair number of samples were analysed and the degradation study was the largest such study to date.
2. Unlabelled primers that gave results when optimised initially and analysed using gel electrophoresis did not give similar results when they were fluorescently labelled. These primers were sent to the vendor company where they were resynthesized at additional cost.
3. The genetic analyser broke down at the crucial stage of the study and this took about 4 months to be rectified. This slowed down the project a great deal as it was the main equipment used to generate final EPG of gene expression in the body fluids.

If I have to repeat this study, the following will be put into consideration in order to save time, cost of primer synthesis and reagents.

1. Enhanced education of prospective participants. This could be achieved by organising a presentation to keep them informed of the nature of the project as well as what is required of them. This procedure is necessary as poster advertisement was could not contain all the required information.
2. The intending participants would be reassured that other than the intended scope of the study, the results generated will not be submitted into the police database as this was a major concern for them.
3. A test sample of all reagents and consumables required will be requested from respective companies concerned. This will prevent unforeseen costs should any of their products fail.

6.4 Scope for Future Studies

With additional time and resources, the following is recommended:

a) RNA analogues with a high affinity and 'locked' ribose ring could be used for more sensitivity and specificity of the designed assay. In its chemistry, for every incorporated nucleic acid probe, there is an increase of 2-8 ^{o}C in melting temperature (*T*m) of the duplex. The overall high *T*m thus increases affinity for the complementary strand which overall would increase the specificity of the markers in PCR.

b) Next Generation Sequencing (NGS) has gained popularity in body fluid identification recently; the same could be implemented and compared in parallel with the performance of the developed assay.

c) Samples obtained and analysed for effects of environmental insults on body fluids prepared at different times compared to the controlled degradation experiments carried out in the lab. With more time and available resources, a parallel experiment could be used to compare the effects of both conditions simultaneously on body fluid identification.

d) Also, it is important to incorporate more markers, both miRNA and mRNA could increase the robustness of the assay. However, adequate care would be required to avoid cross-reactivity with non-target samples.

e) Measure total protein in each body fluid sample.

f) Investigate the effect of environmental insults on the different samples and then measure the different genes in each body fluid.

CHAPTER SEVEN

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Appendix

This section highlights the appendices related to the reported project.

Appendix 1: COSHH and HIRA training.

Appendix 2: UCLan ethical committee research approval.

Appendix 3: Consent form template for voluntary participants.

Appendix 4a: EPGs of semen sample on glass slide incubated at 36 ^{0}C for 2 weeks.

Appendix 4b: EPGs of blood sample spotted on glass slide, incubated at 36 ^{0}C for 4 weeks.

Appendix 4c: EPGs of blood sample spotted on facial mask, incubated at room temperature for 2 weeks.

Appendix 4d: EPGs of blood sample spotted on a sterile scalpel, incubated at room temperature for 2 weeks.

Appendix 4e: EPGs of blood sample spotted on a plastic bag, incubated at room temperature for 4 weeks.

Appendix 4f: EPGs of saliva sample spotted on a glass slide, incubated at 36 ^{0}C for 4 weeks.

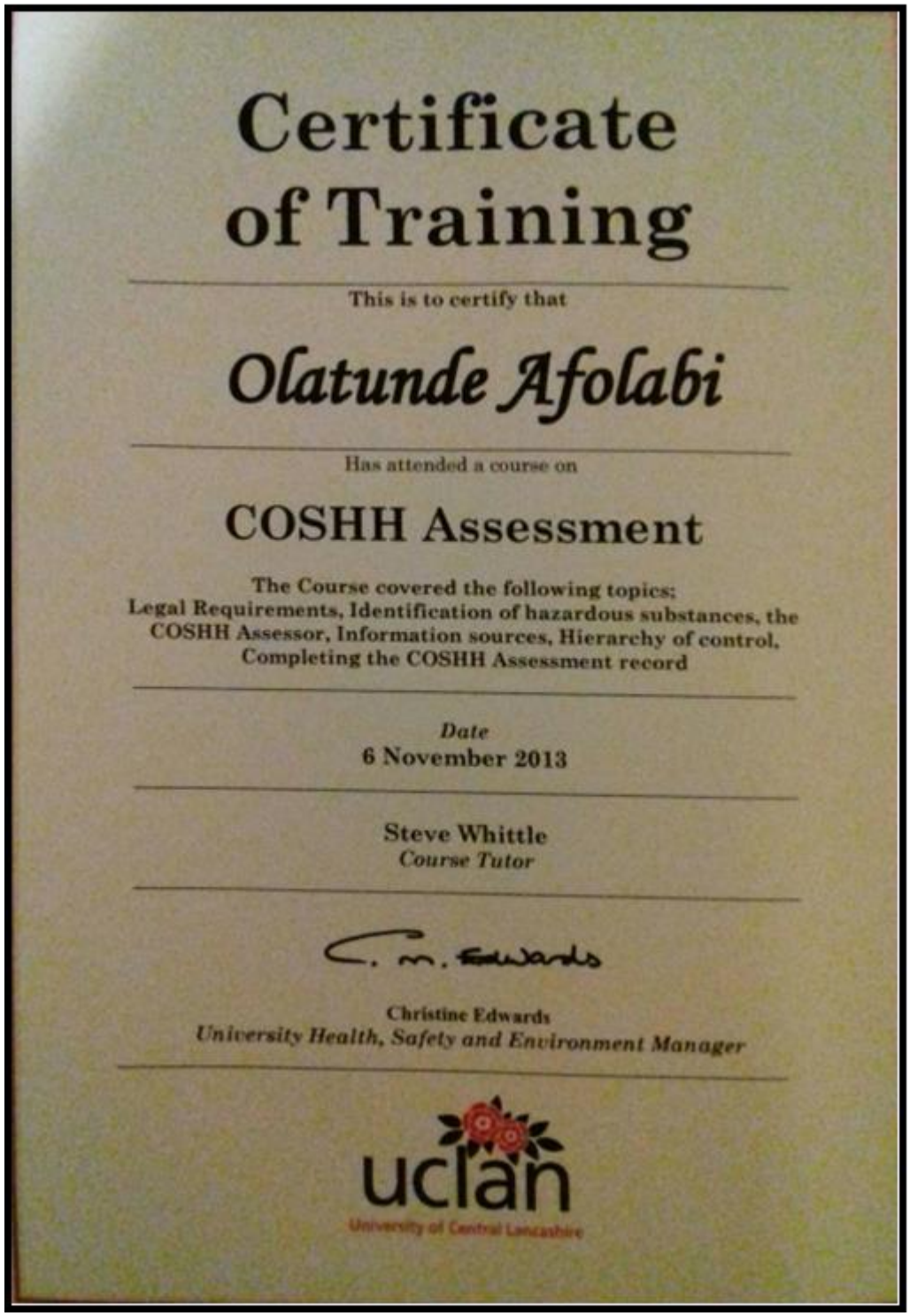
Appendix 4g: EPGs of saliva samples spotted on a straw and incubated at room temperature for 6 weeks.

Appendix 4h: EPGs of saliva samples spotted on a can of coke and incubated at room temperature for 6 weeks.

Appendix 5: Conferences attended and Proceedings.

Appendix 6: Publications.

Appendix 1: COSHH assessment training certificate



Certificate of Training

This is to certify that

Olatunde Afolabi

Has attended a course on

COSHH Assessment

The Course covered the following topics;
Legal Requirements, Identification of hazardous substances, the COSHH Assessor, Information sources, Hierarchy of control, Completing the COSHH Assessment record

Date
6 November 2013

Steve Whittle
Course Tutor

C. M. Edwards

Christine Edwards
University Health, Safety and Environment Manager

uclan
University of Central Lancashire

Appendix 2: UCLan ethical approval letter from Health, Safety and Ethics Committee

uclan
University of Central Lancashire

21 July 2014

S Hadi / Abimbola Olatunde Afolabi
School of Forensic & Investigative Sciences
University of Central Lancashire

Dear Hadi / Olatunde

Re: STEMH Ethics Committee Application
Unique Reference Number: STEMH 232

The STEMH ethics committee has granted approval of your proposal application 'AN EVALUATION OF GENETIC MARKERS FOR FORENSIC IDENTIFICATION OF HUMAN BODY FLUIDS'. Approval is granted up to the end of project date* or for 5 years from the date of this letter, whichever is the longer.

