The Use of Genetic Materials, RNA, to Determine of the Age of Saliva Stains

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PhD thesis

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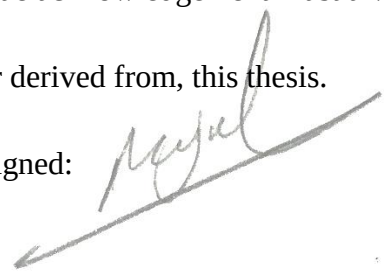
Signed:  Date: 16/12/2013

Abstract

DNA analysis, it does not provide any information about the time of the deposition of biological stain.

In this work, the relationship between the age of the saliva stains that were aged for specific periods of time and the degradation rate of specific RNA molecules were assessed to find the correlation between them. Two types of approaches and two types of RNA molecules were used for this purpose. In the first approach, the relationship assessment was carried out between the saliva's age and the relative quantity ratio (RQR) of two species of the housekeeping genes (β-actin mRNA and 18S rRNA) that were used in a previous study assessed on bloodstains. In the second pilot approach, the relationship assessment was carried out between the saliva's age and the relative quantities of two different-sized segments on the same saliva-specific mRNA markers quantified were assessed with using a pair of TaqMan® Gene Expression Assays (TGEAs).

Correlations were found between the age of the saliva stain and the RQR of the β-actin mRNA to 18S rRNA in the first approach and between the age of the saliva stains and the relative quantities of two different-sized segments of some saliva-specific mRNA markers in the second approach and the age of the sample can be approximated by both. In addition, use of saliva-specific RNA in the second approach offers the advantage of simultaneous age determination and body fluid identification for both saliva and blood. In addition factors affecting the determination of the age of biological stains by means of the analysis of ribonucleic acids such as mixture status, using of RNA*later*® stabiliser, were also evaluated.

Finally, methods that may be employed to apply the findings to casework, taking into account the level of accuracy achieved, are discussed.

Dedication

I dedicate this thesis work to my beloved mother, Gaitha Alsubhi, who has been faithful in her daily prayers for me while I was studying.

I also dedicate this work with sincere gratitude is to my wife, Marwah, who had been very patient with my late-night work, and me being busy all the time. I want to thank her for her unconditional support. I'm sincerely thankful to you, and this PhD is yours too.

My two little angels, Fahad and Ragad, I regret I was too busy and I may did not spend enough time with you, but I worked hard to get this PhD so you could be proud of your dad. You are the beautiful part of these eventful years. The cure for any tiredness I felt was your hugs when I opened the door, and your pure beautiful stories.

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To my unforgettable Saudi friends from whom I have learned a lot, I give my warmest regards. Also I would like to thank all my colleagues and the staff in the centre for their cooperation, help, and their entertaining and helpful discussions.

Finally, I'm grateful to the Ministry of Interior in Saudi Arabia for awarding me a scholarship, allowing me to pursue one of my life long dreams.

Publications and presentations relating to this research

Publications

Alrowaithi M, McCallum N, and Watson N., *A method for determining the age of a bloodstain.* Submitted on June 18, 2013. status in press in Forensic Science International.

Alrowaithi M, Tate R, and Watson N., *Saliva-Blood Adulteration May Impair Age of Stain Determination by RNA Species.* Submitted on April 30, 2013. status under review in Forensic Science International Genetics.

Alrowaithi M, Watson N., *Determination of Time since Deposition of Saliva Stains Using qRT-PCR.* status under preparation.

Alrowaithi M, Watson N., *Simultaneous determination of the age and type of saliva stain.* status under preparation.

Alrowaithi M, Watson N., *Simultaneous determination of the age and type of bloodstain.* status under preparation.

Oral presentation

Alrowaithi M, and Watson N., *Alternative Approach to Genetic Human Identification Using Microcapillary Devices*. Presented at the 5th Saudi International conference 2011 (SIC2011), Coventry, UK.

Alrowaithi M, and Watson N., *Determination of Time Since Deposition of Saliva Stains Using qRT-PCR.* Presented at the 6th Saudi International conference 2012 (SIC2012), London, UK.

Poster presentation

Alrowaithi M, Watson N., *Evaluation of Microcapillary Devices for Genetic Identity Testing.* Presented at the 21st ISHI conference. 2010: San Antonio, Texas, USA

Alrowaithi M, Watson N., *Determination of the age of saliva stain using RT-PCR.* Presented at the 6th PGBiomed-2011 Conference, Glasgow University, 14-16 August

Alrowaithi M, Watson N., *Evaluation of Microcapillary Devices for Genetic Identity Testing.* Presented at the 5th Saudi International conference 2011 (SIC2011), Coventry, UK.

Alrowaithi M, Watson N., *Determination the age of saliva stain using qRT-PC,* Presented at the University research day UDR2012

Alrowaithi M, Watson N., *Determination of Time since Deposition of Saliva Stains Using qRT-PCR.* Presented at the 6th EAFS2012 conference, Netherlands, 20-24 August.

Alrowaithi M, Watson N., *RNAlater® does more than preserve RNA in saliva stain.* Accepted to be presented at FSSoc and CAC joint Autumn Conference 2013, Manchester, 6-8 November.

List of abbreviations

°C	Degrees Celsius
3' UTR	3' untranslated regions
A	Adenine
A_{230}	Absorbance, in optical densities, at 230 nm
A_{260}	Absorbance, in optical densities, at 260 nm
A_{280}	Absorbance, in optical densities, at 280 nm
ALAS2	Aminolevulinate, delta-, synthase 2
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
AMICA1	Adhesion molecule, interacts with CXADR antigen 1
ANK1	Ankyrin 1, erythrocytic
ANOVA	Analysis of Variance
AQP9	Aquaporin 9
ARHGAP26	Rho GTPase activating protein 26
Bp	Base pairs
C	Cytosine
C1QR1	CD93 molecule
C5AR1	Complement component 5a receptor 1
CASP1	Caspase 1, apoptosis-related cysteine peptidase
CD3G	CD3 gamma molecule
Ct	Cycle threshold
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dRn (ΔRn)	Normalised reporter signal minus the baseline

DTT	Dithiothreitol
EDNAP	European DNA Profiling Group
EPG	Electropherogram
G	Guanine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GCF	Gingival crevice fluid
GYPA	Glycophorin A
HBA1	Alpha subunit of haemoglobin1
HBB	Beta subunit of haemoglobin
HBD-1	Human beta-defensin 1
hnRNA	Heterogeneous nuclear RNA
HPLC	High-Performance Liquid Chromatography
HTN3	Histatin 3
ILS600	Internal lane standard 600
KLK	Kallikrein 3
KPCR	Kinetic polymerase chain reaction
KRT4	Keratin 4
LCM	Laser-Capture Microdissection
LR	Likelihood Ratio
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
mL	Millilitre
mM	Millimolar
MMP-11	Matrix metalloproteinase 11
MMP-7	Matrix metalloproteinase 7

MNDA	Myeloid cell nuclear differentiation antigen
mRNA	Messenger RNA
MUC4	Mucin 4
NCF2	Neutrophil cytosolic factor 2
ncRNA	Non-coding RNA
ng	Nanogram
nM	Nanomolar
OD	Optical Density
-OH	Hydroxyl group
PBGD	Porphobilinogen deaminase
PCR	Polymerase chain reaction
pg	Picogram
PRB1	Proline-Rich protein BstNI subfamily 1
PRB 2	Proline-Rich protein BstNI subfamily 2
PRB 3	Proline-Rich protein BstNI subfamily 3
PRB 4	Proline-Rich protein BstNI subfamily 4
PRM1	Protamine 1
PRM2	Protamine 2
Q-PCR/qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase-PCR
RER	Relative Expression Ratio
Rfu	Relative fluorescence unit
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
Rn	Normalised reporter

RNA	Ribonucleic acid
RNAi	RNA interference
RNases	Ribonucleases
ROX	6-carboxy-X-rhodamine
RQR	Relative Quantity Ratio
rRNA	Ribosomal RNA
-RTcontrol	Minus Reverse Transcription Control
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
sec	Second
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SPTB	Spectrin, beta, erythrocytic
sRNA	Small regulatory RNA
STATH	Statherin
STR	Short Tandem Repeat
T	Thymine
TGEA	TaqMan® Expression Assay
Tm	Melting temperature
tRNA	Transfer RNA
U	Uracil
β-ME	Beta-Mercaptoethanol
µL	Microlitre
µM	Micromolar

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1 General introduction

The estimation of the age of a biological stain, i.e. the time elapsed between the formation of a stain and the time it is acquired by the investigator or subjected to analysis, can be useful to the investigation of crimes. It may be useful to the elimination of stains unconnected with the crime. This estimation of a biological stain's age has been attempted by a number of means. In 2005 a method using differences in the rate of denaturation of different species of RNA was reported and is described in section 1.5.2.2 of this thesis. The research reported here is concerned with an investigation of the extension of this technique to saliva stains and a consideration of certain factors that can affect the interpretation of such analyses.

1.1 Chapters overview

Chapter 1 gives an overall introduction to the concepts covered in this thesis. The science of RNA and its uses in forensic genetic field are explained as well as basic terminologies used in RNA analysis.

Chapter 2 explains the materials and methods of the studies used in this thesis.

In Chapter 3, the ability to determine the age of saliva stains stabilised using a commercially available product, called RNA*later*® which is used to stabilise RNA degradation in laboratory samples in clinical analysis, is discussed. In this chapter the relationship between the age of saliva stains and the relative quantity ratio RQR values of the relative quantities of two species of RNA; a messenger RNA of a housekeeping gene, β-actin, to a ribosomal RNA molecule 18S, is studied using saliva stains stabilised using the RNA*later*® reagent after they reach specific ages.

In Chapter 4, the applicability of the RNA*later*® reagent in forensic practice and its effect on the RQR values of β-actin to 18S are discussed.

In Chapter 5, the ability to determine the age of un-stabilised-saliva stain depending on the RQR values of β-actin to 18S is discussed. In this chapter, the relationship between the age of saliva stains and the RQR values is studied using saliva stains analysed immediately upon reaching the desired ages.

In Chapter 6, the effect of the mixture of saliva and blood on the RQR values of β-actin to 18S is discussed.

In Chapter 7, a novel method of simultaneously determining the age of saliva stains and identifying their type using saliva-specific mRNA markers is demonstrated.

Chapter 8 applying of the novel method of simultaneously determining of the age and type of the body fluid on bloodstains using blood-specific mRNA markers is discussed.

1.2 Ribonucleic acids: nature and biological function

Ribonucleic acid (RNA) is a genetic material that plays a biological role in the flow of genetic information and is particularly involved in cellular protein synthesis [1, 2]. RNA is involved in two mechanisms of protein synthesis: transcription and translation i.e. transcribing the DNA nucleotide sequence into an RNA nucleotide sequence and translating the nucleotide sequence into an amino acid sequence, respectively. RNA molecules play an important role in converting DNA code into proteins, which is the central dogma of molecular biology.

RNA is a long, unbranched polymer of ribonucleoside monophosphate moieties that are linked to each other by phosphodiester bonds. These polymers form single-stranded molecules in both eukaryotic and prokaryotic cells. RNA is similar but not identical, to a single strand of deoxyribonucleic acid (DNA).

1.2.1 RNA structure

The nucleotide is the structural unit of RNA and consists of three key components: five-carbon sugar pentose, nitrogenous base, and at least one phosphate group [3-5]. Ribose sugar is a five-carbon sugar. Its carbon atoms are numbered from 1' to 5'. It binds with a nitrogenous base at position 1'. This nitrogenous base is usually one of the four bases—adenine (A), guanine (G), cytosine (C), or uracil (U). At position 3' it binds to a phosphate group, which also binds to the next ribose, bound through position 5' (Figure 1-1) [6, 7]. The compound composed of a five-carbon sugar

(pentose) and a nitrogenous base is called a nucleoside. Addition of a phosphate group to the nucleoside results in a composite called a nucleotide.

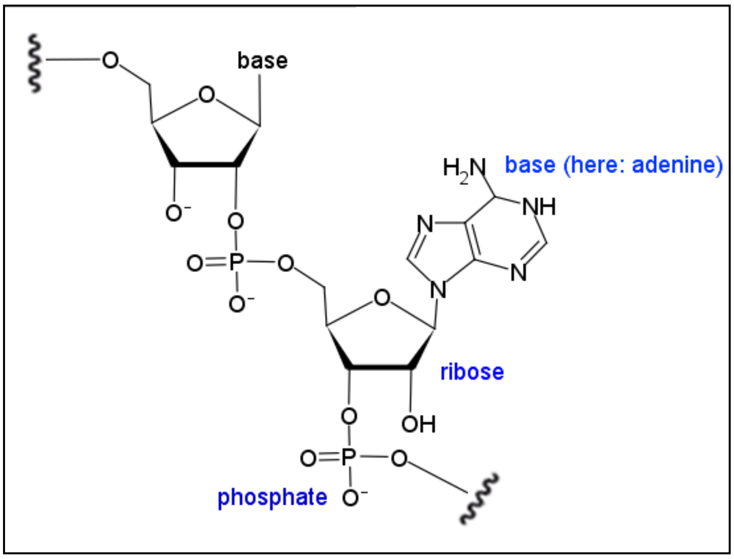

Figure 1-1: Chemical structure of RNA [8]

The presence of a phosphate group, which has a negative charge at the physiological pH, makes the RNA a polyanion charged molecule. The presence of the hydroxyl group at position 2’ is considered a significant structural characteristic of RNA, whereby the RNA helix adopt its particular geometrical form [9, 10].

1.2.2 Difference between RNA and DNA

Although DNA and RNA are composed of the same structural unit, there are certain differences between them (Table 1-1). The key chemical difference is the presence of a hydroxyl group (-OH) that binds to the 2’carbon of the ribose sugar, while it is absent in DNA at the 2’carbon of the ribose sugars (called deoxyribose). The second difference is the substitution of uracil with thymine in RNA. However, in certain situations, uracil might be found in DNA, such as in the case of direct misincorporation of uracil in place of thymine by DNA polymerase during DNA replication and repair [11]. The third difference is that RNA is a gathering of ribonucleotide precursors, while DNA is a gathering of deoxyribonucleotide precursors [3, 5, 12].

Table 1-1: Differences between DNA and RNA [3, 12, 13].

		RNA	DNA
Key nucleotide components		Ribose sugar Nitrogenous base + Phosphate group(s)	Deoxyribose sugar Nitrogenous base + Phosphate group(s)
Common nitrogenous bases	Purines	- Adenine (A) - Guanine (G)	- Adenine (A) - Guanine (G)
	Pyrimidines	- Cytosine (C) - Uracil (U)	- Cytosine (C) - Thymine (T)
Strand type **Hydrolysis**		- Single strand More prone to hydrolysis due to presence of more hydroxyl groups	Double strands Less prone
Structure		Consists of short helices packed together	Consists of long double helices wrapped around the histone protein

1.2.3 RNA synthesis

The process by which a single-stranded RNA molecule is synthesised from a specific chromosome locus is called transcription [5]. This process occurs in the nucleus and the mitochondria of the eukaryotic cell, as well as in plastids in plants. Generally, the transcription process passes through five steps which are as follows: pre-initiation, initiation, promoter clearance, elongation, and termination. In the pre-initiation, initiation, and promoter clearance steps, RNA polymerase enzymes bind to a promoter sequence in the template DNA (usually upstream of the gene). This binding occurs in the presence of different specific transcription factors, resulting in the formation of a complex composed of the promoter, RNA polymerase, transcription factors, and DNA helicase enzyme, as well as activators and repressors. The helicase enzyme starts to unzip the double DNA strands where it breaks the hydrogen bonds between nitrogenous bases. After the formation of the first bond, RNA polymerase clears the promoter [14, 15]. In the elongation step, the RNA polymerase forms RNA from the template DNA by using complementary bases. Extension occurs in the 5' to 3' direction, resulting in the formation of a similar copy of the coding strand, with the exception of thymine base replacing uracil base, and the presence of deoxyribose sugar instead of ribose sugar. In the last step (termination step), the new transcript is

cleaved and then polyadenylated by the addition of adenine bases (As) at its new 3' end. After transcription, other modification processes take place, such as addition of a 5' cap and removal of introns. The termination of transcription in the eukaryotic cell is still not fully understood [5, 16, 17].

1.2.4 Types of RNA and their functions

Many types of RNA have been discovered in cells, and these have been broadly classified into various categories: messenger RNA (mRNA), heterogeneous nuclear RNA (hnRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and microRNA (miRNA) (Table 1-2). mRNA is called coding RNA, because it encodes protein, while other RNA species are called non-coding RNA, because they do not encode protein. The most prominent non-coding RNA molecules are tRNA and rRNA.

Each RNA type is synthesised by a different RNA polymerase and plays a different role in the cell. Some RNA molecules play a role in protein synthesis, and other molecules act as catalysts in many biochemical reactions. In addition, some RNA molecules play complex regulatory roles in the cells. The amount of each RNA type in a cell varies according to the physiology of the cell.

Table 1-2: RNA types and their functions. This table addressed the types of RNA in both prokaryotic and eukaryotic and the basic functions of those RNA types [5].

RNA type	Symbol	Basic function	Prokaryotic	Eukaryotic
Messenger RNA	mRNA	Template for the synthesis of protein	Yes	Yes
Ribosomal RNA	rRNA	Forms backbone of the ribosomal subunits	Yes	Yes
Transfer RNA	tRNA	Transfers amino acids to the ribosome to support translation	Yes	Yes
Heterogeneous nuclear RNA	hnRNA	Large unspliced precursor of mRNA (pre-mRNA)	No	Yes
Small nuclear RNA	snRNA	Facilitates splicing of hnRNA into functional mRNA	No	Yes
Small nucleolar RNA	snoRNA	Processes immature rRNA transcripts in the nucleus	No	Yes
Small cytoplasmic RNA	scRNA	Facilitates protein trafficking and secretion	Yes	Yes
MicroRNA	miRNA	Short antisense RNAs that participate in the regulation of gene expression	No	Yes
RNase P RNA	-	Catalytic RNA component of the enzyme/RNA complex that processes tRNA molecules	Yes	No
Telomerase RNA	-	RNA component of the enzyme/RNA complex that repairs chromosome telomeres	No	Yes

1.2.4.1 Messenger RNA (mRNA)

Messenger RNA (mRNA) is a copy of the DNA base sequence of genes in the DNA after capping, addition of 3' tail, and splicing. It works as a carrier of the information that ultimately will be expressed as proteins. Functional mRNA is derived from a

premature form, large, unspliced precursor of mRNA called heterogeneous nuclear RNA (hnRNA) or pre-mRNA, which is copied directly from the DNA. hnRNA contains the coding and non-coding regions of the copied DNA sequence. This molecule undergoes a process of modifications in the nucleus that includes splicing of non-coding regions, capping, and addition of a 3' tail. This modification results in the formation of mature mRNA, which is directed to the cytoplasm to carry information to the ribosome for protein synthesis. The amount of mRNA that exists inside a cell at a specific time is regulated by the cell itself, where endogenous ribonuclease enzymes catalyse the degradation of mRNA [5].

There are different mRNA species expressed in all cells, and different mRNA species expressed in specific types of tissues. mRNAs expressed in all tissue types are transcripts of the housekeeping genes that are constitutive genes found in all cells and are needed to maintain the basic functions of the cells.β-actin gene and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) gene are examples of housekeeping genes. β-actin mRNA was chosen for investigation in this study. However, many hundreds of mRNA species are expressed in specific tissues due to the specific function and phenotype of the cell in that tissue.

1.2.4.2 Ribosomal RNA (rRNA)

Ribosomal RNA (rRNA) is the most abundant RNA type in the cell. It is the RNA part of the ribosome. The rRNAs in eukaryotic cells can be broken down into two subunits: large subunit (60S) and small subunit (40S). The large subunit contains three species: 5S, 5.8S and 28S. The small subunit mostly contains 18S. Each species classification is based on its sedimentation rates, which are affected by the shape and the mass of the subunit. The S in each subunit name represents the measure of sedimentation rate. S unit is a Svedberg unit, which is a non-international system physical unit used for sedimentation coefficients [5].

The rRNA molecules form the scaffolding of ribosomes [5]. Ribosomes are the protein manufacturing machinery of all living cells; they provide the site and carry the enzymes necessary for decoding mRNA into amino acids. At present, there are about 82 known eukaryotic ribosomal proteins [5].

1.2.4.3 Transfer RNA (tRNA)

Transfer RNA (tRNA) molecules are small molecules consisting of 74-95 bases, three of which are called anticodons; and one amino acid. They are responsible for transporting amino acid to the ribosome in order to support protein synthesis in the cell.

There are at least 20 different tRNAs, one for each amino acid. Each of them 'reads' the mRNA codon by using its own anticodon until the appropriate match of the anticodon with the codon occurs [18].

1.2.4.4 MicroRNA (miRNA)

MicroRNAs (miRNAs) are a class of small non-coding RNA (ncRNA) molecules with a length of 18 to 24 nucleotides. They play an important regulative role in many cellular processes. In human, more than 700 miRNAs have been identified and about 800–1000 miRNAs in the human genome are predicted by bioinformatics estimations. This approaches about 2–3% of all protein-coding genes. An official 'miRBase' database is now available and it contains the actual sequence data for miRNA molecules (precursor and mature) and further information, such as hints of putative functions. Any newly discovered miRNAs are entered into the database sequentially numbered after with a specific number [12].

miRNAs play an important role in the regulation of gene expression by a mechanism called RNA interference (RNAi). They can down-regulate gene expression by binding to the 3' untranslated regions (3' UTR) of target messenger RNAs (mRNAs) [19-21]. A study shows that miRNAs may function as positive regulators in some cases [22]. Moreover, it has been found that miRNAs play a crucial biological role in immune regulation, fat metabolism [23], glucose homeostasis, and aging [23, 24].

1.2.4.5 Small regulatory RNA (sRNA)

Small regulatory RNA (sRNA) molecules are another example of non-coding RNA species. These non-coding RNA molecules are classified into a number of

subcategories: microRNA (miRNA), small nuclear RNA (snRNA), small interfering RNA (siRNA), and small nucleolar RNA (snoRNA) [25].

a. Small nuclear RNA (snRNA)

Small nuclear RNA (snRNA) is an important sRNA subcategory. These molecules are found in the nucleus, binding to protein to form a complex called the 'small nuclear ribonucleoproteins', or snRNPs, complex [26]. It has been found that snRNA molecules play an important role in the splicing process, in which intron sequences are removed from the pre-mRNA transcript to give rise to a mature mRNA. The most abundant snRNA molecules are called the U1, U2, U5, and U4/U6 particles.

b. Small interfering RNA (siRNA)

Small interfering RNAs (siRNAs), another subgroup of small regulatory RNA molecules, have a length of 21 to 25 base pairs [27, 28].

siRNAs play a role in the inhibition of gene expression, where one strand of siRNA binds to a multiprotein complex called the 'RNA-induced silencing complex' (RISC) to form an RNA-containing complex. This complex uses the siRNA molecules as a template to recognise complementary mRNA. When it binds to the complementary strand, it inhibits the transcription of mRNA molecules by activating the RNase and then cleaving already transcripted mRNA molecules [10, 28].

c. Small nucleolar RNA (snoRNA)

rRNA processing and ribosomal assembly take place in the nucleolus. Small nucleolar RNAs (snoRNAs) are abundant in the nucleolus and are isolated from the nucleolar extract [29, 30]. They are about 70–250 nucleotides in length [6, 8]. snoRNAs play a role in guiding the chemical modification of other RNA species-such as rRNA, tRNA, and snRNA. These chemical modifications include methylation* and pseudouridylation† [29]. There are two classes of snoRNA: the C/D box and H/ACA box snoRNA families. C/D box snoRNAs are associated with methylation, where they mediate the addition of methyl groups to the RNA

* Methylation is addition of methyl groups

† Pseudouridylation is isomerisation of uridine in immature rRNA molecules

molecules. H/ACA box snoRNA are associated with pseudouridylation, where they mediate the isomerisation of uridine in immature RNA molecules [29, 31, 32].

1.3 Uses of RNA in forensic science

In the last 20 years, DNA has become the predominant issue in molecular forensic science research, due to the development of sophisticated DNA technology and the adoption of revolutionised high-standard policies. On the other hand, RNA has been notorious for rapidly degrading post-mortem and in vitro, resulting in some forensic scientists carrying out limited research on RNA and avoiding investing money and time in this field [33]. Nonetheless, many research studies have been conducted in this field, which have shown promising possible valuable applications of RNA in molecular forensic science. Some studies have demonstrated extracted RNA suitable for RT-PCR from various human tissues, such as the brain, several days after death [34, 35]. Other studies have extracted RNA from blood spots on filter paper stored for several years [36, 37]. Therefore, RNA is more stable than formerly believed [38].

Many studies show the possible and practical uses of RNA techniques in the forensic field. One of these possible applications is the use of RNA in determining the post-mortem interval, as it has been noticed that RNA exhibits great stability in some tissue up to 96 hours after death [34, 39-41]. Another possible use for RNA is in determining the age of a wound; it is believed that identification of mRNA markers involved in cell reaction to injury and validation of these markers will move forensic pathology into a new era [42, 43]. In addition to those possible applications, there are other uses for RNA in the analysis of samples collected from crime scenes. RNA can be used to determine types of stains, and good progress has been achieved in this field [43-47]. It also can be used in determining the age of a bloodstain and hair sample [48, 49]. Using RNA to determine the age of a bloodstain and using it in the identification of body fluids will be discussed in the following sections.

1.4 Identification of body fluids

Traditional DNA analysis allows for an indication of the source of the sample collected at the crime scene, but it is unable to determine the type of that sample. Due to the importance of identifying types of body fluid, many physical and chemical techniques have been developed, such as the Takayama test, which is a crystal test used to identify blood, and other techniques that depend on immunochemical or immunochromatographic means, in which a human-specific antiserum to serum proteins or haemoglobin is used to identify body fluid [50, 51]. However, there are many disadvantages to using conventional body fluid identification techniques: they are labour-intensive and are considered costly in terms of time and sample usage [45]. Moreover, although some of these disadvantages have been avoided in some new techniques, such as using Raman spectroscopy to identify blood fluid, these techniques remain unable to differentiate between blood and menstrual blood [52].

Using RNA to identify body fluid can provide a feasible sensitive and specific method of identification, as there are many advantages to using RNA over other methods. The high sensitivity results from the possibility of using tests involving an amplification step, using the polymerase chain reaction (PCR), and the high specificity is due to the fact that RNA represents the gene-specific expression of the cells, which is unique for the functional status of cells and organs. Moreover, RNA can be simultaneously isolated with DNA from a sample, which is considered another advantage [33, 53, 54].

Research in identification of body fluid using RNA has shown valuable progress. A number of studies have demonstrated that it is possible to extract mRNA from samples stored for up to 15 years [48]. However, poor storage conditions may affect the stability of RNA more than DNA, which may result in failure of RNA extraction [33].

Many mRNA markers have been identified for a variety of tissues: blood, menstrual blood, semen, saliva, and vaginal secretion (Table 1-3). The choice of these mRNA markers was based on the differences in their functions and the specificity of the

tissue expression in each tissue. For example, it is possible to differentiate menstrual blood from blood by the presence of metalloproteinases, which has been found to be expressed only in menstruating endometrium [44, 55, 56]. The ability to identify menstrual blood is a great achievement, given that bloodstains are the most commonly encountered samples at crime scenes [33].

Table 1-3: Evaluated mRNA markers for body fluid identification. This table details some published mRNA markers that can be used for body fluid identification [33].

Body fluid	RNA markers
Blood	β-spectrin (SPTB) [45]
	Porphobilinogen deaminase (PBGD) [45]
	Haemoglobin alpha locus 1 (HBA) [46]
Menstrual blood	Matrix metalloproteinase 7 (MMP-7) [44],[45]
	Matrix metalloproteinase 11 (MMP-11) [44, 56]
Saliva	Statherin (STATH) [45]
	Histatin 3 (HTN3) [45]
Semen	Protamine 1 (PRM1) [45, 53]
	Protamine 2 (PRM2) [45],[53]
	Kallikrein 3 (KLK = PSA) [46]
Vaginal secretions	Human beta-defensin 1 (HBD-1) [45]
	Mucin 4 (MUC4) [45, 46]

One of the great advances in body fluid identification is the multiplexing of these markers to enable all body fluid markers to be identified in one reaction [45, 57, 58]. In addition, a series of inter-laboratory studies were performed by the European DNA Profiling Group (EDNAP) as collaborative exercises on RNA/DNA co-analysis for body fluid identification and STR profiling. The aim of these inter-laboratory studies is to evaluate the sensitivity, specificity and performance of the body fluid identification using RNA profiling with casework samples to evaluate specific mRNA markers for this purpose in singleplex and multiplex reactions [59-61].

Moreover, many studies have moved to miRNA markers to identify body fluids; it is believed that miRNA shows an advantage over mRNA in that is it is less prone to degradation than mRNA due to its very small size. Therefore, miRNA should be

more suitable for degraded or compromised specimen samples, which are frequently encountered at crime scenes [47, 62].

1.5 Determination of the age of bloodstains

Although DNA analysis in forensic science has progressed and is able to indicate the source of bloodstains and spatially link a suspect to a crime scene, it does not provide any information about the time of the deposition of the bloodstain, except that it was deposited prior to collection from the crime scene [63]. This deficiency in the temporal link between a bloodstain and the time of the commission of a crime may have a negative effect on the investigation process, where an innocent person might be accused of committing a crime or a criminal might be acquitted. The importance of this temporal link increases in crimes where there is a close personal tie between the victim and the suspect. In such situations, biological stains from the suspect are expected to be found in the victim's home and other locations associated with the victim. Similarly, it is also expected that biological stains from the victim will be found in locations associated with the suspect. If the time of the deposition of the biological stain is not identified, it is impossible to temporally link the commission of the crime with the collected biological stain. This may result in an inability to solve the crime accurately.

Such cases highlight the advantages of determining the time of deposition of biological stains. The first advantage is that it is possible to determine the time of a crime if, by using another independent tool, it is known that the biological stain was deposited at the time of the commission of the crime. Another advantage is that the source of the biological stain could be potentially excluded if the age of the sample does not fit with the exact time of the commission of the crime when that time has been determined using another independent means [49].

Many studies have been carried out to determine the time of bloodstain deposition. These studies can be divided into two types. The first type is a study that depends on the physical and chemical features of the bloodstain to determine its age. The second type is a study that depends on the genetic materials in the bloodstain.

1.5.1 Studies depending on physical and chemical features of bloodstains

The majority of these studies depended on the physical and chemical changes that accompany the transformation of haemoglobin into its derivatives.

In 1930, Schwarzacher noticed that the solubility of a bloodstain in water decreased with increasing age, and he tried to make a correlation between the age of the bloodstain and its solubility in water to estimate the age of the bloodstain [64]. Schwarz (1934) attempted to determine the age of bloodstains based on the intensity of the blue colour resulting from using Guaiacum as a substrate for the peroxidase enzyme in the presence of haemoglobin. It has been suggested that the colour intensity increases with an increase in the amount of haemoglobin, which means the colour intensity will decrease as the age of the bloodstain increases [65]. In another study, the age of a bloodstain was correlated with the size of the black border around the bloodstain, which results from diffusion of Cl^- and fixing it as AgCl. It has been suggested that the size of the black border increases as the age of the bloodstain increases [65-67].

Various methods and techniques have been used to determine the age of bloodstains. In some studies, high-performance liquid chromatography (HPLC) was used to determine the age of a bloodstain by analysing the decomposition peaks and correlating them with the age of the sample [68]. Remission analysis, immunoelectrophoresis, and analysis of absorption spectra have been used in other studies [69-73].

However, there are many disadvantages to using this type of method in determining the age of a bloodstain. Firstly, the time window offered by most of these methods is too narrow and is therefore not suitable for forensic casework. Secondly, these methods are unable to differentiate between samples from different species. Thirdly, the results of these methods might be misleading or non-existent, due to the difference in sample sizes [49, 74-76]. In addition, it seems likely that the results of these tests will be affected by the storage conditions of the stains.

1.5.2 Studies depending on analysis of the genetic materials of bloodstains

Limited studies have been carried out to determine the age of biological samples using genetic materials; most of them were carried out on bloodstains.

1.5.2.1 Study carried out by Bauer *et al.* (2003)

The aims of this study were to illustrate the possibility of extracting RNA suitable for RT-PCR from blood samples stored for several years and to use this RNA to indicate the age of that blood. To achieve these aims, a number of blood samples stored for several years (up to five, eight, and 15 years) were used, as well as samples that were stored under different controlled conditions. These samples were analysed and RNA was quantified using two RT-PCR assays: semi-quantitative duplex RT-PCR assay and competitive RT-PCR assay. The semi-quantitative duplex RT-PCR assay was used with an internal standard where two fragments of β-actin mRNA were amplified in a single reaction by using four specific primers. In this assay, the author assumed that the RT-PCR products of the segment nearer to the 3' end would be greater than that nearer to the 5' end in the degraded sample. In the competitive RT-PCR assay, engineered mouse cyclophilin mRNA was used as an external standard to compete endogenous cyclophilin mRNA in the RT-PCR. Using this external standard, the specificity of the PCR can be controlled and the efficiency of the real-time and RNase contamination can be checked [48].

This study demonstrated that it is possible to extract RNA from blood samples stored for up to 15 years. It also proved that the methods used were able to quantify the RNA in such samples. Finally, the study concluded that it is possible to use mRNA as an indicator to estimate the age of a bloodstain [48].

However, this study was criticised because the method used is not applicable in forensic casework, given that it requires four to five years' difference between samples to notice a significant difference in RNA degradation [77].

1.5.2.2 Study carried out by Anderson *et al.* (2005)

The aim of this study was to develop a reliable genetic experiment to determine the age of a bloodstain that is applicable for forensic casework. This study was carried out on a number of bloodstain samples over a 150-day period under controlled conditions. In this study, two RNA molecules from different RNA types were chosen to be analysed by real-time reverse transcriptase PCR, and the ratio between them was used as an indicator of the ageing of the bloodstain [49].

The first RNA molecule is the β-actin mRNA molecule, which is a transcript of a housekeeping gene that expresses in all cell types at a relatively high level. It is not combined with a protective protein complex; therefore, it does not have environmental protection. The second RNA molecule is 18S rRNA, which is part of the ribosomal RNA and it is also a product of a housekeeping gene. 18S rRNA is a very abundant species, with thousands of copies per cell. It is less prone to degradation than mRNA because, as hypothesised by Anderson *et al.* [49], it is always present in a complex of ribosomal proteins that provide environmental protection [49]. Because of these differences in characteristics between these chosen two RNAs, it is expected that β-actin mRNA will degrade faster than 18S rRNA. Therefore, the ratio of the quantities between them changes with time, which can be used as an indicator of the age of the bloodstain [49].

This study suggested that the ratio of relative quantities between β-actin mRNA and 18S rRNA could be used to predict the age of a bloodstain, and it concluded by illustrating the advantages of this technique over the previous approaches. The first advantage is that this technique is more precise and accurate in determining the age of bloodstains. Another advantage is that the experiment can be applied to other tissue types besides blood, because the genes of the chosen RNAs are products universally expressed. The analysis is not affected by the size of the sample, as it examines the RNA ratio, and only a small quantity of the sample is needed for the test. Using the Species-specificity of the primers and probes eliminates false signals generated due to contamination. Finally, in this technique, DNA and RNA are extracted simultaneously, which allows for identifying the person and estimating the age of the sample by using a small sample [49]. However, the effects of

unpredictable environmental conditions on the bloodstain were not addressed in this study [33]. A recent study [73] used this method of determining the age of bloodstain to demonstrate the gender- related difference in the RNA ratio.

Although the method used in the Anderson *et al.* [49] study is more applicable in forensic casework than molecular methods, the study was criticised and received many comments. The first comment was that the study was restricted only to bloodstains. In addition, the time estimated by this method was crude. Another comment was that the samples were examined only once every month. The approach used in the study was rudimentary in that only the Ct value was used to measure the 18S rRNA to β-actin ratio. The last comment was that the study did not give any information about the type or the extent of the RNA degradation in the bloodstain [77].

1.5.2.3 Study carried out by Ballantyne (2008)

This study was carried out to assess the degradation of genetic material (i.e. RNA and DNA), analysis of protein (i.e. haemoglobin), and assessment of the changes in the enzymes' activity to accurately estimate the age of a bloodstain.

This study was carried out on genetic materials aimed to find a correlation between the time since deposition of a bloodstain and RNA degradation, as well as to find a correlation between the time since deposition of the bloodstain and DNA correlation. Unfortunately, the study was unable to find any correlation between the time since deposition of a bloodstain and the degradation of 15 chosen blood-specific mRNAs (from bloodstains stored at room temperature for 5 min and for three months). Moreover, the study was not able to determine the direction of the RNA degradation (5'-end or 3'-end). The assessment of the correlation between the time since deposition and DNA degradation was not performed, because the author thought it advantageous after the failure to find a correlation between time since deposition and RNA degradation [77].

However, the study developed a novel method of determining bloodstain age by analysis of the haemoglobin and assessment of the changes in the enzymes' activity in the bloodstain.

1.5.2.4 Further study carried out by Anderson *et al.* (2011)

This study is a continuation of their previous study [49], but with some modifications in the methodology. In this study, the ages of blood samples were estimated based on multivariate analysis. Many amplicon combinations (18S 501 bp to 171 bp, β-actin 169 bp to 18S 171 bp, and β-actin 301 bp versus 89 bp) were assayed at different age periods. Incorporating the results from several amplicon sets offered a robust method of predicting the age of bloodstains [78].