It is your responsibility to ensure that

- the project is carried out in line with the information provided in the forms you have submitted
- you regularly re-consider the ethical issues that may be raised in generating and analysing your data
- any proposed amendments/changes to the project are raised with, and approved, by Committee
- you notify roffice@uclan.ac.uk if the end date changes or the project does not start
- serious adverse events that occur from the project are reported to Committee
- a closure report is submitted to complete the ethics governance procedures (Existing paperwork can be used for this purposes e.g. funder's end of grant report; abstract for student award or NRES final report. If none of these are available use e-Ethics Closure Report Proforma).

Yours sincerely

Tal Simmons
Chair
STEMH Ethics Committee

* for research degree students this will be the final lapse date

NB - Ethical approval is contingent on any health and safety checklists having been completed, and necessary approvals as a result of gained.

Appendix 3: Consent forms for voluntary participants

Consent Form

Consent form for research project "An evaluation of genetic markers for forensic identification of human body fluids" as explained in information sheet dated 25/06/2014.

One copy of this consent form will be given to participant and one kept by the researcher.

I, the undersigned, confirm that (please tick box and initial them as appropriate):

1. I have read and understood the participant information sheet given to me with this form for the above study. ☐
2. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
3. I understand that my participation in this study is voluntary. ☐
4. I understand that I have a week after the collection of the sample to withdraw from the study without giving any reason. ☐
5. The procedures regarding confidentiality have been clearly explained to me. ☐
6. The use of my sample and data in this study, and publications, has been explained to me and I have no objection to that use. ☐
7. I agree to participate in this study. ☐

_____ ___/___/___

Name of Participant Date

_____

Signature

_____ ___/___/___

Signature of one of the below: Date

Research Student: Abimbola Olatunde Afolabi (AOAfolabi@uclan.ac.uk)

Research Supervisor: Sibte Hadi (shadi@uclan.ac.uk)

An evaluation of genetic markers for forensic identification of human body fluids

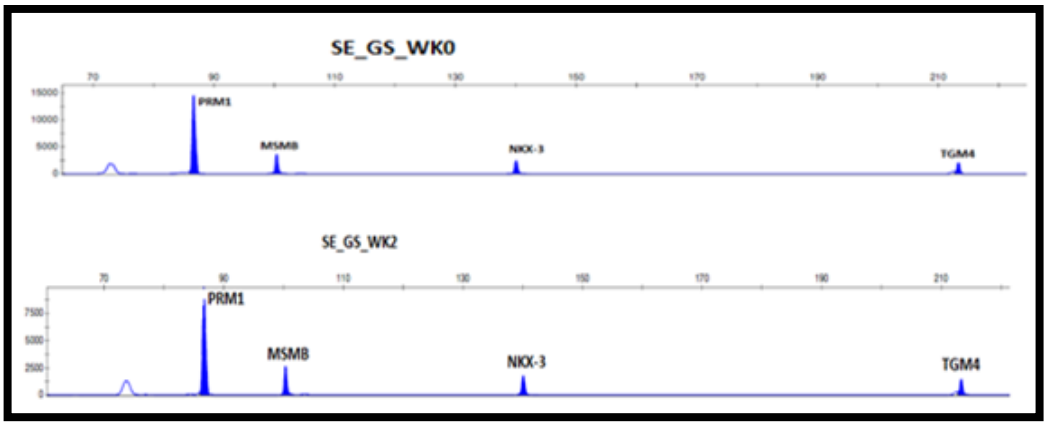
Thank you for agreeing to participate in our study. Your unique reference number is: BFId (Se/Sa/Mb/Vs/Bl) _ _ _ _ _. If you wish to withdraw your data set from the experiment, please contact with your unique reference number:

Dr. Sibte Hadi, School of Forensic and Investigative Sciences, University of Central Lancashire, Preston PR1 2HE

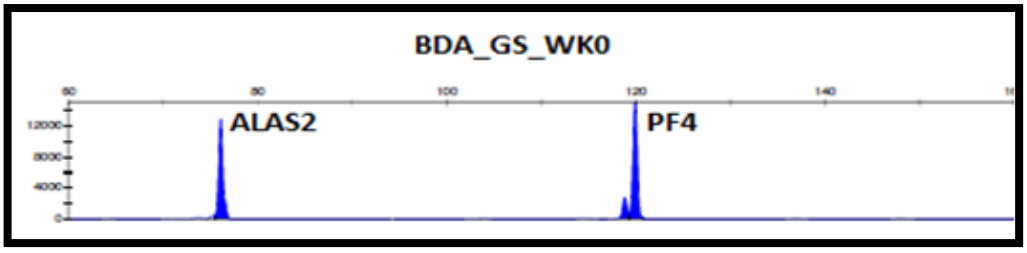
E-mail: shadi@uclan.ac.uk

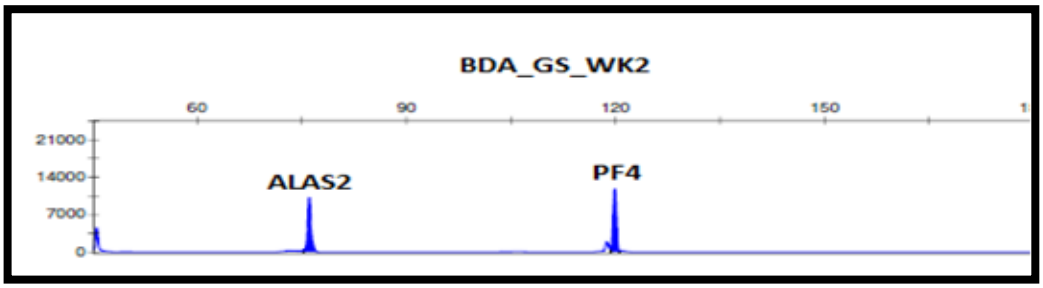
Office Telephone No: 01772894395

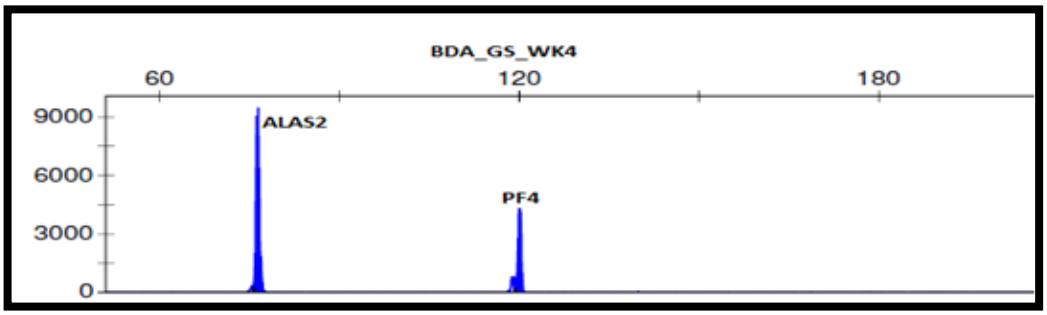
Appendix 4a: EPGs of semen sample spotted on glass slide and incubated at 36 ^{0}C for 2 weeks.



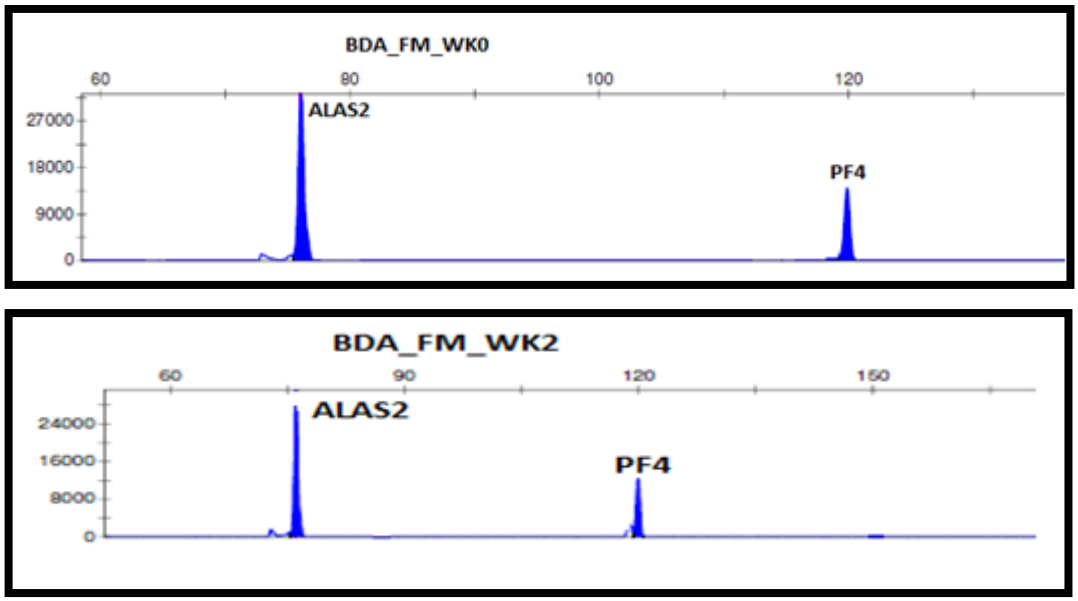
Appendix 4b: EPGs of blood sample spotted on glass slide and incubated at 36 ^{0}C for 4 weeks.



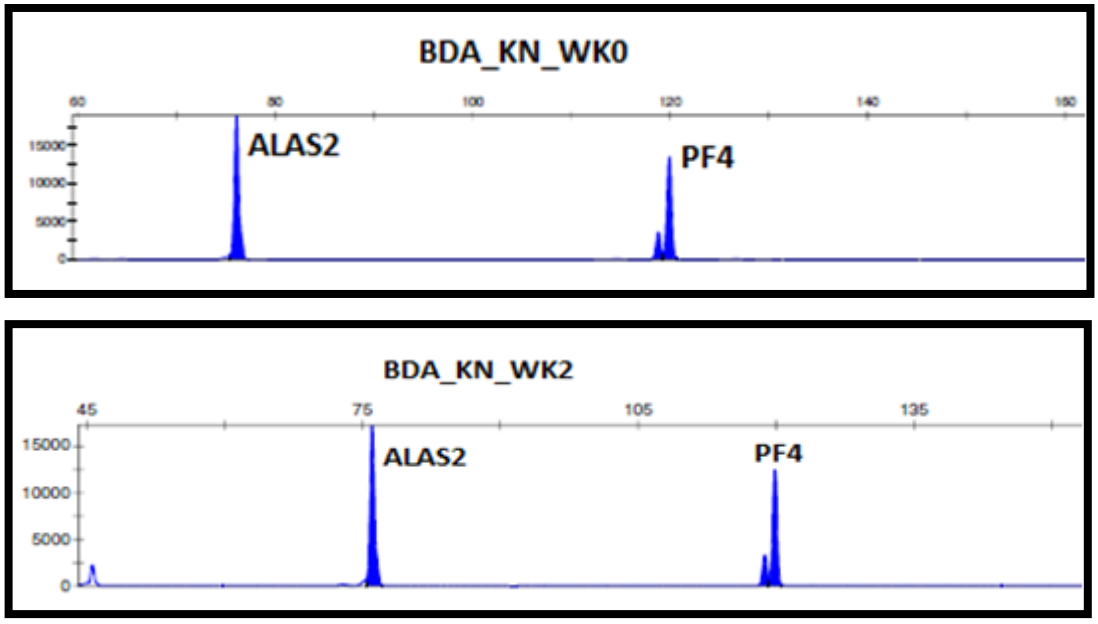




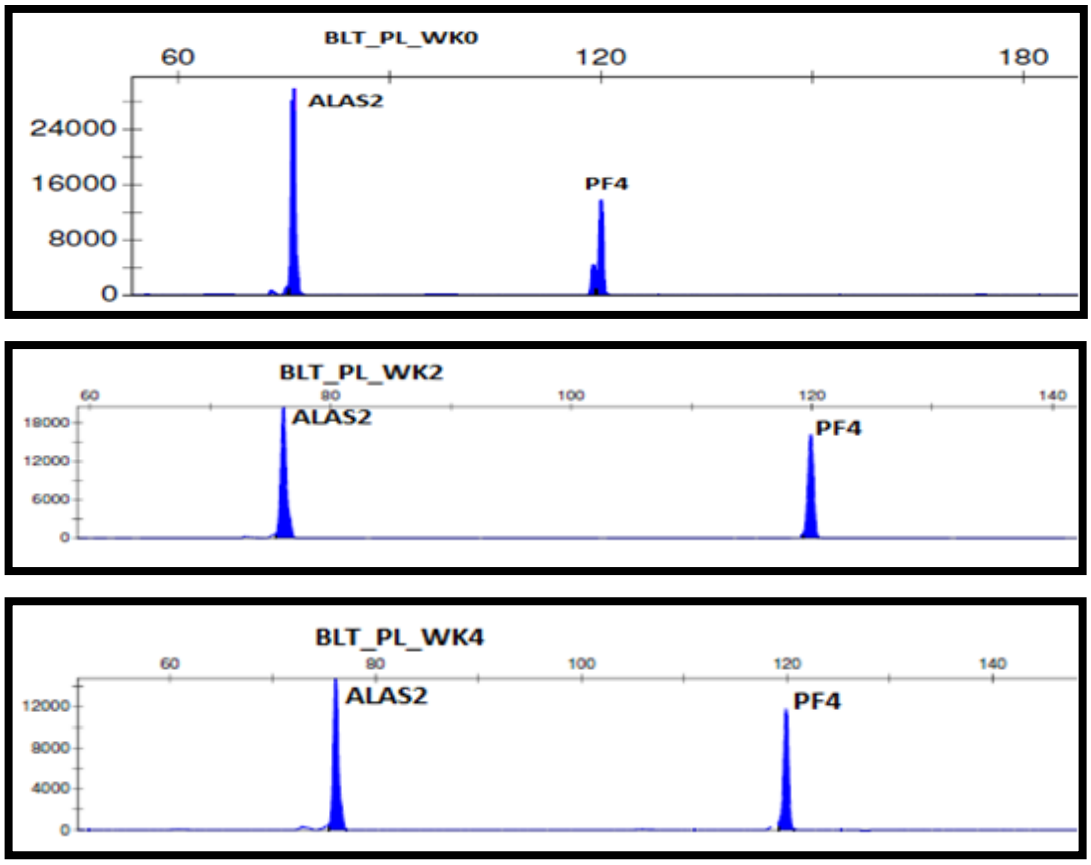
Appendix 4c: EPGs of blood sample spotted on facial mask and incubated at room temperature for 2 weeks.