1.5.2.5 Study carried out by Hampson *et al.* (2011)

The aim of this study was to predict the age of a hair sample by applying the method used by Anderson *et al.* [49] for determining the age of a bloodstain in which the ratio between β-actin and 18S rRNA was used for estimation. Applying this method to a hair sample demonstrated that the age of hair also can be estimated using Anderson *et al.*'s method [75]. This confirms the hypothesis of the suitability of the approach to other tissue types besides blood. In this study, the efficiency of the amplification reaction was taken into consideration, and statistical software was used to correct variations, which is considered a positive addition to the method.

1.6 Saliva

Saliva is a heterogeneous fluid composed of water (~ 99%) and solid (1%) with the solid part made up of organic substances such as lingual lipase, L-amylase, kallikrein, lysosymes , and mucin, and of inorganic substances such as Na^+, Cl^-, K^+, and HCO_3^- [79]. Saliva also contains epithelial cells (~4.3×10^5 cells/mL) and a large number of bacteria (~1.7×10^5 bacteria/mL) [80].

Saliva is secreted by three pairs of salivary glands (the parotid, submandibular, and sublingual glands) (Figure 1-2). These glands secrete about one to two litres of saliva per day. The rate of saliva secretion varies according to the physical and

physiological status of the body; about 0.1mL/min is secreted at rest while during active stimulation saliva secretion up to 4mL/min [81].

Saliva serves five major categories of functions including taste and digestion, lubrication and mucosal protection, maintenance of tooth integrity, microbial control and pH maintenance [82].

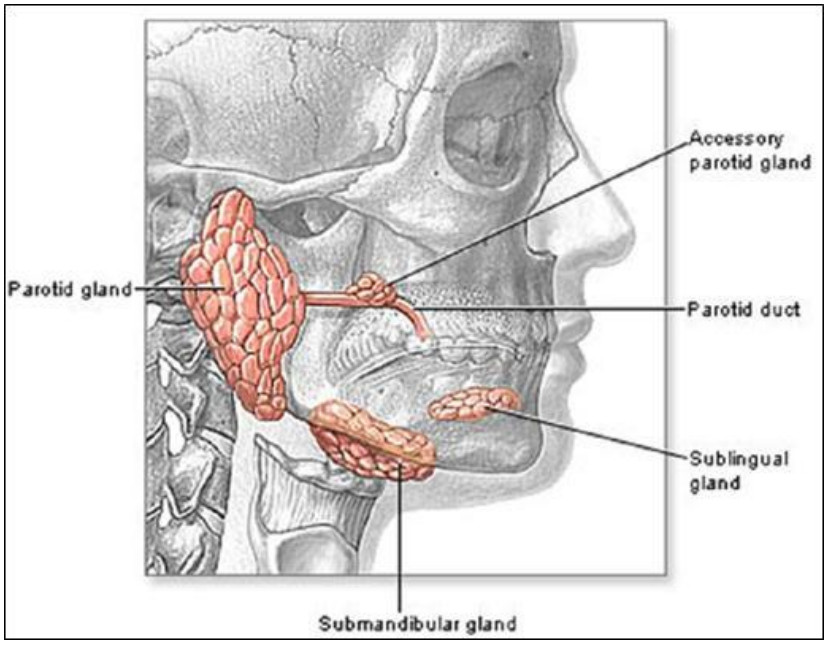

Figure 1-2: Salivary glands in a human. The figure shows the major salivary glands (parotid, submandibular, and sublingual glands), as well as the minor salivary glands [83].

Saliva is considered a clinically informative biological fluid and it is useful for many applications in clinical practice such as disease prognosis and laboratory or clinical diagnosis [84]. One of the main advantages of using saliva in clinical practice is that disease biomarkers and human DNA can be obtained noninvasively in comparison to blood that requires an expert phlebotomist. The amount of DNA in liquid saliva is about 1,000-10,000 ng/mL [85].

In forensic practice, saliva with blood, semen and vaginal secretions are considered to be the most frequently encountered body fluids in crime scene. Analysis of these body fluids may help forensic scientists and pathologists to prove or exclude an association between a suspect, victim and a crime scene [86]. In addition, identification of the type of body fluid found in a crime scene may give an indication

about the criminal act, which can be an important clue to reconstructing that crime scene. In addition, analysing saliva found in crime scene plays an important role in many forensic casework where enough DNA amount was retrieved to obtain DNA profile from saliva stains on different materials stored for different time [85].

Many techniques were developed to determine the type of saliva in crime scene such as alternate light source and those depending on α-amylase enzyme such as Phadebas® test [87]. However, these tests even the most-widely used Phadebas® test are used as presumptive tests and have the potential for false positive results. No confirmatory test for saliva identification is currently adopted in forensic practice. Many approaches using more sophisticated techniques have been suggested to identify saliva type, such as s Raman spectroscopy and approaches depending on genetic materials: RNA, miRNA and DNA methylation [62, 88-90].

The presence of a large number of bacteria, in saliva plays a role in increasing the level of nucleic acid degradation in saliva compared to blood and semen. The degradation rate of nucleic acid in saliva varies between individuals due to the difference in the species and the number of bacteria in saliva of different individuals [91, 92].

However, DNA and RNA were successfully found in saliva either fresh or old, despite the variety of ribonucleases present in saliva. More than 3000 human mRNAs were detected in cell-free saliva from healthy individuals. Moreover, many viral and bacterial RNAs were also detected in some infected individuals [93-97]. In addition, RNA was also successfully detected in six-year-old saliva stains [98].

In the study carried out by Kumar *et al.* [99]. no mRNA species were detected either in the cellular or non-cellular fraction of saliva using microarray and RT-PCR [99]. However, this study was criticised by Ballantyne [100], who referred the inability to detect mRNA in saliva to a problem in the technique used where mRNA was detected in saliva in many studies from both fresh and old saliva samples [45, 55, 101].

1.6.1 Source of RNA in saliva

RNA can enter the oral cavity through three sources. The first source is the major salivary glands (parotid, submandibular, and sublingual glands) and the minor glands, where the RNAs originate from secreting cells or may originate from other cells elsewhere in the body and transfer to the gland via the circulatory system [102-105]. The second source is the gingival crevice fluid (GCF), which contains various types of cells, including the cells of the gingival pockets and blood cells: leukocytes and erythrocytes [106]. The third source is desquamated oral epithelial cells.

Some studies carried out on salivary RNA have shown that most of the salivary RNA is partially degraded, but some appears intact. Much of salivary RNA possesses a poly (A) tail. Some studies have shown that some of the RNA molecules extracted from saliva have higher stability due to association of these RNA molecules with macromolecules [107]. Surprisingly, it has been found that endogenous mRNA is protected from direct degradation in the same way as cell free RNA in plasma [107, 108].

1.6.2 β –actin mRNA and 18S rRNA in saliva

β-actin mRNA and 18S rRNA were found in saliva in a detectable level [109-111]. It has been found that β-actin transcripts are present in high concentrations in saliva [94]. Most of the detected β-actin mRNA were partially degraded. β-actin mRNAs are associated with macromolecules and appear more stable than exogenous β-actin mRNAs, which degrade more slowly [107].

1.7 RNA extraction

Extraction of RNA is more difficult than DNA, because RNA is chemically and biologically more labile, especially at high temperatures and in alkali media [5]. In addition to these intrinsic handling difficulties, the aggressive activity of a variety of resilient ribonucleases (RNases) represents a significant challenge. These RNases exist in the environment as well as inside cells, sequestered inside organelles and vacuoles [112]. Therefore, to isolate the RNA, these factors should be taken into account. The first consideration is that all reagents used in the isolation process must

be free of RNase contamination. The second consideration is the effect caused by the fact that endogenous RNase activity must be avoided by formulating extraction tactics with sufficient management of endogenous RNase activity upon disruption of the organelles and vacuoles [5].

There are many methods of RNA extraction, and they yield cytoplasmic RNA, nuclear RNA, or a mixture of both, which is total or cellular RNA. In these methods, protocols begin with cellular lysis, using a buffer that falls into one of two categories. The first buffer category consists of harsh cytoplasmic agents that disrupt the cytoplasmic membrane and subcellular organelles and inactivate RNase. Sodium dodecyl sulphate, guanidinium salts, urea, phenol, chloroform, and N-laurylsarcosine (sarcosyl) are examples of this buffer category. The second buffer category consists of agents that gently solubilise the plasma membrane and keep the integrity of the nucleus and other organelles. Examples of this buffer category are non-ionic hypotonic lysis buffers [5].

Some of the methods of RNA extraction were modified to enable extraction of RNA and DNA simultaneously, which saves time, effort, and reagents [113, 114].

To sum up, more consideration should be taken with RNA extraction, as it is more unstable than DNA and given the aggressive activity of RNase. The method of RNA extraction should be chosen according to the type of the RNA that needs to be isolated.

In this work three types of extraction kits were used: TRI reagent, RNeasy® Micro Kit and AllPrep® DNA/RNA Mini Kit. All these kits consist of harsh cytoplasmic agents.

1.8 Total RNA quantitation (RNA quality and quantity)

It is necessary to ascertain the approximate quantity of total RNA extracted and to assess its quality. The accurate assessing of the quality and quantity of the total RNA has a real benefit in assessing and validating real-time PCR results. In the techniques used in gene using expression analysis such as TaqMan®, RT-PCR, southern and/or

microarray analysis, to obtain an accurate gene expression profile, it is necessary to know accurately the integrity and quantity of the starting materials.

There are many methodologies used in assessing the quality and quantity of RNA: spectrophotometric methods, electrophoretic profile of RNA, UV shadowing, sample capacity to support RT-PCTR, Northern analysis, and sample analysis to support *in vitro* translation [5, 115].

Spectrophotometric analysis, which is the most common approach, is used to assess RNA quality and quantity by measuring ultraviolet (UV) absorption. This method is the easiest and the most rapid. Pure RNA samples have a characteristic absorbance profile between 230-320 nm and result in a standard curve shape (Figure 1-3). Any deviation from this shape indicates the presence of contaminants: excess salt, contaminating proteins, and/or carryover organic solvents.

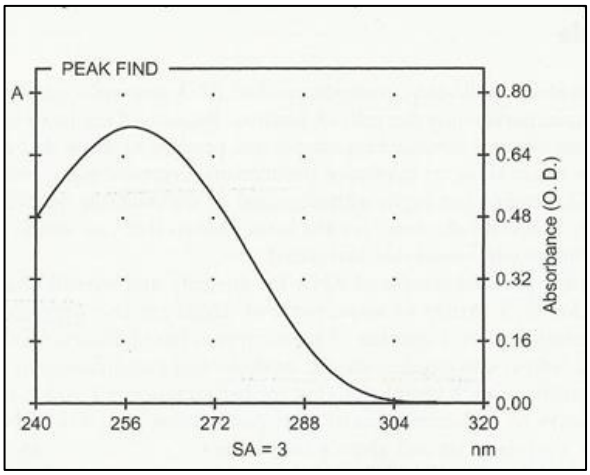

Figure 1-3: Typical UV absorbance spectrum of purified RNA [5].

RNA concentration is determined based on this equation:

$$[\text{RNA}]\mu g/ml = A_{260} \times \text{dilution} \times 40 \qquad \text{Equation 1-1}$$

where

A_{260} = absorbance, in optical densities, at 260 nm (OD_{260})

Dilution = dilution factor

40 = average extinction coefficient of RNA (40 µg/OD_{260})

The purity and quality of the sample are determined by measuring the ratio between UV absorbance at different wavelengths. The presence of contaminants is calculated from the ratio (A_{260}/A_{280}). In the pure sample, the ratio is 2.0 ± 0.1 (Table 1-4 shows the ratios between UV absorbance at different wavelengths).

Table 1-4: Strategic indicators of RNA purity [5].

Ratio	Pure Sample	Problem
A_{260}/A_{280}	2.0	Ratio below 1.8 suggests protein contamination
A_{260}/A_{230}	2.0 – 2.4	Ratio below 1.8 suggests organic compound contamination
A_{260}/A_{240}	1.4	Ratio below 1.4 suggests an excessive amount of salt in the sample

There are many advantages of using this method which has long been used as a standard in RNA quantitation. It is characterised with simple sample preparation where there is no need for additional mixing of reagents. The good reproducibility is another advantage of this approach. However, there are some disadvantages to using it, the major disadvantage being the false quantification reading resulting from the contaminants that absorb at 260 nm such as genomic DNA or phenol. In addition, a relatively large amount of sample is required for the analysis, which is considered another disadvantage of this approach [116].

The NanoDrop™ product line (NanoDrop Technologies, Wilmington, DE) is one of the amazing improvements in spectrophotometric instrumentation. Analysis of several samples in rapid succession can be accomplished with these instruments by measuring full-spectrum visible and UV wavelengths [5]. They require a small quantity of RNA for measurement, which is considered a great advantage.

The other method used in RNA quantitation is the Ribogreen analysis system which is an intercalating fluorescent analysis system. In this method, a fluorescent dye interacting with nucleic acid is used. Fluorescent detectors such as microplate reader or spectrofluorometer measure the emitted fluorescence. The Ribogreen system is a commercially provided RNA calibration standard. This system is highly sensitive

and requires small amounts of sample. However, its reproducibility is poor compared with UV. In addition it does not differentiate between RNA and genomic DNA and its results are affected with contaminants such as phenol [116].

The other method to assess RNA quality and quantity is the electrophoretic profile of RNA. The indicator used in gel electrophoresis for the purity of RNA is the measurement of the fluorescence ratio between 28S rRNA and 18S rRNA. However, conventional gel electrophoresis is labour-intensive and time-consuming and requires a large amount of RNA sample. However, the introduction of microchip electrophoresis makes this method easier and more feasible, as microchip electrophoresis requires small quantities of RNA sample (1 µL) and the analysis is carried out in a short period of time (about half an hour). Agilent's 2100 Bioanalyzer™ and the Bio-Rad Experion™ are examples of microchip electrophoresis that can be used to assess RNA quality and quantity [13]. In this method, a micro-fabricated chip is used to electrophoretically separate RNA samples that are detected via laser induced fluorescence detector. The RNA band size is estimated by using a RNA ladder during electrophoresis while the RNA integrity is visually assessed from the 18S and 28S ribosomal RNA bands [117, 118]. The high 18S and 28S rRNA peaks with a small amount of 5S RNA are indicative of preparation of intact RNA while the RNA degradation appears as an elevation in the threshold baseline and a decreasing in the 28S:18S ratio [118]. In addition, both Bioanalyzer 2100 and Experion software programs automatically generate the 28S/ 18S ratios. The perfect ribosomal ratio is 2, which is difficult to obtain, particularly for RNA extracted from clinical samples [117, 118]. The software programs also classify the RNA quality on a numbering system called the RNA Integrity Number (RIN) in which the RNA integrity is ranked from 1 to 10 with 1 which is most degraded and 10 which is most intact [115, 117, 118].

1.9 RNA stabilisation

Due to the instability of RNA, it is crucial to stabilise the RNA in order to obtain reliable gene expression analysis results. There are many methods used for RNA stabilisation.

1.9.1 Rapid freezing method

Rapid freezing method is a traditional method of RNA stabilisation where the RNA is stabilised by rapid freezing of the sample using liquid nitrogen or on dry ice [5, 119]. However there are some limitations in this method [5]. It needs special equipment, such as insulated vacuum flasks, for handling cold materials. It is important to rapidly freeze the sample and this may not happen in a large sample volume. This method can only help stabilise the sample after sample harvest and does not stabilise the sample at other stages of RNA analysis for example during centrifugation. In addition, ice crystals that are generated in the sample during freezing can break open the organelles and release nucleases inside them. Multiple cycles of freezing and thawing may cause further RNA degradation [5].

1.9.2 RNA stabilisation reagents for tissues

RNA stabilising reagents are commercially available reagents used to stabilise RNA such as RNA*later*® RNA Stabilisation Reagent [5, 120]. Using these reagents enables rapid and reliable gene-expression analysis. RNA becomes more stable with these reagents for example using RNA*later*® rRNA Stabilisation Reagent stabilise the RNA for 1 day at 37°C, 7 days at 18 to 25° C, 4 weeks at 2 to 8° C and for archival storage at -20 or -80°C. These reagents allow for sample cutting and handling at room temperature prior to RNA extraction without the need for ice or liquid nitrogen [5].

1.9.3 Strong denaturing reagents

Using chaotropic agents, such as guanidine isothiocyanate and guanidine hydrochloride, in RNA purification inactivates RNases and prevents RNA degradation. However, although these reagents are very effective during RNA purification, this effect is limited because they are added only after sample harvest [5].

1.9.4 Other stabilisation methods

There are reagents that are designed for specific purposes. For example RNA stabilising reagents are used for specific tissue sample, such as The PAXgene Blood RNA System which used for blood. Other reagents are designed to stabilise RNA from bacteria such as RNAprotect® Bacteria Reagent (Qiagen) [5].

1.10 Real-time PCR

Real-time PCR is also known as quantitative real-time polymerase chain reaction (Q-PCR/qPCR/qRT-PCR) or kinetic polymerase chain reaction (KPCR). Real-time PCR is a technique in which data is collected through the amplification process of a target DNA. This allows for the combining of amplification and quantification into a single step.

In real-time PCR, two common methods for detection are used: non-specific and specific detection methods. In the non-specific detection method, non-specific fluorescent dyes intercalating with any double-stranded DNA are used [121-124]. In the specific detection method, a sequence-specific probe is used [125-128]. This probe is labelled with a fluorescent reporter which allows detection only DNA target that hybridised with its complementary probe [129]. Each of these methods has its own advantages and disadvantages (Table 1-5).

Table 1-5: Pros and cons of specific and non-specific real-time PCR detection method [129]**.**

	Non-specific real-time PCR detection method	Specific real-time PCR detection method
Pros	- Relatively inexpensive - Can be incorporated into optimised and long-established protocols - Can be used to monitor the amplification of any double-stranded DNA sequence.	- Specific - Huge choice of chemistries - Multiplexing
Cons	- Non-specific (may generate false positive signals) - Requires post-PCR dissociation curve analysis - No multiplexing	- Expensive - Different probes are required for different sequences

A variety of fluorescent chemistry techniques are used to correlate the concentration of the PCR products to the fluorescence intensity [130]. Real-time PCR is characterised by displaying a PCR cycle at which the target amplification is first detected as a value. This value is called the cycle threshold (Ct), "the number of PCR cycles when the fluorescent signal is significantly above that of background noise and still in the exponential growth region of the curve" (i.e. the intersection between the amplification curve and the baseline) (Figure 1-4) [78]. Therefore, a higher concentration of target DNA will result in a lower Ct value, due to the faster significant increase in the fluorescence signal [131].

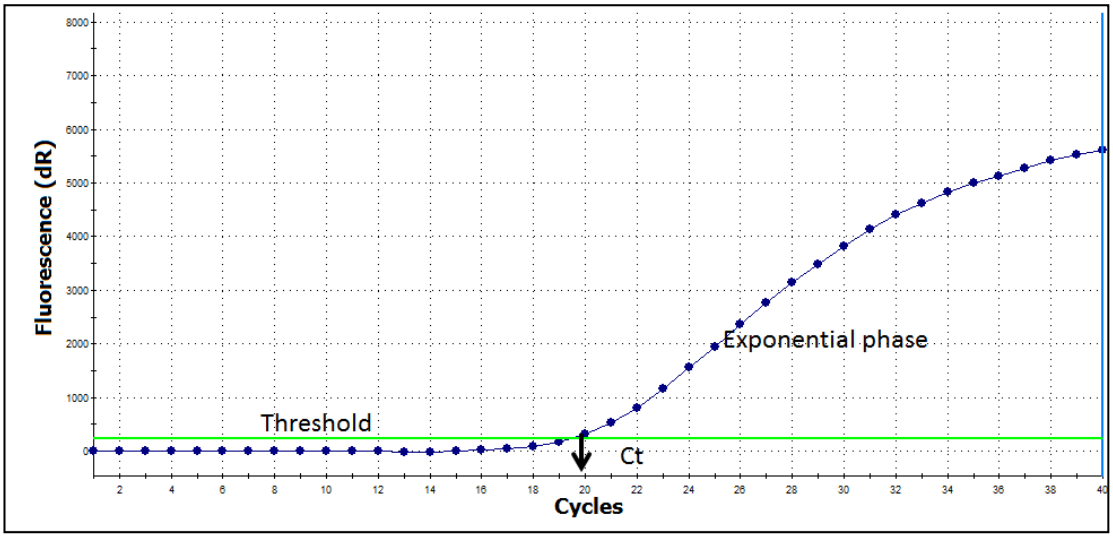

Figure 1-4: Graphical representation of real-time PCR data. Ct is the Cycle threshold and represents the intersection between the amplification curve and the baseline (Original figure from my data).

The values that are usually represented in the graphical representation of real-time PCR data include Ct value, normalised reporter (Rn) value, and delta Rn normalised reporter (ΔRn or dRn) value. The Rn value is the ratio of the intensity of fluorescence emission of the reporter dye to the intensity of fluorescence emission of the passive reference dye. ΔRn value is the normalisation of Rn obtained by subtracting the baseline:

$$\Delta Rn = Rn - \text{baseline} \qquad \text{Equation 1-2}$$

A PCR amplification curve can be divided into four phases: linear ground phase, early exponential phase, log-linear (exponential) phase, and plateau phase

(Figure 1-5). In the linear ground phase, the fluorescent emission does not increase above the background and spans the first 10-15 cycles, where the baseline is calculated. The phase when the fluorescent emission has become significantly higher than the background and has reached the threshold is called the early exponential phase, during which Ct value is detected. In the linear-log phase, the PCR products double each cycle under ideal conditions. This phase is also called exponential growth phase. The last phase is the plateau phase, when no significant fluorescent emission is detected, due to the consumption of the amplification products [132].

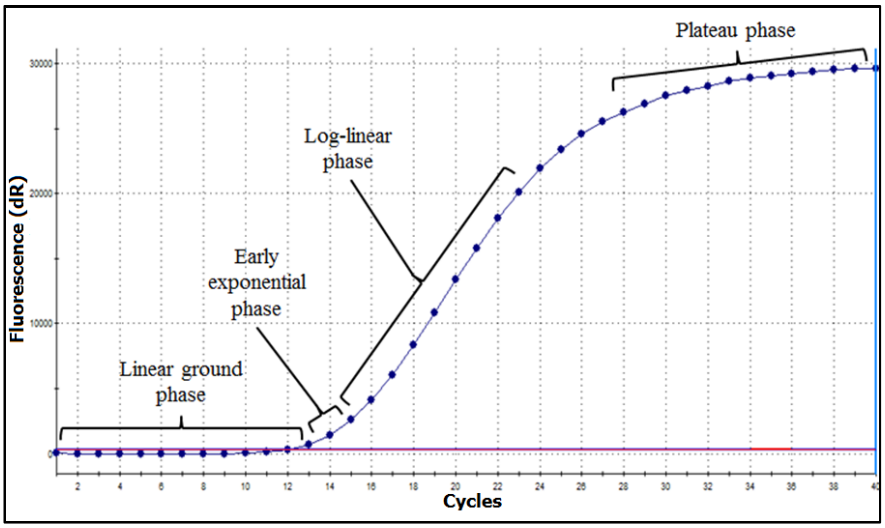

Figure 1-5: Phases of PCR amplification curve. The amplification curve can be divided into four phases:, the linear ground, early exponential, log-linear, and plateau phases [133].

Nowadays, real-time PCR has become the most widely used method for measuring gene expression [129]. There are many advantages to using real-time PCR over other methods of quantification gene expression analysis. Compared with other methods, real-time PCR requires a very small quantity of RNA templates and has relatively high throughput. It can detect even a single copy of a specific transcript [134]. It also can differentiate between mRNA molecules with almost identical sequences. It does not need post-amplification manipulation. In addition, it is significantly more sensitive than other methods [135, 136]. For example, it is more sensitive than RNase protection assays by 10,000- to 100,000-fold and more than dot blot hybridization by 1000-fold [135, 136]. The accurate dynamic range of the

quantitative data that is provided by real-time PCR is 7 to 8 log orders of magnitude [137].

Moreover, it can reliably detect small variances in gene expression between samples (as small as 23%) [138, 139].

In contrast, there are also some disadvantages of using real-time PCR. The high price of the equipment and reagents is considered one of these disadvantages. In addition, because of the extremely high sensitivity of real-time PCR, a rigorous experimental design and a full understanding of normalisation techniques are inevitably required in order to obtain an accurate conclusion.

In gene expression analysis, used to determine what genes are being expressed in a cell or tissue, real-time PCR can be carried out as a one-step quantitative reverse transcriptase-PCR (qRT-PCR) or as a two-step qRT-PCR. In a one-step reaction, both reverse transcription and PCR amplification are carried out in a single tube, while in the two-step reaction each is conducted in separate tubes (Table 1-6 shows the pros and cons of each method) [140].

Table 1-6: Pros and cons of one-step qRT-PCR versus two-step qRT-PCR [133, 140, 141].

	One-Step qRT-PCR	Two-Step qRT-PCR
Pros	- Minimise experimental variation because both enzymatic reactions occur in a single tube - Minimise the number of handling and pipetting steps - No contamination between RT and qPCR steps - Lower background in SYBR® assays - Best option for high-throughput screening (less time-consuming)	- More efficient because random primers and oligo d(T) can be used - More flexible (separate optimisation possible for the two reactions) - Possibility of stocking cDNA to quantify several targets - Data is quite reproducible
Cons	- No possibility to use UNG carry-over prevention - Usually less sensitive - Not suitable when the same sample is assayed on several occasions over a period of time	- RNase inhibitors can influence the PCR reaction after the RT - Higher background when performing a SYBR® assay

1.10.1 Real-time PCR quantification methods

There are two types of methods for real-time PCR quantification: absolute quantification and relative quantification. In the absolute quantification method, the concentration of the unknowns is determined depending on a standard curve generated by using a series of diluted standards of known concentration. The standard curve shows the relationship between the Ct value and the initial amount of total RNA, cDNA, or DNA. Therefore, the concentration of the unknown can be measured by determining its Ct value [131]. In this method, it is assumed that the sample and standards have equal efficiency [142]. The second method is the relative quantification method, in which the change in gene expression is measured based on a calibrator. The calibrator is either an external standard or an internal reference gene [143]. The result of this method is expressed as a ratio (target/calibrator). Many mathematical methods are employed to calculate this ratio (Table 1-7), and the results differ depending on the mathematical methods used [144, 145]. In general, each method of quantification has advantages and limitations.

Table 1-7: Mathematical methods used for measurement of gene expression in real-time PCR relative quantification [133].

Methods	Amplification Efficiency Correction	Amplification Efficiency Calculation	Amplification Efficiency Assumptions	Automated Excel-Based Program	Reference
Standard Curve	No	standard curve	no experimental sample variation	No	[146]
Comparative C_t ($2^{-\Delta\Delta C_t}$)	Yes	standard curve	reference = target	No	[143]
Pfaffl *et al.*	Yes	standard curve	sample = control	REST‡	[147]
Q-Gene	Yes	standard curve	sample = control	Q-Gene	[145]
Gentle *et al.*	Yes	raw data	researcher defines log-linear phase	No	[138]
Liu and Saint	Yes	raw data	reference and target genes can have different efficiencies	No	[144]
DART-PCR§	Yes	raw data	statistically defined log-linear phase	DART-PCR	[148]

‡ Relative expression software tool
§ Data analysis for real-time PCR

1.10.2 Relative Expression Ratio (RER) and Relative Quantity Ratio (RQR)

In many situations of gene expression analysis, the determination of the absolute copy number of the transcript is unnecessary and it is more relevant to report the relative change in expression of that gene compared to other genes which are expressed at relatively constant levels. Therefore, a relative quantification method is the preferable method to measure gene expression. In this method, the change in the gene expression is quantified based on either internal or external calibrators where the result is displayed as a ratio called the relative expression ratio (RER). Many software tools were established to compute this RER either between one sample and one control or between one sample and a control group. The RER is used in clinical applications where it shows the physiological change in gene expression levels. In clinical practice, the change in the RER may indicate a physiological change or a clinical condition [149-151]. Therefore, a gene expression analysis is carried out under strict conditions to avoid *ex vivo* degradation, which may affect the results [129, 147, 152].

The relative quantification method was used also to identify the relative quantity between 18S rRNA and β-actin in an aged bloodstain and a hair sample [49, 75]. The results of these studies demonstrated the relationship between the age of the samples and relative quantity of examined RNA molecules. Using this method and these two genes gives an advantage to this method when applied to other tissue types. In addition, the ratio is not affected by the size of the sample.

However, Hampson *et al.* [75] has used the term RER to describe the relative quantity of those RNA despite the fact this quantity may not represent the actual gene expression (because most of the RNA is degraded). Therefore, the use of this term when dealing with aged or degraded samples, such as that used in these studies, could be misleading.

The relative quantity ratio (RQR) is another term suggested in this thesis for preferred use in the case of dealing with aged samples or degraded RNA to more accurately reflect the RNA status. RQR gives an indication about the relative quantity of the RNA in the sample, regardless of the expression status of the gene.

1.10.3 Amplification efficiency

One of the important considerations in relative quantification is the amplification efficiency of the reaction. In the old methods of measuring gene expression, it was assumed that the efficiency is 1 and the PCR products double in each cycle in the exponential phase [153]. In fact, this assumption cannot be applied to all amplification reactions, where many reactions are not carried out under ideal conditions. Therefore, such assumptions will lead to incorrect estimation of gene expression. However, current mathematical methods take the amplification efficiency into consideration and always require precise measurement of it to measure gene expression accurately.

There are two ways of calculating amplification efficiency. In the first way, efficiency is calculated from a standard curve using the following formula:

$$\text{Efficiency} = 10^{(-1/\text{slop})} \qquad \text{Equation 1-3}$$

However, because efficiency varies during the exponential phase, where it starts relatively stable and then gradually declines to zero, calculating efficiency using this method does not always accurately represent changes in efficiency and overestimates it. This overestimation has a large effect on the quantification of gene expression. In the second way, efficiency is calculated from the raw data calculated during a PCR. This method is more accurate than the first method [143, 145-147].

1.10.4 Controls

There are many factors affecting the integrity of the real-time PCR process. Therefore, various controls are used to ensure the integrity of every step. For example, minus reverse transcription control (-RT control) is used to ensure no DNA contamination. In -RT control, the reverse transcription process is carried out using all its components except the reverse transcriptase enzyme. However, this control can be substituted by using primers span exon/exon junction. Table 1-8 shows these controls and their purposes.

Table 1-8: Controls used to correct variations in the real-time PCR process [133, 154].

Control	Example/Method	Purpose
Minus reverse transcription control (-RT control)	Run reverse transcription step but without reverse transcriptase	Used to ensure no DNA contamination
Endogenous control	Nucleic acid already present in the examined sample	Accounts for 1- the variation in the amount of the starting RNA and 2- the variation in the efficiency of the reverse transcriptase enzyme
Passive reverence dye	ROX dye	Accounts for the delicate variations in 1- PCR master mix volumes 2- non-PCR-related fluctuations in fluctuations in fluorescence signal
Exogenous control	RNA spike control kit	Accounts for the variations in PCR master mix itself

Endogenous control is important to correct the sample-to-sample variations resulting from the differences between individuals in tissue mass or cell number, RNA integrity or quantity, or experimental treatment. This process of correction is called normalisation. The main feature for the ideal control gene is its expression in an unchanging fashion. However, no gene has this feature in all experimental conditions. Therefore, before using a control gene for normalisation, its expression stability should be validated for the specific requirements of the experiment [154].

There are many approaches for normalising gene expression data. The first approach is normalisation against the mRNA housekeeping genes, such as GAPDH and β-actin. This approach has traditionally been used because of the previous assumption of the stable expression of those genes [155]. However, the high sensitivity and the high dynamic range of real-time PCR changed this assumption when it was found that the expression of these genes are affected by different factors including biological processes, treatments, and even different tissues or cell types [156-160].

Therefore, validation of the expression stability of a housekeeping gene is necessary for a specific requirement of the experiment [132]. The second approach is normalisation against ribosomal RNA (rRNA), particularly 28S and 18S rRNA. However, because of the structural difference between rRNA and mRNA and the

difference in the polymerases that transcript both of them, their expression may not be affected equally by treatments that significantly change mRNA expression [161]. The third approach is normalisation against total RNA concentration [132]. However, this method does not consider important factors: the effect of the cellular processes on total RNA and RNA quality and reverse transcription efficiency [133]. The fourth approach is normalisation against multiple mRNAs. This is a new method in which stability expression is measured in multiple housekeeping genes in the tested sample, and the most suitable genes for a specific experiment are identified. There are many software programs for measuring the expression stability of these control genes, such as geNorm and BestKeeper [162, 163]. However, this approach is the most labour-intensive approach.

The PCR product accumulated during the real-time PCRT process is detected using many methods of detection chemistries (Table 1-9 shows and compares these detection chemistries).

Table 1-9: Characteristics of detection chemistries. Presentation of and comparison between the characteristics of detection chemistry (+++, very expensive; ++, moderately expensive; +, inexpensive) [133].

Detection Chemistries	Example	Specificity	Multiplex Capability	Specific Oligonucleotide Required	Allelic Discrimination	Cost
DNA Binding Dyes	SYBR Green dye	two PCR primers	No	No	No	+
Hybridisation Probes	Four Oligonucleotide Method	two PCR primers; two specific probes	Yes	Yes	Yes	+++
	Three Oligonucleotide Method	two PCR primers; one specific probe	Yes	Yes	Yes	+++
Hydrolysis Probes	TaqMan Chemistry	two PCR primers; one specific probe	Yes	Yes	Yes	+++
Hairpin Probes	Molecular Beacons	two PCR primers; one specific probe	Yes	Yes	Yes	+++
	Scorpions	one PCR primer; one primer/probe	Yes	Yes	Yes	+++
	Sunrise Primers	two PCR primers	Yes	Yes	Yes	+++
	LUX Primers**	two PCR primers	Yes	Yes	No	++

** LUX, light upon extension

1.10.5 Causes of variation in real-time PCR

Although the high sensitivity of real-time PCR has given it an advantage over other gene expression analyses, this high sensitivity is considered the biggest obstacle in real-time PCR. This is because a minor variation during the PCR process, such as minor changes in the reaction components or in the thermal cycling conditions, may result in a large change in the overall amount of PCR product. Therefore, a large effort should be made to minimise factors causing variations in order to obtain the most accurate data possible. The variations in real-time PCR can occur during the reverse transcription step or the PCR step, or due to the difference between individuals performing the test (Table 1-10 shows the causes of variations in real-time PCR).

As a consequence of these causes, experimental variations are inevitable, and the effort is to minimise as many of them as possible. Therefore, the results obtained from any experiment should be validated by calculating the intra- and the inter-assay variations.

Table 1-10: Causes of real-time PCR variations. The table shows the causes of the variations in real-time OCR data and the suggested solution [133].

Real-time PCR step	Cause of variation	Example	Suggested solution
RT-PCR	PCR inhibitor	Transcriptase enzyme acts as PCR inhibitor	- Using a cDNA precipitation protocol - Omitting DTT from the reaction
		Dithiothreitol (DTT) acts as PCR inhibitor	
		Inhibitors in complex biological sources	
		PCR inhibitors carried over during sample preparation	
	Variable reverse transcription-PCR efficiency because of oligonucleotides used for priming	Gene-specific primers cannot assay both a target and a control gene from the same cDNA template	Using a mixture of both oligo(dT) and random hexamer primers during the reverse transcription reaction
		oligo(dT) priming may not effectively transcribe the 5′ end of long transcripts	
		Random and specific hexamers may not effectively transcribe the 5′ end of long transcripts	
	RNA template structure and concentration	RNA secondary structure and protein complexes can interfere with the reaction	Raising reaction temperature above 47°C (65)
	Structure and concentration of the reverse transcriptase enzyme	Different reverse transcriptase enzymes have differing abilities to read through secondary structure	Using reverse transcription enzyme with the greatest efficiency and accuracy e.g. SuperScript™ RT II (Invitrogen)
	Biological sample itself	In cases where whole tissue is assayed, measuring several different cell types within a single sample yields an average expression value of the different cell types	Using techniques such as laser-capture microdissection (LCM)
PCR step	Assay design	Have an effect on the amplification efficiency	Assay optimisation for specific experiment
	PCR equipment	Inaccurate or incorrect temperature	- Measuring any positional effect - Routine maintenance
	PCR reagents (even the same manufacturer)	Have an effect on the amplification efficiency	- Assay optimisation for specific experiment - Using the same batch of enzymes, buffers, and master mixes
External	Human performance of experiment	Inaccurate pipetting	- Precision pipetting and pipette calibration - Running a standard curve during every reaction - Using the same pipettes - Test performed by the same individual

1.11 TaqMan® Gene Expression Assays

Real-time PCR is often considered to be the touchstone or "gold standard" for nucleic acid quantification [164-166]. It offers many advantages compared to other techniques in terms of sensitivity and the specificity. It also offers a large dynamic range and high precision and reproducible quantitation [133, 167, 168]. TaqMan® based real-time PCR has become the preferred method to quantify gene expression and confirmation of microarray results due to its performance capabilities and ease-of-use [164, 169, 170].

TaqMan® chemistry is one of the methods of detection chemistries that are used in real-time PCR (Table 1-9 shows the methods of detection used in real-time PCR). In TaqMan® Gene Expression Assays(Applied Biosystems, Foster City, CA), the 5' nuclease activity of AmpliTaq Gold® DNA polymerase is utilised to hydrolyse an oligonucleotide (TaqMan® probe) hybridised to its target amplicon during PCR (Figure 1-6) [171]. TaqMan® Gene Expression Assays consist of a pair of unlabelled sequence-specific primers and a TaqMan® probe. The TaqMan® probe is labelled with a FAM™ or VIC® dye label on the 5' end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end. Using the primer set together with the TaqMan® probe affords two levels of sequence specificity. In addition, designing primers and probes spanning at least one intron of the genomic sequence will help in minimising problems associated with DNA contamination.