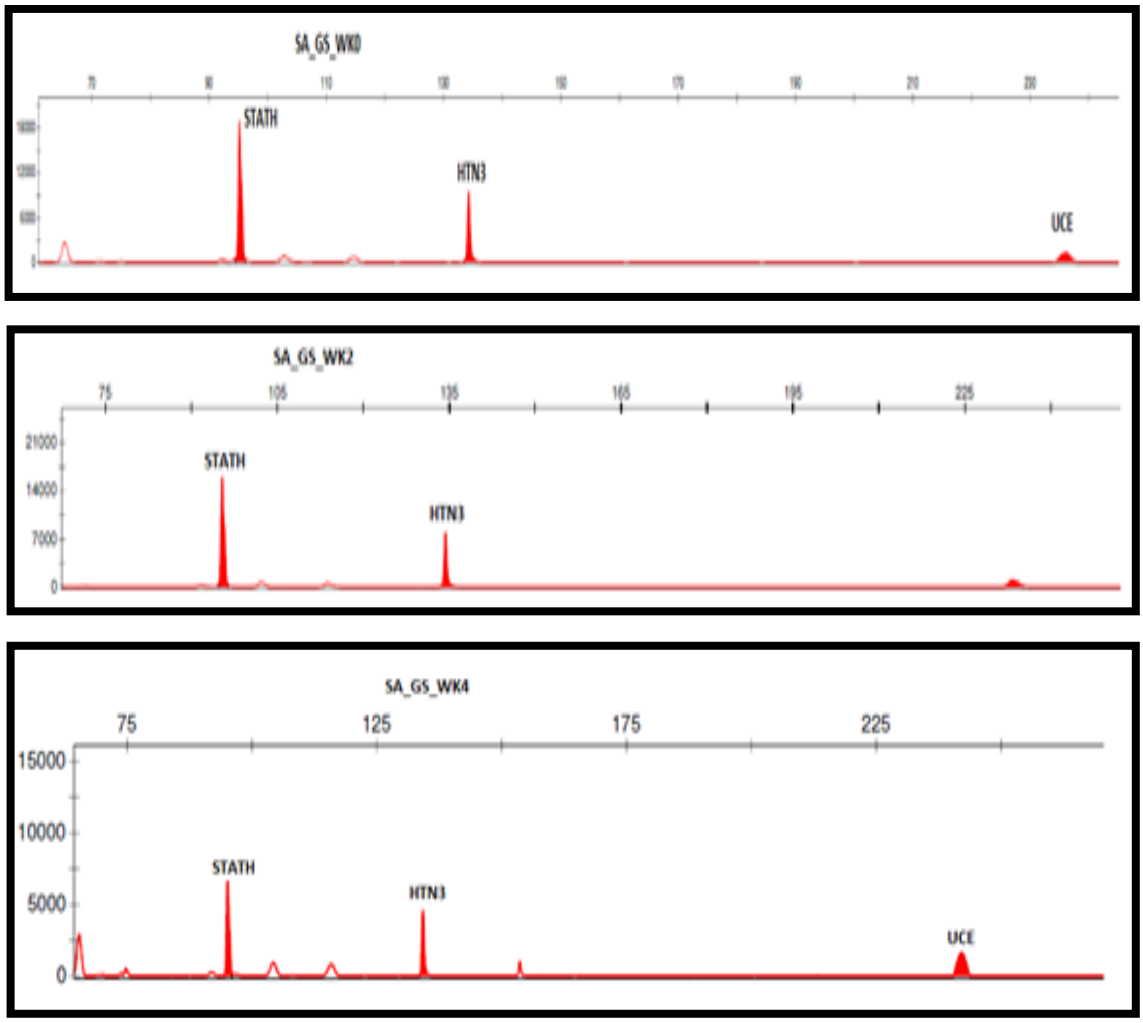
Appendix 4d: EPGs of blood sample spotted on sterile scalpel and incubated at room temperature for 2 weeks.



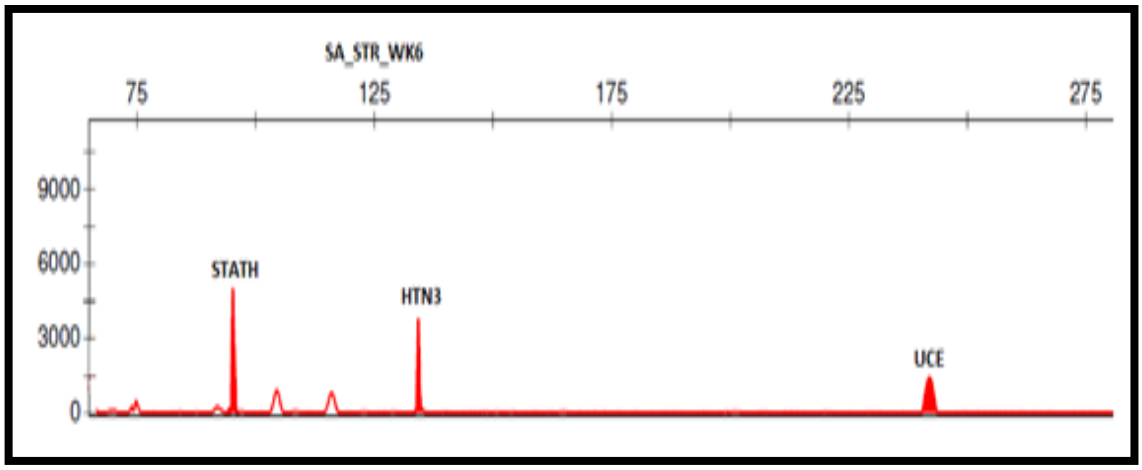
Appendix 4e: EPGs of blood sample spotted on plastic bag and incubated at room temperature for 4 weeks.



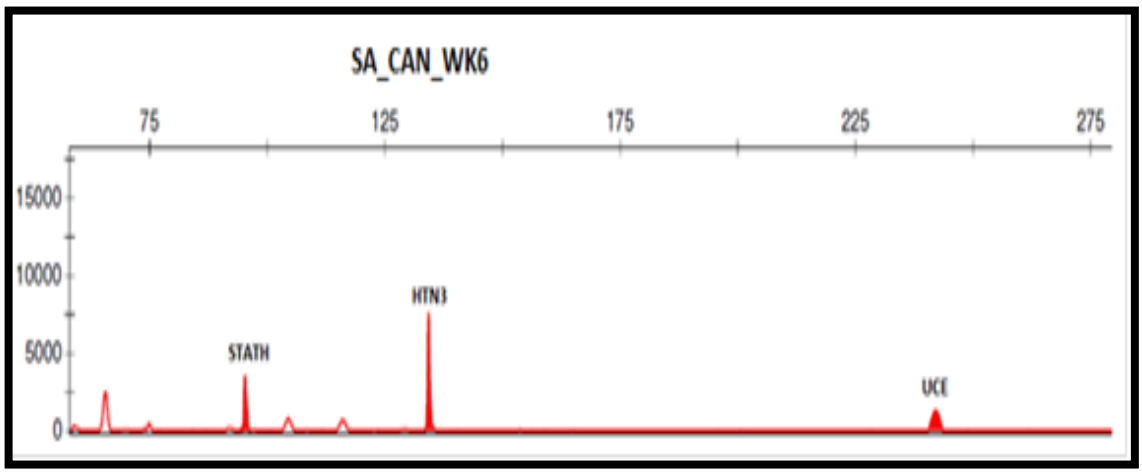
Appendix 4f: EPGs of saliva sample spotted on glass slide and incubated at 36 ^{0}C for 4 weeks.



Appendix 4g: EPG of saliva sample spotted on a straw and incubated at room temperature for 6 weeks.



Appendix 4h: EPG of saliva sample spotted on a can of coke and incubated at room temperature for 6 weeks.



Appendix 5: CE results of primer-mix sent to an independent laboratory for testing.

sample	reverse transcription positive				reverse transcription negative			
	Sample File Name	**Marker**	**Size**	**Height**	**Sample File Name**	**Marker**	**Size**	**Height**
Semen	A01_1.fsa	ACTB	69.6	1441				
3 ul	A01_1.fsa	MSMB	~100	~3600				
	B01_2.fsa	NKX3-1	~139	~3600				
	A01_1.fsa	PF4	118.3	1918				
	A01_1.fsa	PRM1	84.03	3412				
	A01_1.fsa	TGM4	212.05	3850				
	A01_1.fsa	UCE	237.63	3923				
Semen	B01_2.fsa	ACTB	70.23	2667				
3 ul on swab	B01_2.fsa	MSMB	99.49	3861				
	B01_2.fsa	NKX3-1	138.41	3890	H04_2-.fsa	NKX3-1	139.08	643

	B01_2.fsa	PF4	116.05	1099	H04_2-.fsa	PF4	117.06	1547
	B01_2.fsa	PRM1	83.91	2861				
	B01_2.fsa	TGM4	212.36	3988				
	B01_2.fsa	UCE	237.64	2870				
Semen	C01_3.fsa	ACTB	70.46	1607				
5 ul	C01_3.fsa	MSMB	98.83	4009				
	C01_3.fsa	NKX3-1	138.22	3792				
	C01_3.fsa	PRM1	84.13	3744				
	C01_3.fsa	TGM4	212.85	2249				
	C01_3.fsa	UCE	237.68	3964				
Semen	D01_4.fsa	ACTB	70.29	2632				
5 ul on swab	D01_4.fsa	MSMB	99.07	3898				
	D01_4.fsa	NKX3-1	138.42	4023				

	D01_4.fsa	PF4	116.57	1114				
	D01_4.fsa	PRM1	84.01	3081				
	D01_4.fsa	TGM4	211.95	4128				
	D01_4.fsa	UCE	237.73	3909				
Semen	E01_5.fsa	ACTB	70.36	748				
10 ul	E01_5.fsa	MSMB	99.79	434				
	E01_5.fsa	NKX3-1	138.93	1950				
	E01_5.fsa	PF4	115.58	1297				
	E01_5.fsa	PRM1	83.91	3362				
	E01_5.fsa	TGM4	212.6	~170				
	E01_5.fsa	UCE	237.65	250				
Semen	F01_6.fsa	ACTB	71.57	2339				
10 ul on swab	F01_6.fsa	MSMB	99.25	4216				
	F01_6.fsa	NKX3-1	138.24	3822	D05_6-.fsa	NKX3-1	139.08	1210

	F01_6.fsa	PRM1	83.69	3447				
	F01_6.fsa	TGM4	212.02	3963				
	F01_6.fsa	UCE	237.85	3881				
menstrual	G01_7.fsa	ACTB	68.65	2029				
blood	G01_7.fsa	ALAS2	75.3	223				
vaginal swab	G01_7.fsa	CRYP2B7P1	201.44	4402				
	G01_7.fsa	MUC4	141.6	4344				
	G01_7.fsa	NKX3-1	~170	139	E05_7-.fsa	NKX3-1	139	1362
	G01_7.fsa	SFTA-2	187.08	4543				
	G01_7.fsa	UCE	238.11	1076				
menstrual	H01_8.fsa	ACTB	70.6	1684				
blood	H01_8.fsa	ALAS2	75.42	371				
vaginal swab	H01_8.fsa	CRYP2B7P1	202	1207				

	H01_8.fsa	MUC4	142.15	1506				
	H01_8.fsa	NKX3-1	139.09	2200	F05_8-.fsa	NKX3-1	139	2219
	H01_8.fsa	PF4	117.58	2818				
	H01_8.fsa	SFTA-2	187.42	747				
	H01_8.fsa	UCE	238.06	423				
menstrual	A02_9.fsa	ACTB	70.07	2261				
blood	A02_9.fsa	ALAS2	74.87	3356				
vaginal swab	A02_9.fsa	CRYP2B7P1	202.11	4408				
	A02_9.fsa	MUC4	141.54	3645				
	A02_9.fsa	NKX3-1	138.71	3647	G05_9-.fsa	NKX3-1	139	3971
	A02_9.fsa	PF4	117.24	2920				
	A02_9.fsa	SFTA-2	187.13	3408				

	A02_9.fsa	UCE	237.67	104				
menstrual	B02_10.fsa	ACTB	70.45	2210				
blood	B02_10.fsa	CRYP2B7P1	202.19	4519				
vaginal swab	B02_10.fsa	MUC4	141.54	4239				
	B02_10.fsa	NKX3-1	138.93	254	H05_10-.fsa	NKX3-1	139.08	1160
	B02_10.fsa	PF4	117.59	943				
	B02_10.fsa	SFTA-2	187.16	4348				
	B02_10.fsa	UCE	237.68	578				
menstrual	C02_11.fsa	ACTB	69.52	931	A06_11-.fsa	ALAS2	74.93	665
blood	C02_11.fsa	CRYP2B7P1	201.88	752				
vaginal swab	C02_11.fsa	MUC4	142.18	1100				

	C02_11.fsa	NKX3-1	139.52	2073	A06_11-.fsa	NKX3-1	138.17	2875
					A06_11-.fsa	PF4	117.86	1087
	C02_11.fsa	SFTA-2	187.03	157				
menstrual	D02_12.fsa	ACTB	70.38	1499	B06_12-.fsa	ACTB	69.69	858
blood	D02_12.fsa	CRYP2B7P1	201.51	4413				
vaginal swab	D02_12.fsa	MUC4	142.79	3465				
	D02_12.fsa	NKX3-1	139	3553	B06_12-.fsa	NKX3-1	138.52	3982
	D02_12.fsa	SFTA-2	187.19	3947				
	D02_12.fsa	UCE	237.64	965				
vaginal	E02_13.fsa	Lcrispatus	307.32	4018	C06_13-.fsa	Lcrispatus	306.96	4243
secretion	E02_13.fsa	NKX3-1	139	228	C06_13-.fsa	NKX3-1	139	359

vaginal swab	E02_13.fsa	PF4	116.17	1079				
vaginal	F02_14.fsa	ACTB	69.09	1255	D06_14-.fsa	ACTB	69.55	875
secretion	F02_14.fsa	CRYP2B7P1	202.01	4557				
vaginal swab	F02_14.fsa	MUC4	141.46	3660				
	F02_14.fsa	NKX3-1	138.25	3463	D06_14-.fsa	NKX3-1	138.43	3941
	F02_14.fsa	PF4	117.25	349				
	F02_14.fsa	SFTA-2	187.45	4374				
	F02_14.fsa	UCE	237.89	833				
vaginal	G02_15.fsa	ACTB	70.56	2771	E06_15-.fsa	ACTB	70.46	165
secretion	G02_15.fsa	CRYP2B7P1	201.31	4571				
vaginal swab	G02_15.fsa	MUC4	141.54	4407				

	G02_15.fsa	NKX3-1	139	1283				
	G02_15.fsa	PF4	117.28	290				
	G02_15.fsa	SFTA-2	186.77	4647				
	G02_15.fsa	UCE	237.67	2622				
vaginal	H02_16.fsa	ACTB	69.4	1886	F06_16-.fsa	ACTB	70.27	304
secretion	H02_16.fsa	CRYP2B7P1	201.9	5208				
vaginal swab	H02_16.fsa	Lcrispatus	306.68	1933	F06_16-.fsa	Lcrispatus	305.47	4427
	H02_16.fsa	MUC4	141.51	4603				
	H02_16.fsa	NKX3-1	139.18	997	F06_16-.fsa	NKX3-1	138.92	2319
	H02_16.fsa	SFTA-2	187.34	5132				
	H02_16.fsa	UCE	238.18	1908				

vaginal	A03_17.fsa	ACTB	70.41	3227				
secretion	A03_17.fsa	CRYP2B7P1	201.09	2313				
vaginal swab	A03_17.fsa	MUC4	141.4	4382				
					G06_17-.fsa	NKX3-1	139.08	1411
	A03_17.fsa	SFTA-2	186.38	2375				
	A03_17.fsa	UCE	237.51	2576				
vaginal	B03_18.fsa	ACTB	70.45	3630				
secretion	B03_18.fsa	CRYP2B7P1	201.57	2261				
vaginal swab					H06_18-.fsa	Lcrispatus	305.93	455
	B03_18.fsa	MUC4	142.52	4687				
	B03_18.fsa	SFTA-2	186.91	2285				

	B03_18.fsa	UCE	237.55	595				
blood	C03_19.fsa	ACTB	70.69	841				
3 ul blood	C03_19.fsa	ALAS2	74.48	1242				
	C03_19.fsa	NKX3-1	138.92	383				
	C03_19.fsa	PF4	116.73	2018				
	C03_19.fsa	UCE	237.6	2647				
blood	D03_20.fsa	ACTB	70.51	1394				
3 ul blood	D03_20.fsa	ALAS2	74.51	1115				
	D03_20.fsa	NKX3-1	138.5	~140				
	D03_20.fsa	PF4	116.91	3742				
	D03_20.fsa	UCE	237.85	2604				

blood	E03_21.fsa	ACTB	70.57	1235				
3 ul blood	E03_21.fsa	ALAS2	74.5	1485				
	E03_21.fsa	NKX3-1	139	2593				
	E03_21.fsa	PF4	117.78	3727				
	E03_21.fsa	UCE	237.82	2604				
blood	F03_22.fsa	ACTB	70.68	2136				
3 ul blood	F03_22.fsa	ALAS2	75.36	391				
	F03_22.fsa	NKX3-1	138.5	~170				
	F03_22.fsa	PF4	118.31	3437				
	F03_22.fsa	UCE	237.16	2575				
blood	G03_23.fsa	ACTB	70.46	274				

3 ul blood	G03_23.fsa	ALAS2	74.59	1544				
	G03_23.fsa	NKX3-1	139	282				
	G03_23.fsa	PF4	117.08	3536				
	G03_23.fsa	UCE	237.24	2652				
blood	H03_24.fsa	ALAS2	75.28	1361				
1 ul blood on cotton fabric	H03_24.fsa	PF4	116.4	1375				
saliva	A04_25.fsa	ACTB	70.34	3991				
10 ul saliva on swab	A04_25.fsa	STATH	96.79	1699				
saliva	B04_26.fsa	ACTB	70.34	3532	H07_26-.fsa	ACTB	70.47	378