In this assay, during the extension step, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe. Once the probe is separated from the quencher, fluorescence emits from the reporter dye and increases from one cycle to the next due to exponential amplification of the PCR products. Fluorescence will be detected and measured using a real-time PCR machine. Cycle by cycle measuring of PCR products is carried out where a highly accurate and precise quantitation of gene expression over a large, dynamic range is provided during the highly reproducible exponential phase of PCR.

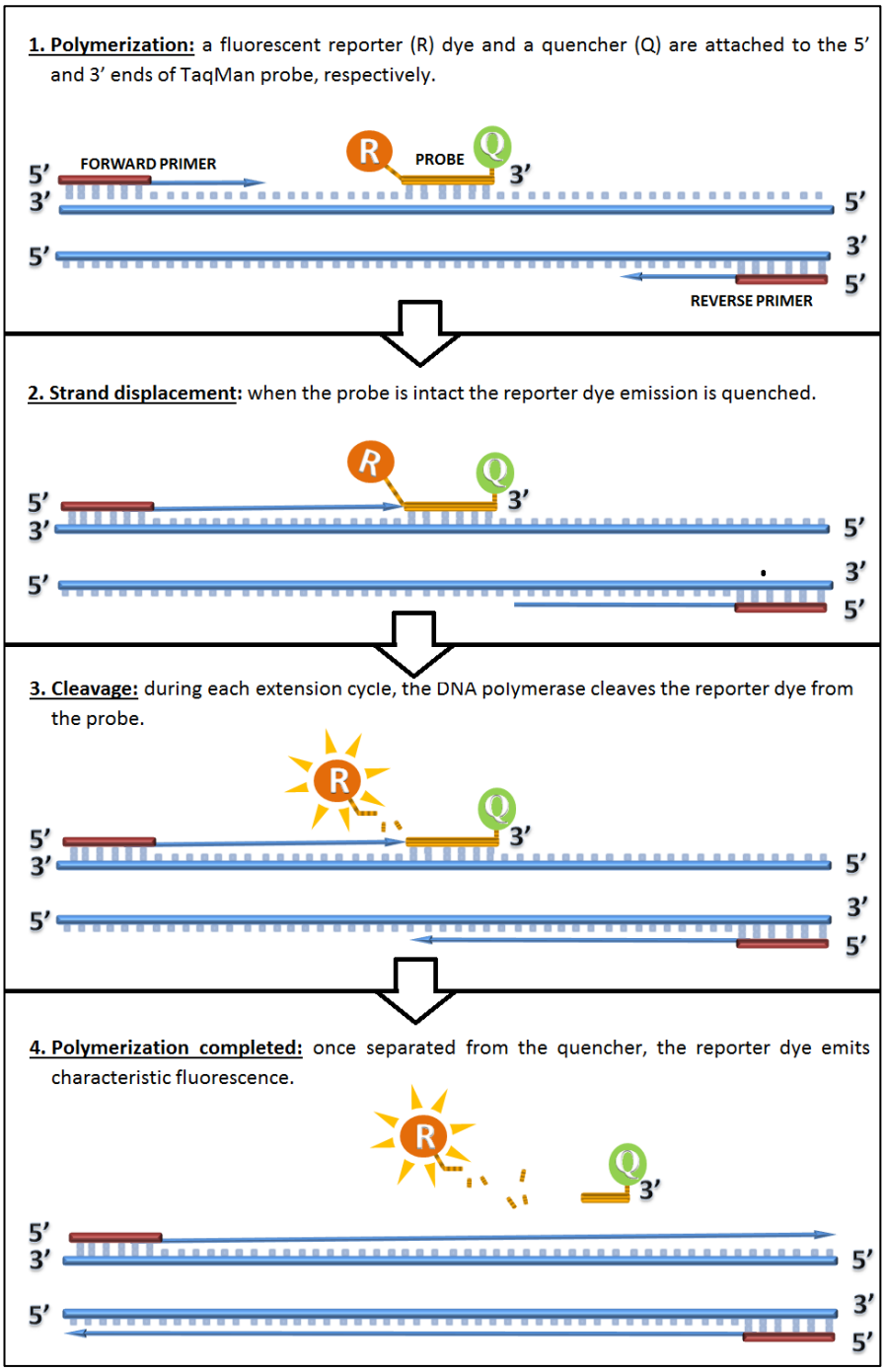

Figure 1-6: Scheme of TaqMan® Gene Expression Assay based Real-time PCR. A TaqMan® probe is designed to hybridise to a specific sequence between the forward and reverse PCR primers. The probe is labelled with a fluorescent reporter dye label and a quencher on the 5' and 3' end respectively. The reporter dye emission is quenched when the probe is intact. During the extension step, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe because of its 5' to 3' nuclease activity. Once the probe is separated from the quencher, fluorescence emits from the reporter dye. Because the amount of fluorescent signal emitted during each cycle of amplification is proportional to that generated by product, this provides the basis for the quantitative measurements of gene expressions. Original figure is based on the information from Wang *et al.* [171].

1.12 GenEx statistical software

GenEx statistical software (version 4.3.7; BioEPS GmbH, Munich, Germany) is software that was developed by MultiD Analyses AB (MultiD) company. It is software for real-time quantitative PCR (qPCR) expression profiling. The software offers many advantages in analysing the qPCR data. By using this software the real-time qPCR data were analysed easily. The analysis involves correction of the raw Ct values, normalising the data to many factors and performing statistics [172].

The raw Ct values obtained in reaction with efficiency lower than 100% can be corrected just by entering the actual reaction efficiency of the reaction in the software. The software was developed to be able to normalise the real-time qPCR data to many factors. The data can be normalised to the expression of reference gene (-s). The data obtained from different runs can be normalised to the inter-plate calibrator sample where the same sample is used on the different plates to ensure consistency in results taken from different plates. It will correct for any small variation between batches of reagents. The data can be also normalised against the qPCR repeats. In addition, the data obtained from samples containing inhibitors can be corrected using the GenEx software based on the Ct values of the spike in the samples and reference sample. Moreover, the data obtained can be statistically analysed including descriptive statistics, t-tests, non-parametric test and nested ANOVA [172].

Note that the term “spike” is used here to refer to a control consisting of a different DNA or RNA species to that of the unknown. It is amplified using a different set of primers to that of the experiment. The amplification of the spike is conducted in the presence of the extract used in the experiment. If inhibitors are indeed present in the experimental sample extract, then a reduction in the amplification of the spike should be noticed when compared to the amplification of a spike with no extract material present [172].

1.13 Aims

The overall purpose of this thesis is to assess and study the application of RNA analysis to establish the time since deposition of saliva. A number of different aspects of this work were studied and are described in the following passage.

The first piece if work reported here was to examine the hypothesis that the work carried by Anderson *et al.* [49] to determine the age of bloodstain could be extended to saliva stains stabilised using RNA*later*® reagent. This study is reported in Chapter Three. To test this hypothesis, the work reported by Anderson *et al.* [49] was firstly replicated to establish the procedure in the Centre for Forensic Science at Strathclyde and evaluate it for possible limitations. The method was then applied using more advance reagents, kits and statistical software that were not available at the time of the original work, i.e. preceding publication in 2005. These include the use of RNeasy® Micro Kit (Qiagen), and SuperScript® III First-Strand Synthesis System. In addition the real-time PCR data obtained was normalised, the GenEx statistical software was used for this.

The second hypothesis tested the effect of the RNA*later*® reagent on the stability of RNA molecules in saliva stains and consequently on the RQR values calculated. This hypothesis is described in Chapter Four. In order to study this hypothesis, the effect of the RNA*later*® reagent on the results from real-time PCR of RNA in RNA*later*® stabilised saliva stains was studied. This was extended to include the effect of the RNA*later*® reagent on the efficiency of end point PCR and, in turn, on the RNA profile.

The third hypothesis tested was if there is a relationship between the age of unstabilised saliva stains and the RQR of β-actin mRNA to 18S rRNA. This is discussed in Chapter Five. In this chapter the advantage of using of the co-isolation extraction kit (AllPrep® DNA/RNA Mini Kit) and QIAshredder homogeniser (Qiagen) was also evaluated.

The fourth hypothesis was to test if the RQR values of β-actin mRNA to 18S rRNA, found from stain analysis, are affected in cases where there is a mixture of saliva and

blood. This work is reported in Chapter Six where the effect of possible mixture combinations using specific volumes of 'fresh' and 'aged' saliva and 'fresh' and 'aged' blood was evaluated.

The fifth hypothesis tested concerns the use of tissue-specific mRNA species as targets for TaqMan gene expression assays, TGEAs. Specifically, there a relationship between the age of the saliva stains and the relative quantities of two different, and differently sized, segments of the same saliva-specific mRNA markers. This method allows simultaneous determination of the age and type of the stain using only mRNA specific to saliva. This type of test should circumvent difficulties anticipated where generic, housekeeping genes shared by different body fluids, are used. The testing of this hypothesis is reported in in Chapter Seven.

The final hypothesis to be considered is if the tissue specific test can be applied to blood stains simultaneously with that for saliva. The method is described in Chapter Seven.

1.14 Experimental strategies

To achieve the above aims, some strategies were implemented. Firstly, appropriate training for RNA extraction, reverse transcription and real-time PCR were carried out to ensure the validity of the results. Secondly, the work was carried out as a series of studies to identify the drawbacks and obstacles in each study in order avoid them in the next study. The third strategy was to follow restricted precautionary protocols to prevent contamination with genomic DNA, RNA and RNase and to decrease the possibility of operator error. These included using RNase-free gloves and changing them periodically, decontaminating the work area with the RNase decontamination solution RNAzap® (Ambion) and using RNase-free reagents and disposable RNase-free plastic ware. To ensure no genomic DNA contamination, RNA extracts were treated with DNase I enzyme and minus-RT control was run in real-time PCR. In addition, no-template control was run in all steps of the experiment: extraction, reverse transcription and real-time PCR. Samples were run in duplicate in reverse transcription and in duplicate or triplicate in real-time PCR to be

confident about the results. Real-time PCR sample run in duplicate was repeated if the difference between the results was more than one. Fourthly, DNA profiling was carried out for all samples extracted using kits that simultaneously extracted DNA and RNA in order to assess the success of co-isolation and to identify any potential DNA contamination.

Although the first part of the work in this thesis is based on Anderson *et al.'s* work [49], some innovations were applied in order to improve the work. These innovations include using more efficient purification kits for RNA extraction i.e. RNeasy® Micro Kit and AllPrep® DNA/RNA Mini Kit. These kits allow for fast extraction of RNA from very small amounts of starting materials with high purity and quality. Further innovation is the use of RNA stabiliser i.e. RNA*later*® RNA stabilisation reaction. This RNA stabiliser offers some advantages if it used in real cases, such as storing samples without further RNA degradation, which will save the evidence and enable retesting of samples collected from crime scene (a frequent requirement in forensic cases). It is also offers an advantage of saving the sample of the experiment from further RNA degradation when it reaches the desired age. Because the experiment is about predicting the age of a biological sample, it was essential to extract the RNA from the sample at the exact desired time because any delay would lead to more degradation in the RNA and the results would be inaccurate. No samples would be available for confirmatory repeating on a different day because the age would be different. In addition, examination of a sample at the desired age may be missed in case of failure of RNA extraction or presence of contamination at those samples. All these factors, and the fact that this research area is totally new, considering the department and the researcher, means that there is a higher likelihood of errors occurring. Therefore, use of RNA stabiliser to stop the RNA degradation process in samples when they reach desired age will help in the following area: controlling the age of the sample; performing the experiment at any time; and the possibility of repeating RNA analysis for the same sample, where the sample will be aliquoted and the RNA in more than one aliquot will be stabilised at specific age, with one used for analysis and other used for repeating. Finally, a statistical software program (GenEx software) was used to correct the variations which resulted from the high sensitivity

of the real-time PCR and differences in efficiency between reactions in order to obtain many valid results as possible.

2 Materials and methods

This chapter explains the materials and methods of the studies used in this thesis.

2.1 Samples collection

Biological samples were provided by volunteers after obtaining ethics approval and gaining consent from all the volunteers concerned.

2.1.1 Blood

Two methods of blood collection were used.

2.1.1.1 Blood collection via finger stick

Blood collected by finger stick (50 µL) was dropped on a clean sterile cotton swabs. Samples were stored at room temperature in a dry place to simulate natural aging until they reached the desired ages (0, 7, 14, 21 and 30 days).

2.1.1.2 Blood collection via venepuncture

Blood Collection and Sampling 5 mL of blood was collected by standard venepuncture from two men and one woman on two different occasions. 20 µL aliquots were immediately spotted on the clean sterile cotton swabs and stored at room temperature in a dry place to simulate natural aging until they reached the desired ages (0, 7, 14, 21 and 30 days). When the samples reach the desired age, one of the proceeded to next step (extraction) and one stabilise using RNA stabiliser.

2.1.2 Saliva

Saliva samples were collected from healthy volunteers with no history of malignancy. Collected saliva samples were dried by different methods. In the first method, 50 µL of saliva was dried into sterile cotton clothes with size 0.5 × 0.5 mm. In the second method, RNase/DNase free cotton swabs were dipped into saliva samples. Samples in both methods were stored at room temperature in a dry place to stimulate natural aging until they reach the desired ages (0, 1, 2, 3, 7, 14, 21, 28, 35

and 60 days). When samples reach the desired age, either immediately proceeded to next step (extraction) or stabilised using RNA stabiliser.

2.2 RNA stabilisation

About 350-450 µL of the RNA stabiliser, called RNA*later*® (Qiagen, Valencia, CA), was added to each blood and saliva sample when they reached the desired ages to stop further RNA degradation.

2.3 RNA Extraction

RNA was extracted from two types of samples; blood and saliva. Different extraction techniques were used with each type of samples, procedures 2.3.1 and 2.3.2 for blood and saliva respectively. The extracts were passed directly to the next steps of RT-PCR and RNA quantitation or stored at -80˚C to prevent RNA degradation. A negative control sample was run simultaneously with each assay this negative control is called extraction no template control (Ext-NTC) sample.

2.3.1 RNA extraction from blood using TRI reagent

2.3.1.1 Materials

Materials used were TRI reagent (Molecular Research Center) (cat # T3809-100ML), polyacryl carrier (Molecular Research Center) (cat # PC152), RNase free water, 1-bromo-3-chloropropane (B9673-200ML), isopropanol and 75% ethanol.

2.3.1.2 Procedure

The bloodstain sample was added to a solution consisting of 200 µL of water and 3 µL of a polyacryl carrier and then 750 µL of TRI Reagent BD was added. The sample was briefly vortexed and then incubated at 50 ˚C for 10 min. 100 µL of 1-bromo-3-chloropropane was added. The sample was vortexed for 15 seconds and then incubated at room temperature for 3 min. The sample was then centrifuged for 15 min at 4 ˚C at 12,000 × g.

The upper aqueous layer which contains RNA molecules was added to a new tube (~500 µL) while the lower phase, containing DNA molecules was stored for the purpose of DNA extraction as shown in section 2.8.1.

500 µL cold isoproponal was added to the upper aqueous layer. Sample was inverted twice and then incubated at room temperature for 7 min. The sample was then centrifuged for 8 min at 4 ˚C at 12,000 × g. The liquid supernatant was discarded. 1 ml of 75% ethanol was added to wash the RNA pellet. The sample was briefly vortexed and centrifuged for 5 min at 4 ˚C at 12,000 × g. The liquid supernatant was removed and the RNA pellet was allowed to air-dry for 5 min at room temperature. Finally, 40 µL of RNase free water was added to resuspend the RNA pellet and the samples was incubated at 55˚8C for 10 min.

2.3.2 RNA extraction from saliva sample

2.3.2.1 RNA extraction from Saliva using RNeasy® Micro Kit

2.3.2.1.1 Materials

The material used were RNeasy® Micro Kit (Qiagen, Valencia, CA) (cat #74004), β-mercaptoethanol (cat # M7154-25ML) and 70% and 80% ethanol.

2.3.2.1.2 Procedure

The RNeasy® Micro Kit manufacturer's instructions were followed with minor modifications. These modifications included incubation of the sample after the step of addition of the 350 µL of Buffer RLT containing β- mercaptoethanol. The samples were incubated for three hours at 56 ˚C in a thermoshaker at a speed of 900 rpm. In addition, DNase I enzyme was used to eliminate genomic DNA.

2.3.2.2 RNA extraction from saliva using AllPrep® DNA/RNA Mini Kit

2.3.2.2.1 Materials

The materials used were All/Prep DNA/RNA Mini Kit (Qiagen, Valencia, CA) (cat # 80204), β-Mercaptoethanol (cat # M7154-25ML) and 70% Ethanol.

2.3.2.2.2 Procedure

The All/Prep Mini Kit manufacturer's instructions were followed with minor modifications. These modifications include the incubation of the sample after the step of addition of the 350 µL of Buffer RLT containing β-mercaptoethanol. The samples were incubated for three hours at 56 ˚C in a thermoshaker at a speed of 900 rpm. In addition, the QIAshredder spin columns (Qiagen) were used where lysate directly pipetted into the column and centrifuged for 4 min at 13,000 rpm.

2.3.3 DNase treatment of RNA using Ambion® TURBO DNA-free™

2.3.3.1 Materials

The material used was Ambion TURBO DNA-free™ (cat #AM1907M).

2.3.3.2 Procedure

For 35 µL reaction volume, 3.5 µL 10X TURBO DNase Buffer and 1 µL TURBO DNase were added to 30.5 µL of RNA. The tube was then mixed gently and incubated at 37°C for 20–30 min. A 3.5 µL resuspended DNase Inactivation Reagent was then added and mixed well. Thereafter, it was incubated for 5 min at room temperature, with occasional mixing. The tube was then centrifuged at 12,000 rpm for 1.5 min. Finally, the RNA was transferred to a fresh tube.

2.4 Total RNA quantitation

2.4.1 Total RNA quantitation using Nanodrop

Total RNA in present elutes was quantified using NanoDrop 1000 Spectrophotometer according to manufacturer's instructions using 1.5 µL of the extract. The RNA quantitation runs in triplicate.

2.4.2 Total RNA quantitation using Agilent RNA 6000 Pico LabChip Kit

Total RNA present in elutes was quantified using Agilent RNA 6000 Pico LabChip Kit, according to manufacturer's instructions, using 1 µL of the extract. The RNA quantitation runs in duplicate.

2.5 Reverse transcription

Reverse transcription has been carried out using two types of kits: TaqMan® Gold Reverse Transcription Reagent from applied Biosystems and SuperScript® III First-Strand Synthesis System from Invitrogen.

2.5.1 Reverse transcription using TaqMan® Gold Reverse Transcription Reagent Kit

2.5.1.1 Materials

The material used was the TaqMan® Gold Reverse Transcription Reagent (Applied Biosystems) (cat # N808-0234).

2.5.1.2 Procedure

Amplification reactions were carried out in a 25 µL reaction volume with a final concentration of the reverse transcriptase master mix; 1X TaqMan® buffer A, 5.5 mM $MgCl_2$, 500 µM each dNTP and 2.5 µM random hexamer, 31.25 U Multiscript transcriptase and 10 U RNase inhibitor. Minus reverse transcriptase (-RT) control was used with each assay to ensure there was no contamination with genomic DNA.

Samples were placed in thermal cycler under the following conditions (25 ˚C for 10 min, 48 ˚C for 30 min and 95 ˚C for 5 min).

2.5.2 Reverse transcription using SuperScript® III

2.5.2.1 Materials

The material used was SuperScript® III First-Strand Synthesis System (Invitrogen) (cat # 18080-051).

2.5.2.2 Procedure

Amplification reactions were carried out in a 20 µL reaction volume. Manufacturer's instructions were followed for preparation of cDNA synthesis mix and RNA/primer mixture as shown in the Table 2-1.

Table 2-1: Reverse transcriptase master mix preparation using SuperScript® III (Invitrogen). A) preparation of 10 µL cDNA synthesis mix. B) preparation of 10 µL RNA/primer mix.

A

cDNA synthesis mix		
	1X (µL)	
10X RT buffer	1	
25mM $MgCl_2$	4	
DTT	2	
RNase OUT	1	
Superscript	1	
Total	10	

B

RNA/primer mixture		
	1X (µL)	
Random hexamer	1	
dNTP	1	
Water	2	
	4	
Sample (RNA)	6	
Total	10	

Finally a 20 µL volume was prepared by adding 10 µL of cDNA synthesis mix to 10 µL of RNA/primer mixture. Then samples were placed in thermal cycler under the following conditions: 25 ˚C/10 min, 50 ˚C/50 min, 85 ˚C/5 min. Unwanted RNA molecules were removed by adding 1 µL RNase H to each tube and incubating at 37 ˚C for 20 min.

To confirm the purity of the extract from any contaminating DNA, the same reverse transcription method but without using reverse transcriptase enzyme was carried out on a portion of each extracted RNA sample.

2.6 Real-time PCR

Real time PCR was carried out using two types of kits; TaqMan® Universal PCR Master Mix Kit and TaqMan® Universal Master Mix II Kit from applied Biosystems.

2.6.1 Materials

The materials used were β-actin control reagent (Applied Biosystems) (cat # 401846), 18S rRNA control reagent (Applied Biosystems) (cat #4308329), and either TaqMan® Universal PCR Master Mix (Applied Biosystems) (cat # 4304437) or TaqMan® Universal Master Mix II (Applied Biosystems) (cat # 4440040).

2.6.2 Procedure

Amplification reactions were carried out in a 25 µL reaction volume with a final concentration of real-time PCR master mix: 1X TaqMan® buffer, 100 nM 18S rRNA forward and reverse primer, 100 nM VIC dye, 300 nM β-actin forward and reverse primers and 200 nM FAM dye. Samples were placed in Stratagene Mx3005P™ multiplex quantitative PCR system (Stratagene) and run under the following conditions: 50 ˚C for 2 min, and 95 ˚C for 10 min and then 40 cycles of 95 ˚C for 15 seconds and 60 ˚C for 1 min).

To ensure efficient real-time PCR, there were a number of steps. First, each cDNA sample from each time point for each individual was run in either duplicate or triplicate. Second, standards in 10-fold dilution series with five points were run in duplicate with each batch in order to calculate the efficiency of the reaction in that batch. Third, randomly chosen cDNA samples were run at three points of 10-fold dilution in order to assess the efficiency of the individual qPCR. Forth, a control cDNA (i.e. same cDNA aliquot, also called inter-plate calibrator) was run with all real-time runs in order to control the variability of primers and probe, and master mix as well as machine. A negative control sample was run simultaneously with each run.

2.6.3 Data analysis

Real-time PCR data were analysed using GenEx statistical software (version 4.3.7; BioEPS GmbH, Munich, Germany). This software normalises the raw Ct values against individual efficiency rates, interpolate calibrators and qPCR repeats.

All statistical data analyses and graphs were prepared using the Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA) and Microsoft Excel statistics program (Microsoft, USA).

2.7 Optimisation of primer and probe for real time PCR

2.7.1 Materials

The materials used were the β-actin control reagent (Applied Biosystems) (cat # 401846), 18S rRNA control reagent (Applied Biosystems) (cat #4308329) and TaqMan® Universal Master Mix II (Applied Biosystems) (cat # 4440040).

2.7.2 Procedure

Singleplex optimisation was carried out for each primer pair (β-actin primers pair and 18S rRNA primer pair). Each amplification reaction was carried out in a 25 µL reaction volume using 1× TaqMan® Universal Master Mix II (Applied Biosystems), forward and reverse primers, 3 µL cDNA sample, and 200 nM probe. Table 2-2 and Table 2-3 show the concentrations that are evaluated in the optimisation study for the forward and reverse primers. After primer optimisation, probes (FAM dye-labelled β-actin probe and VIC dye-labelled 18S rRNA probe) were optimised using the optimal obtained primer concentrations in a 25 µL reaction volume. The concentrations that were evaluated in optimisation study for each probe are 100, 150, 200 and 250 nM. Each sample was carried out in triplicate. Samples were placed in Stratagene Mx3005P™ multiplex quantitative PCR system (Stratagene) and run under the following conditions: 50 ˚C for 2 min, and 95 ˚C for 10 min and then 40 cycles of 95 ˚C for 15 seconds and 60 ˚C for 1 min.

Table 2-2: Concentration evaluated in optimisation study of forward and reverse β-actin primers.

		Forward primer (nM)			
		300	200	100	50
Reverse primer (nM)	300	✓	✓	✓	✓
	200	✓	✓	✓	✓
	100	✓	✓	✓	✓
	50	✓	✓	✓	✓

Table 2-3: Concentration evaluated in optimisation study of forward and reverse 18S rRNA primers.

		Forward primer (nM)				
		250	200	150	100	50
Reverse primer (nM)	250	✓	✓	✓	✓	✓
	200	✓	✓	✓	✓	✓
	150	✓	✓	✓	✓	✓
	100	✓	✓	✓	✓	✓
	50	✓	✓	✓	✓	✓

2.8 Optimisation of TaqMan® Gene Expression Assay, TGEA, multiplexing

2.8.1 Materials

The materials used were TaqMan® Gene Expression Assay (Applied Biosystems) and TaqMan® Universal Master Mix II (Applied Biosystems) (cat # 4440040).

2.8.2 Procedure

A multiplex reaction was carried out using a pair of TGEA. Each amplification reaction was carried out in a 20 µL reaction volume using 1×TaqMan® Universal Master Mix II (Applied Biosystems), 4 µL cDNA sample, and either 0.5 µL or 1 µL of each of the 20×TaqMan® Gene Expression Assays pair.

At the same time, a singleplex reaction was carried out for each TGEA according to manufacturer's instructions (i.e. using 1 µL of TGEA). Table 2-4 shows the volumes that are used and evaluated in the optimisation study for each TGEAs pair. Each sample was carried out in triplicate. Samples were placed in Stratagene Mx3005P™ multiplex quantitative PCR system (Stratagene) and run under the following conditions: 50 ˚C for 2 min, and 95 ˚C for 10 min and then 40 cycles of 95 ˚C for 15 seconds and 60 ˚C for 1 min. Finally, the results of the multiplex reaction were compared with those of the singleplex reactions.

Table 2-4: Amplification components for the TGEA pair multiplexing. Volumes shown are for each TGEAs multiplex prepared

PCR reaction mix component	Volume per 20-µL reaction (µL)	
	For 1 µL TGEA	For 0.5 µLTGEA
2×TaqMan® Gene Expression Master Mix	10	10
20×TaqMan® Gene Expression Assay 1	1	0.5
20×TaqMan® Gene Expression Assay 2	1	0.5
Template (1 to 100 ng)	4	4
Water	5	6
Total	20	20

2.9 DNA extraction

2.9.1 DNA extraction from blood using TRI Reagent

2.9.1.1 Materials

The materials used were DNAzol® Genomic DNA Isolation Reagent (Molecular Research Center, Cincinnati, OH) (Cat. # DNA 127) polyacryl carrier (Molecular Research Center) (Cat. # PC152), DNase/RNase-free water, sodium dodecyl sulphate (SDS), proteinase K and 95% ethanol.

2.9.1.2 Procedure

DNA was extracted from the lower phase of the bloodstain processed in section 2.3.1.2. The lower phase comprises the interphase and organic layers. The blood-

stained cotton in the organic layer was removed and washed in 500 µL of DNAzol and was then placed in mixture of 1 mL of DNAzol, 10 µL of 10% sodium dodecyl sulphate (SDS) and 20 µL of 20 mg/ml of proteinase K. The samples were then incubated for two days at room temperature with occasional vortexing. The samples were then spun for 10 min at 10,000 × g. The supernatant was removed. To each sample, 3 µL of polyacryl carrier and 500 µL of 95% ethanol were added. The tubes were inverted several times and incubated for 3 min at room temperature. Then, samples were spun for 5 min at 5000 × g and washed with 1 mL of ethanol and spun for 2 min at 2000 × g, followed by washes. After discarding the supernatant, the DNA pellet was air dried for 5 min. Finally, 20 µL of nuclease-free water was used to resuspend the DNA pellet.

2.9.2 DNA extraction from saliva using AllPrep® DNA/RNA Mini Kit

2.9.2.1 Materials

The material used was All/Prep DNA/RNA Mini Kit (Qiagen, Valencia, CA) (cat # 80204).

2.9.2.2 Procedure

DNA was extracted from saliva samples processed in section 2.3.2.2 according to the All/Prep Mini Kit manufacturer's instructions.

2.10 DNA quantification using Investigator® Quantiplex Kit

2.10.1 Materials and method

DNA was quantified using the Investigator® Quantiplex Kit according to the manufacturer's instructions.

2.11 DNA profiling using Investigator Decaplex SE Kit

2.11.1 Materials and methods

DNA profiling was obtained using Investigator Decaplex SE Kit according to the manufacturer's instructions.

Electrophoresis was performed in duplicate using the 3130 Genetic analyser (Life Technologies, UK). A 1 µL of each amplified sample was added to 12 µL Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 µL of DNA Size Standard 550 (BTO). Tubes were heated at 95˚C for 3 min and snap cooled on ice for at least 3 min. Samples were injected through the capillary using 3130 POP-7 run module. The electrophoresis parameters used for all the samples are described in Table 2-5.

Table 2-5: Electrophoresis parameters used to run samples amplified using Investigator Decaplex SE Kit.

Electrophoresis parameters	Condition
Injection time	5 s
Oven temperature	60°C
Injection Voltage	15kv
Run time	35 min

3 Determination of the age of saliva stains stabilised in RNA*later*® reagent

3.1 Introduction

This chapter discusses the determination of the age of saliva stains preserved in RNA*later*® which is commercially available products used to stabilise RNA degradation in laboratory samples in clinical analysis. The method used to determine the age of the stains is the method proposed to determine the age of biological stains depending on the relative quantity ratio (RQR) between two different species housekeeping gene RNA molecules namely, β-actin mRNA and 18S rRNA molecules. This method has been used to determine the age of bloodstain and the age of hair sample [49, 75]. However, some modifications were applied to the method used, including the extraction technique and reverse transcription kit used.

The extraction method used was RNeasy® Micro Kit. There are many reasons to adopt column-based nucleic acid purification instead of the phenol/chloroform method applied to the bloodstain and hair sample [49, 75]. Firstly, column based extraction techniques are widely used and indeed have been used in the inter-laboratory studies carried out by the European DNA Profiling group (EDNAP). Secondly, there are advantages that are exhibited by this method over the conventional phenol/chloroform method with respect to time and safety, as well as the column-based extraction method's ability to extract RNA from very small amounts of starting material with a high purity and quality.

The reverse transcription kit used was the SuperScript® III First-Strand Synthesis System. This kit was used in order to obtain higher cDNA yields and also because its high sensitivity enables detection of template molecules from as little as 1.0 pg.

Furthermore, RNA*later*® was used in this experiment to stabilise RNA molecules in saliva stains when they reach the desired ages. Many factors contributed to use of RNA*later*®. First, it was important to extract the RNA from the sample at precisely the desired time, delays lead to more RNA degradation and inaccurate results. Second, no samples would be available for confirmatory repeating on different days

due to the inevitable age difference. Third, examination of a sample at the desired age may be missed by failure of RNA extraction or the presence of contamination in the samples.

All these reasons, combined with the fact that this there is a little prior research in this area, increase the likelihood of error occurring. Therefore, the use of RNA stabiliser to stop the RNA degradation process in samples when they reach a desired age will help to: control the age of the sample; perform the experiment at any time; and provide the potential of repeating RNA analysis for the same sample, where that sample will be aliquoted and the RNA in more than one aliquot will be stabilised at a specific age with one used for analysis and another used for repeating. In addition, RNA*later*® stabiliser offers some advantages if it is used in real cases. For example, the storage of samples without further RNA degradation, thus preserving the evidence and enabling samples collected from crime scenes to be tested, which is a frequent requirement in forensic cases. It also offers the advantage of saving the sample of the experiment from further RNA degradation when it reaches the desired age.

The work in this chapter will be carried out in two stages. In the first stage, the optimisation of the primers and probes that will be used in the real time-PCR step of the study will be carried out to ensure efficient qPCR amplification and this stage will be discussed in section 3.2. In the second stage, the method developed by Anderson *et al.* [49] will be applied on the saliva stain using the optimal obtained concentrations of primers and probes and this stage will be discussed in section 3.3.

3.2 Optimisation of primers and probes

3.2.1 Materials and method

3.2.1.1 Sampling, RNA extraction and reverse transcription

100 µL of fresh saliva sample was collected in a clean sterile tube. RNA extraction was carried out using RNeasy® Micro Kit (Qiagen) as described in section 2.3.2.1. The reverse transcription was carried out using SuperScript® III (Invitrogen) as described in section 2.5.2.

3.2.1.2 Real-time PCR

The β-actin primers and 18S rRNA primers and their probes were run in real time PCR using the procedure described in section 2.6.

3.2.1.3 Data analysis

All statistical data analyses and graphs were carried out in the Microsoft Excel statistics program (Microsoft, USA).

3.2.2 Results and discussion

As a different body fluid (i.e. saliva stain) was used, and in order to obtain the optimum results of real time PCR, this study began with assay optimisation of the primers and probes to ensure efficient qPCR and to correct any limitations. Singleplex optimisation studies on the probes and multiple primer combinations were carried out. The probes and primer combinations that had greatest efficiency (i.e. lowest Ct value and dRn value) were chosen and tested as a multiple assay. Singleplex optimisation studies showed that the most efficient concentration for both forward and reverse β-actin primers was 300 nM, which is similar to the results of Anderson *et al.* [49] and Hampson *et al.* [75], while the efficient concentration for 18S rRNA (forward and reverse primers) was 150 nM (Figure 3-1 and Figure 3-2). Moreover, the results also showed that the most efficient concentration for the FAM probe was 200 nM, while the most efficient concentration for the VIC probe was 100 nM (Figure 3-3). Therefore, a multiplex of the primers was prepared from probes and primer combinations showing the greatest efficiency (i.e. lowest Ct value and largest dRn) in the singleplex optimisation studies. The multiplex from these concentrations results in a qPCR with high efficiency (β-actin: 83.6% and 18S rRNA: 95.2%) (Figure 3-4).

Although the qPCR of both β-actin mRNA and 18S rRNA showed high efficiency, it was kept in mind that this small change in the efficiency has a pronounced effect on Ct values and in turn on the RQRs due to the high sensitivity of the real time PCR. Therefore, these obtained efficiencies were used in the GenEx software to correct the raw Ct values (Table 3-2). Comparing the raw Ct values of the samples in Table 3-1,

the corrected Ct value in Table 3-2 demonstrates the significant impact of a small change in reaction efficiency on the amplification reaction, and shows the advantage of using statistical software, such as GenEx, to correct such a limitation.

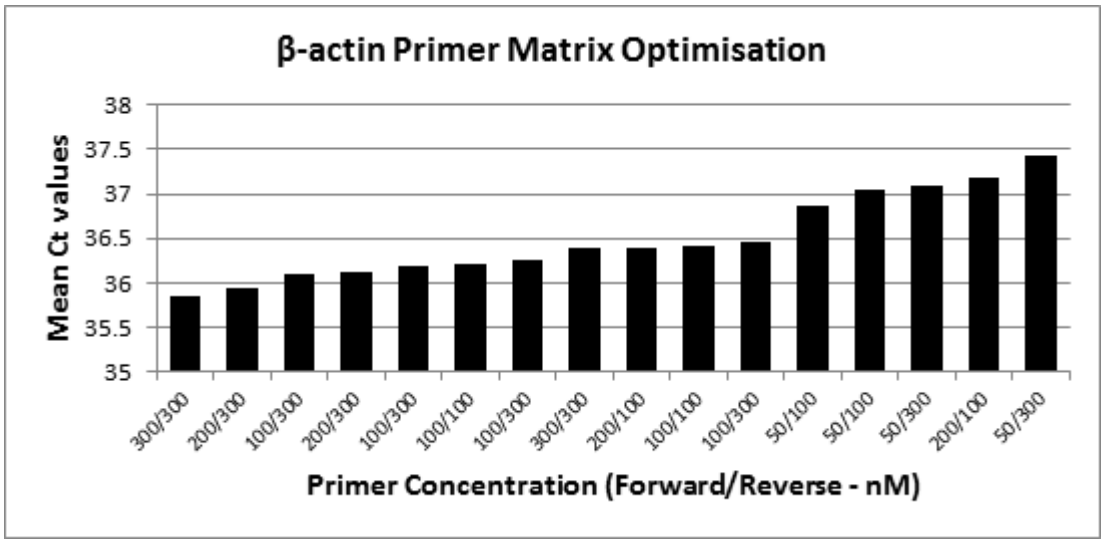

Figure 3-1: β-actin primer optimisation. Primer concentrations of 300 nM for both forward and reverse primers produced the most efficient reaction rate. The bar chart was obtained using the Microsoft Excel statistics program (Microsoft, USA).