5 ul saliva on swab	B04_26.fsa	HTN3	135.99	3902				
	B04_26.fsa	NKX3-1	139	~170				
	B04_26.fsa	STATH	97.26	2827				
saliva	C04_27.fsa	ACTB	70.36	3784				
5 ul saliva	C04_27.fsa	HTN3	135.67	3490				
	C04_27.fsa	MUC4	142.06	635				
	C04_27.fsa	NKX3-1	139	291				
	C04_27.fsa	STATH	97.79	3740				
saliva	D04_28.fsa	ACTB	70.4	270				
5 ul saliva	D04_28.fsa	NKX3-1	138.93	554	B08_28-.fsa	NKX3-1	139	265
saliva	E04_29.fsa	ACTB	70.39	4023				

buccal swab	E04_29.fsa	HTN3	135.79	4028				
	E04_29.fsa	STATH	98.33	3616				
saliva	F04_30.fsa	ACTB	70.32	2619				
buccal swab	F04_30.fsa	HTN3	136.1	3430				
	F04_30.fsa	MUC4	142.17	1657				
	F04_30.fsa	NKX3-1	138.92	762				
	F04_30.fsa	PF4	117.71	1400				
	F04_30.fsa	SFTA-2	186.99	251				
	F04_30.fsa	STATH	97.42	2730				
	F04_30.fsa	UCE	237.41	163				
	C05_neg.fsa	ALAS2	75.94	336				

Appendix 6: Conferences and proceedings

Poster Presentation: Reference genes study: A requisite in qRT-PCR experiments at ISFG conference, Krakow, August 2015.

Attendee/Participant: American Academy of Forensic Sciences (AAFS), Las Vegas, February 2016.

Poster Presentation: Evaluation of genetic markers for forensic identification of human body fluids at Postgraduate Research Conference, University of Central Lancashire, Preston, September, 2016.

Appendix 7: Publications

Reference gene study for Forensic body fluid identification (2015) *Forensic Science International: Genetics Supplement Series* 5, Pages e167-e169.

Current advances in Forensic Body Fluid Identification Techniques: A review (Under preparation).

Developmental validation of a 14-marker mRNA multiplex assay for Forensic Identification of Human Body Fluids (Under preparation).

Evaluation of Genetic Markers for Forensic Identification of Human Body Fluids

O.A. Afolabi[1], A.D. Roeder[2], A. Iyengar[1], S. Hadi[1]

[1]School of Forensic and Applied Sciences, University of Central Lancashire, Preston, UK.

[2]Cellmark Forensic Services, Abingdon, Oxfordshire, UK.

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Aim: To develop a messenger RNA (mRNA) multiplex assay for body fluid identification and investigate its sensitivity and specificity on commonly encountered forensic body fluids.

Introduction

Body fluids such as blood, semen, saliva, vaginal secretion, and menstrual blood are frequently encountered and the adequate identification of the nature of these poses a challenge, especially when evidential samples with minute biological cells are encountered [1].

While DNA profiling tends to identify the perpetrator of a crime, RNA profiling is needed to establish what tissue source the evidential sample originates. This can be achieved by using genetic markers (mRNA) that are highly specific for forensic body fluids. Since each of these body fluids have a unique expression profile, this when studied can aid in unambiguous identification of the body fluids [2].

In this study, a total of 32 common body fluid markers and 10 reference genes were evaluated for their utility in body fluid identification. All markers were studied for specificity, sensitivity and cross reactivity. Expression of the body fluid markers were assayed in 0-6 months old samples stored at room temperature. Furthermore, mock crime scene samples were prepared on glass slides, hand gloves, scalpel blades, facial masks, can of drinks, drinking straw and plastic bags. These were left undisturbed for 6 weeks in a dark, cool cupboard, and incubator at 37 ºC and 56 ºC. All samples were run in duplicate and negative controls were prepared for each batch of samples and analysed.

Materials and Methods

Samples

- Following ethical approval, consented samples were collected from different individuals at University of Central Lancashire, Preston, UK.
- Vaginal secretion and menstrual blood were collected on sterile swabs and dried at room temperature.
- Blood was collected by both fingerpick and venepuncture (without anticoagulant treatment).
- Semen and saliva were collected in 1.5ml Eppendorf tubes and stored at -80 ºC.

RNA extraction and Reverse Transcription

- Total RNA was isolated using RNeasy Mini Plus kit (Qiagen, UK Ltd) according to manufacturers' recommended instructions.
- Post RNA purification and DNase treatment was carried out using Turbo DNA-free kit™ (Life Technologies Corp. UK)
- RNA was quantified using NanoDrop ND-2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE)
- For the reverse transcription reaction, random primers and Superscript III reverse transcriptase (Life technologies, UK) was used according to the manufacturer's recommended protocol.

Cycling parameters

cDNA samples were amplified using initial denaturation at 95 ºC for 15 min. This was followed by 33 cycles of denaturation at 94 ºC for 15s; annealing at 58 ºC for 60s; extension at 72 ºC for 60s and final extension at 72 ºC for 20 min.

Results

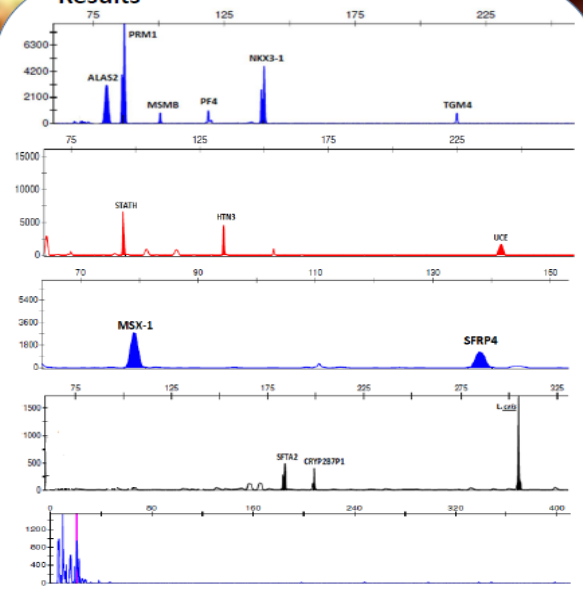

Fig. 1: Electropherogram (EPG) showing positive amplifications of (a) Blood markers: ALAS2, PF4; (b) Semen markers: PRM1, MSMB, NKX3-1, TGM4; (c) Saliva markers: STATH, HTN3; (d) Menstrual blood markers: MSX-1, SFRP4; (e) Vaginal secretion markers: SFTA2, CRYP2B7P1, L.*cris*; (f) Reference genes: UCE and Negative control.

Conclusion

- A 14 marker robust mRNA multiplex assay has been developed for body fluid identification.
- All mRNA markers were highly specific with no cross-reactivity among them and could be detected down to 25 pg input RNA.
- Stability study carried out on samples stored over 6 months demonstrated stable expression of body fluid specific mRNA markers (see Figure 1) and reference genes.
- All mock casework stains were correctly diagnosed for the specific body fluid using the multiplex.

References

1. A. Lindenbergh, M. de Pagter, G. Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Science International: Genetics. 6 (2012) 565-577.
2. Roeder, A.D., Haas, C., mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification. 127 (2013) 707-21.

Acknowledgements

We would like to thank voluntary participants that donated various samples towards this project.

Reference Genes Study: A Requisite in qRT-PCR Experiments

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[1]School of Forensic and Investigative Sciences, University of Central Lancashire, Preston, UK.

[2]Cellmark Forensic Services, Abingdon, Oxfordshire, UK.

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Aim: To investigate the efficiency, sensitivity and stability of reference genes in five commonly encountered Forensic body fluids using SYBR Green and Taqman assay detection methods.

Introduction

Body fluids such as blood, semen, saliva, vaginal secretion, and menstrual blood are frequently encountered by forensic investigators on crime scenes. In Forensic case work, adequate identification of the origin of biological stains is required [1].

Reference genes are constitutive genes used in forensic body fluid identification studies for normalisation of data generated and control of variations that may occur in experiments and samples [2]. Ideally, they are meant to be stably expressed irrespective of experimental or environmental conditions. The use of reference genes thus improves the reliability of qRT-PCR and fragment analysis experimental results. In normalisation experiments, the data is ascertained by comparing the expression data of target gene and a reference gene [3].

In this study, 10 common reference genes used in Forensic body fluid identification studies were selected from relevant literature. These include UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and 515. qRT-PCR efficiency, sensitivity and limit of detection (LOD) were investigated. Taqman probes were then designed for the markers with 95%-110% efficiency (slope= -3.33±10%) and used to assess the expression of all the reference genes. Stability test was carried out on nine blood samples, eight semen and menstrual blood samples, ten saliva samples and seven vaginal secretion samples stored over a period of 6 months at room temperature. These were from different individuals and all reactions were carried out in duplicates.

Materials and Methods

Samples

- Following ethical consideration, 51 consented samples of different individuals was collected at the University of Central Lancashire, Preston, UK.
- Vaginal secretion and menstrual blood were collected on sterile swabs and dried at room temperature.
- Blood was collected by both fingerpick and venepuncture (without anticoagulant treatment).
- Semen and Saliva were collected into 1.5ml Eppendorf tubes and stored frozen at -80 ºC.

RNA extraction and Reverse Transcription

- Total RNA was isolated using RNeasy Mini Plus kit (Qiagen, UK Ltd) according to manufacturers' recommended instructions.
- Post RNA purification and DNase treatment was carried out using Turbo DNA-free kit™ (Life Technologies Corp. UK)
- RNA was quantified using the NanoDrop ND-2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE)
- For the reverse transcription reaction, random primers and Superscript III reverse transcriptase (Life technologies, UK) was used according to the manufacturer's recommended protocol.

SYBR Green assay

- Cycling parameters

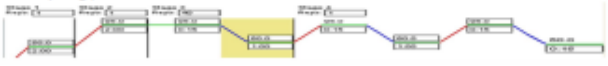

Fig. 1: SYBR Green assay cycling parameters used to amplify body fluid samples.

Taqman probe assay

- Cycling parameters

Fig. 2: Taqman probes assay cycling parameters used to amplify body fluid samples.

Results

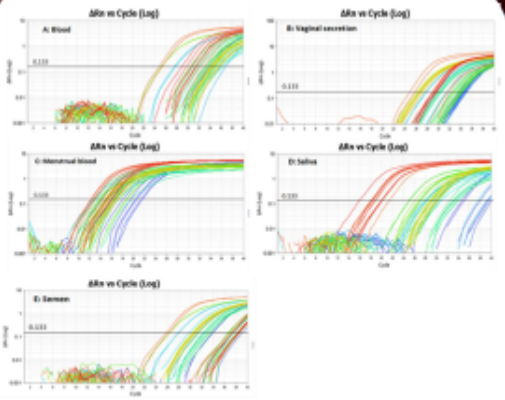

Fig. 3: Amplification plots of ACTB, RPS 29, TEF, B2M and UCE on five body fluid samples; A: Blood, B: Vaginal secretion, C: Menstrual Blood, D: Saliva, E: Semen at 25pg, 50pg, 100pg, 500pg, 1ng and 2ng input RNA concentrations. Each reaction comprises donors' samples (in duplicates) at different input RNA concentrations.

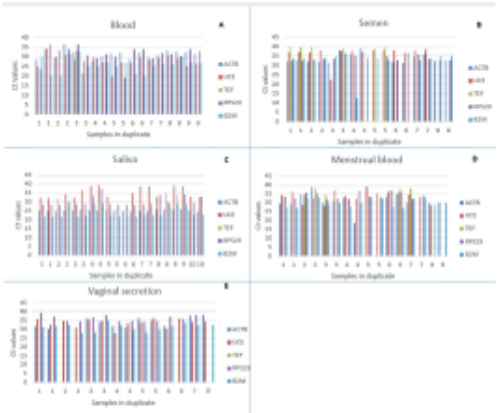

Fig. 4: Histogram plot. Expression stability of RNA stains in over 6 month old samples (a) blood, (b) semen, (c) saliva, (d) menstrual blood, (e) vaginal secretion, with no modification made to the storage conditions to control RNA degradation. All genes except TEF were expressed in at least 80% of the analysed samples for each body fluid.

Conclusion

- From the total of 10 reference genes analysed across five body fluid samples, five (UCE, TEF, ACTB, B2M, and RPS29) attained the required 95% - 110% efficiency, with a slope of -3.33±10% using a serial dilution of the body fluids and generating Ct values from the amplification curve.
- All the markers except TEF displayed high sensitivity and were detected down to 25 picogram of RNA input.
- Stability study carried out on samples stored over 6 months using Taqman probe assays evaluated for the reference genes demonstrates **B2M, ACTB, RPS29 and UCE** as robust markers in this study.
- Furthermore, this study reiterates that the use of reference genes be based on adequate validation for any specified study other than their reported use in similar studies.

References

1. A. Lindenbergh, M. de Pagter, G. Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Science International: Genetics. 6 (2012) 565-577.
2. J. Vandesompele, K.D. Preter, F. Pattyn, B. Poppe, N.V. Roy, A.D. Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes., 3 (2002) 34. 3 (2002) 34.
3. C. Tricarico, P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, et al., Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies, Anal. Biochem. 309 (2002) 293-300.

Acknowledgements

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Reference gene study for forensic body fluid identification

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ABSTRACT

Reference genes are used in forensic body fluid identification studies to normalise data generated during gene expression experiments. The use of reference genes improves the reliability of qRT-PCR. In this study, 10 most common reference genes UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and S15 widely used in forensic body fluid identification studies were selected from relevant literature and qPCR efficiency and sensitivity of all the reference genes was tested using SYBR Green detection. Stability was also assayed using samples stored at room temperature for 6 months using Taqman assay probes. All the markers except TEF displayed high sensitivity and were detected down to 25 pg of RNA input. Stability study demonstrates that B2M, ACTB, RPS29, and UCE are ideal markers for normalization in forensic body fluid identification studies. The study confirms that reference genes should be selected only upon adequate validation of their suitability.

1. Introduction

Body fluids such as blood, semen, saliva, vaginal secretion, and menstrual blood are frequently encountered by forensic investigators on crime scenes. In Forensic case work, adequate identification of the origin of biological stains is required [1]. Study of genes preferentially expressed in various body fluids has attracted the attention of forensic geneticists in recent years and a number of gene expression assays have been designed [1,2]. As a result of sample-to-sample variations, variations due to experimental and environmental conditions; reference genes (otherwise known as house-keeping genes) are used to normalise data generated in gene expression studies [3]. These are expected to be stably expressed in samples irrespective of the impact of experimental or environmental conditions. For optimal reliability of qRT-PCR and fragment analysis results, normalisation of data is achieved by comparing the expression data of target gene and a reference gene [4]. It is vital to establish the suitability of a chosen reference gene before its usage. In this study, UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and S15 were investigated for efficiency, sensitivity and stability in order to evaluate their suitability as reference genes for forensic body fluid data normalization studies.

2. Material and methods

Body fluid samples were collected from 51 different voluntary donors following procedures approved by the ethical committee of the University of Central Lancashire, Preston, UK. RNA was isolated using RNeasy Mini Plus kit (Qiagen, UK Ltd.) according to manufacturers' recommendations, with a final elution of 30 μl RNase-free H_2O. Final elution was repeated twice for optimum yield. Post RNA purification and DNase treatment was done using Turbo DNA-free kit™ (Life Technologies Corp. UK) to remove traces of genomic DNA. RNA was quantified using the NanoDrop ND-2000c UV–vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Purified RNA was reverse transcribed to cDNA using random primers and Superscript III reverse transcriptase (Life technologies, UK).