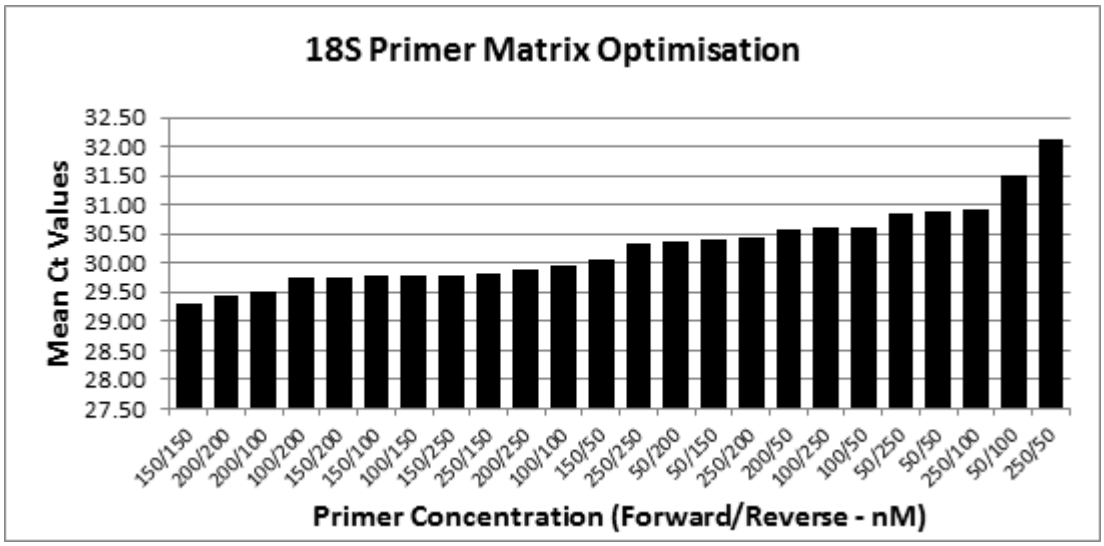

Figure 3-2: 18S primer optimisation. Primer concentrations of 150 nM for both forward and reverse primers produced the most efficient reaction rate. The bar chart was obtained using the Microsoft Excel statistics program (Microsoft, USA).

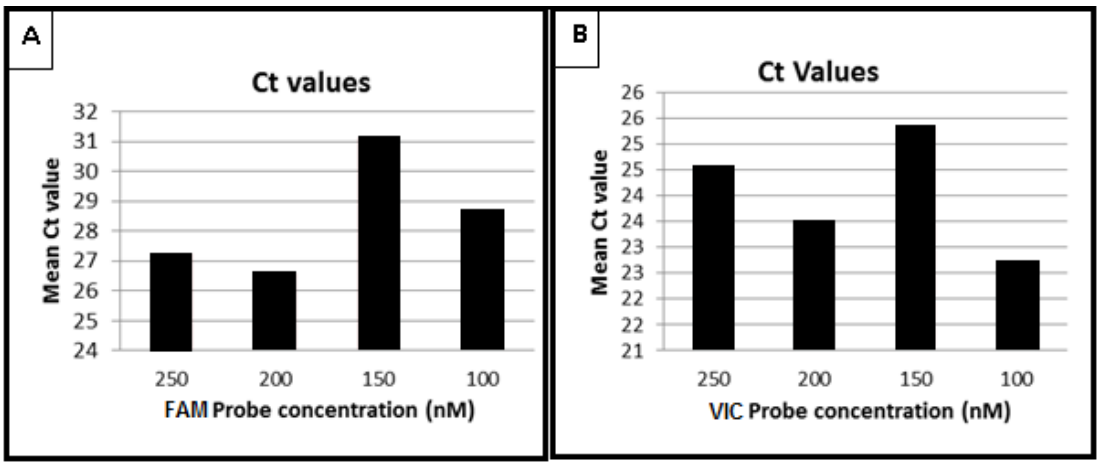

Figure 3-3: Optimisation of FAM and VIC probes. (A) Matrix optimisation of FAM probe. (A) The graph shows the Ct values obtained at each concentration of FAM probe. The concentration of 200 nM was the most efficient as indicated by the lowest mean Ct value (B) The graph shows the Ct values obtained at each concentration of VIC probe. The concentration of 100 µM was the most efficient as indicated by the lowest mean Ct value. The bar chart was obtained using the Microsoft Excel statistics program (Microsoft, USA).

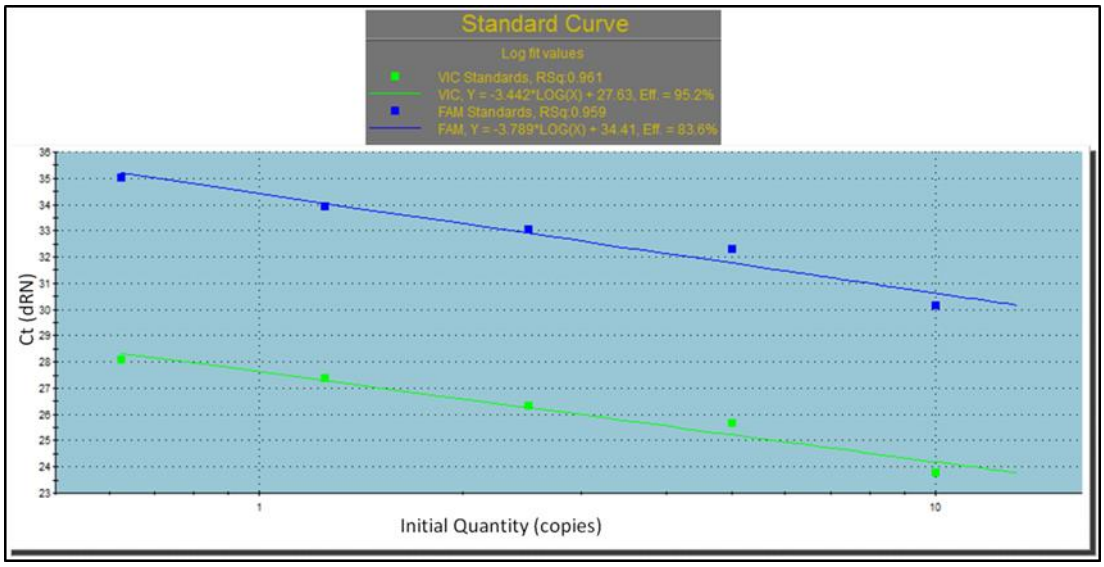

Figure 3-4: Standard curves for β–actin and 18S rRNA using 10-fold dilution series of RNA. The efficiency for β–actin is 83.6% while the efficiency for 18S is 95.2%. The blue line represents the standard curve of the β–actin where FAM was used as a reporter dye for the β–actin. The green line represents the standard curve of the 18S where VIC was used as a reporter dye for the 18S. The values which were obtained by Stratagene Mx3005P™ to form these standard curves are shown in Appendix 1.

3.3 Saliva age determination using RQR values of β-actin mRNA to 18S rRNA

3.3.1 Materials and method

3.3.1.1 Preparation of samples

Saliva was collected from six volunteers (three males and two females) with no history of malignancy. The stains were made by immediate spotting of 100 µL of a saliva sample on clean cotton swabs and allowed to dry at room temperature in a sterile hood. The stains were stored at room temperature, in a dry place, to simulate natural aging until they reach the desired ages (0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days). Stains were immersed in 350 µL RNA*later*® (Qiagen) to preserve RNA from degradation until the extraction phase.

3.3.1.2 RNA extraction and reverse transcription

RNA extraction was carried out using RNeasy® Micro Kit (Qiagen) as in section 2.3.2.1. The reverse transcription was carried out using SuperScript® III (Invitrogen) as in section 2.5.1.

3.3.1.3 Real-time PCR

The real-time PCR was carried out using β-actin control reagents and 18S rRNA control reagents (Applied Biosystems), as in section 2.6.

3.3.1.4 Data analysis

The raw fluorescence Ct values were processed using GenEx statistical software (Version 5.4.0.512; BioEPS GmbH, Munich, Germany) in order to normalise the raw Ct values against interplate calibrators, individual efficiency rates and qPCR technical repeats. Then the normalised Ct values were used to calculate the RQR of β-actin to 18S rRNA by dividing Ct value of β-actin mRNA to the Ct value of 18S rRNA. All statistical data analyses and graphs were prepared using the Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA) and Microsoft Excel statistics program (Microsoft, USA). The level of significance (α) for any hypothesis test was 0.05. Normality was determined using the Anderson-

Darling normality test. Determination of the correlation between the RQR values and the age of the stain was done using Spearman's test [173]. A value close to one or a negative one indicated a strong correlation. A value close to zero indicated a weak correlation. Both nonparametric methods were used because they could effectively deal with small sample sizes.

A general linear mixed model was used to analyse the results of the study. The factor included in this model are gender; donor, which was nested within gender: donor (gender); sample age, which was nested within donor and gender; age (donor [gender]); and age of saliva crossed with gender: (age*gender) (Figure 3-5). All factors, except age of saliva and sex, were taken to be random. Because the factors nested within each other, the F-test was used with the model to determine significance.

The results of the study were analysed using a nested analysis of variance. For the analysis, donor was nested within gender: donor (gender). *Ex vivo* age of saliva was nested within donor: age (donor [gender]). All factors, except age of saliva and sex, were taken to be random.

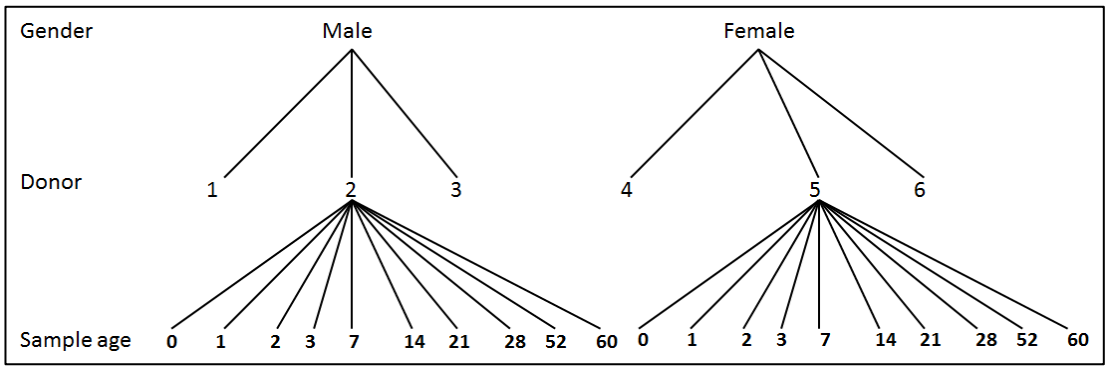

Figure 3-5: Schematic diagram of nested data structure. The figure shows that the donor was nested within gender and sample age was nested within donor and gender.

3.3.1.5 Materials and method for repeating of Anderson *et al.'s* work [49] on bloodstains

To repeat Anderson *et al.* [49] work, blood from three volunteers was collected by finger stick (50 µL) dropped on a clean sterile cotton swabs. Four bloodstains were

made for each volunteer. Half of the bloodstains made were extracted directly (i.e. 0-day age bloodstains) while the other half were stored and extracted after a month of storage (i.e. 30-day age bloodstains). The method used was similar to that used in Anderson *et al.* [49] work. Extraction is described in section 2.3.1, reverse transcription is described in section 2.5.1 and real time PCR is described in section 2.6.

3.3.2 Results

Table 3-1 shows the raw fluorescent Ct values of β-actin mRNA and 18S rRNA measured by real-time PCR at 0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days. These raw Ct values were corrected with the efficiency of the reaction using GenEx statistical software (version 4.3.7; BioEPS GmbH, Munich, Germany) (Table 3-2).

Table 3-3 shows the RQRs calculated from the corrected Ct values for saliva samples that were aged for up to 60 days under uncontrolled conditions (room temperature, in a dry dark place). Table 3-4 displays the statistical analysis of the RQR of β-actin mRNA to 18S rRNA at 0 1, 2, 3, 7, 14, 21, 28, 52, and 60 days. This statistical analysis was conducted with the Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA).

Table 3-1: Mean raw fluorescent Ct values of β-actin mRNA and 18S rRNA at 0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days for six samples before efficiency correction. The mean Ct value is calculated by the software of Stratagene Mx3005P™ where each sample is run in triplicate. The Ct value of each individual sample is shown in Appendix 2.

Samples *n*=6	Mean Ct Values																			
	day 0		day 1		day 2		day 3		day 7		day 14		day 21		day 28		day 52		day 60	
	β-actin	18S	β-actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S
1	39.37	25.23	38.87	27.12	38.03	32.81	39.93	36.19	39.19	33.02	33.66	23.34	37.01	27.70	38.81	29.12	35.87	25.12	37.37	25.81
2	34.52	26.21	31.12	24.72	37.10	32.72	36	25.67	38.87	26.91	33.37	22.9	36.92	27.52	38.74	26.44	35.26	25.16	36.19	24.87
3	35.94	25.7	33.33	25.68	37.38	33.94	36.00	25.83	36.99	26.79	36.79	25.27	34.66	25.28	33.3	23.26	31.92	21.72	35.14	23.02
4	35.61	25.28	34.65	25.73	38.61	33.83	34.86	28.61	34.95	26.64	36.57	24.84	35.13	26.01	33.09	22.82	32.16	21.41	34.45	22.71
5	33.69	25.51	38.17	26.00	39.77	26.69	35.22	28.73	39.95	34.2	34.90	23.88	36.53	25.25	36.02	23.08	36.88	23.95	35.00	23.90
6	34.82	25.39	36.71	25.87	30.61	24.55	36.78	32.41	34.79	33.87	35.09	24.28	36.61	24.8	34.46	22.27	35.93	23.71	34.68	23.72

Table 3-2: Corrected Ct values of β-actin mRNA and 18S rRNA at 0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days for the six samples after efficiency correction using GenEx software. The data in this table are obtained from correcting the mean raw Ct values in Table 3-1.

Samples *n*=6	Ct Values																			
	day 0		day 1		day 2		day 3		day 7		day 14		day 21		day 28		day 52		day 60	
	β-actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S	β -actin	18S
1	36.92	25.12	37.74	25.73	36.92	25.12	38.77	29.22	38.05	26.66	32.68	18.84	35.93	22.36	37.68	23.51	34.82	20.28	36.28	20.84
2	36.02	24.04	30.21	23.45	36.02	24.04	34.95	20.72	37.74	21.73	32.40	18.49	35.84	22.22	37.61	21.35	34.23	20.31	35.14	20.08
3	36.29	24.20	32.36	24.36	36.29	24.20	34.95	20.85	35.91	21.63	35.72	20.40	33.65	20.41	32.33	18.78	30.99	17.54	34.12	18.59
4	37.48	26.09	33.64	24.41	37.48	26.09	33.84	23.10	33.93	21.51	35.50	20.05	34.11	21.00	32.13	18.42	31.22	17.29	33.45	18.34
5	38.61	25.32	37.06	24.66	38.61	25.32	34.19	23.20	38.79	27.61	33.88	19.28	35.47	20.39	34.97	18.63	35.81	19.34	33.98	19.30
6	29.72	19.29	35.64	24.54	29.72	19.29	35.71	26.17	33.78	27.35	34.07	19.60	35.54	20.02	33.46	17.98	34.88	19.14	33.67	19.15

Table 3-3: Relative quantity ratios of β-actin mRNA to 18S rRNA for six samples at 0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days. RQRs are calculated from the mean Ct values in Table 3-2.

n=6	Relative Quantity Ratio									
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 52**	**Day 60**
Sample 1	1.53	1.47	1.47	1.33	1.43	1.73	1.61	1.60	1.72	1.74
Sample 2	1.30	1.29	1.50	1.69	1.74	1.75	1.61	1.76	1.69	1.75
Sample 3	1.37	1.33	1.50	1.68	1.66	1.75	1.65	1.72	1.77	1.84
Sample 4	1.38	1.38	1.44	1.47	1.58	1.77	1.62	1.74	1.81	1.82
Sample 5	1.30	1.50	1.53	1.47	1.40	1.76	1.74	1.88	1.85	1.76
Sample 6	1.35	1.45	1.54	1.36	1.24	1.74	1.78	1.86	1.82	1.76

Table 3-4: Statistical analysis of the relative quantity ratios of samples at 0, 1, 2, 3, 7, 14, 21, 28, 52, and 60 days. Statistical calculations were carried out using Minitab® software.

	Mean RQR	**SD[a]**	**CV%[b]**	**Variance**
day 0	1.37	0.09	6.35	0.008
day 1	1.40	0.08	6.03	0.006
day 2	1.50	0.14	2.52	0.019
day 3	1.50	0.18	10.16	0.031
day 7	1.51	0.17	12.31	0.028
day 14	1.75	0.16	0.75	0.027
day 21	1.67	0.12	4.30	0.014
day 28	1.77	0.18	5.69	0.033
day 52	1.78	0.18	3.61	0.033
day 60	1.77	0.16	2.29	0.025

[a] Standard deviation [b] Coefficient of variation

3.3.3 Discussion

3.3.3.1 RQRs of β-actin mRNA to 18S rRNA

Figure 3-6 represents RQR values obtained from saliva stains that had been aged for a period up to 60 days under uncontrolled conditions (room temperature, in a dry dark place) to simulate natural aging.

The results of this study show the relationship between RQRs of β-actin to 18S rRNA, extracted from a saliva sample, and the age of the sample. It was found that RQRs of β-actin to 18S rRNA increase with increasing sample age where the RQRs acted in a linear fashion (Figure 3-6). The increase in the RQRs with time is steady at early ages, becomes slightly marked at seven to 14 days before steadily increasing again at older ages.

In addition, Figure 3-6 shows a wide range of RQR values at each time period, with the exception of 14-day-old stains. The range of RQR values diversity increases with an increase in the stains age from zero to seven days, then, the diversity decreases with an increase in the stain's age from 28 to 60 days.

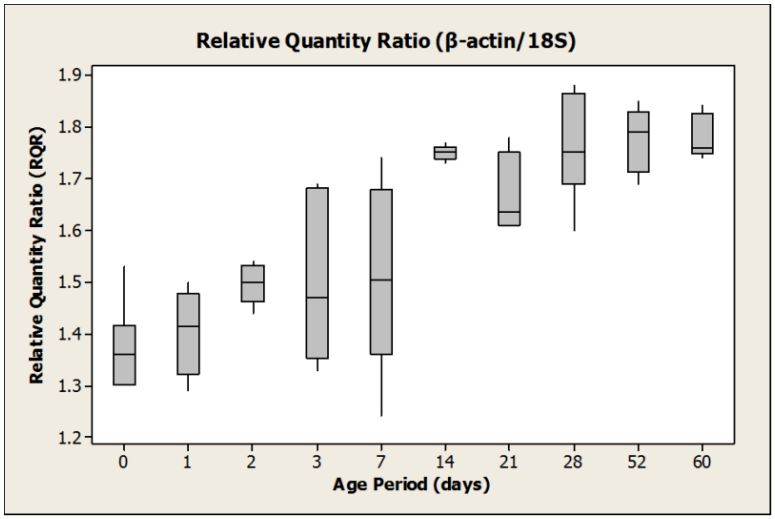

Figure 3-6: A boxplot showing RQRs by age of an *ex vivo* saliva stain. Boxplot obtained from the data shown in Table 3-3using Minitab® 16 software.

3.3.3.2 Combined data and trend analysis

The relationship between the age of the saliva stain and the obtained mean RQR values of β-actin to 18S was statistically calculated. The Anderson-Darling normality test was carried out on the mean RQR values and on the aging period. The test shows that one of the variables, aging period, was not normally distributed (Figure 3-7). Therefore, nonparametric analysis was adopted for statistical analysis. Spearman's correlation indicates that there is a strong linear positive correlation between the mean RQR values of β-actin to 18S and the age of saliva stains (Spearman's r = 0.96; p-value= 0.000004), although, this correlation does not specify which value affects the other.

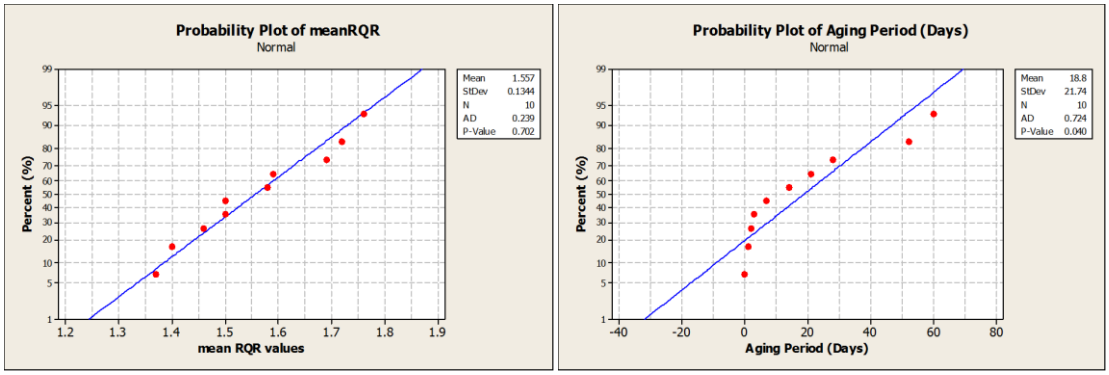

Figure 3-7: A normal probability plot using the Anderson-Darling normality test on mean RQR values and the aging period.

To estimate the relationship between the mean RQR values of β-actin to 18S rRNA and the age of saliva stains, a regression analysis was applied on the data obtained by plotting the mean RQR values against the age of saliva. Fitting the data on a simple linear model demonstrates that the RQR values increase with an increase in the age of the stain (R^2= 67.6%) (Figure 3-8). In addition, fitting the data on a cubic model shows a higher R^2 value (R^2= 73.6%) (Figure 3-9). However, the cubic model appears less reliable where the 95% confidence interval shows a wider confidence strip than that of the linear model, thus making the linear model a more reliable prediction model than the cubic model, depending on the data plotted (Figure 3-10).

The age of saliva stains can be estimated based on these RQR values using a linear model (R^2= 67.6%):

$$y = -157.0 + 109.8\,x \qquad \text{(Equation 3-1)}$$

where y is the age of the saliva stain in days and x is the RQR of β-actin to 18S rRNA.

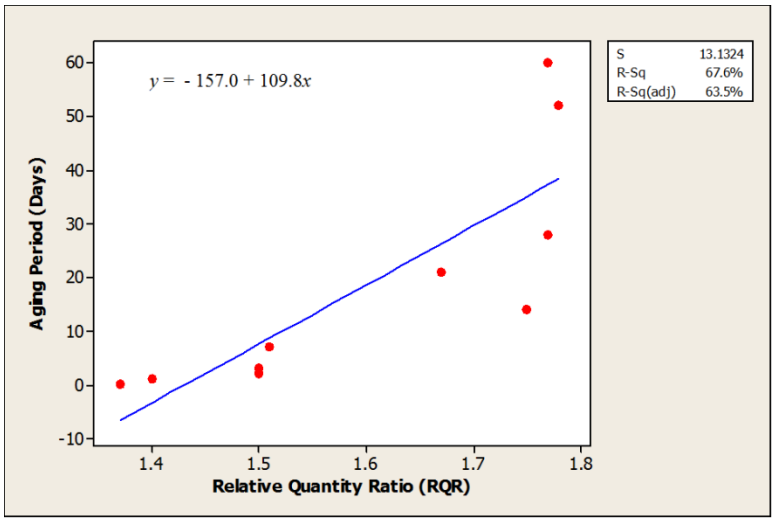

Figure 3-8: Time-wise trend in the RQR of saliva samples over time shown on linear model. A simple linear regression curve obtained from the data shown in Table 3-4 using Minitab® 16 software.

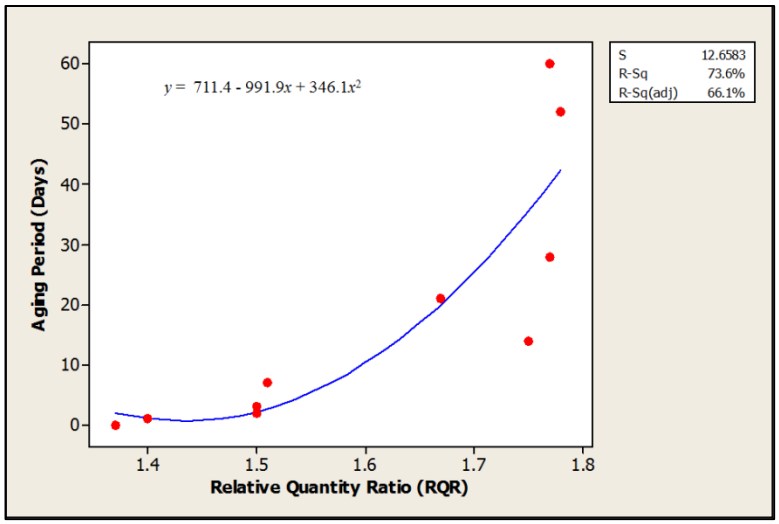

Figure 3-9: Time-wise trend in the RQR of saliva samples over time shown on cubic model. A second-order polynomial curve obtained from the data shown in Table 3-4 using Minitab® 16 software.

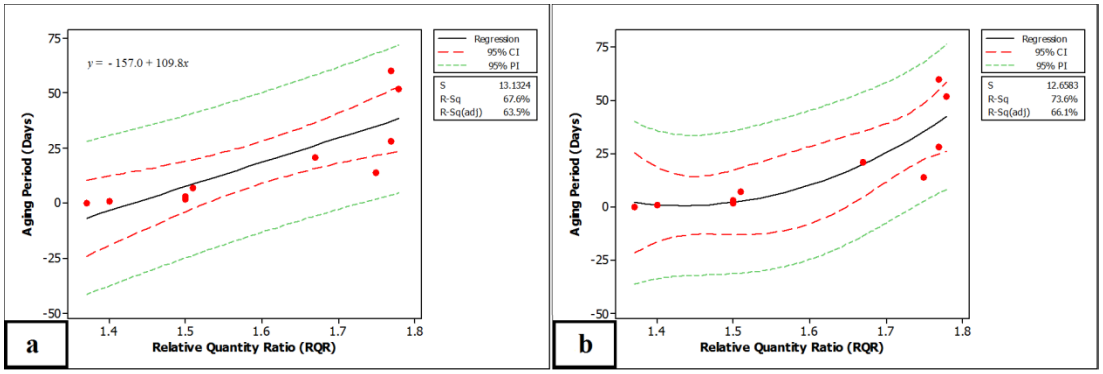

Figure 3-10: Time-wise trend in the RQR of saliva samples over time with 95% confidence interval and 95% prediction interval. a) Linear regression b) Cubic regression.

To evaluation the effect of the ages of saliva and the individuals on the mean RQR values at specific two-way analysis of variance (ANOVA) test was carried. The results of the two-way ANOVA test performed on the mean RQRs showed that the difference in the means of different sample ages was highly significant (*p value* <0.001). The results also showed that the difference in means between individuals at specific ages of the saliva stain is not significant (*p value* >0.05). The results of ANOVA test are shown in Appendix 3 and Appendix 4. These results indicated that the age of the saliva stain was the only significant factor contributing to the differences in the RQR values of samples.

The nested analysis of variance results showed that the largest source of variability in the RQR values of β-actin mRNA to 18S rRNA is due to the age of saliva stains where R^2 values of 0.75 (75%) were produced (Figure 3-11). The remainder of the difference (25%) was accounted for by other factors such as inter-person, intra-person, and residual effects. The residual effects are those not assigned to the other possible sources of variability and for which the exact cause is unknown. The variability in the RQR values accounting for the inter-person variability was 1.4%, where it produced R^2 values of 0.014 (5.52% of the 25% because of non-age related effects). In addition, a small gender effect was found on the RQR values. This effect was 0.16% (0.64% of the 25% because of non-age related effects).

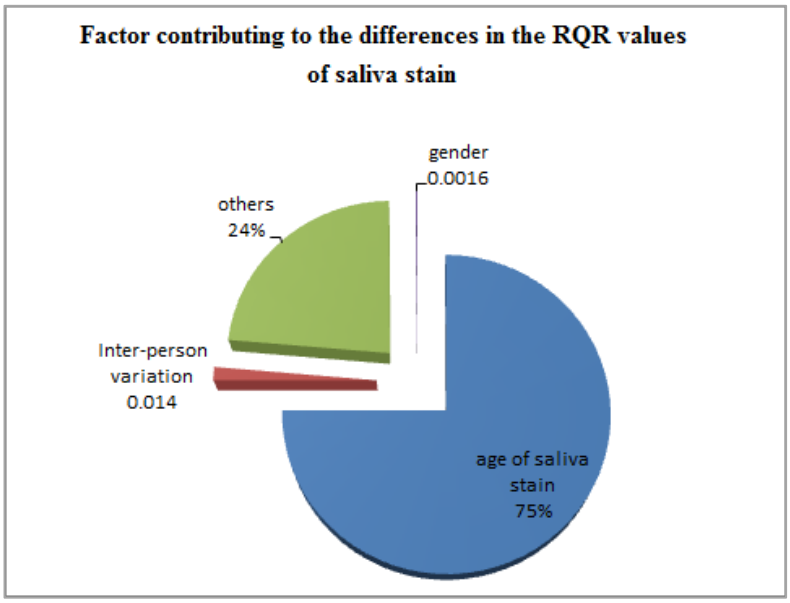

Figure 3-11: Factors contributing to the differences in the RQR values of saliva stain. The figure shows that the difference in the RQR value mostly resulted from age of saliva itself.

3.3.3.3 The inter-person variation at each age period

The inter-person variation at each age period was statistically calculated using RQRs of the corrected Ct values (i.e., normalised qPCR data). A number of observations were obtained from this set of data. First, the inter-person variation ranged from 0.75% (14 days) to 12.31% (seven days) with a mean value of 5.40% across all aging times (Table 3-5 and Figure 3-12). The results of the inter-person variations show a slightly large variation at the beginning of the study, and this variation increased at three to seven days, then decreased for the rest of the study (Figure 3-6). The variation at the beginning of the study indicates there are levels of natural biological variance *in vivo* and exposure of RNA molecules to various types of ribonucleases in the mouth before spitting. There are many possible explanations for the following fluctuation in the inter-person variations. First, there is an inherited inter-person difference in the composition of ribonucleases in saliva, as saliva contains a large number of endogenous and exogenous ribonucleases with variable activity, and these may degrade RNA molecules at different rates and by different mechanisms [93, 97, 107]. Second, the RNA stabiliser may have had an effect on the RNA extraction from saliva samples. Laboratory errors cannot be eliminated as a source of the noted variations. The difference may result from incorrect calculation of RQRs because of

incomplete normalisation of the RNA expression. Although efficiency correction of the Ct value was carried out, the individual efficiency was not measured for each individual sample. This may lead to Ct value being corrected to an incorrect reaction efficiency, as the reaction may be affected by a constituent of the sample absent in the other sample. These possibilities merit the need for further investigation with a larger volunteer population size.

Table 3-5: Inter-person variations over time. Mean RQR calculated from results by day given in **Table 3-3** using Minitab® software. (Equation used to calculate coefficient of variation (CV) is CV= standard deviation / mean × 100).

	Mean RQR ***n*=6**	**Inter-person variation (Coefficient of variation, %)**
Day 0	1.76	2.29
Day 1	1.69	3.61
Day 2	1.72	5.69
Day 3	1.59	4.30
Day 7	1.58	0.75
Day 14	1.50	12.31
Day 21	1.46	10.16
Day 28	1.50	2.52
Day 52	1.40	6.03
Day 60	1.37	6.35

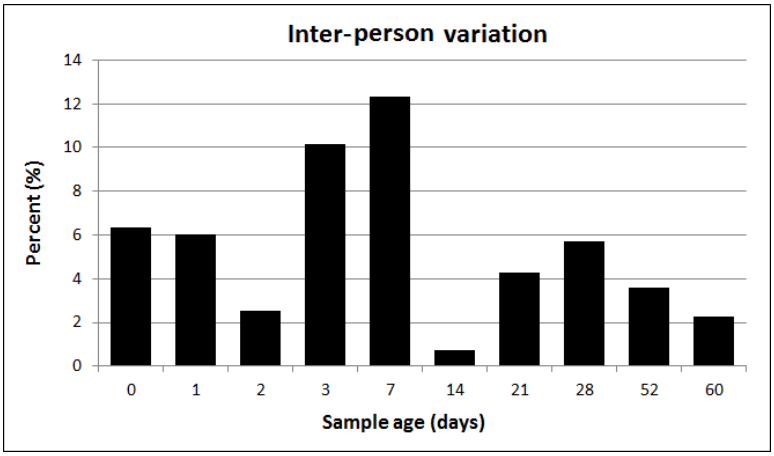

Figure 3-12: Inter-person variation samples over time. The bar chart was obtained from the data shown in Table 3-3 using the Microsoft Excel statistics program (Microsoft, USA).

3.3.3.4 Comparing the results with those obtained from previous studies [49, 75] carried out on bloodstains and hair samples

When the results of this study were compared with previous studies [49, 75] carried out on bloodstains and hair samples, a number of points were noticed. Firstly, the method used in calculating the RQRs in this study is based on calculating the ratio of β-actin mRNA to 18S rRNA. This method of calculation differs from that cited in Anderson *et al.* [49] study where their RQR values were calculated from the ratio of 18S rRNA to β-actin mRNA. In this study, when the ratio of β-actin mRNA to 18S rRNA was used, the correlation between the RQR values and the age of saliva stains was similar to that obtained by Anderson *et al.* [49], despite using the ratio in reverse. This means that either the relationship between the RQR values and saliva stains is opposite to that between the RQR values and bloodstains, or there is a mistake in one of the calculation methods used. This discrepancy will be discussed in following paragraphs to identify the confounding points, and to identify which ratio should be used.

First of all, Anderson *et al.* [49] proposed that different RNA species degrade, *ex vivo*, at different rates. They succeeded in finding a correlation between the age of bloodstain and ratio of two different species RNAs (i.e. β-actin mRNA and 18S rRNA). They referred the difference in the degradation rate of these RNAs to the RNA's structure difference. The β-actin mRNA is not combined with a protective

protein complex and does not have environmental protection, while 18S rRNA is a part of the ribosomal RNA and is less prone to degradation. Therefore, it is expected that β-actin mRNA will degrade faster than 18S rRNA. The results of Anderson *et al.* and Hampson *et al.* [49, 75] studies confirm this hypothesis. However, according to this hypothesis, calculation of the ratio using *Ct* values of 18S rRNA to that of β-actin mRNA should result in a decreasing trend with time. This is because, in this method, β-actin will be the denominator of the ratio and, as β-actin degrades faster than 18S rRNA, the increase in the Ct values of β-actin with time are larger than that of 18S rRNA. Thus, the denominator becomes higher with time compared to the nominator, resulting in a downward trend rather than an increase as shown in Anderson *et al.* [49] study.

The second, the raw Ct values data provided in Anderson *et al.* [49] study show that the Ct values of β-actin are higher than that of the 18S rRNA. This means the ratio should be less than one, if the ratio is calculated from 18S rRNA to β-actin. This ratio should also become lower as the stain's age increases. However, the results of Anderson *et al.* [49] study are inconsistent with this finding, where the mean ratio is 1.475 at age 0, becoming higher with increasing the of the stain's age.

The third, in the discussion section, Anderson *et al.* [49] cited that

> "the *Ct* value of 18S did not appreciably change over the course of 150 days but the *Ct* values for β-actin became significantly reduced as a function of time thus the relative ratio of 18S rRNA to β-actin mRNA increased over time".

This statement opposes their original hypothesis. The unstable nature of β-actin mRNA should in theory cause a reduction in the template quantity over the degradation interval, which manifests as an increase in β-actin *Ct*. If the C*t* value for β-actin did indeed reduce, as this quote indicate, this implies more β-actin mRNA was recovered from bloodstains after a prolonged degradation interval than at the start of the time trial, an implication which is obviously on the contrary to the conclusions made in the Anderson *et al.* [49].

The fourth, the Anderson *et al.* [49] approach was replicated for this in the lab using the same method published on a blood sample at ages 0 and 30 days old. The results of this replication show the same trend of increasing when the ratio was calculated from β-actin mRNA to 18S rRNA, with values similar to that obtained by Anderson *et al.* [49]. The results of this replication study are shown in Appendix 5.

Because of these four addressed points, for this thesis, it was decided to use the ratio of β-actin mRNA to 18S rRNA instead of 18S rRNA to β-actin mRNA, because it is believed that the 18S rRNA to β-actin mRNA ratio was not used in Anderson *et al.* [49] study, combined with the findings of this thesis, indicate a high likelihood that the Anderson *et al.* [49] study used the ratio of β-actin mRNA to 18S rRNA.

Secondly, this aging study of saliva is based on the findings of an aging study of bloodstains [49]; the same primers and genes were used. However, the RQRs of the saliva samples at a specific age may be not the same as the RQRs of blood samples at the same age because the housekeeping genes (β-actin mRNA and 18S rRNA) are differently expressed in tissues (expressed at different rates in different tissues).

Thirdly, the findings of this study confirm the correlation between the RQRs and the sample age and are concordant with the findings of other studies carried out on blood and hair [49, 75]. Furthermore, the shape of this relationship is similar where the relationship between the RQR values and the age of stains behaves in linear fashion [49, 75].

However, a wide range of RQR values were noticed at each age period as shown in Figure 3-6. There are many suggested explanations for this. Firstly, the degradation status of RNA molecules in saliva may result in this diversity [93, 97]. It was found that most of the RNA molecules in fresh saliva are partially degraded [174]. Degraded and partially degraded RNA molecules are common in fresh saliva samples because they emerge from several origins, including dead cells, and are exposed to various types of ribonucleases from bacteria in the mouth. Thus, a wide range of RQR values in saliva at day zero is expected because it contains degraded RNA derived from several origins, including dead cells, and due to exposure of these RNA to various types of ribonucleases in saliva.