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http://dx.doi.org/10.1016/j.fsigss.2015.09.067
1875-1768/

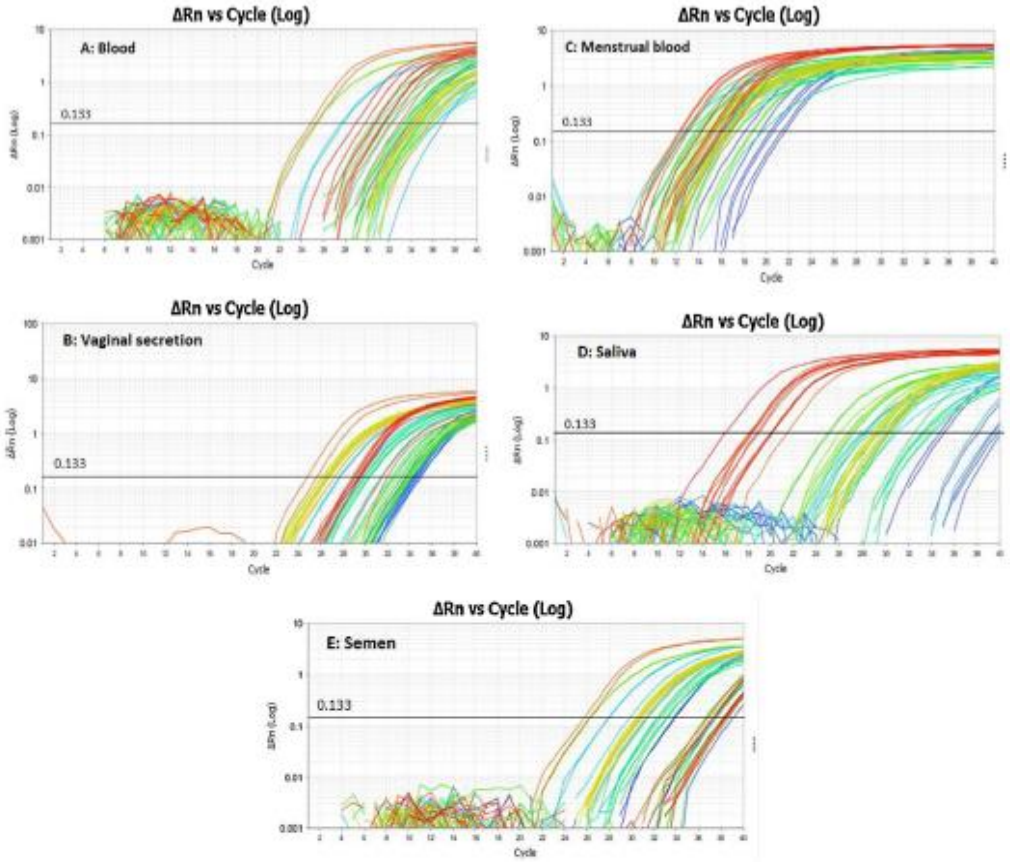

Fig. 1. Amplification plots of ACTB (blue), RPS 29 (orange), TEF (red), B2M (green) and UCE (pink) on five body fluid samples; A: blood, B: vaginal secretion, C: menstrual blood, D: saliva, E: semen at 25 pg, 50 pg, 100 pg, 500 pg, 1 ng and 2 ng input RNA concentrations. Each reaction comprises donors' samples (in duplicate) at different input RNA concentrations.

3. Realtime PCR assays

3.1. SYBR Green and Taqman probes

Samples were prepared in duplicate and cycle threshold (Ct) values were generated on the 7500 Realtime PCR system (Applied Biosystems by Life Technologies Corp.). The expression of each reference gene was assessed by analyzing the cycle threshold (Ct) value with a default instrument threshold of 0.133.

4. Results and discussion

Using serial dilution of samples, we were able to successfully evaluate the qRT-PCR efficiencies of UCE, TEF, GAPDH, 18S rRNA, ACTB, B2M, B-Actin, OAZ1, RPS 29 and S15 using SYBR Green method (data not shown). Five markers: UCE, TEF, ACTB, B2M, and RPS29 attained a set threshold of 95–110% efficiency, with a slope of −3.33 ± 10%. Sensitivity of these five markers was estimated using Taqman probes with different concentrations of RNA input (25 pg, 50 pg, 100 pg, 500 pg, 1 ng, 2 ng). All the markers except TEF displayed high sensitivity and were detected down to 25 pg input RNA (Fig. 1). In samples stored at room temperature over 6 months, all markers except TEF were expressed in more than 80% of all stored body fluid samples. B2M was found to be most stable with stability decreasing across ACTB, RPS29 and UCE respectively (Fig. 2).

In contrast to other studies, we could not achieve the set efficiency threshold for GAPDH, 18S rRNA, B-Actin, OAZ1 and S15. The sensitivities and stabilities of B2M, ACTB, RPS29 and UCE in our study proves their robustness for qRT-PCR body fluid identification studies. We are currently investigating these reference genes under various environmental conditions. Also, more research is ongoing to identify and validate robust body fluid specific markers which will allow unambiguous identification of forensic body fluids.

Conflict of interest

None.

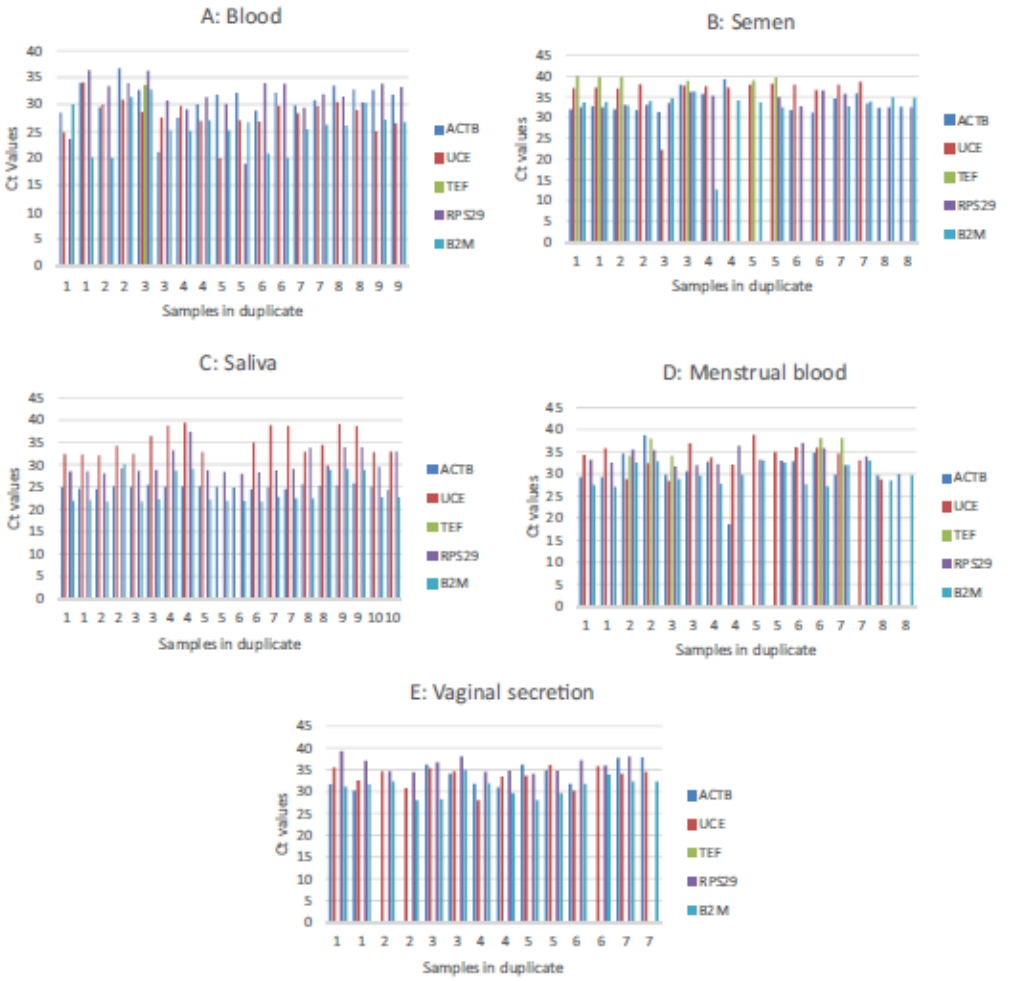

Fig. 2. Histogram plot. Expression stability of RNA stains in over 6 month old samples (a) blood, (b) semen, (c) saliva, (d) menstrual blood, (e) vaginal secretion, with no modification made to the storage conditions to control RNA degradation. All markers except TEF were expressed in at least 80% of the analysed samples for each body fluid.

Acknowledgement

The authors would like to thank the voluntary participants for various samples donated to undertake this research.

References

[1] A. Lindenbergh, M. de Pagter, G. Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Sci. Int.: Genet. 6 (2012) 565–577.

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