Secondly, ribonucleases in saliva may also play a role in this diversity. The presence of various types of endogenous and exogenous ribonucleases in saliva affects the integrity of the RNA molecules [93, 97]. Ribonucleases in saliva affect the integrity of RNA molecules at different levels depending on their quantity and the chemical property of saliva e.g. pH level [93, 97]. This means that, although RNA in fresh saliva is partially degraded, the extent of the degradation process in the following days depends on the ribonuclease types and saliva constitution.

Thirdly, the RNA stabiliser (RNA*later*®) used may have its own effect on the results obtained. RNA*later*® was used in this experiment as an attempt to apply RNA stabiliser to saliva stains that were stored under conditions to partially simulate a crime scene. The use of this stabiliser has many advantages, as addressed in the introduction section 1.9. The RNA stabiliser (RNA*later*®) used in this experiment to stabilise RNA molecules after the saliva stains reach their desired age, may have its own effect on the RQR values, resulting in the wide range of RQR values at each age period. It is reported that the ammonium sulphate content of RNA*later*® does more than just preserve RNA where it increases cellular membrane permeability and changes intracellular salt concentration [175]. Finally, experimental errors cannot be eliminated as a potential source of the wide range of RQR values noticed at each specific age.

3.4 Conclusion

In this study, the approach proposed by Anderson *et al.* [49] to determine the age of the bloodstain was applied on RNA*later*®-stabilised saliva stains in order to evaluate the suitability of this approach for saliva stains. The study was initiated by optimising the primers and probes in a singleplex reaction. The most efficient concentrations were chosen to be used in a multiplex assay. The multiplex from these concentrations resulted in a qPCR with a high efficiency that was used to correct the Ct values obtained using GenEx software. The results of repeating the method used by Anderson *et al.* [49] demonstrates an ability to repeat the work and obtain similar results. However, the results indicate that the preferred ratio for determining the age

of bloodstain should be the ratio of β-actin mRNA over 18S rRNA, and not the converse as suggested by Anderson *et al.* [49].

The results of the aging study of saliva stains demonstrate that there is a relationship between the age of saliva and the RQRs of β-actin mRNA to 18S rRNA and the age of the saliva stain. It was found that RQRs of β-actin mRNA to 18S rRNA increase with an increase in the age of the stains. In addition, the correlation between the age of saliva samples and the RQR values confirms the hypothesis that this approach is suitable when applied to tissue types other than blood. However, this result suggests further validation study to be used for prediction at high degree of accuracy and precision.

Moreover, this study shows that the plot of the results of this experiment was linear in shape, and this is similar to the relationships found in previous studies on blood and hair.

Although this method for the aging study could be used as an indicator for the age of biological samples, a wide range of RQR values was noticed at each age period and this range shows an increasing trend in the early ages, followed by a decreasing trend in samples older than 28 days. This wide range of RQR values may be due to the degradation status of RNA molecules, types of ribonucleases in saliva or due to practical reasons. In addition, the RNA*later*® reagent was used to stabilise RNA molecules in saliva stain and its possible effect on the results of the study should be taken into consideration.

In conclusion, this study supports the suitability of RNA for forensic analysis. The results of the aging study of saliva stabilised using RNA*later*® demonstrate the relationship between the age of saliva and RQRs, and this can be used as an indicator to predict the age of the sample. However, further investigation is required in order to assess the effect of RNA*later*® stabiliser, age and gender variation on the RQRs and to evaluate the intra-person variation.

Therefore, the experiment will be extended to address the points found in the study that are reported here. The issues that will be discussed in the next chapters are: the

effect of RNA*later*® stabiliser on the RQR values; the effect of contamination of a mixture of more than one body fluid on the RQR values due to use of housekeeping genes that are expressed in all tissue types; the effect of the age and gender variations on the RQRs and the causes resulting in the difference in the shape of the plot; and finally, an evaluation of the intra-person variation.

4 Evaluation of the stability of RNA molecules in old dried saliva stored in RNA*later*®

4.1 Introduction

In Chapter 3, the method of using of RQR values to determine the age of bloodstains was applied on saliva stains stabilised in RNA*later*® (Qiagen). This chapter will go on to discuss the appropriateness of using RNA*later*® to stabilise RNA in dried saliva stains in order to evaluate the accuracy of the results obtained in the previous chapter. Evaluation of the suitability of RNA*later*® will be carried out on two forensic applications. The first application is the determination of the age of biological stains depending on the RQR values of β-actin to 18S rRNA. An evaluation will be carried out by comparing the accuracy of determining the RQR values of old saliva stains immediately extracted and those extracted after preservation in RNA*later*®. The second application is the identification of saliva type depending on saliva-specific mRNA markers. An evaluation will be carried out by comparing STATH mRNA marker profiling obtained for saliva stains immediately extracted and those extracted after preservation in RNA*later*® reagent [55].

4.2 Materials and method

4.2.1 Preparation of samples

Saliva was collected from five volunteers who gave their informed consent. Six 25 µL aliquots from each donor were immediately spotted onto clean cotton swabs and dried at room temperature inside a sterile hood. Two sets of swabs, each consisting of three swabs from each donor, were formed. One set was labelled as stabilised stain, and the second set was labelled as un-stabilised stain. Both sets were stored in a dry place at room temperature and were used as a source for RNA extraction at *ex vivo* ages of 15, 30 and 60 days. The swabs from set one were immersed in 350 µL RNA*later*® (Qiagen) and stored for 60, 40 and 15 days, respectively, prior to extraction. The swabs from set two were extracted immediately upon reaching *ex vivo* ages of 15, 30 and 60 days. The total number of samples in each set was 15 (five

volunteers × three *ex vivo* ages) and two negative controls. RNA extraction and reverse transcription

RNA Extraction was carried out using AllPrep® DNA/RNA Mini Kit (Qiagen) as described in the procedure in section 2.3.2.2. All samples were treated with TURBO DNase I (Ambion) as described in the procedure in section 2.3.3. The reverse transcription was carried out using SuperScript® III (Invitrogen), as described in the procedure in section 2.5.2.

4.2.2 Real-time PCR

The real-time PCR was carried out using β-actin control reagents and 18S rRNA control reagents (Applied Biosystems), as described in the procedure in section 2.6.

4.2.3 Amplification and electrophoresis

A volume of 2 μL of the RT-reaction was amplified using a primer pair for STATH in a total reaction volume of 20 μL. The sequence of the STATH primer pair used, and the predicted size of the amplified products, is presented in Table 4-1. The PCR master mix components and their volumes used in the reaction are shown in Table 4-2. Negative control sample was included to accompany the run. The PCR was carried out on a 2720 thermal cycler (Life Technologies, UK) following the amplification cycle protocol in Table 4-3.

Electrophoresis was performed in triplicate using the 3130 Genetic Analyser (Life Technologies, UK). A 1 μL of each amplified sample was added to 9.5 μL Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 μL of internal lane standard 600 (ILS600, Promega). Tubes were heated at 95˚C for 3 min and snap cooled on ice for at least 3 min. Samples were injected through the capillary using 3130 POP-7 run module. The electrophoresis parameters used for all the samples are described in Table 4-4.

Table 4-1: Sequence of STATH primer pair used to evaluate the effect of RNA*later*® on saliva type identification and predicted size of amplified products.

Primer	Sequence (5'-3')	Tm (°C)	Product size (bp)	Ref
STATH (For)	CTTCTGTAGTCTCATCTT G	49.0	198	[55]
STATH (Rev)	TGGTTGTGGGTATAGTGGTTGTTC	65.5		

Table 4-2: Reaction volume of each component used in STATH PCR reaction. Multi mix was prepared for *n* + 1 for *n* number of samples.

Components	Volume per sample (µL)
2× AmpliTaq Gold PCR Master Mix	10
STATH forward primer (4 µM)	2
STATH reverse primer (4 µM)	2
Sample	2
ddH_2O	4
Total	20

Table 4-3: Amplification protocol using STATH primer pair with 2720 thermal cycler.

1 cycle	30 cycles			1 cycle
95	95	54	70	72
5 min	15 s	50 s	60 s	7 min

Table 4-4: Electrophoresis parameters used to run samples amplified with STATH primer pair.

Electrophoresis parameters	Condition
Injection time	5 s
Oven temperature	60°C
Injection Voltage	15kv
Run time	35 min

4.2.4 Data analysis

I) Real-time PCR data:

The Relative Quantitative Ratios (RQR) of β-actin to 18S rRNA were calculated by dividing the *Ct* value of β-actin mRNA with the *Ct* value of 18S rRNA. Statistical analysis was conducted with the Minitab® statistical software package (version 16, Minitab® Inc., State College, PA, USA) and with the Microsoft Excel statistics program (Microsoft, USA). The paired t-test was used to analyse the data obtained.

II) Genetic analyser data:

The electropherograms (EPGs) obtained were analysed using the GeneMapper v3.2.1 software and the peak height data were transferred into Microsoft Excel for further data manipulation. The mean peak height for each set of samples was obtained by averaging the average peak height of the three repeats of each sample in the set. Statistical analysis was also conducted using Microsoft Excel and Minitab® 16 software.

4.3 Results and discussion

4.3.1 Evaluation of the effect of RNA*later*® on the RQR values of β-actin mRNA to 18S rRNA

The raw Ct values of β-actin mRNA and 18S rRNA of saliva stains were measured using TaqMan® real time PCR. The raw Ct values obtained were tabulated in Appendix 6. The data obtained were used to calculate the RQR values of β-actin mRNA to 18S rRNA of the analysed samples and the resulting RQR values were tabulated in Table 4-5 and Table 4-6.

Table 4-5: Relative quantitative ratios of β-actin mRNA to 18S rRNA for stabilised and un-stabilised old saliva stains. The un-stabilised saliva stains were immediately extracted without stabilisation and the stabilised saliva stains were extracted after RNA*later*® stabilisation. RQRs are calculated from the mean Ct values in Appendix 6.

	Un-stabilised saliva stain			Stabilised saliva stain		
	Replicate 1	**Replicate 2**	**mean**	**Replicate 1**	**Replicate 2**	**mean**
1	1.87	1.91	1.89	2.25	2.11	2.18
2	2.05	1.64	1.85	2.19	2.14	2.16
3	1.83	1.80	1.81	2.31	2.19	2.25
4	1.92	1.94	1.93	2.09	2.09	2.09
5	2.11	2.10	2.11	2.05	1.47	1.76
6	1.83	1.85	1.84	2.06	2.09	2.08
7	1.72	1.74	1.73	2.00	1.93	1.97
8	1.89	1.91	1.90	1.96	1.97	1.96
9	1.83	1.80	1.82	1.96	1.99	1.98
10	1.82	1.84	1.83	1.98	1.99	1.99
11	1.98	1.95	1.96	2.02	2.02	2.02
12	1.81	1.84	1.82	1.98	1.98	1.98
13	1.78	1.73	1.76	2.06	2.00	2.03
14	1.93	1.88	1.91	2.07	2.04	2.05
15	1.90	1.83	1.86	2.01	2.01	2.01

Table 4-6: Mean RQRs of β-actin mRNA to 18S rRNA for stabilised and un-stabilised old saliva stains. The un-stabilised saliva stains were immediately extracted without

stabilisation and the stabilised saliva stains were extracted after RNA*later*® stabilisation. RQRs are calculated from the mean Ct values in Table 4-5.

	Un-stabilised saliva stain	stabilised saliva stain
1	1.89	2.18
2	1.85	2.16
3	1.81	2.25
4	1.93	2.09
5	2.11	1.76
6	1.84	2.08
7	1.73	1.97
8	1.90	1.96
9	1.82	1.98
10	1.83	1.99
11	1.96	2.02
12	1.82	1.98
13	1.76	2.03
14	1.91	2.05
15	1.86	2.01

Table 4-7 shows the statistical evaluation of the RQR values of the two sets of saliva samples: un-stabilised saliva stain set and stabilised saliva stain set. It was noticed that the average RQR value of the stabilised stain set differed from the average RQR value of the un-stabilised stain set.

Table 4-7: Average RQR values and standard deviations obtained from the un-stabilised saliva stain set and stabilised saliva stain set.

Stain set	No. of samples	Mean value	SD[a]	CV%[b]
Un-stabilised stain set	15	1.87	0.09	0.05
Stabilised stain set	15	2.03	0.11	0.06

[a] Standard deviation [b] Coefficient of variation

Real time PCR was carried out in duplicate for each sample in each saliva set where RQR values were calculated from these *Ct* values obtained. Values used were the

average of the RQR values of each sample [*n*=30]. The RQR values were plotted on the scattergraph to visually compare the two sets of the saliva stains (Figure 4-1). The scattergraph shows that the RQR values of 12 out of 15 stains (80%) noticeably increased after stabilising saliva with RNA*later*® while two RQR values of 15 stains (~13%) slightly increased after RNA*later*® stabilisation. In addition, the RQR value decreased in only one stain (~6.6%).

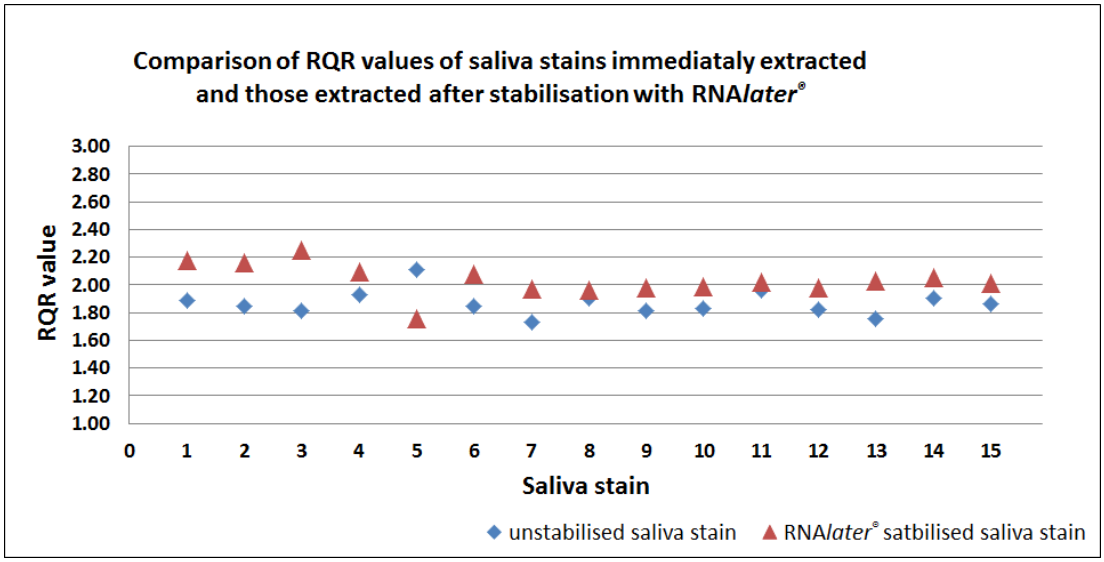

Figure 4-1: Scattergraph of RQR values of two sets of saliva stains. The scattergraph shows the RQR values from un-stabilised saliva stain set and stabilised saliva stain set.

The data were used to formulate a boxplot as shown in Figure 4-2, to also compare the results between the un-stabilised saliva stains and stabilised saliva stains for RQR values. The median RQR value for un-stabilised saliva stains was 1.85 with a minimum of 1.73 and maximum of 2.11, while for stabilised saliva stains, the median RQR value was 2.02 with a minimum and maximum of 1.76 and 2.25 respectively.

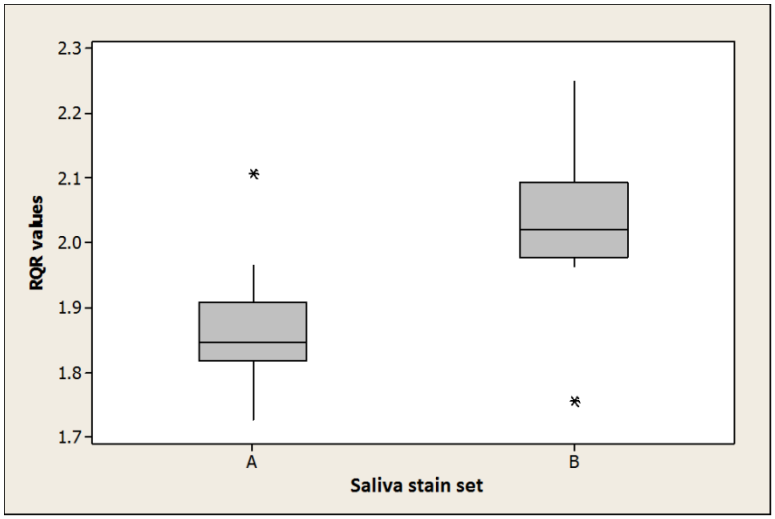

Figure 4-2: Boxplot of RQR values based on set of saliva stains analysed. A: un-stabilised saliva stain set; B: stabilised saliva stain set. The whiskers indicate the minimum and maximum values for A: 1.73 to 2.11, B: 1.76 to 2.25; interquartile range for A: 0.09, B: 0.12; and median of A: 1.85, B: 2.02. Outlier is indicated by asterisk.

The normality of RQR values of the two sets of stains was tested using the Anderson-Darling normality test (Figure 4-3). Results showed that RQR values were normally distributed. Therefore, parametric analysis was adopted for statistical analysis.

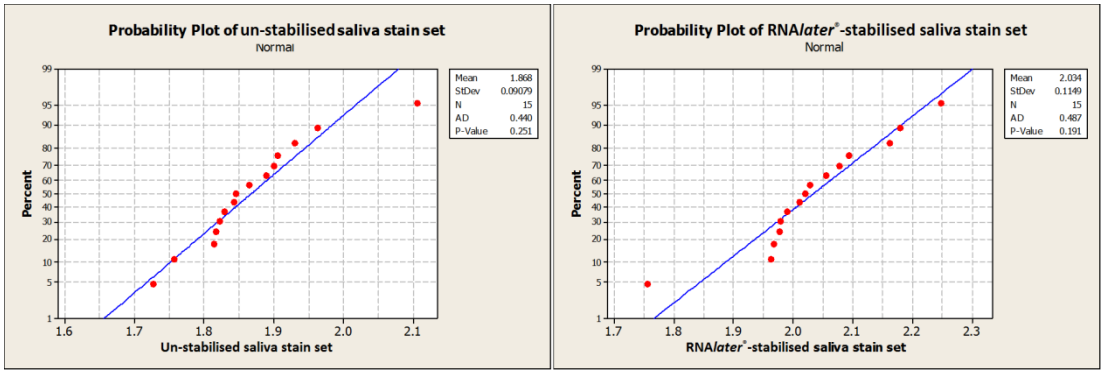

Figure 4-3: A normal probability plot using the Anderson-Darling normality test on RQR values of the two sets of saliva stain.

Paired *t*-test was carried out on the RQR values of the two saliva stain sets to observe whether there was a significant difference between the two sets. A *p*-value of 0.002 for the un-stabilised saliva stain set versus the stabilised saliva stain set indicates that there is a highly significant difference in the mean of the two sets' RQR values.

The overall result of this study demonstrated that the RQR values of β-actin to 18S rRNA of dried saliva stains increase if they are stabilised in RNA*later*® for a period of time. This increase in the RQR values was statistically significant. The suggested explanations of this change in the RQR values are that the RNA*later*® reagent stabilises RNA species, i.e. β-actin mRNA and 18S rRNA, in a different efficiency. This difference in the stabilisation efficiency between these two RNA species causes the change in their ratio with time.

There are two possible causes of this difference in the stabilisation efficiency of the RNA*later*® reagent between the two RNA species. The first possible cause is the structural difference between β-actin mRNA and 18S rRNA where they are from structurally different RNA species. In addition, the type and structure of the proteins that bind to them differs: 18S rRNA makes a complex with ribosomal protein, while salivary β-actin mRNA associates with other different macromolecules [5, 107]. These associated molecules protect the RNA species from degradation in different degrees. This may increase the RNA*later*® stabilisation efficiency of one of RNA species over the other. The second possible cause is the difference in the size of the stabilised molecules where the efficiency of RNA*later*® to stabilise RNA molecules depends on their size. In the study carried out by Michaud *et al.*, RNA*later*® shows a higher efficiency when stabilising short DNA amplicons than large amplicons [119]. This means, due to the difference in the amplicon sizes of the products of the TaqMan® reagent kits used for β-actin mRNA and for 18S rRNA (300 bp for β-actin 187 bp for 18S rRNA), RNA*later*® stabilises 18S rRNA amplicons more efficiently than β-actin amplicons. Both suggested causes expect more stabilisation of 18S rRNA than β-actin in RNA*later*® and may explain the increase in the RQR values. The ratio used in calculating RQR values locates β-actin as nominator and 18S rRNA as denominator, and the higher stability of 18S RNA in RNA*later*® results in an increase of the nominator over the denominator, resulting in a higher RQR value.

4.3.2 Evaluation of the effect of RNA*later*® on the peak height of STATH mRNA

STATH profiling was carried out in triplicate for each sample (*n*=90). Only peak heights of 500 rfu and above were used in the statistical calculations. The analysis of the un-stabilised saliva stain showed that the peak height of STATH mRNA in the stain decreased with time (Figure 4-4). This finding confirms the finding of a previous study [111], which showed the tendency of RNA saliva-specific markers, namely STATH mRNA and HTN3 mRNA, in saliva stains stored at room temperature to decrease with time.

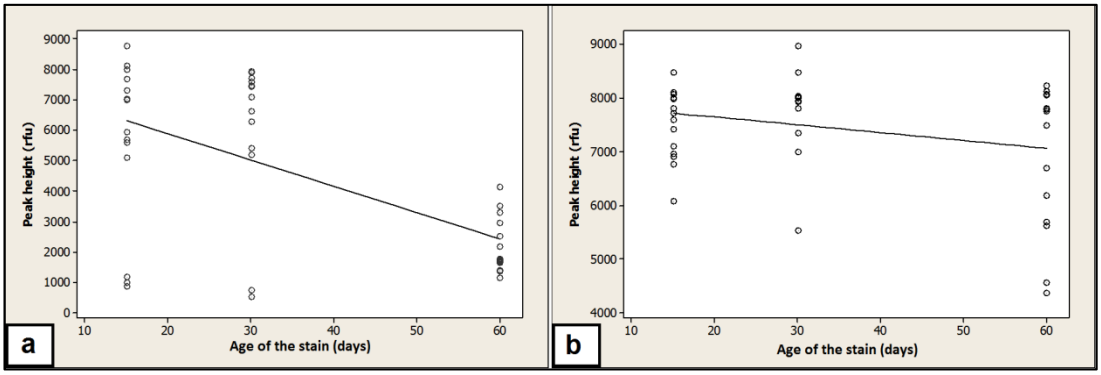

Figure 4-4: End-point PCR of STATH marker results in up to 60-day-old. a) un-stabilised saliva stains b) RNA*later*®-stabilised saliva stain. The trendline of the STATH peak heights illustrate a higher tendency to decrease with time in the un-stabilised saliva stain than the stabilised stain.

Table 4-8 shows the statistical evaluation of the peak heights of both sets of saliva samples: un-stabilised saliva stain set and RNA*later*®-stabilised saliva stain set. It was noticed that the average peak heights value of the RNA*later*-stabilised stain set is obviously larger than the average peak heights value of the un-stabilised stain set.

Table 4-8: Average peak heights and standard deviations obtained from the un-stabilised saliva stain set and stabilised saliva stain set.

Stain set	No. of samples	Mean value	SD[a]	CV%[b]
Un-stabilised stain set	45	4620	2902	0.62
Stabilised stain set	45	7429	979	0.13

[a] Standard deviation [b] Coefficient of variation

The STATH mRNA profile was obtained from all the stains preserved in RNA*later*®, including those preserved for 60 days. The bar chart was used to visually compare the peak heights of the two sets of the saliva stains (Figure 4-5). The bar chart in this figure shows that the peak heights increased in ~93% of the samples compared to the un-stabilised samples (14 of 15 samples). The mean peak heights of saliva stains stabilised in RNA*later*® for 60 days and 30 days were greater by ~27% and 26% respectively, while the peak heights of those stabilised for 15 days were greater by ~68%. One sample, stabilised in RNA*later*® for 60 days, showed a minor decrease of 3.5%.

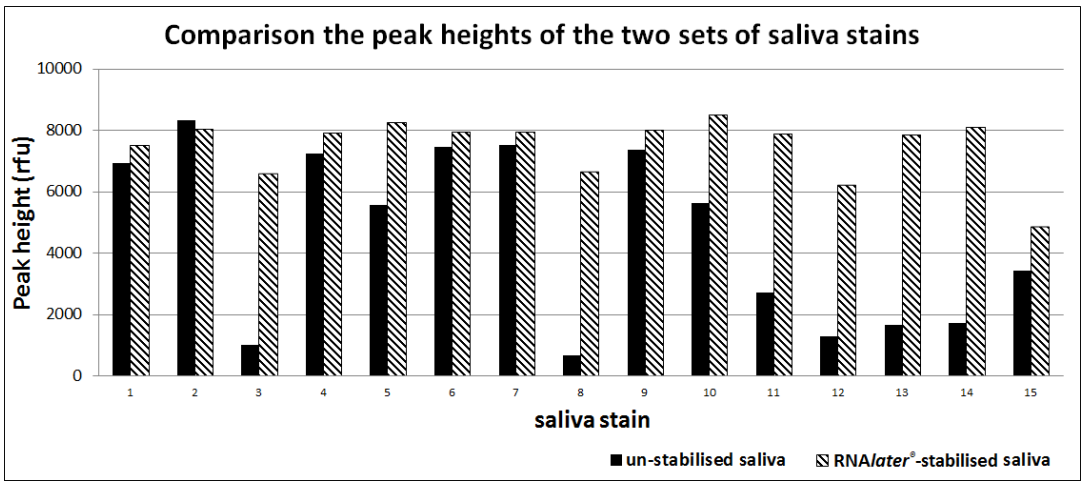

Figure 4-5: Comparison of peak heights (rfu) of the two sets of saliva stains: un-stabilised saliva stain and stabilised saliva stain. Sample numbers 1-5 stabilised for 60 days; sample numbers 6-10 stabilised for 30 days; and sample 11-15 stabilised for 15 days.

The peak height results of the un-stabilised saliva stains and stabilised saliva stains were compared using a boxplot as shown in Figure 4-6. The median peak height for un-stabilised saliva stains was 5573 rfu with a minimum of 661 rfu and maximum of

8337 rfu, while for stabilised saliva stains, the median peak height was 7867 with a minimum and maximum of 4848 and 8487 respectively.

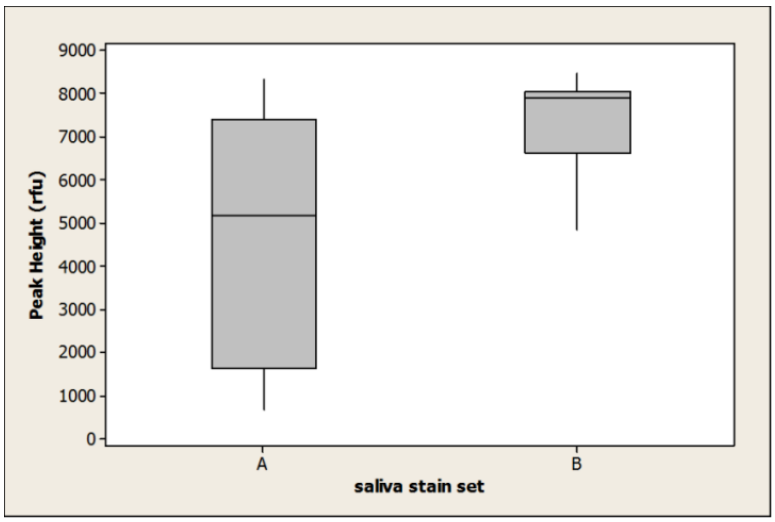

Figure 4-6: Boxplot of peak height values based on set of saliva stains analysed, A: un-stabilised saliva stain set; B: stabilised saliva stain set. The whiskers indicate the minimum and maximum values for A: 661 to 8337, B: 4848 to 8487; interquartile range for A: 5801, B: 1423; and median of A: 5573, B: 7867.

The normality of data of the two sets of stains was tested using the Anderson-Darling normality test (Figure 4-7). Results showed that peak heights of the un-stabilised saliva stains were not normally distributed. Therefore, non-parametric analysis was used for statistical analysis.

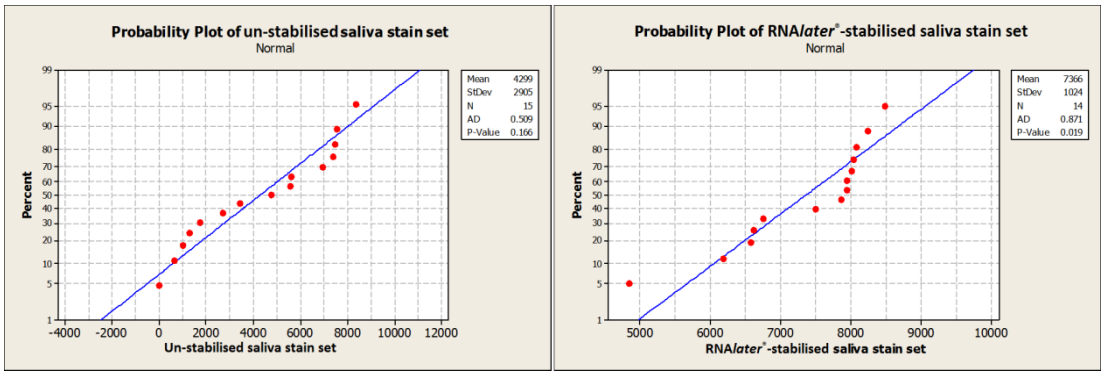

Figure 4-7: A normal probability plot using the Anderson-Darling normality test on peak heights of the two sets of saliva stain.

A Mann–Whitney U test was carried out on the peak heights of the two saliva stain sets to detect any significant difference between the two sets. A *p*-value of 0.002 for the un-stabilised saliva stain set versus the stabilised saliva stain set indicates that

there is a highly significant difference in the mean of the RQR values of the two sets. The result of this study showed a significant increase in the peak heights of dried saliva stains after their stabilisation in RNA*later*® for a period of time.

Samples were divided into three groups according to their ages (15, 30 and 60 days) to identify which of them was affected most by RNA*later*® (Figure 4-8). The bar charts in the figure show that the oldest samples (60 days old) were mostly affected by RNA*later*®. In addition, the peak heights of 60-day-old samples significantly increased (*p* value = 0.0122). However, no significant change was found in stains 15 days old and 30 days old (*p* value was 0.2963 and 0.0601 respectively).

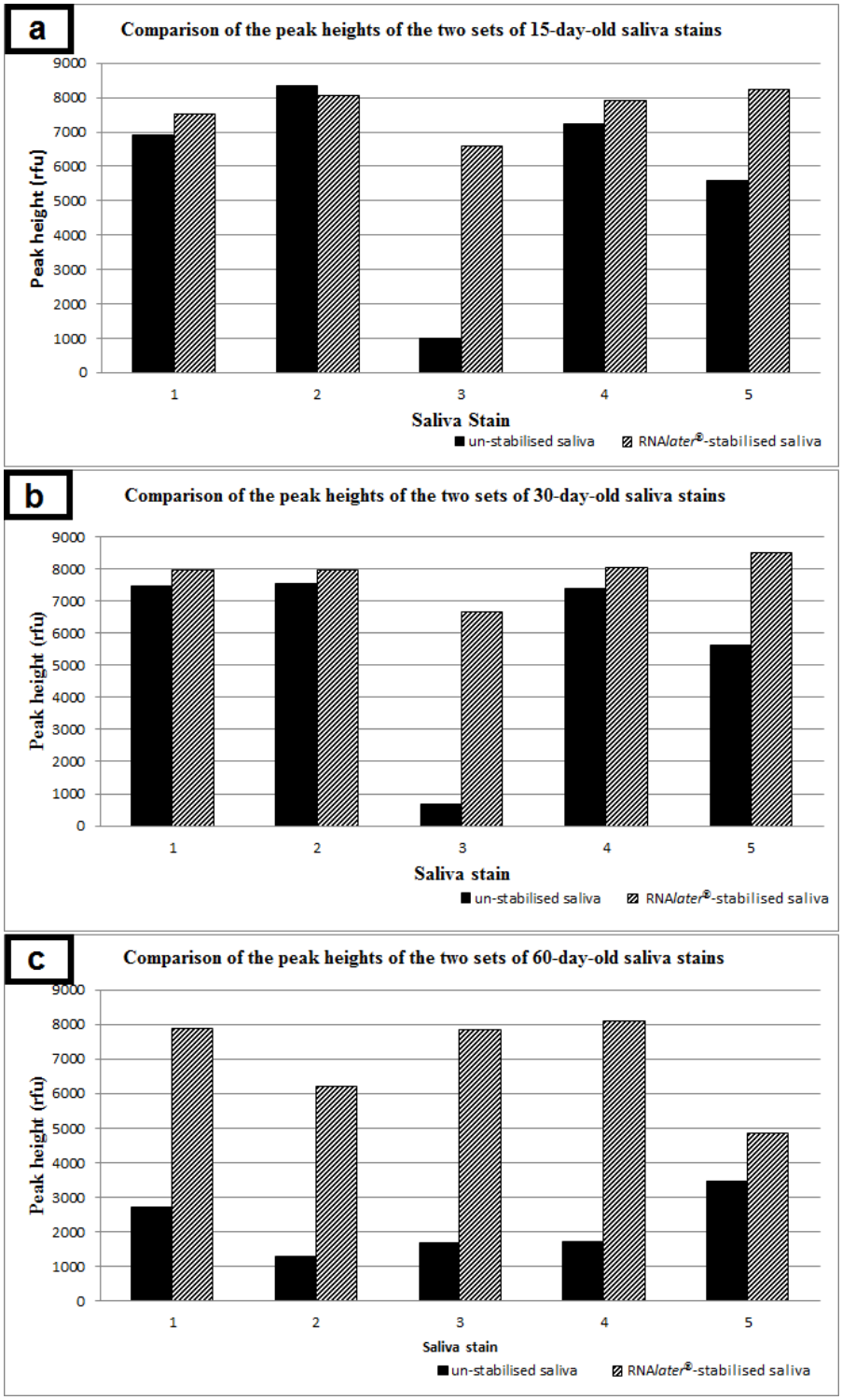

Figure 4-8: Comparison of peak heights (rfu) of the two sets of saliva stains according to their ages (15, 30 and 60 days). Solid bars represent un-stabilised saliva stains and diagonal hatched bars represent stabilised saliva stains. a) Comparison of peak heights (rfu) of 15-day-old stains. b) Comparison of peak heights (rfu) of 30-day-old stains. c) Comparison of peak heights (rfu) of 60-day-old stains.

The possible explanations for this statistically significant increase in the peak heights are either an improvement in RNA purification; or an improvement in PCR amplification of STATH mRNA after RNA*later*® stabilisation. RNA*later*® is composed of concentrated ammonium sulphate solution. The improvement in the PCR amplification may result from ammonium sulphate which is one of the main constituents of the RNA*later*® stabiliser [175]. Ammonium sulphate has its own effect on the nucleic acid purification and on the PCR amplification. Due to the presence of ammonium, intracellular salt concentration in tissue alerts, and this results in leakage of cellular components. In addition, RT-PCR performance improved in the presence of ammonium sulphate where it mitigates or eliminates the inhibitory and cleaving properties of many agents [175]. Moreover, a recent study illustrated that the amplification of the GC-rich DNA sequence was improved and better facilitated by ammonium sulphate [176]. The aforesaid characteristics of ammonium sulphate may suggest PCR amplification improvement due to RNA*later*® which could explain the increase in the peak heights of STATH mRNA profile.

It is known that salivary RNA is exposed to various types of endogenous and exogenous ribonucleases affecting its integrity [93, 97]. In addition, saliva contains various substances (cell debris, food particles, bacteria, etc.) and some of these substances may have an inhibitory effect on the PCR. These factors, combined with the unsuitable storage condition before sample analysis make salivary RNA more prone to degradation even in dried stains, appearing as a decrease in peak height of mRNA with time [14]. Therefore, using RNA*later*® to stabilise RNA in dried saliva stains may help in prevent further RNA degradation and increase the PCR amplification. The success in stopping RNA degradation in saliva stains will help to avoid false negative results due to RNA degradation of molecules after sample collection. It will also help term storage of samples.

4.4 Conclusion

As RNA*later*® was used in the previous chapter to stabilise saliva stains analysed in order to determine the correlation between RNA degradation and the age of saliva samples, the aim of this study was to evaluate the viability of using RNA*later*® to

stabilise RNA in dried saliva stains and, in turn, the validity of the results obtained. The results of this evaluation study showed that RQR values of the stains stabilised with RNA*later*® significantly differed from those undergoing RNA*later*® stabilisation. This difference may be the result of unequal stabilisation of the RNA molecules in saliva, where RNA*later*® stabilises RNA species at different efficiency rates due to differences in their structure or size. As RQR values show the relative quantity of one RNA molecule in relation to other RNA molecules, unequal stabilisation of the two molecules will result in a change of the RQR value. These real-time PCR results reveal that the relative quantity of RNA molecules in dried saliva stains is significantly changed after RNA*later*® stabilisation. This demonstrates that RNA*later*® is unable to stabilise salivary transcriptome even in dried saliva stains, where it was demonstrated that RNA*later*® was unable to stabilise such stains in fresh liquid saliva [120, 177].

This study proceeded to evaluate the suitability of using RNA*later*® to stabilise salivary RNA for another forensic applications using end point PCR, i.e. identification of saliva type. Interestingly, comparing the STATH profiles obtained from un-stabilised saliva stains and stabilised saliva stains shows that the peak heights of STATH significantly increased when saliva stains underwent RNA*later*® stabilisation. This increase in peak heights may result from the presence of ammonium sulphate in RNA*later*®, where it was found that ammonium sulphate improves nucleic acid purification and the PCR amplification. The role of ammonium sulphate in reducing or eliminating the inhibitory effect of substances may become more noticeable with saliva because it contains various substances (cell debris, food particles, bacteria, etc.) and some of these substances may have an inhibitory effect on the PCR.

To conclude, this chapter results show that RNA*later*® significantly changes the RQR values of saliva stains. Therefore, the results of RQR values of saliva stains obtained in the previous chapter may not express the actual RQR values, due to the effect of RNA*later*®. Moreover, it has been found that RNA*later*® has a positive effect on the peak heights of STATH profile. This may therefore indicate a possible

application for RNA*later*® in the forensic field for the purpose of preserving saliva stains, collected from crime scenes, for subsequent body fluid identification.

In the next chapter the correlation between the age of saliva stains and RQR values will be evaluated from saliva stains without using RNA*later*® to obtain the actual RQR values. This therefore avoids the effect of RNA*later*® on the RQR value and on the estimated age of the stains.

5 Determination of the age of un-stabilised saliva stain

5.1 Introduction

Previous chapters discussed the determination of the age of saliva stains stabilised in RNA*later*® using the method of RQR of β-actin to 18S rRNA and the effect of RNA*later*® on the age determination. This chapter discusses the determination of the age of unstabilised saliva stains by applying the method of RQR of β-actin to 18S rRNA with minor modifications on the extraction method used in the previous chapter. These modifications include using AllPrep® DNA/RNA Mini Kit and QIAshredder homogeniser. The reason for adopting AllPrep® DNA/RNA Mini Kit is to simultaneously co-extract DNA and RNA as well as other advantages of column-based nucleic acid purification which were addressed in section 3.1). QIAshredder spin column was used to ensure sample homogenisation and avoid column blocking.

In this study, all volunteers donated samples on the same day on three occasions. This means that the samples from all volunteers on each occasion will be at the same age throughout the experiment. Therefore, extraction of the samples of all volunteers at each age period will be carried out in the same batch in order to avoid batch to batch variations.

5.2 Materials and method

5.2.1 Preparation of samples

Saliva was collected from five volunteers (two males and three females) with no history of malignancy. Volunteers were asked not to eat or smoke and not to use antiseptic mouthwash or brush their teeth in the hour prior to saliva collection. Two mL of saliva were collected in sterile tubes on three separate occasions from each donor. The stains were made by immediate spotting of 100 µL of the saliva sample on clean cotton swabs and were allowed to dry at room temperature in a sterile hood. Then, stains were stored at room temperature in a dry place to simulate natural aging until they reach the desired ages (0, 7, 14, 35, and 60 days) where they immediately proceeded to the extraction step. As the samples were simultaneously collected from

all volunteers on each occasion, the same batch of samples from all volunteers at each age period on each occasion proceeded to extraction.

5.2.2 RNA extraction and reverse transcription

RNA extraction was carried out using the AllPrep® DNA/RNA mini Kit (Qiagen) as described in section 2.3.2.2. All samples were treated with TURBO DNase I (Ambion) as described in section 2.3.3. The RNA quantity and quality were determined spectrophotometrically using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) (Procedure) as described in section 2.4.1 and using the Agilent RNA 6000 Pico Kit for the 2100 Bioanalyzer (Agilent Technologies) as described in section 2.4.2. The reverse transcription was carried out using SuperScript® III (Invitrogen) as described in section 2.5.2.

5.2.3 Real-time PCR

The real-time PCR was carried out using β-actin control reagents and 18S rRNA control reagents (Applied Biosystems), as described in section 2.6.

5.2.4 DNA extraction, quantification and profiling

DNA extraction was carried out using AllPrep® DNA/RNA mini Kit (Qiagen) as described in section 2.9.2. The DNA quantification was carried out using the Investigator Quantiplex kit as described in section 2.10. DNA profiling was carried out using Investigator Decaplex SE Kit as described in section 2.11.

5.2.5 Data analysis

The raw fluorescence Ct values were processed using GenEx statistical software (Version 5.4.0.512; BioEPS GmbH, Munich, Germany) in order to normalise the raw Ct values against interplate calibrators, individual efficiency rates and qPCR technical repeats. Then the normalised Ct values were used to calculate the RQR of β-actin to 18S rRNA by dividing Ct value of β-actin mRNA to the Ct value of 18S rRNA.

All statistical data analyses and graphs were prepared using the Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA) and Microsoft Excel statistics program (Microsoft, USA). The level of significance (α) for any hypothesis test was 0.05. Normality was determined using the Anderson-Darling normality test. Determination of the correlation between the RQR values and the age of the stain was done using Spearman's test [173]. A value close to one or a negative one indicated a strong correlation. A value close to zero indicated a weak correlation. Both nonparametric methods were used because they could effectively deal with small sample sizes.

A general linear mixed model was used to analyse the results of the study. The factors dealt with in this model included gender; donor, which was nested within gender: donor (gender); occasion, which was nested within donor and gender; occasion (donor [gender]); sample age, nested within occasion: age (occasion [donor (gender)]) and age of saliva crossed with gender: (age*gender). All factors, except age of saliva and sex, were taken to be random. Because the factors nested within each other, the F-test was used with the model to determine significance.

The results of the study were analysed using a nested analysis of variance. For the analysis, donor was nested within gender: donor (gender). Occasion was nested within donor: occasion (donor [gender]). *Ex vivo* age of saliva was nested within occasion: sample age (occasion [donor (gender)]) (Figure 5-1). All factors, except age of saliva and sex, were taken to be random.

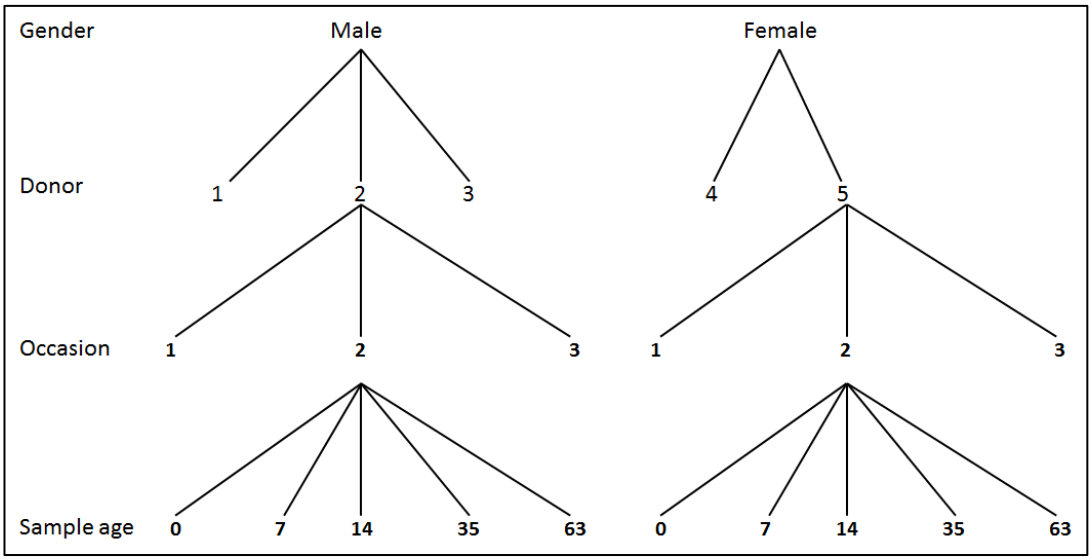

Figure 5-1: Schematic diagram of nested data structure. The figure shows that the donor was nested within gender, occasion was nested within donor and gender, and sample age was nested within sample age, donor and gender.

5.3 Results

5.3.1 RQRs of β-actin mRNA and 18S rRNA

Table 5-1 shows the raw fluorescent Ct values of β-actin mRNA and 18S rRNA measured by real-time PCR at 0, 7, 14, 35, and 60 days. These raw Ct values were corrected with the efficiency of the reaction using GenEx statistical software (version 4.3.7; BioEPS GmbH, Munich, Germany) (Table 5-2).

Table 5-3 shows the RQRs calculated from the corrected Ct values for saliva samples, aged for up to 60 days, under uncontrolled conditions (room temperature, in a dry, dark place). Table 5-4 displays the statistical analysis of the RQR of β-actin mRNA to 18S rRNA at 0, 7, 14, 35, and 60 days obtained using Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA).

Table 5-1: Mean raw fluorescent Ct values of β-actin mRNA and 18S rRNA at 0, 7, 14, 35, and 63 days before efficiency correction for 15 samples from five donors on three occasions. The mean Ct value is calculated using the software of Stratagene Mx3005P™, where each sample is run in duplicate. The Ct value of each individual sample is shown in Appendix 7.

Donors *n*=5	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
1	1	25.85	20.18	No Ct	32.85	25.59	18.03	37.01	30.80	37.46	28.76
	2	30.55	27.45	28.01	21.48	29.51	25.84	30.96	23.86	35.55	27.39
	3	36.32	28.70	29.43	22.72	33.08	25.95	29.80	20.11	No Ct	32.84
2	1	31.10	21.76	30.46	21.29	34.85	27.63	30.61	20.59	38.00	25.86
	2	35.00	26.14	38.03	27.21	28.06	18.66	38.26	27.13	No Ct	37.46
	3	26.86	16.65	No Ct	32.49	40.80	30.97	35.39	24.22	36.82	29.52
3	1	31.22	24.26	34.49	26.33	36.42	27.57	33.98	24.99	31.85	20.70
	2	32.68	23.82	39.94	29.82	38.08	27.25	40.77	26.32	37.71	24.86
	3	34.48	20.21	34.64	26.11	No Ct	No Ct	34.65	23.89	31.95	21.41
4	1	24.69	18.75	31.38	26.30	23.98	17.21	29.82	23.43	28.75	21.19
	2	29.07	24.21	32.54	25.22	32.92	25.04	33.42	25.62	32.32	23.05
	3	29.90	24.09	31.17	24.18	37.06	29.26	30.39	21.85	29.98	21.88
5	1	30.98	26.62	31.15	25.21	33.55	26.96	34.90	28.09	31.68	23.50
	2	27.25	20.61	28.83	23.05	35.26	27.84	31.64	22.01	35.69	27.42
	3	34.34	26.83	35.22	29.11	31.58	24.54	33.42	24.58	32.56	24.87

Table 5-2: Corrected Ct values of β-actin mRNA and 18S rRNA at 0, 7, 14, 35, and 63 days after efficiency correction using GenEx software for 15 samples from five donors on three occasions. The data in this table are obtained from correcting the mean raw Ct values in Table 5-1.

Donors *n*=5	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
1	1	24.15	19.84	No Ct	32.30	23.90	17.72	34.58	30.29	35.00	28.28
	2	28.54	26.99	26.17	21.12	27.57	25.41	28.92	23.46	33.22	26.93
	3	33.93	28.22	27.50	22.34	30.90	25.51	27.84	19.77	No Ct	32.29
2	1	22.63	21.40	28.46	20.93	32.56	27.16	28.60	20.24	35.50	25.43
	2	32.70	25.70	35.53	26.76	26.21	18.34	35.75	26.68	No Ct	36.83
	3	16.91	16.37	No Ct	31.94	38.12	30.45	33.06	23.82	34.40	24.75
3	1	29.17	23.86	32.22	25.89	34.02	27.11	31.74	24.57	29.76	20.35
	2	25.85	23.42	37.31	29.32	35.58	26.79	38.09	25.88	35.23	24.44
	3	24.27	19.87	32.37	25.67	No Ct	No Ct	32.37	23.49	29.85	21.05
4	1	23.07	18.44	29.32	25.86	22.40	16.92	27.86	23.03	26.86	20.84
	2	27.16	23.80	30.40	24.79	30.76	24.62	31.22	25.19	30.19	22.66
	3	27.94	23.68	29.12	23.78	34.62	28.77	28.39	21.48	28.01	21.51
5	1	28.94	26.18	29.10	24.79	31.34	26.51	32.61	27.62	29.59	23.11
	2	25.46	20.27	26.93	22.67	32.94	27.38	29.56	21.64	33.35	26.96
	3	32.08	26.38	32.90	28.62	29.51	24.13	31.23	24.16	30.42	24.45

Table 5-3: Relative quantity ratios of β-actin mRNA to 18S rRNA for 15 samples at 0, 7, 14, 35, and 63 days. RQRs were calculated from the mean Ct values in Table 5-2.

Donors *n*=5	Occasions	Relative Quantity Ratio				
		day 0	day 7	day 14	day 35	day 63
1	1	1.22	1.23	1.35	1.14	1.24
	2	1.06	1.24	1.09	1.23	1.23
	3	1.20	1.23	1.21	1.41	1.24
2	1	1.06	1.36	1.20	1.41	1.40
	2	1.27	1.33	1.43	1.34	1.30
	3	1.03	1.33	1.25	1.39	1.39
3	1	1.22	1.24	1.26	1.29	1.46
	2	1.10	1.27	1.33	1.47	1.44
	3	1.22	1.26	1.29	1.38	1.42
4	1	1.25	1.13	1.32	1.21	1.30
	2	1.14	1.23	1.25	1.24	1.33
	3	1.18	1.22	1.20	1.32	1.30
5	1	1.11	1.17	1.18	1.18	1.28
	2	1.26	1.19	1.20	1.37	1.24
	3	1.22	1.15	1.22	1.29	1.24

Table 5-4: Statistical analysis of the relative quantity ratios of samples at 0, 7, 14, 35, and 63 days. Statistical calculations were carried out using Minitab® software.

	Mean RQR	SD[a]	CV%[b]	Variance
day 0	1.17	0.08	6.81	0.006
day 7	1.23	0.07	5.30	0.004
day 14	1.25	0.09	6.82	0.007
day 35	1.31	0.10	7.33	0.009
day 63	1.32	0.08	6.13	0.007

[a] Standard deviation [b] Coefficient of variation

5.3.2 DNA profile

A complete DNA profile was obtained for all samples amplified using investigator Decaplex SE Kit and no evidence of contamination was noticed in any of the samples.

5.4 Discussion

In this study, real time PCR were carried out using the primers' and probes' optimal concentrations obtained in the study carried out in Chapter 3. The high reaction efficiency was also obtained for both β-actin (81-85%) and 18S rRNA (92.1-96.3%). Reaction efficiency obtained in each batch was used to correct the Ct values for β-actin and 18S rRNA in the batch samples.

5.4.1 RQRs of β-actin mRNA to 18S rRNA

The results of this study also demonstrate the relationship between RQRs of β-actin to 18S rRNA extracted from a saliva sample and the age of the sample. Table 5-4 shows that the means of RQR values increase with an increase in the sample age. Figure 5-2 illustrates also this increase in the RQRs of β-actin to 18S rRNA with increasing sample age. In addition Figure 5-2 shows a wide range of RQR values at each time period.

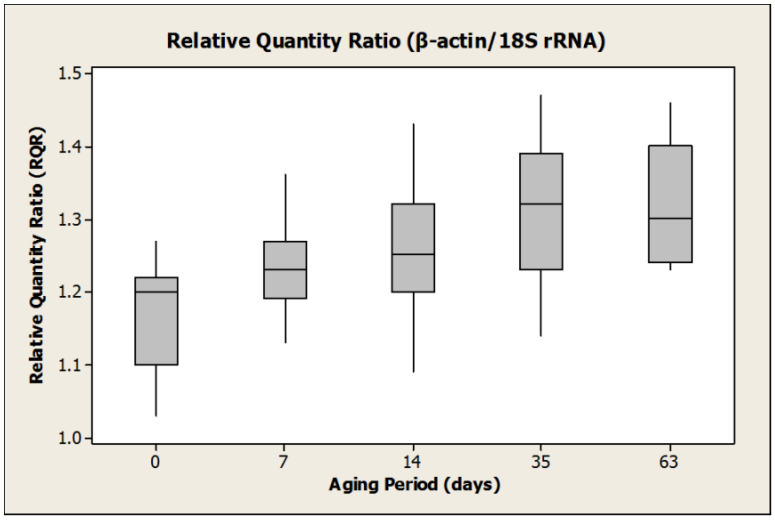

Figure 5-2: A boxplot showing RQRs by age of an *ex vivo* saliva stain. Boxplot was obtained from the data shown in Table 5-3 using Minitab® 16 software.

5.4.2 Combined data and trend analysis

The relationship between the age of saliva stain and the obtained mean RQR values of β-actin to 18S rRNA was statistically calculated. The Anderson-Darling normality test was carried out on the mean RQR values and on the aging period to test the

normality of the data. The test showed that the variables were normally distributed. Therefore, parametric analysis was adopted for statistical analysis. Pearson's correlation indicates that there is a strong linear positive correlation between the mean RQR values of β-actin to 18S rRNA and the age of saliva stains (Pearson's r = 0.901; p-value= 0.037). However, this correlation does not specify which value affects the other.

Regression analysis was applied on the data obtained by plotting the mean RQR values against the age of saliva in order to estimate the relationship between the mean RQR values of β-actin to 18S rRNA and the age of saliva stains. Fitting the data on a simple linear model demonstrates the increase in RQR values with the stain's age increase (R^2= 81.2%) (Figure 5-3 and Figure 5-5). In addition, fitting the data on a cubic model shows a higher R^2 value (R^2= 91.9%) (Figure 5-4). However, the cubic model appears less reliable where the 95% confidence interval shows a wider confidence strip than that of the linear model. This means that the linear model is a more reliable prediction model than the cubic model, depending on the data plotted (Figure 5-5).

The age of the saliva stain can be estimated based on these RQR values using a linear model (R^2= 81.2 %):

$$y = - 446.0 + 374.1\, x \qquad \text{(Equation 5-1)}$$

where y is the age of the saliva stain in days and x is the RQR of β-actin to 18S rRNA.

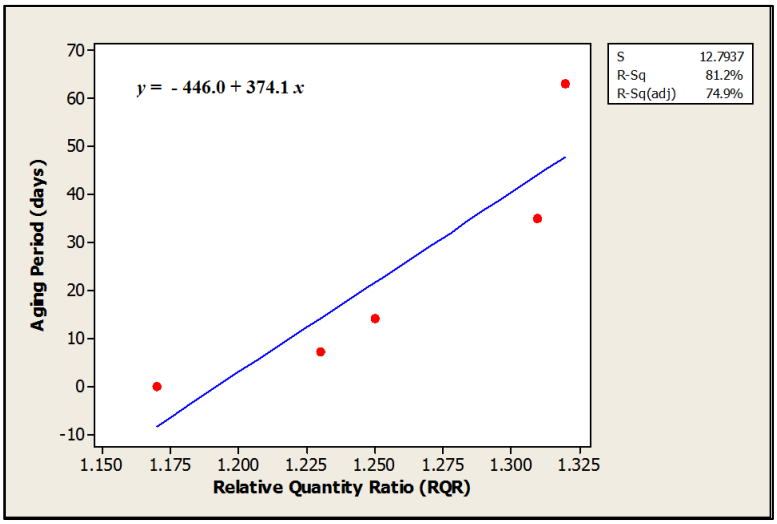

Figure 5-3: Time-wise trend in the RQR of saliva samples over time shown on linear model. The curve was obtained from the data shown in Table 5-4.

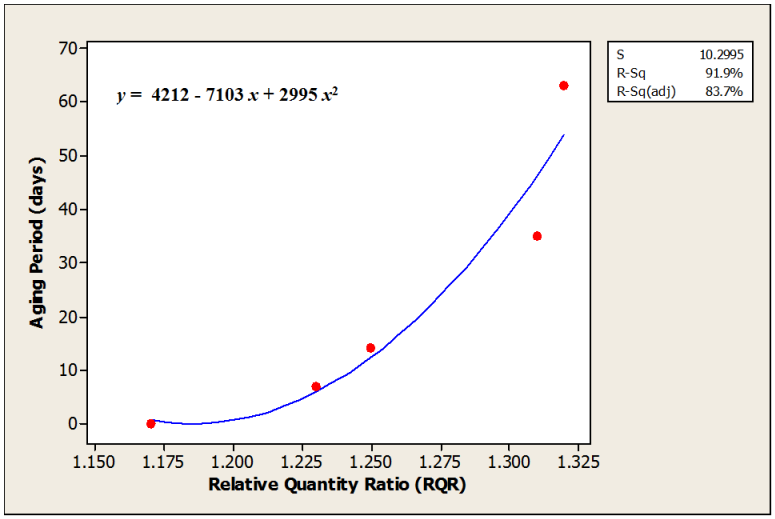

Figure 5-4: Time-wise trend in the RQR of saliva samples over time shown on cubic model. A second-order polynomial curve obtained from the data shown in Table 5-4 using Minitab® 16 software.

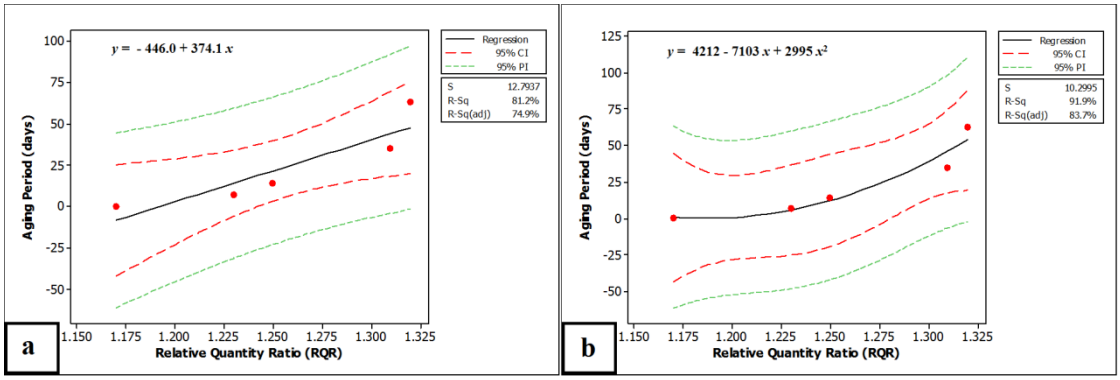

Figure 5-5 Time-wise trend in the RQR of saliva samples over time with 95% confidence interval and 95% prediction interval. a) Linear regression b) Cubic regression.

To evaluation the effect of the ages of saliva and the individuals on the mean RQR values at specific two-way analysis of variance (ANOVA) test was carried. The results of the two-way ANOVA showed that the difference in the means of different sample ages was highly significant (*p value* <0.00001). The results also showed that the difference in means between individuals at specific ages of saliva stains is also significant (*p value* <0.05) (results of ANOVA test are shown in Appendix 3 and Appendix 4).

The nested analysis of variance results showed that the largest source of variability in the RQR values of β-actin mRNA to 18S rRNA is due to the age of saliva stains where it produced R^2 values of 0.65 (65%) (Figure 5-6). The remainder of the differences between populations (35%) was accounted for by other factors such as inter-person, intra-person, and residual effects. The residual effects are those not assigned to the other possible sources of variability and for which the exact cause is unknown. The variability in the RQR values accounting for the inter-person variability was 1.8%, and for the intra-person variability was 1.4%. In addition, the gender effect on the RQR values that was found was 2.8%.

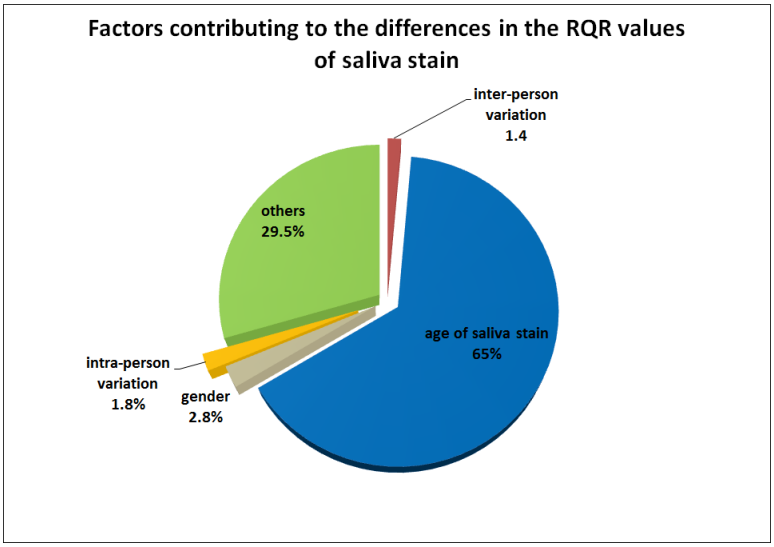

Figure 5-6: Factors contributing to the differences in the RQR values of saliva stain. The figure shows that the difference in the RQR values mostly results from the saliva's age.

5.4.3 The inter-person variation at each age period

The inter-person variation at each age period was statistically calculated using RQRs of the corrected Ct values (i.e., normalised qPCR data). It was found that the inter-person variation ranged from 5.3% (seven days) to 7.33% (35 days) with a mean value of 6.48% across all aging times (Table 3-5 and Figure 3-12). The results of the inter-person variations show higher variations at the beginning of the study compared to those reported for blood and hair [49, 75]. The high inter-person variation at the beginning may be due to the natural biological variance *in vivo* and the constitutional components of saliva. As saliva contains various endo- and exo-ribonucleases from many sources and different extraneous substances this makes saliva content vary from person to person [93, 107]. This variation stays almost at the same level for all age periods except at 35 days where it shows a slightly lower variation.

In this study, RNA stabiliser RNA*later*® was not used in order to avoid its effect on the RQR values. The extraction of all samples at the same age was carried out in one batch in order to minimise errors resulting from the batch to batch variation.

As a result of these amendments in the study, the intensive fluctuations in the results of the inter-person variations with increase of the sample age, which have been

noticed in the inter-person variation results in Chapter 3, decreased. However, a high inter-person variation was noticed over all age periods.

Table 5-5: Inter-person variations over time. Mean RQR calculated from results by day given in Table 5-3 using Minitab® software. (Equation used to calculate coefficient of variation (CV) is CV= standard deviation / mean × 100).

	Mean RQR *n*=15	Inter-person variation (Coefficient of variation, %)
Day 0	1.17	6.81
Day 7	1.23	5.30
Day 14	1.25	6.82
Day 35	1.31	7.33
Day 60	1.31	6.13

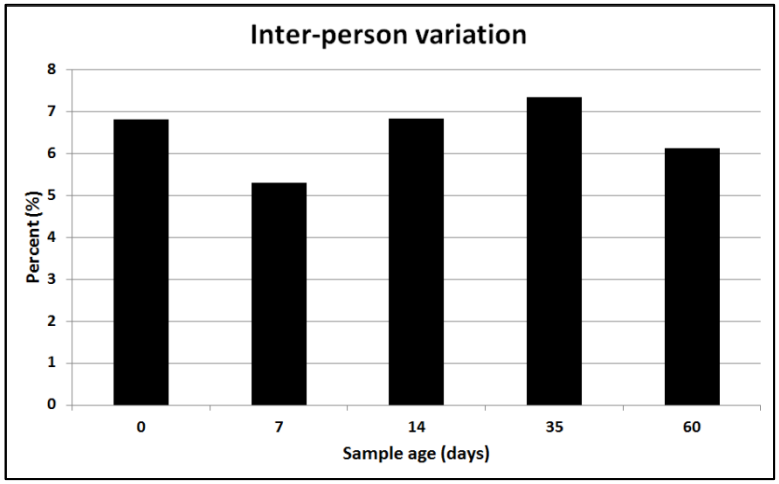

Figure 5-7: Inter-person variation samples over time. The bar chart was obtained from the data shown in Table 5-3 sing the Microsoft Excel statistics program (Microsoft, USA).

5.4.4 Intra-person variation

Intra-person variation varied from 4.74% (donor 4) to 24.2% (donor 3) with an overall average of 12.76%. The high intra-person variation may be a direct reflection on the composition of saliva varying from time to time due to physiological and chemical causes. It is known that once salivary RNA is exposed to various types of endogenous and exogenous ribonucleases its integrity is affected [93, 97]. In addition, saliva contains various substances (cell debris, food particles, bacteria, etc.)

that may also affect the salivary RNA integrity. It should be taken into consideration that this high level of variation was obtained despite all volunteers abstaining from eating, smoking or brushing their teeth in the hour prior to saliva collection. This requirement was stipulated in order to decrease the external effect of eating, smoking or teeth-brushing on salivary RNA. Due to the high level of variation observed, the precision and accuracy of determining the age of saliva. In turn, this could be considered as a limitation to application of this method in casework. The specific conditions (i.e. no food, drink, cigarettes etc.) which the volunteers adhered to, yet which still yielded a high level of variation, may not be present in actual casework. In real cases, saliva may be exposed to various extraneous substances in the mouth before saliva spitting and these factors may affect the RNA status and ribonucleases and, in turn, increase inter- and intra-person variation.

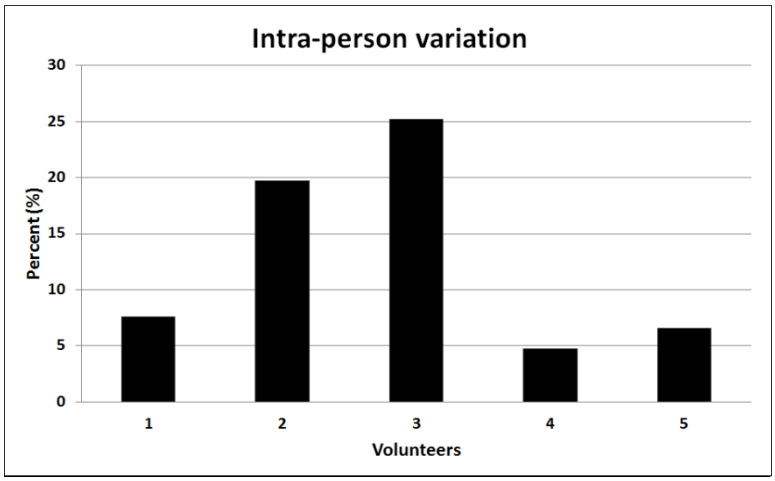

Figure 5-8: Intra-person variation of the five volunteers.

Regardless of the accuracy and precision of the results obtained, successful application of this approach on saliva stains (because of using housekeeping genes RNA) poses an important issue which merits further investigation. This issue is the effect of the saliva on the precision and accuracy of determining the age of the bloodstain in cases where blood is mixed with saliva. As the mixture between different body fluids, either from one or more people, is commonly observed in

forensic practice [85], the effect of the mixture status between blood and saliva on the RQR values of both blood and saliva stains will be discussed in the next chapter.

5.4.5 Comparing the results with those obtained from previous studies [49, 75] carried out on bloodstains and hair samples

Points that were noticed when comparing the results obtained in this study with those of previous studies [49, 75] carried out on bloodstains and hair samples include: firstly, in this study, the RQRs increase with increasing sample age. This trend is similar to those trends obtained in previous studies [49, 75]. In addition, the same trend was obtained despite the ratio used being the opposite to that used by Anderson *et al.* [49]. This confirmed our finding (discussed in Chapter 3) about the Anderson *et al.* [49] mistake in explaining the ratio used to obtain their results. Secondly, in this study, the shape of the relationship between RQRs and the sample age behave in a linear fashion. This finding is concordant with the findings of other studies carried out on blood and hair [49, 75]. This finding confirms the hypothesis proposed by Anderson *et al.* [49] that "different types of RNAs decay, *ex vivo,* at different rates". It also confirms the applicability of the method suggested by Anderson *et al.* [49] on saliva stains.

Thirdly, in this study, high levels of inter- and intra-person variations were noticed compared to those previously reported about bloodstains and saliva stains [49, 75]. Eady *et al.* [178] imputed the inter-person variation in gene expression in blood among healthy individuals to the difference in the gender, age and body mass between individuals, and to the difference in the leukocyte subsets analysed. In hair, the small inter-person variation was attributed to natural biological variance *in vivo* [75]. In this study, the high inter-person variation noticed in saliva at the beginning may be due to the natural biological variance *in vivo* and the constitutional components of saliva. It was found that most of the RNA molecules in fresh saliva are partially degraded [107]. In contrast to blood and hair, saliva contains various endo- and exo-ribonucleases from many sources, as well as the presence of different extraneous substances [93, 107]. In addition, the types and the quantity of ribonucleases and the extraneous substances may vary in the same individual from

time to time for a variety of reasons, such as the cleanliness of the mouth, the use of antiseptic mouthwash or presence of food types. This makes saliva content vary among individuals and, in the same individual from time to time, resulting in the high inter and intra-individual variations noticed.

5.4.6 Comparing the results with those obtained from RNA*later*®-stabilised saliva stain.

In this study, the RNA*later*® reagent was not used and the saliva stain was immediately extracted upon reaching the desirable age, in order to avoid the RNA*later*® reagent's effect on the RQR values discussed in the previous chapter. In addition, the extraction of all samples at the same age was carried out in one batch to decrease errors resulting from batch to batch variation as much as possible. Many points were noticed when comparing the results obtained in this study with those obtained in Chapter 3, in which RNA*later*®-stabilised saliva stains were analysed.

Firstly, it was noticed that the RQR values of the unstabilised saliva stain obtained were lower than those obtained from the same age RNA*later*®-stabilised saliva stain discussed in Chapter 3 (Table 5-6). This finding is harmonious with the finding regarding the effect of the RNA*later*® reagent on the RQR values of saliva stains discussed in Chapter 4 (see Figure 4-1).

Table 5-6: Mean relative quantity ratios of β-actin mRNA to 18S rRNA for unstabilised saliva stains analysed in this chapter and for RNA*later*® stabilised saliva stains analysed in Chapter 3.

Sample age	Mean RQR	
	Un-stabilised saliva stain	RNA*later*-stabilised saliva stain
day 0	1.17	1.37
day 7	1.25	1.51
day 14	1.31	1.75
day 60-63	1.31	1.77

Secondly, the results of the inter-person variations obtained in this study vary from those obtained following analysis of RNA*later*®-stabilised saliva stains studied in

Chapter 3. In this study, a high inter-person variation was noticed for all age periods while, in the previous study (i.e. analysing RNA*later*®-stabilised saliva stains), the inter-person variations showed inconsistent fluctuations with an increase of the sample age. Thus, these inconsistent fluctuations obtained in the previous study may be imputed to the effect of the RNA*later*® reagent, or may result from batch-to-batch variability where the sample at each age period was analysed in different batches at different times.

Thirdly, in this study, simultaneous extraction of RNA and DNA was carried out using the AllPrep® DNA/RNA Mini Kit which offers an important benefit over the RNeasy® Micro Kit with regard to determining the age of the stain. The AllPrep® DNA/RNA Mini Kit provides a convenient way of analysing RNA for determining stain's age and determining the DNA profile of the donor. This will help to evaluate sample contamination with the sample of another individual.

Apart from these points, this study and the study carried out on RNA*later*® stabilised saliva stains confirm the applicability of the method developed by Anderson *et al.* [49] on saliva stains.

5.5 Conclusion

In this study, the approach proposed by Anderson *et al.* [49] to determine the age of a bloodstain was applied on unstabilised saliva stains (i.e. immediately extracted upon reaching the desirable ages) in order to evaluate the suitability of this approach on saliva stains and in order to avoid the possible effect of the RNA*later*® reagent on the RQR results. The results of this aging study also demonstrated the relationship between the age of saliva and the RQRs of β-actin mRNA to 18S rRNA, and the age of saliva. It was found that RQR values of β-actin mRNA to 18S rRNA increase with an increase in the age of the stain. This supports the hypothesis that there are differences in *ex vivo* RNA degradation rates and confirms the hypothesis proposed by Anderson *et al.* [49] on the potential for the application's approach on tissue types other than blood. However, the wide prediction interval resulted in a low precision of the age prediction.

In this study, inter-and intra-person variations were determined. A high level of variations was noticed compared to that observed in blood and hair. This high level of variation could be due to the variation between individuals in the degradation status of RNA molecules in saliva and due to the presence of various types of ribonucleases in saliva. In addition, this high level of variation was found despite setting controlled conditions for collection in an attempt to decrease the effect of some external materials, such as food and smoke. This high variation resulted in low precision and accuracy in determining saliva age, which can be considered as a limitation to apply this approach in casework.

The variation associated with gender and age was not addressed in this study because the data obtained are not extensive enough, due to the small number of volunteers involved in the study (five volunteers: two males and three females).

Because the approach proposed by Anderson *et al.* [49] can be applied on both blood and saliva through use of housekeeping genes RNA, the effect of the mixture on the RQR value of both blood and saliva was pointed out as an important issue that should be discussed.

Therefore, the effect of contamination of a mixture of blood and saliva on the RQR values of each pure stain type's pure form, resulting from using housekeeping genes expressed in all tissue types, will be discussed.

6 Effect of body fluid mixture on RQR values used to determine the age of biological stains

In previous chapters, the applicability of the use of the RQR method for saliva stains was discussed. This chapter discusses the effect of mixing two different types of body fluid (blood and saliva) on the RQR values of β-actin to 18S rRNA. The work also considers the impact on the test results of mixtures of body fluids where one contributor to the mixture is older than the other. This is to simulate, under tightly controlled conditions, a circumstance that might realistically be experienced with stains found at crime scenes. As blood will be analysed in this study, the organic extraction method TRI Reagent BD was used.

6.1 Materials and method

6.1.1 Sample collection

Stains of blood, saliva and a mixture of both were made on clean cotton swabs.

Firstly, pure blood and pure saliva stains were prepared as follows:

Stains made from one type of body fluid, or 'pure' stains were prepared from blood and saliva collected from five volunteers, with informed consent, via standard venepuncture for blood and spitting for saliva. From each volunteer, two pure stains for blood (20 µL) and two for saliva (50 µL) were made on clean cotton swabs to create ten stained swabs of each type of body fluid. Each stain was allowed to dry at room temperature inside a sterile hood and was then extracted as a dry, fresh, pure stain.

In addition, from blood and saliva collected from four volunteers, eight more pure stains were prepared for each type of body fluid sample from the same volunteers. These additional stains were kept for specific "ages" (i.e., two stains for 10 days, two stains for 30 days and four stains for nine months) before the preparation of mixed stains by the addition of fresh blood or saliva, as described below.

Secondly, four types of mixture stains from the volunteers were prepared from the blood and saliva that had been collected when the stains of pure body fluids were prepared.

The mixture stains consisted of:

1. Fresh blood and fresh saliva were prepared by adding 50 µL of fresh saliva to 20 µL of fresh aliquoted blood on cotton swabs. These were extracted after drying.

2. Fresh blood and aged saliva were prepared by adding 20 µL of fresh blood to previously prepared aged saliva stains.

3. Aged blood and fresh saliva were prepared by adding 50 µL of fresh saliva to previously prepared aged bloodstains.

4. Aged stains of blood and saliva were prepared by mixing fresh blood and saliva and stored to reach various ages, as specified above, and left in a dry place until they reached specific *ex vivo* ages prior to extraction.

All mixtures of blood and saliva are shown in Table 6-1. Each of the following mixture combinations were prepared in duplicate, using blood and saliva from five volunteers for Mixture type 1. Other mixture types were prepared using samples from four volunteers giving 34 mixture stains in all.

Table 6-1: Combinations used for mixture study.

Types	Composition of Mixture stain	Mixture Preparation
Mixture type 1	Fresh blood and fresh saliva	Mixture prepared by mixing fresh blood and saliva and then extracted directly
Mixture type 2	Fresh blood and aged saliva	Mixture prepared by addition of fresh blood to aged saliva stain and then extracted
Mixture type 3	Aged blood and fresh saliva	Mixture prepared by addition of fresh saliva to aged bloodstain and then extracted
Mixture type 4	Aged blood and aged saliva	Mixture prepared by mixing fresh blood and saliva and stored until specific age reached, then extracted.

Figure 6-1 shows the timeline of collection and preparation of stains prior to extraction.

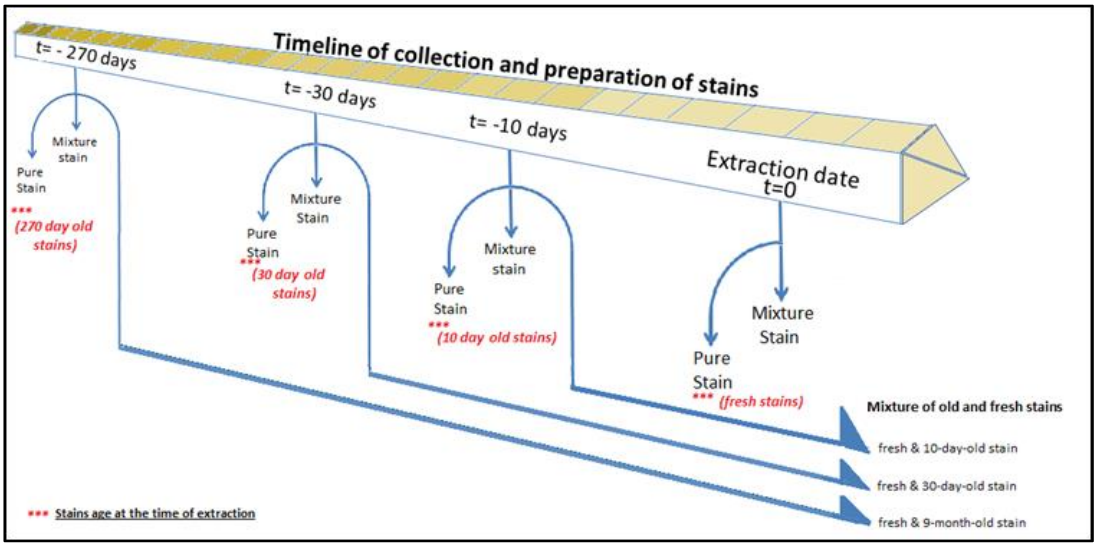

Figure 6-1: Timeline of collection and preparation of pure blood and saliva stains and mixture of both of them.

6.1.2 RNA extraction and reverse transcription

RNA extraction was carried out using the TRI reagent as described in section 2.3.1. The reverse transcription was carried out using SuperScript® III (Invitrogen) as described in section 2.5.2.

6.1.3 Real-time PCR

The real-time PCR was carried out using β-actin control reagents and 18 S rRNA control reagents (Applied Biosystems), as described in section 2.6. The Ct value accepted as valid in order to avoid false-positive samples, was any value lower than 40.

Due to the number of samples, duplication of each sample and running of negative controls, samples were run in batches. Each batch contains pure blood, pure saliva stains and all mixture types made from that stains. An inerplate calibrator was used with each batch in addition to the negative control.

6.1.4 Data analysis

The Relative Quantitative Ratios (RQR) of β-actin to 18S rRNA were calculated by dividing the Ct value of β-actin mRNA with the Ct value of 18S rRNA. All statistical data analyses and graphs were done using the Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA). The level of significance (α) for any hypothesis test was 0.05. Normality was determined by the Anderson-Darling normality test. Kruskal-Wallis non-parametric ANOVA [173] was used to compare between the RQR values of all combination of mixtures between blood and saliva samples. If a p-value of less than 0.05 was obtained, the Mann-Whitney U test [173] was carried out to show the pairwise comparisons of the three stain types.

6.2 Results

The raw Ct values of β-actin mRNA and 18S rRNA of pure blood, pure saliva, and a mixture of blood and saliva were measured using TaqMan® real time PCR. The data obtained were used to calculate RQR of β-actin mRNA to 18S rRNA of the analysed samples.

The raw Ct values were tabulated based on a combination of the Ct values results of each mixture type with those of stains composed of pure types from the same volunteer. Each table was constructed to show the biological repeat in a vertical arrangement and the technical repeat in a horizontal arrangement. Each table shows the Ct values results of each mixture type and the pure types of the constituent stains, and was followed with a table showing the RQR values resulting from the Ct values results. The raw Ct values obtained for each mixture type stain and the pure types of constituent stains are showing in Table 6-2, Table 6-4, Table 6-6 and Table 6-8. In addition, the RQR values calculated from the Ct values are shown in Table 6-3, Table 6-5, Table 6-7 and Table 6-9.

Table 6-2: The Ct values of β-actin mRNA and 18S rRNA for mixture type-1 stains and the pure types of the constituent stains. For each donor A to E, two pure biological samples were analysed in parallel i.e. technical repeat, where each sample was tested in duplicate at the qPCR stage, i.e. technical repeats. This gives four results for each stain, i.e. 2 biological × 2 technical, for each stain from each donor for both blood and saliva. Asterisk (∗) indicates stain made of a pure form of body fluid. Double asterisks (∗∗) indicates stains made by mixing of two pure body fluids shown in (∗).

Donor no	Test no	Pure fresh bloodstain (∗)				Pure fresh saliva stain (∗)				Mixture type 1 (∗∗) (fresh blood & fresh saliva)			
		Replicate 1		Replicate 2		Replicate 1		Replicate 2		Replicate 1		Replicate 2	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
A	1	29.59	19.73	29.01	19.09	38.90	25.26	38.92	25.61	29.40	18.85	28.20	17.85
	2	28.85	18.86	27.90	18.72	39.99	26.31	39.46	25.29	28.47	19.11	29.12	20.08
B	3	32.78	23.25	32.87	22.36	38.98	29.31	37.99	28.56	31.80	21.06	31.80	21.06
	4	29.89	20.90	29.77	20.25	39.53	30.64	38.78	31.02	37.39	25.44	37.25	25.00
C	5	30.50	21.03	30.04	21.61	34.97	23.16	35.37	23.74	29.47	19.14	29.50	19.41
	6	31.77	21.76	30.95	20.91	33.86	21.85	32.93	21.52	27.82	17.61	26.98	17.08
D	7	34.67	26.07	34.12	25.65	37.81	28.43	37.85	28.89	31.16	22.74	32.03	23.73
	8	35.55	26.33	34.75	26.94	37.53	27.60	36.65	27.77	30.21	23.06	30.22	22.39
E	9	28.52	21.44	28.12	21.14	29.77	22.38	28.70	21.91	24.76	18.52	24.95	18.04
	10	27.83	21.41	27.58	20.58	35.12	26.01	35.01	26.32	23.44	17.36	22.43	17.12

Table 6-3: Relative Quantity Ratios of β-actin mRNA to 18S rRNA for mixture type-1 stains and the pure types of the constituent stains. RQRs were calculated from the Ct values in Table 6-2.

Donor no	Test no	Pure Fresh bloodstain			Pure fresh saliva stain			Mixture type 1 (fresh blood & fresh saliva)		
		Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR
A	**1**	1.50	1.52	**1.51**	1.54	1.52	**1.53**	1.56	1.58	**1.57**
	2	1.53	1.49	**1.51**	1.52	1.56	**1.54**	1.49	1.45	**1.47**
B	**3**	1.41	1.47	**1.44**	1.33	1.33	**1.33**	1.51	1.51	**1.51**
	4	1.43	1.47	**1.45**	1.29	1.25	**1.27**	1.47	1.49	**1.48**
C	**5**	1.45	1.39	**1.42**	1.51	1.49	**1.50**	1.54	1.52	**1.53**
	6	1.46	1.48	**1.47**	1.55	1.53	**1.54**	1.58	1.58	**1.58**
D	**7**	1.33	1.33	**1.33**	1.33	1.31	**1.32**	1.37	1.35	**1.36**
	8	1.35	1.29	**1.32**	1.36	1.32	**1.34**	1.31	1.35	**1.33**
E	**9**	1.33	1.33	**1.33**	1.33	1.31	**1.32**	1.34	1.38	**1.36**
	10	1.30	1.34	**1.32**	1.35	1.33	**1.34**	1.35	1.31	**1.33**
Mean RQR				**1.41**			**1.40**			**1.45**
SD [a]				**0.08**			**0.11**			**0.10**
Variance				**0.006**			**0.012**			**0.010**
CV% [b]				**5.55**			**7.8**			**6.79**
Median				**1.43**			**1.34**			**1.48**

[a] Standard deviation, [b] Coefficient of variation

Table 6-4: The Ct values of β-actin mRNA and 18S rRNA for mixture type-2 stains and the pure types of the constituent stains. For each donor A to D, two pure biological samples were analysed in parallel i.e. technical repeat, where each sample was tested in duplicate at the qPCR stage, i.e. technical repeats. This gives four results for each stain, i.e. 2 biological × 2 technical, for each stain from each donor for both blood and saliva. Aged stains for donor A is 10 days, for donor B is 30 days, for donor C and D are more than nine months. Asterisk (∗) indicates stain made of a pure form of body fluid. Double asterisks (∗∗) indicates stains made by mixing of two pure body fluids shown in (∗). No Ct indicates that Ct values were above the cut-off cycle threshold (40).

Donor no	Test no	Pure fresh bloodstain (∗)				Pure aged saliva stain (∗)				Mixture type 2 (∗∗) (fresh blood & aged saliva)			
		Replicate 1		Replicate 2		Replicate 1		Replicate 2		Replicate 1		Replicate 2	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
A	1	32.78	23.25	32.87	22.36	30.99	20.80	30.06	21.02	29.89	20.90	30.63	20.84
	2	29.89	20.90	29.77	20.25	34.63	20.99	35.57	21.30	30.02	19.62	31.10	21.16
B	3	30.50	21.03	30.04	21.61	37.36	20.64	36.58	20.21	32.78	20.36	32.12	20.46
	4	31.77	21.76	30.95	20.91	33.72	17.94	32.84	17.85	31.33	18.87	30.85	19.04
C	5	34.67	26.07	34.12	25.65	No Ct	No Ct	No Ct	No Ct	29.59	21.44	30.48	21.77
	6	35.55	26.33	34.75	26.94	No Ct	No Ct	No Ct	No Ct	28.95	19.43	29.84	22.44
D	7	28.52	21.44	28.12	21.14	No Ct	No Ct	No Ct	No Ct	28.85	21.06	27.93	20.09
	8	27.83	21.41	27.58	20.58	No Ct	No Ct	No Ct	No Ct	28.61	20.29	27.34	19.12

Table 6-5: Relative Quantity Ratios of β-actin mRNA to 18S rRNA for mixture type-2 stains and the pure types of the constituent stains. RQRs were calculated from the Ct values in Table 6-4. ND indicates the RQR value cannot be measured.

Donor no	Test no	Pure Fresh bloodstain			Pure aged saliva stain			Mixture type 2 (fresh blood & aged saliva)		
		Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR
A	1	1.41	1.47	**1.44**	1.49	1.43	**1.46**	1.43	1.47	**1.45**
	2	1.43	1.47	**1.45**	1.65	1.67	**1.66**	1.53	1.47	**1.50**
B	3	1.45	1.39	**1.42**	1.81	1.81	**1.81**	1.61	1.57	**1.59**
	4	1.46	1.48	**1.47**	1.88	1.84	**1.86**	1.66	1.62	**1.64**
C	5	1.33	1.33	**1.33**	ND	ND	**ND**	1.38	1.40	**1.39**
	6	1.35	1.29	**1.32**	ND	ND	**ND**	1.49	1.33	**1.41**
D	7	1.33	1.33	**1.33**	ND	ND	**ND**	1.37	1.39	**1.38**
	8	1.30	1.34	**1.32**	ND	ND	**ND**	1.41	1.43	**1.42**
Mean RQR				**1.39**			**1.70**			**1.47**
SD [a]				**0.07**			**0.18**			**0.10**
Variance				**0.004**			**0.032**			**0.009**
CV% [b]				**4.74**			**10.59**			**6.55**
Median				**1.38**			**1.74**			**1.44**

[a] Standard deviation, [b] Coefficient of variation

Table 6-6: The Ct values of β-actin mRNA and 18S rRNA for mixture type-3 stains and the pure types of the constituent stains. For each donor A to D, two pure biological samples were analysed in parallel i.e. technical repeat, where each sample was tested in duplicate at the qPCR stage, i.e. technical repeats. This gives four results for each stain, i.e. 2 biological × 2 technical, for each stain from each donor for both blood and saliva. Aged stains for donor A is 10 days, for donor B is 30 days, for donor C and D are more than nine months. Asterisk (∗) indicates stain made of a pure form of body fluid. Double asterisks (∗∗) indicates stains made by mixing of two pure body fluids shown in (∗). No Ct indicates that Ct values were above the cut-off cycle threshold (40).

Donor no	Test no	Pure aged bloodstain (∗)				Pure fresh saliva stain (∗)				Mixture type 3 (∗∗) (aged blood & fresh saliva)			
		Replicate 1		Replicate 2		Replicate 1		Replicate 2		Replicate 1		Replicate 2	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
A	1	37.67	22.69	37.62	22.66	38.90	25.26	38.92	25.61	38.67	26.13	39.01	28.27
	2	37.75	22.34	36.76	22.01	39.99	26.31	39.46	25.29	38.38	24.29	38.56	26.78
B	3	37.90	20.71	38.30	21.16	38.98	29.31	37.99	28.56	35.78	22.94	36.12	22.30
	4	35.06	18.75	36.01	18.85	39.53	30.64	38.78	31.02	35.00	19.77	35.19	22.13
C	5	No Ct	No Ct	No Ct	No Ct	34.97	23.16	35.37	23.74	29.59	21.44	30.48	21.77
	6	No Ct	No Ct	No Ct	No Ct	33.86	21.85	32.93	21.52	28.95	19.43	29.84	22.44
D	7	No Ct	No Ct	No Ct	No Ct	37.81	28.43	37.85	28.89	28.85	21.06	27.93	20.09
	8	No Ct	No Ct	No Ct	No Ct	37.53	27.60	36.65	27.77	28.61	20.29	27.34	19.12

Table 6-7: Relative Quantity ratios of β-actin mRNA to 18S rRNA for mixture type-3 stains and the pure types of the constituent stains. RQRs were calculated from the Ct values in Table 6-6. ND indicates the RQR value cannot be measured.

Donor no	Test no	Pure aged bloodstain			Pure fresh saliva stain			Mixture type 3 (aged blood & fresh saliva)		
		Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR
A	1	1.66	1.66	**1.66**	1.33	1.33	**1.33**	1.46	1.40	**1.43**
	2	1.69	1.67	**1.68**	1.29	1.25	**1.27**	1.53	1.49	**1.51**
B	3	1.83	1.81	**1.82**	1.51	1.49	**1.50**	1.56	1.62	**1.59**
	4	1.87	1.91	**1.89**	1.55	1.53	**1.54**	1.71	1.65	**1.68**
C	5	ND	ND	**ND**	1.33	1.31	**1.32**	1.35	1.35	**1.35**
	6	ND	ND	**ND**	1.36	1.32	**1.34**	1.28	1.32	**1.30**
D	7	ND	ND	**ND**	1.33	1.31	**1.32**	1.45	1.39	**1.42**
	8	ND	ND	**ND**	1.35	1.33	**1.34**	1.43	1.51	**1.47**
Mean RQR				**1.76**			**1.37**			**1.47**
SD [a]				**0.11**			**0.10**			**0.12**
Variance				**0.012**			**0.009**			**0.015**
CV% [b]				**6.29**			**6.99**			**8.44**
Median				**1.75**			**1.34**			**1.45**

[a] Standard deviation, [b] Coefficient of variation

Table 6-8: The Ct values of β-actin mRNA and 18S rRNA for mixture type-4 stains and the pure types of the constituent stains. For each donor A to D, two pure biological samples were analysed in parallel i.e. technical repeat, where each sample was tested in duplicate at the qPCR stage, i.e. technical repeats. This gives four results for each stain, i.e. 2 biological × 2 technical, for each stain from each donor for both blood and saliva. Aged stains for donor A is 10 days, for donor B is 30 days, for donor C and D are more than nine months. Asterisk (∗) indicates stain made of a pure form of body fluid. Double asterisks (∗∗) indicates stains made by mixing of two pure body fluids shown in (∗). No Ct indicates that Ct values were above the cut-off cycle threshold (40).

Donor no	Test No	Pure Aged bloodstain (∗)				Pure Aged saliva stain (∗)				Mixture type 4 (∗∗) (aged blood & saliva)			
		Replicate 1		Replicate 2		Replicate 1		Replicate 2		Replicate 1		Replicate 2	
		β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
A	1	37.67	22.69	37.62	22.66	30.99	20.80	30.06	21.02	37.78	22.69	36.84	22.06
	2	37.75	22.34	36.76	22.01	34.63	20.99	35.57	21.30	37.94	22.50	38.62	21.82
B	3	37.90	20.71	38.30	21.16	37.36	20.64	36.58	20.21	38.73	20.49	37.94	20.29
	4	35.06	18.75	36.01	18.85	33.72	17.94	32.84	17.85	38.75	21.88	39.69	20.35
C	5	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
	6	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
D	7	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
	8	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

Table 6-9: Relative Quantity Ratios of β-actin mRNA to 18S rRNA for mixture type-4 stains and the pure types of the constituent stains. RQRs were calculated from the Ct values in Table 6-8. ND indicates the RQR value cannot be measured.

Donor no	Test No	Pure aged bloodstain			Pure aged saliva stain			Mixture type 4 (aged blood & saliva)		
		Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR	Replicate 1 RQR	Replicate 2 RQR	Mean RQR
A	**1**	1.66	1.66	**1.66**	**1.49**	1.43	**1.46**	1.71	1.67	**1.69**
	2	1.69	1.67	**1.68**	**1.65**	1.67	**1.66**	1.75	1.77	**1.76**
B	**3**	1.83	1.81	**1.82**	**1.81**	1.81	**1.81**	1.89	1.87	**1.88**
	4	1.87	1.91	**1.89**	**1.88**	1.84	**1.86**	1.91	1.95	**1.93**
C	**5**	ND	ND	ND	**ND**	ND	**ND**	ND	ND	**ND**
	6	ND	ND	ND	**ND**	ND	**ND**	ND	ND	**ND**
D	**7**	ND	ND	ND	**ND**	ND	**ND**	ND	ND	**ND**
	8	ND	ND	ND	**ND**	ND	**ND**	ND	ND	**ND**
Mean RQR				**1.76**			**1.70**			**1.82**
SD [a]				**0.11**			**0.18**			**0.11**
Variance				**0.012**			**0.032**			**0.012**
CV% [b]				**6.29**			**10.59**			**6.04**
Median				**1.75**			**1.74**			**1.82**

[a] Standard deviation, [b] Coefficient of variation

Table 6-10 shows the summary of average RQR values and standard deviations of all analysed samples, including pure blood, pure saliva and a mixture of blood with saliva. It was noticed that the average RQR value of the mixture differed from the samples composed of only one type of body fluid, i.e. the pure samples. Moreover, there is a slight but apparent difference between the RQR values of pure bloodstains and pure saliva stains.

Table 6-10: Summary showing average RQR values and standard deviations. Data were obtained from blood, saliva and mixture samples. Blood indicates pure bloodstains (n=10 fresh and 8 aged), Saliva indicates pure saliva stains (n=10 fresh and 8 aged) and mixture indicates all mixture types (n=10 for type-1 and 8 for each of the other mixture types).

Stain type	No. of samples	Mean	Median	SD[a]	CV(%)[b]	Variance
Blood	18	1.51	1.46	0.185	12.28	0.034
Saliva	18	1.45	1.34	0.198	13.62	0.039
Mixtures	34	1.44	1.45	0.207	14.35	0.043

[a] Standard deviation, [b] Coefficient of variation

The RQR values were obtained for all samples except those older than nine months being the pure blood, the pure saliva stain and the mixtures. Interestingly, RQR values were obtained for those nine month old samples when they were mixed with fresh materials.

6.3 Statistical analysis

Non-parametric analysis was used for statistical analysis of the data where the results of the Anderson-Darling normality test carried out on the RQR values indicated that the samples were not normally distributed.

Statistical analysis was carried out in two steps. In the first step, all data were divided into three groups according to the type of the stain regardless of the age of the stain and statistical analysis was carried out. Kruskal-Wallis independent samples test was carried out on the RQR values of the three stain types (i.e. blood, saliva and mixture stains) to observe if there was a significant difference between them. The results of

the statistical analysis performed for all samples show that there is no significant difference found between the three groups (p-value > 0.05).

In the second step, the comparison was carried out between the three types of stains in each mixture type separately, using the Kruskal-Wallis test to examine if there was a significant difference between them. If a significant difference was found, pairwise comparisons were carried out using Mann-Whitney test to identify which two stain types significantly differ. The results of the statistical evaluation of each of the four mixture types showed that the RQR values of some of the mixture types differ from those of pure stains (Table 6-11).

In the first type of mixture (i.e. mixtures of fresh blood and fresh saliva), the Kruskal-Wallis test indicated that there was a significant difference between these three stain types (p-value <0.05). Pairwise comparison indicated that there was a significant difference between the RQR values of mixture and pure saliva samples (p-value <0.05). In addition, it indicated that there was no difference found between the RQR values of either the mixture and pure blood samples or the pure blood and saliva samples (p-value >0.05).

In the second type of mixture (i.e. mixtures of fresh blood and aged saliva), the Kruskal-Wallis test also showed that there was a highly significant difference between these three stain types (p-value <0.05). Pairwise comparison indicated to a highly significant difference between aged saliva samples and both mixture and fresh pure blood samples (p-value=0.034 and p-value=0.014 respectively). However, there was no significant difference found between the RQR values of fresh blood samples and mixture samples (p-value >0.05). Interestingly, it was found that the RQR values of pure saliva stains older than 30 days significantly changed when contaminated with fresh blood.

In the third type of mixture (i.e., mixtures of aged blood and fresh saliva), the Kruskal-Wallis test showed that there was a highly significant difference between these three stain types (p-value <0.01). Pairwise comparison indicated that aged blood samples significantly differ from both mixture and fresh saliva samples (p-value=0.017 and p-value=0.008 respectively). However, there was no significant

difference found between the mixture and the pure saliva samples (*p*-value >0.05). As in the previous case, the RQR values of pure bloodstains older than 30 days significantly changed when contaminated with fresh saliva.

Interestingly, in the fourth type of mixture (i.e., mixtures of aged blood and saliva), no significant differences were found between mixture stains and either blood or saliva stains (*p*-value >0.05). However, no RQR values were obtained for mixture, blood or saliva stains older than nine months old.

Table 6-11 Pairwise comparisons of the RQR values of each type of mixture between bloodstains and saliva stains using Mann-Whitney tests. Pairwise comparison was carried out between each two of the three examined stains types (bloodstain saliva stain and mixture stain) in order to evaluate all four types of mixture. Type 1 is a mixture made of fresh blood and fresh saliva. Type 2 is a mixture made of fresh blood and aged saliva. Type 3 is a mixture made of aged blood and fresh saliva. Type 4 is a mixture made of aged blood and aged saliva. Asterisk (*) indicates that the difference is significant at the <0.05 level.

Mixture type	Stain (1)	Stain (2)	Sig	Confidence Interval (95%)	
				Lower Bound	Upper Bound
Type 1: Fresh blood and fresh saliva	Fresh blood	Mixture	0.171	-0.150	0.060
	Fresh saliva	Mixture	0.021*	-0.210	-0.010
	Fresh blood	Fresh saliva	0.159	-0.010	0.150
Type 2: Fresh blood and aged saliva	Fresh blood	Mixture	0.127	-0.180	0.030
	Aged saliva	Mixture	0.034*	-0.010	0.440
	Fresh blood	Aged saliva	0.014*	-0.530	-0.040
Type 3: Aged blood and fresh saliva	Aged blood	Mixture	0.017*	0.090	0.470
	Fresh saliva	Mixture	0.103	-0.240	0.030
	Aged blood	Fresh saliva	0.008*	0.180	0.560
Type 4: Aged saliva and aged blood	Aged blood	Mixture	0.471	-0.270	0.200
	Aged saliva	Mixture	0.312	-0.470	0.170
	Aged blood	Aged saliva	0.563	-0.200	0.430

6.4 Discussion

The RQR method of determining the age of bloodstains can be considered the most promising molecular method of determining the age of a bloodstain collected from a crime scene [74]. Through this method, the age is estimated by measuring the RQR of β-actin mRNA to 18S rRNA. Both of these RNAs are housekeeping RNA genes, which means that they are expressed in all tissues and can be found in all body fluids. This feature was considered to be an advantage because the method can be applied to other tissue types. They will be present in mixtures of different body fluids, such as those found at crime scenes. These mixtures may be from the same individual or from different individuals. This study investigated the effect of mixing bloodstains with another bodily fluid, namely saliva, on the RQR values and, in turn, their effect on bloodstain age estimation.

It was found that the average RQR values of pure stains whether blood or saliva, differ when that body fluid mixes with another body fluid type. This difference becomes more noticeable when stains are mixed with body fluid of a different age. Figure 6-2 illustrates that the average RQR values of aged pure bloodstains differ prominently when these stains are mixed with fresh saliva. This may result in the RQR values being underestimated and, in turn, may lead to the age of bloodstain being underestimated. In addition, RQR values were obtained for nine month-old bloodstains when they were mixed with fresh saliva, despite no RQR values being obtained when the bloodstains were in the pure form. Similar findings were obtained for the average RQR values of aged saliva stain when they mixed with blood. Interestingly, the RQR values of aged mixture stains, which were composed of same age blood and saliva, were larger than that of pure form stains.

Indeed, as the aim of this study was to evaluate the effect of a mixture of two body fluids on the RQR values used to estimate the age of one of the two body fluids, it was intended to measure the RQR values of a mixture of blood and saliva at different ages and to compare them with the RQR values of their pure forms at an age similar to that of the mixture. The results demonstrate the effect of a mixture of blood and saliva on the RQR values of their pure forms. However, a large number of mixture

stains should be analysed to estimate the average RQR value of mixture status at each age more precisely, and this is beyond scope of this study.

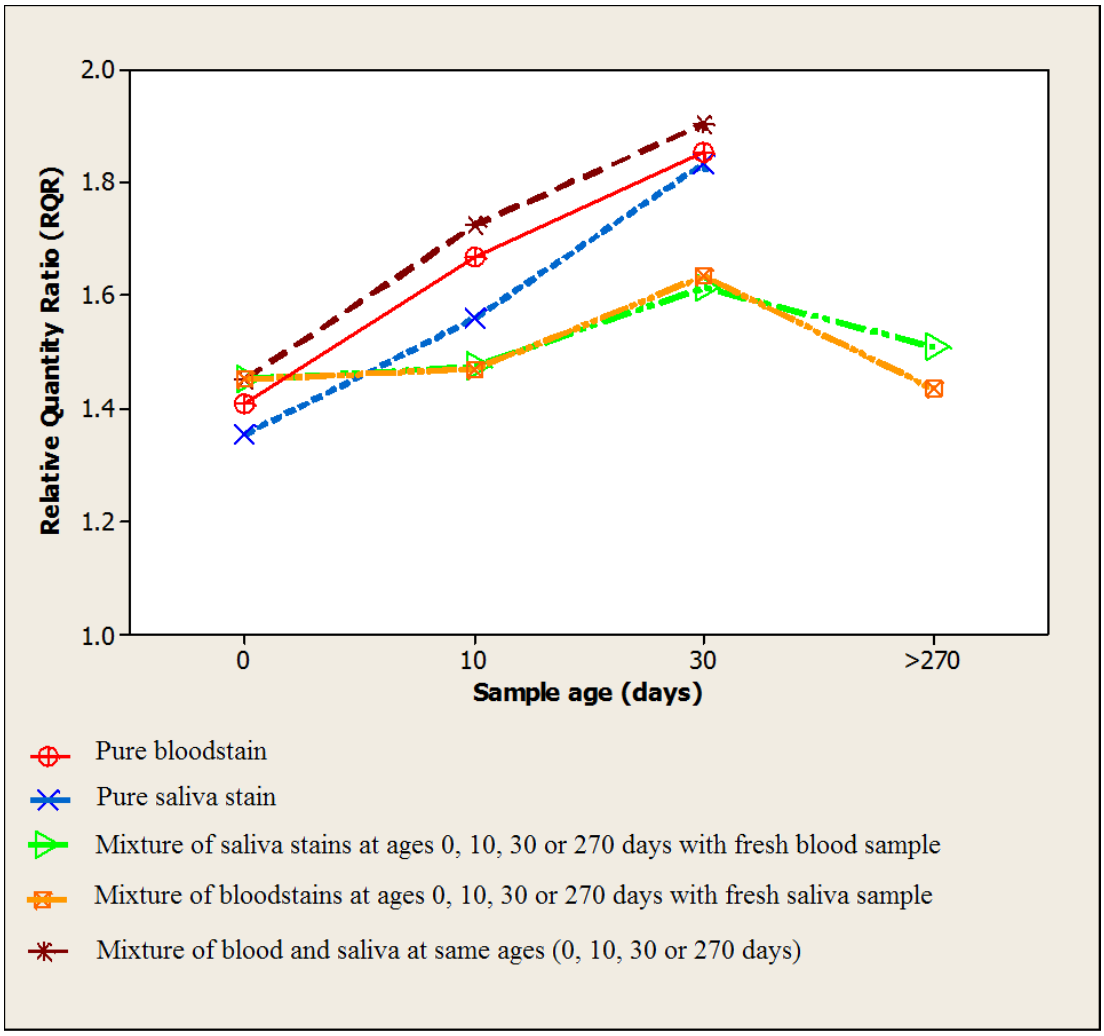

Figure 6-2: Average RQR values of pure blood and saliva stains and mixtures of them plotted by age of stain in days. The figure illustrates the difference between the RQR values of pure blood and saliva stains and mixtures.

Moreover, the statistical analysis of the RQR values of the analysed stains showed that the RQR values of pure blood or saliva samples differed significantly when cross-contaminated. This may lead to an incorrect estimation of the age of the stains.

The results obtained from studying each mixture type separately showed that the effect of one body fluid on the RQR values of the other body fluid depends on the

ages of both stains (Figure 6-3). In mixture type 1, in which two types of fresh samples were mixed, no significant difference was found between the RQR values of the mixture and those of the blood and saliva pure stains (Figure 6-3a). In addition, pure saliva stains show lower RQR values than those of pure bloodstains, which may result from the biological and constitutional differences between them. However, this difference was not statistically significant.

In mixture type 4, in which blood was mixed with saliva and the samples were dried and aged, no significant difference was found between the RQR values of the mixture and those of the blood or saliva pure stains, or between the RQR values of the pure stains themselves (Figure 6-3d). However, a pure aged saliva stain exhibits a larger diversity in RQR values as compared to those of pure bloodstains. This large range may be due to the constitutional components of saliva. It is known that saliva contains various endo- and exo-ribonucleases from many sources, e.g. viral and bacterial ribonucleases, as well as extraneous substances, such as food debris [93, 107]. These substances have different effects on RNA integrity in saliva, which may result in the wide range of RQR values observed.

The greatest effect of mixture on RQR value appeared in mixture types 2 and 3, in which old samples were mixed with a fresh sample of a different type (Figure 6-3b, c). It was found that the RQR values of pure stains that were older than a month differed significantly when mixed with fresh samples. Moreover, this effect appeared clearly in samples older than nine months: the results changed from an inability to obtain an RQR value in pure samples to obtaining an RQR value when a fresh sample of either blood or saliva was added to an old, i.e. more than nine month-old, stain. Moreover, the distribution of the obtained results might be affected by experimental error. Therefore, confirmation by further experiment could be required.

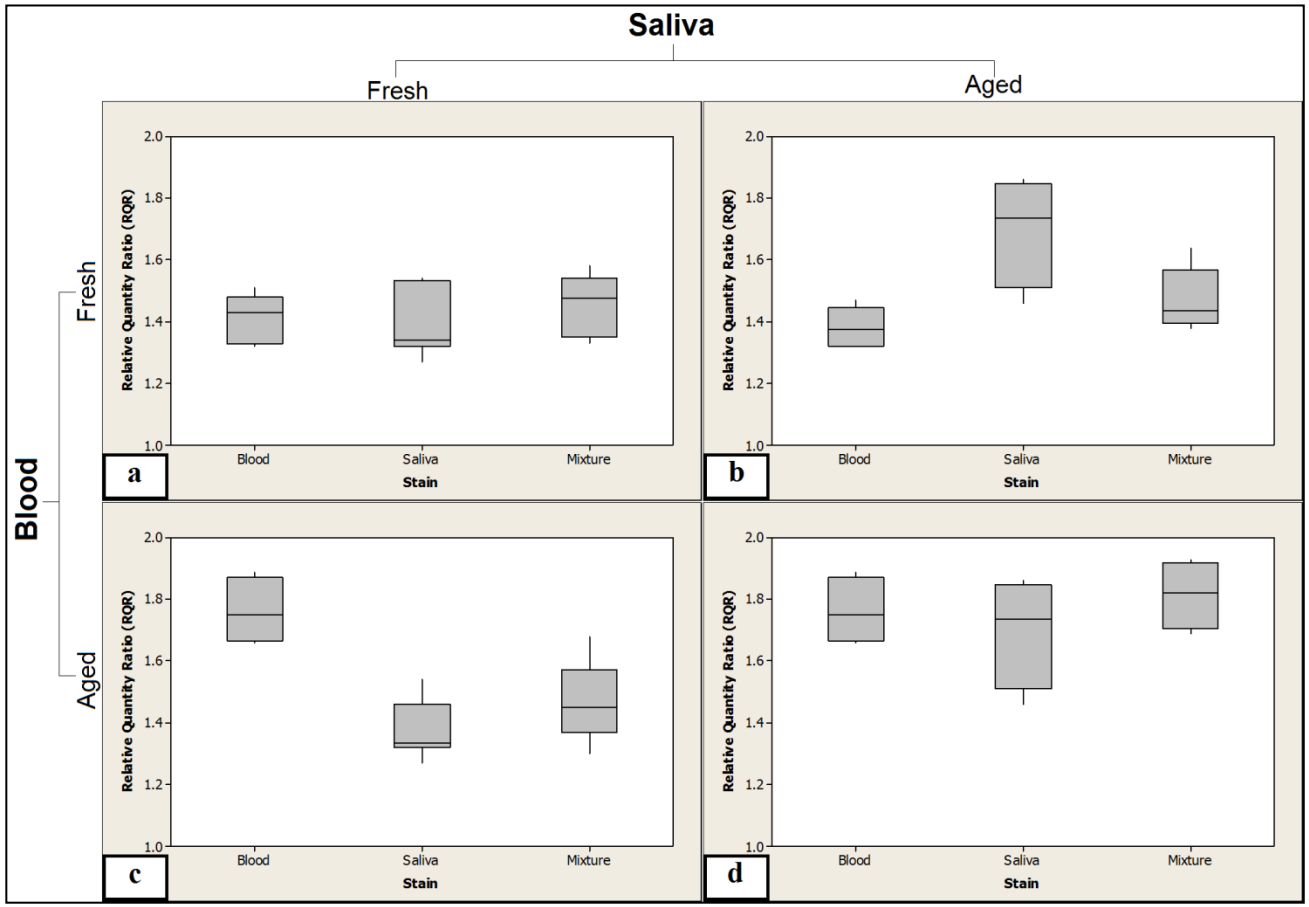

Figure 6-3: RQR values obtained from three stain types (blood, saliva and mixture) in four types of mixtures of blood and saliva demonstrated in a box-and-whiskers plot. a) Mixture type 1: a mixture of fresh blood and fresh saliva. b) Mixture type 2: a mixture of fresh blood and aged saliva. c) Mixture type 3: a mixture of aged blood and fresh saliva. d) Mixture type 4: a mixture of aged blood and aged saliva. One outlier was observed for Mixture type 1.

6.5 Conclusion

In conclusion, although using housekeeping RNA molecules has been considered an advantage of the method developed by Anderson *et al.* [49], the use of these molecules could be a limitation in cases in where two types of body fluids have mixed. The results of this study show the effect of the contamination of bloodstains with saliva on RQR values and, in turn, the effect on their estimated ages. A marked effect of fresh saliva on the RQR values of old bloodstains, and also of fresh blood on the RQR values of old saliva stains, was noticed. This demonstrates the effect of a mixture on the RQR value resulting from the difference in the ages of the stains. The amount of body fluid in the mixed samples may also have an effect on the RQR values of a mixed stain, and this should be studied further. Other body fluids, such as semen and sweat, may have the same effect on the RQR values of bloodstains, and these should also be investigated.

This work used the method developed by Anderson *et al.* [49], which is considered to be the most promising molecular method of bloodstain age determination. However, this work revealed difficulties in the use of housekeeping gene RNA for the purpose of establishing the age of bloodstains that have been mixed with other bodily fluids, whether from the same person or another person. This is a common situation in forensic practice [85]. Therefore, the use of housekeeping gene RNA to determine the age of bloodstains or other body fluids should be accompanied by body fluid identification in order to identify mixtures. As the source of the stains in forensic casework will, by definition, be unknown, the underlying assumptions concerning the composition of the stains must be explicitly stated. The use of tissue-specific genes instead of housekeeping genes to determine the age of bloodstains could be a solution to the effect of mixtures with other body fluids.

In the following chapters, the correlation between the age of saliva stain and some saliva specific mRNA will be assessed. Two regions of the chosen saliva specific gene will be selected for assessment as markers to aid in determination of the age of saliva stains.

7 Analysing two different sized TaqMan® Gene Expression Assays of saliva mRNA markers for simultaneous determination of saliva stain age and type

7.1 Introduction

The work carried out in this chapter evaluated the possibility of using a saliva-specific RNA marker for simultaneous determination of saliva age and type. This evaluation looked for the correlation between the age of the saliva stain and the relative quantity of two different-sized segments of the same saliva-specific mRNA markers, quantified using TaqMan® Gene Expression Assays (TGEA). The quantification method that will be used to determine the relative quantity of those segments is the $2^{-\Delta Ct}$ method [78, 143]. This method was developed by Livak and Scmittgen [143] to analyse the relative changes in gene expression from qPCR experiments (Table 1-7).

In gene expression analysis, the $2^{-\Delta Ct}$ method is used to quantify the expression of target gene in relative to the expression of endogenous reference gene where the amount of target was normalized to an amount of the endogenous. As the same gene will be analysed in this study, it will be dealt with the large amplicon size product as a target and the small amplicon size product as a reference. Therefore, the Ct values results of the large amplicon will be normalized to the results of the Ct values results of the small amplicon. It was hoped that use of this approach would avoid the effects, noted in section 6.5, of mixtures of saliva with other body fluids.

In this study, predesigned off-the-shelf TGEAs will be used. Therefore, specific criteria were set for choosing suitable TGEAs.

7.1.1 Saliva-specific RNA markers

Many studies have been carried out to discover mRNA molecules that can be used as markers to identify body fluid commonly found in crime scenes, i.e. venous blood, menstrual blood, saliva, semen and vaginal secretion [44, 55, 57, 58, 98, 111, 179, 180]. Many mRNA transcripts from a number of genes have been identified as

suitable markers for each body fluid type. In addition, other mRNA molecules have been nominated as markers for skin cells for forensic applications [181, 182].

Eleven transcripts have been reported as being suitable markers for saliva samples in forensic applications. These are Statherin (STATH), Histatin 3 (HTN3), Keratin 4 (KRT4), Keratin 6A (KRT6A), Keratin 13 (KRT13), Small Proline-Rich protein 1A (SPRA1A), Small Proline-Rich protein 2A (SPRA2A), Proline-Rich protein BstNI subfamily 1 (PRB1), Proline-Rich protein BstNI subfamily 2 (PRB 2), Proline-Rich protein BstNI subfamily 3 (PRB 3) and Proline-Rich protein BstNI subfamily 4 (PRB 4) [45, 55, 57, 58, 111, 179, 180, 183].

Some studies developed multiplex assays composed of a number of body fluid-specific transcripts, including some of these saliva-specific transcripts, in order to parallel-test several of the mRNA markers in a single sample extract [45, 57, 58, 61].

7.1.2 Predesigned TaqMan® Gene Expression Assays

Applied Biosystems offers a large family of TaqMan® Gene Expression Assays to meet the increasing need of quantifying gene expression. These assist researchers to perform quantitative gene expression studies on a variety of species. The assays range from off-the-shelf gene-specific probe and primer sets, to Custom TaqMan® probes and primers manufactured to the custom desired sequences, and everything in between. These assays offer the greatest sensitivity, specificity, reproducibility, and the broadest dynamic range. In addition, assays are provided in a ready-to-go format with no optimisation. This eliminates the labour, expense, and bioinformatics expertise required to design quantitative real-time PCR assays.

Each TaqMan® Gene Expression Assay has an assay ID which is a specific nomenclature, specifying each assay using a unique, alphanumeric string that encodes basic descriptive information about the assay. The prefix code of the assay ID, which is composed of two letters, indicates the species to which the TaqMan® Gene Expression Assay is designed, e.g. the prefix code “hs” indicates that the assay is designed for *Homo sapiens*. In addition, the suffix code of the assay indicates the placement of the assay (Table 7-1).

Table 7-1: TaqMan® Gene Expression Assays suffix codes [184].

Suffix	Definition
_m	An assay whose probe spans an exon junction.
_s	An assay whose primers and probes are designed within a single exon. Such assays, by definition, detect genomic DNA.
_g	An assay that may detect genomic DNA. The assay primers and probe may also be within a single exon.
_mH **_sH** **_gH**	An assay that is designed to a transcript belonging to a gene family that has high sequence homology. The assays are designed to yield a 10- to 15-Ct difference between the target gene and the gene with the closest sequence homology. This means that an assay detects the target transcript with 1000- to 30,000-fold greater discrimination (sensitivity) than the closest homologous transcript, if both transcripts are at the same copy number in a sample.
_u	An assay whose amplicon spans an exon junction, and whose probe binds completely in one of the spanned exons.
_ft	An assay designed to detect fusion transcripts that result from chromosomal translocation. One primer and the probe are on one side of the fusion transcript breakpoint, and the second primer is on the other side of the fusion transcript breakpoint. The assay does not detect gDNA.
_at	An assay that is designed to detect a synthetic RNA transcript with a unique sequence that lacks homology to current annotated biological sequences.

7.1.3 Criteria for choosing appropriate predesigned TGEA pairs for biological stain age determination

For each commonly encountered body fluid at a crime scene, many transcripts have been identified as specific mRNA markers. In addition, many off-the-shelf TGEAs were designed for each of these transcripts. However, no study evaluated these TGEAs for use in determining the age of biological stains. A comprehensive evaluation of the TGEAs was beyond the resources available for this study, and instead a strategy was developed to choose the most appropriate off-the-shelf TGEA

pairs in order to study the age of biological stains. A list of criteria was used to identify the most suitable assays for this study.

These criteria were set to evaluate Applied Biosystems predesigned off-the-shelf TGEAs based on the findings of the previous studies, and to avoid the limitations found in them. The predesigned off-the-shelf TGEAs were evaluated depending on five criteria that take into account some of the characteristics of the available off-the-shelf TGEAs, such as amplicon length, spanning the exon-exon junction and assay location as described in the following paragraphs.

The first criterion was that the chosen off-the-shelf TGEA should have been designed for one of the transcripts that has been assigned as a specific mRNA marker. This will help to simultaneously determine the age of the sample and confirm the tissue type of the analysed sample. In addition, determination of the age of the biological sample using the housekeeping gene may be over- or under-estimated when mixing the analysed sample with other tissue types. Therefore, using a tissue specific marker instead of a housekeeping gene RNA in an aging study will avoid the resulting over- or under-estimation.

The second criterion was that the chosen off-the-shelf TGEA pairs should be for the same tissue-specific mRNA. Using two regions from the same RNA will ensure a ratio of approximately one in the fresh samples, because both regions are present in an almost equimolar amount when the sample is first expelled. This will reduce the difference in expression levels that may occur when comparing different RNAs. This, in turn, will reduce both between people and within person variation [78]. In addition, the difference in the size of the two mRNA regions results in a ratio between them due to their difference in degradation. This ratio can be used as an indicator for the age of the sample, where the age will be determined from the changes in the ratio between these two regions.

The third criterion was that one of the primers or the probe of the assay should span the exon-exon junction. This will help to avoid amplification of genomic DNA. The fourth criterion was that the size of the amplicon of the two chosen off-the-shelf TGEAs should be different. An increase in size difference of the amplicons of the

two TGEAs is preferable. A previous study [185] has shown that a short amplicon is less prone to degradation and more stable than relatively large amplicons. In the multivariate analysis study carried out by Anderson *et al.* [78], the results of the nested analysis of variance for the age of bloodstain showed that the pair of amplicons containing the shortest amplicons from the same mRNA i.e. β-actin 301 versus 89 bp, exhibited the highest correlation with age of bloodstains compared with other pairs [78]. In this study, the Ct values of the short amplicon that exhibit stability with time will be used to normalise the Ct values of the larger amplicon. Therefore, the amplicons chosen will be different in size and consequently subject to different rates of degradation.

The final criterion was that the two chosen off-the-shelf TGEAs should occupy different locations on the transcript. It is preferable that each of the chosen TGEAs is near to one of the ends of the transcript. The position of the chosen amplicons may play a role in the difference in their degradation rate. Therefore, amplicons should be chosen where one of them is near to the start area of degradation while the other is far away from that area, or near the most stable area on mRNA. However, there are many proposed degradation mechanisms for RNA degradation: 3' to 5' direction degradation; poly (A) shortening followed by 3' to 5' direction degradation and random degradation [186-191]. Thus, choosing the appropriate positions cannot be guaranteed because the degradation mechanisms of the chosen mRNA molecules are unknown. Studies of the degradation mechanisms have been carried out on a small number of mRNA molecules but unfortunately, not the same mRNA molecules which were for this thesis. Therefore, it was proposed that the degradation mechanism of the chosen mRNA should be the most common proposed degradation mechanism, in which degradation is started by poly A followed by 3' to 5' direction degradation [186, 189].

7.1.4 The predesigned off-the-shelf TGEAs chosen for saliva stain age determination

Applied Biosystems predesigned TGEAs were evaluated using previous criteria to choose the most appropriate TGEA pairs for aging saliva stains.

1- Saliva-specific RNA markers

At the time of writing, eleven transcripts were identified as suitable RNA markers to identify saliva stains. These were Statherin (STATH), Histatin 3 (HTN3), Proline-Rich protein BstNI subfamily 1-4 (PRB1, PRB2, PRB3 and PRB4), Keratin 4 (KRT4), Keratin 6A (KRT6A), Keratin 13 (KRT13), small Proline-Rich protein 1A (SPRR1A) and small proline-rich protein 2A (SPRR2A) (Table 7-2 addresses the saliva-specific mRNA markers identified). All of these transcripts meet the first criterion which is the use of tissue-specific RNA markers instead of housekeeping gene RNA.

Table 7-2: Saliva-specific mRNA markers. This table shows transcripts that were identified as specific mRNA markers for saliva, their symbols and the location of their genes.

Transcript Name	Symbol	Gene Location	Ref
Statherin	STATH	Chr. 4	[45, 55, 57, 58, 111, 179]
Histatin 3	HTN3	Chr. 4	[45, 55, 57, 58, 111, 179]
Proline-rich protein BstNI subfamily 1	PRB1	Chr. 12	[55]
Proline-rich protein BstNI subfamily 2	PRB2	Chr. 12	[55]
Proline-rich protein BstNI subfamily 3	PRB3	Chr. 12	[55]
Proline-rich protein BstNI subfamily 4	PRB4	Chr. 12	[183]
Keratin 4 mRNA	KRT4	Chr. 12	[98, 180]
Keratin 6A	KRT6A	Chr. 12	[98, 180]
Keratin 13	KRT13	Chr. 17	[98, 180]
Small proline-rich protein 1A	SPRR1A	Chr. 1	[98, 180]
Small proline-rich protein 2A	SPRR2A	Chr. 1	[98, 180]

2- TGEA pairs that occupy the same saliva-specific RNA marker

TGEAs were assessed as pairs of TGEAs from the same transcript in order to evaluate their adherence to the second criterion, in which the chosen TGEA pair

should be on the same saliva-specific RNA markers. Therefore, transcripts for which there was only one available TGEA were excluded.

3- TGEAs that are primer or probe spanning exon-exon junction

As summarised in Table 7-3, two assays which were available for PRB2 mRNA span exon-exon junction, three of four assays which were available for STATH mRNA span exon-exon junction, and two of five assays which were available for KRT13 mRNA span exon-exon junction. In addition, one of the two available assays for HTN3 mRNA and one of the three available assays for KRT4 mRNA span exon-exon junctions. Other assays either do not span exon-exon junctions or they span exon-exon junctions but are not suitable because there is no other companion assay available for that mRNA transcript.

Table 7-3: Available Applied Biosystems TGEAs for saliva-specific mRNA. This table shows the number of available TGEAs and the length of each assay product. Number between brackets shows exon boundaries of the TGEAs. Asterisk (*) marks assay that does not span exon-exon junction.

Saliva-specific mRNA	No. of available TGEA	Amplicon product length of each TGEA arranged from the shortest to the longest (bp)				
		Shortest	2nd shortest	3rd shortest	2nd longest	Longest
STATH	4	67 (2-3)	79 (1-1) *		85 (3-4)	90 (2-3)
HTN3	2	79 (1-1)*	---		---	136 (5-6)
PRB1	1	121 (2-3)	---		---	---
PRB2	2	83 (3-4)	---		---	93 (2-3)
PRB3	1	122 (4-5)	---		---	---
PRB4	1	93 (4-5)	---		---	---
KRT4	3	61 (6-7)	---		76 (5-6)*	80 (8-9)*
KRT6A	2	74 (1-1)*	---		---	83 (1-1) *
KRT13	5	73 (7-7)*	75 (6-7)	77(7-8) *	82 (5-6) *	87 (6-7)
SPRR1A	1	95 (2-2)*	---		---	---
SPRR2A	2	127 (2-2)*	---		---	139 (2-2) *

4- TGEA pairs that show the largest difference in amplicon size (more than 50 bp)

The largest difference in amplicon size was chosen using each TGEA with the two longest amplicon sizes and each TGEA with the two shortest amplicon sizes. This method was selected, rather than restricting the study by using TGEAs with the sole longest amplicon size and the sole shortest amplicon size only to increase the possibility of picking TGEA pairs that also meet other criteria. Figure 7-1 shows the base pair size difference between each of the two longest amplicon products and each of the two shortest amplicon products of each Applied Biosystems TGEA for each saliva-specific marker. An arbitrary limit of 50 bp was set as the smallest for consideration.

As shown in Figure 7-1, only one pair of TGEA for HTN3 mRNA had a size difference of more than 50 bp (57 bp) from all these combinations. All other combinations were less than the chosen limit. The TGEAs for HTN3 mRNA that show this 57 bp difference were the TGEA with the longest amplicon product (assay ID Hs00264790_m1 with amplicon product length 136 bp), and the TGEA with the shortest amplicon product (assay ID Hs00264790_m1 with amplicon product length 79 bp). The next longest combination of transcript targets, with a base pair difference between TGEA of the longest and the shortest amplicon products, was for STATH mRNA (23 bp) followed by KRT4 mRNA (19 bp). Other TGEA combinations between each of the two longest amplicon products and each of the two shortest amplicon products were less than 50 bp (between 7-18 bp). There is only one predesigned TGEA available for PRB1, PRB3, PRB4 and SPRR1A mRNA and therefore none were suitable for this study.

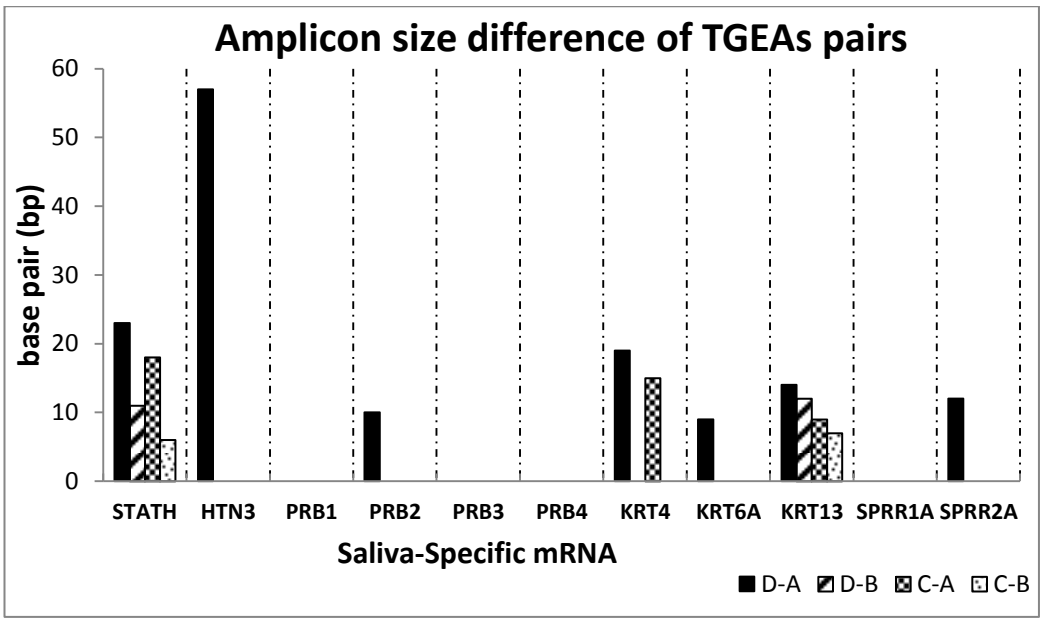

Figure 7-1: Amplicon size difference of the Applied Biosystems predesigned TGEAs pairs for saliva-specific RNA markers. This figure shows the base pair size difference between the two longest amplicon products and the two shortest amplicon products of each Applied Biosystems TGEAs for each saliva-specific marker. (A) shortest amplicon. (B) second shortest amplicon. (C) second longest amplicon. (D) longest amplicon.

5- TGEA pairs that occupy different regions on the mRNA

Table 7-3 shows the exon boundaries of each of the available Applied Biosystems TGEAs for saliva-specific mRNA. This provides a guide about the locations of the TGEAs on the transcript and the distance between their locations. To ensure that each assay of the chosen TGEA pair is near to one end of the transcript and far from the other TGEA, the distance between their locations should be more than a quarter of the size of the transcript (i.e. more than 25% of the mRNA size). This is shown in Figure 7-2, where the distance between the two TGEAs was presented as a percentage of the distance between the locations of each of the two assays.

As shown in Figure 7-2, only three pairs of TGEAs possess a distance between the two assays above a quarter of the size of the whole transcript: KRT4 mRNA (~29.21%), HTN3 (28.62%) and KRT13 (27.94%).

For KRT4 mRNA, the TGEA that shows ~29.21% of the transcript size between the TGEA pair is the TGEA with the longest amplicon product, which is assay ID

Hs00970607_g1, occupying location 1443 bp of the transcript. and the TGEA assay with the shortest amplicon product, occupying location 1085 bp of the transcript (i.e. assay ID Hs00361610_g1).

For HTN3 mRNA, the TGEAs that show 28.62% of the transcript size between the TGEA pair are the TGEA with the longest amplicon product which is assay ID Hs00264790_m1, occupying location 302 bp of the transcript, and the TGEA assay with the shortest amplicon product, occupying location 51 bp of the transcript (i.e. assay ID Hs04194749_g1).

For KRT13 mRNA, the TGEAs that show 27.94% of the transcript size between the TGEA pair are the TGEA with the longest amplicon product, which is assay ID assay ID Hs00357961_g1, occupying location 1091 bp of the transcript, and the TGEA assay with the shortest amplicon product, occupying location 1646 bp of the transcript (i.e. ID Hs02558881_s1).

Other combinations show a distance less than the chosen limit, or the TGEA pairs overlapping locations. In addition, as previously mentioned, PRB1, PRB3, PRB4 and SPRR1A mRNA are not suitable for this study because they each have only one predesigned TGEA.

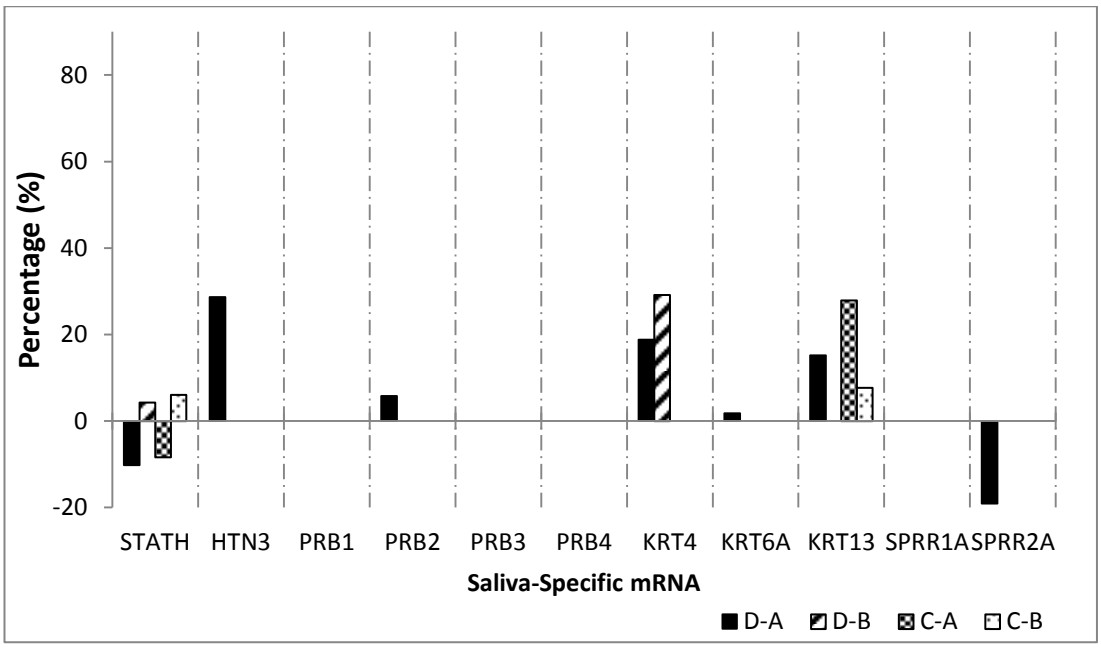

Figure 7-2: Percentage of the distance between the locations of two Applied Biosystems predesigned TGEAs to the whole size of the transcript. This figure shows the distance between the locations of Applied Biosystems predesigned TGEAs on the saliva-specific mRNA as a percentage of the distance between each of the two assays with the longest amplicon products and the two assays with the shortest amplicon products to the whole size of the saliva-specific mRNA. Positive results indicate the locations of the assays differ from one another and they are far from each other; negative results indicate the locations of the two assays overlapped while a zero result arises due to unavailability of the assay. (A) shortest amplicon. (B) second shortest amplicon. (C) second longest amplicon. (D) longest amplicon.

Table 7-4 summarises the results of the evaluation of fulfilling the criteria set in order to choose the appropriate Applied Biosystems predesigned TGEAs for use in determining the age of saliva stains. The results of the evaluation show that some of the TGEAs do not fill some of these criteria. These TGEAs are those designed for PRB1, PRB3, PRB4, KRT6A, SPRR1A and SPRR2A. Therefore, they were excluded from the study. In addition, some TGEAs wholly fulfil some of the criteria while they fulfil other criteria to a lesser extent. These TGEAs are those designed for STATH, HTN3, PRB2 and KRT4.

Table 7-4: Evaluation of Applied Biosystems predesigned TGEAs for saliva age determination. This table shows the summary of the evaluation of the available predesigned TGEAs for this study. (✓) indicates that the TGEA pair definitely meets criterion, (×) indicates that it does not meet criterion and (p) indicates it partially meets criterion.

Saliva-specific mRNA	Criteria				
	1	2	3	4	5
STATH	✓	✓	p	p	P
HTN3	✓	✓	✓	p	P
PRB1	✓	×	×	✓	×
PRB2	✓	✓	p	✓	P
PRB3	✓	×	×	✓	×
PRB4	✓	×	×	✓	×
KRT4	✓	✓	p	p	P
KRT6A	✓	✓	p	×	P
KRT13	✓	✓	p	p	×
SPRR1A	✓	×	×	×	×
SPRR2A	✓	✓	p	×	×

Indeed, as none of the TGEAs definitely fulfil all the set criteria, TGEAs that were designed for PRB2, STATH, HTN3, and KRT4 were considered to be the most appropriate for this study. However, because of time and financial restrictions, the TGEAs of only two transcripts of these four were evaluated. PRB2 was also excluded because the available TGEAs are designed for both PRB1 and PRB2 where they belong to a gene family transcript with high sequence homology. In addition, the distance between the locations of TGEAs of PRB2 on the saliva-specific RNA markers is smaller than those of HTN3 and KRT4. STATH was excluded because only two of the criteria were completely fulfilled while others completely fulfilled three criteria.

Therefore, in this study, TGEA pairs of KRT4 mRNA and HTN3 mRNA were chosen. Table 7-5 shows the TGEA pairs that will be used in this study to determine the age of the saliva stain.

Table 7-5: Chosen TGEA pairs of KRT4 mRNA and HTN3 mRNA to determine the age of the saliva stain. This table shows pairs of TGEA for KRT4 mRNA and HTN3 mRNA. Each pair consists of two different labelled dyes.

TGEA pair	mRNA	Assay ID	Refseq	Assay location	Amplicon length	Labelled dye
1	KRT4	Hs00361611_m1	NM_002272.3	1183	61	VIC
		Hs00361610_g1	NM_002272.3	1058	76	FAM
2	KRT4	Hs00361611_m1	NM_002272.3	1183	61	VIC
		Hs00970607_g1	NM_002272.3	1443	80	FAM
3	HTN3	Hs00264790_m1	NM_000200.2	302	136	VIC
		Hs04194749_g1	NM_000200.2	51	79	FAM

7.2 Optimisation of TGEAs multiplexing

TGEAs are provided as ready-to-use assays with no need for optimisation in a singleplex reaction. In addition, they are provided with their own specific manufacturer's instructions showing the quantity of TGEA that should be used in the reaction. However, because two assays will be used in this study in a multiplex reaction, it was necessary to optimise the combined reaction to obtain the highest reaction efficiency for each assay. This was carried out to determine the quantity of each TGEA to be used for each reaction. Optimisation was carried out for each of the three pairs of TGEAs, as shown in Table 7-5 (KRT4 76 bp - 61 bp, KRT4 80 bp - 61 bp and HTN3 136-79 bp amplicon combinations). As manufacturer's instructions for each assay require using 1 µL of TGEA in each 20 µL singleplex reaction volume, the volumes of TGEA evaluated in this optimisation for each assay in the multiplex are 0.5 and 1 µL of each assay.

7.2.1 Materials and method

7.2.1.1 Sampling, RNA extraction and reverse transcription

100 µL of fresh saliva sample was collected in a clean sterile tube. RNA extraction was carried out using the AllPrep® DNA/RNA mini Kit (Qiagen) as described in section 2.3.2.2. All samples were treated with TURBO DNase I (Ambion) as described in section 2.3.3. The reverse transcription was carried out using SuperScript® III (Invitrogen) as described in section 2.5.2.

7.2.1.2 Real-time PCR

Each of the two pairs of TGEAs of KRT4 (i.e. TGEAs pair 1, which is assay ID Hs00361611_m1 and assay ID Hs00361610_g1, and TGEAs pair 2 which is assay ID Hs00361611_m1 and assay ID Hs00970607_g1) and the pair of TGEAs of HTN3 mRNA (i.e. TGEA pair is assay ID Hs04194749_g1 and assay ID Hs00264790) were run in real time PCR, using the procedure described in section 2.8.

7.2.1.3 Data analysis

All statistical data analysis and graphs were done using the Microsoft Excel statistics program (Microsoft, USA).

7.2.2 Results and discussion

As TGEAs commercially provided for a single reaction will be used in a multiplex reaction, the aim was to evaluate using either 0.5 or 1 µL of each of the TGEA pair in the multiplex reaction. Each of the three pairs of the TGEAs chosen was evaluated separately. As shown in Figure 7-3, Figure 7-4 and Figure 7-5, there was no huge difference in the Ct values for each TGEA in the multiplex reactions using either 0.5 µL or 1 µL from those Ct values in the singleplex using 1 µL.

In TGEA pair 1 of KRT4 (composed of assay ID Hs00361611_m1 and assay ID Hs00361610_g1), using 0.5 µL of each TGEA in the multiplex shows results similar to the results obtained from using 1 µL of each TGEA in a singleplex reaction, where the difference in Ct value was less than 0.5. However, using 1 µL of each TGEA in the multiplex shows a slightly higher Ct value result, compared to the singleplex reaction, i.e. 1.5 Ct value difference between singleplex and multiplex for assay ID Hs00361610_g1.

In TGEA pair 2 of KRT4 (composed of assay ID Hs00361611_m1 and assay ID Hs00970607_g1), using 0.5 and 1 µL of each TGEA in the multiplex shows results similar to the results obtained using 1 µL of each TGEA in the singleplex reaction, where the difference in Ct value was less than 0.5.

In the TGEA pair of HTN3 (composed of assay ID Hs04194749_g1 and assay ID Hs00264790), using 0.5 and 1 µL of each TGEA in the multiplex shows less than 0.5 Ct value difference from using 1 µL of each TGEA in the singleplex reaction.

The experiment was repeated with other saliva samples and similar results were obtained. A volume of 0.5 µL of each TGEA was therefore chosen for use in a multiplex reaction in this study given that the difference in Ct values of using 0.5 µL of each TGEA in multiplex and the recommended volume in the singleplex reaction was less than 0.5. This will help to duplicate when using TGEA where half of the recommended volume will be used from each TGEA. In addition, reaction efficiency will be measured with each real time PCR run to ensure efficient qPCR and to correct any limitation.

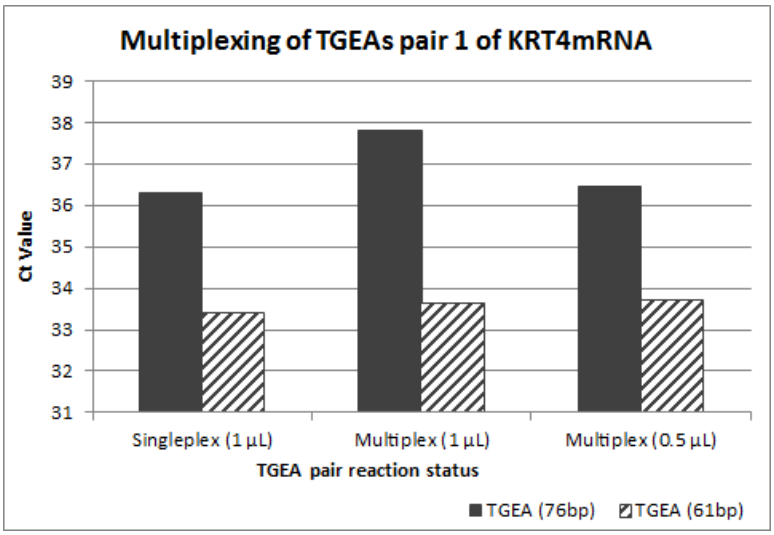

Figure 7-3: Multiplexing of TGEA pair 1 of KRT4 mRNA. TGEA pair 1 is composed of TGEA_ ID Hs00361611_m1 (61 bp) and TGEA_ ID Hs00361610_g1 (76 bp).

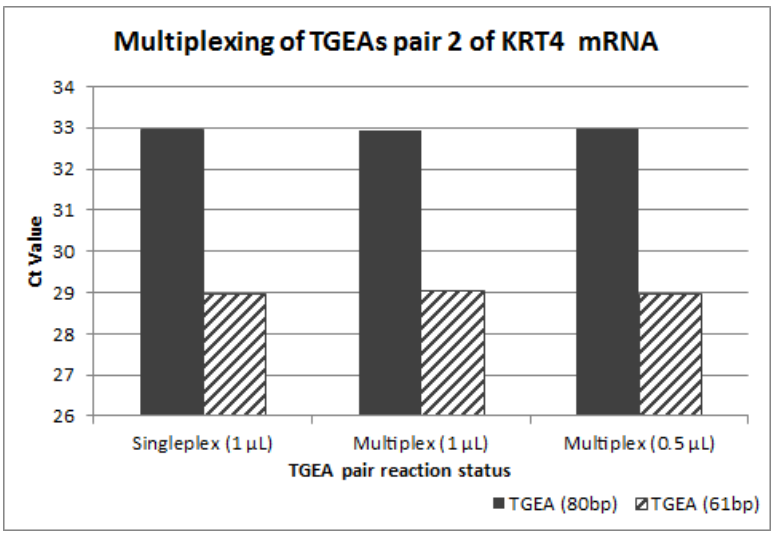

Figure 7-4: Multiplexing of TGEA pair 2 of KRT4 mRNA. TGEA pair 2 is composed of TGEA ID Hs00361611_m1 (61 bp) and TGEA ID Hs00970607_g1 (80 bp).

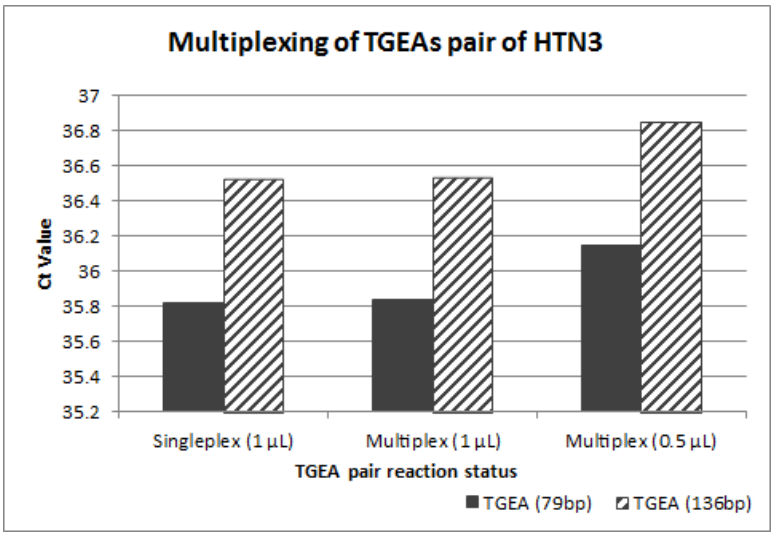

Figure 7-5: Multiplexing of TGEA pair of HTN3 mRNA. TGEA pair is composed of TGEA ID Hs04194749_g1 (79 bp) and TGEA ID Hs00264790_m1 (136 bp).

7.3 Age of saliva stain using saliva-specific mRNA marker

In this section, the relationship between the age of the saliva stain and the $2^{-\Delta Ct}$ values of the three chosen pairs of TGEAs for KRT4 mRNA and for HTN3 will be assessed (Table 7-5). The three TGEA pairs that will be used involve examination of the following amplicon combinations: KRT4 mRNA 76 bp - 61 bp, KRT4 mRNA 80 bp - 61 bp and HTN3 mRNA 136 -79 bp.

7.3.1 Materials and method

7.3.1.1 Preparation of samples

Saliva was collected from five volunteers (three males and two females) with no history of malignancy. Volunteers were asked not to eat or smoke and not to use antiseptic mouthwash or brush their teeth in the hour prior to saliva collection. Two millilitres of saliva were collected in sterile tubes on two separate occasions from each donor. The stains were made by immediate spotting of 100 µL of saliva sample on clean cotton swabs and air dried at room temperature in a sterile hood. The, stains were stored at room temperature in a dry place to simulate natural aging until they reach the desired ages (0, 7, 14, 35, and 60 days) when extracted. As samples were collected from all volunteers at each occasion at the same time, samples from all volunteers at each age period in each occasion were extracted in the same batch.

7.3.1.2 RNA extraction and reverse transcription

RNA extraction was carried out using the AllPrep® DNA/RNA mini Kit (Qiagen) as described in section 2.3.2.2. All samples were treated with TURBO DNase I (Ambion) as described in section 2.3.3. The RNA quantity and quality were determined spectrophotometrically using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) (Procedure) as described in section 2.4.1 and using the Agilent RNA 6000 Pico Kit for the 2100 Bioanalyzer (Agilent Technologies) as described in section 2.4.2. The reverse transcription was carried out u using SuperScript® III (Invitrogen) as described in section 2.5.2.

7.3.1.3 Real-time PCR

Real time PCR were carried out in a multiplex of each pair of TGEA using 0.5 µL of each TGEA where the procedure described in section 2.8 was followed.

7.3.1.4 DNA extraction, quantification and profiling

DNA extraction was carried out using AllPrep® DNA/RNA mini Kit (Qiagen) as described in section 2.9.2. The DNA quantification was carried out using the Investigator Quantiplex kit as described in section 2.10. DNA profiling was carried out using Investigator Decaplex SE Kit as described in section 2.11.

7.3.1.5 Data analysis

The raw fluorescence Ct values were processed using GenEx statistical software (Version 5.4.0.512; BioEPS GmbH, Munich, Germany) in order to normalise the raw Ct values against interplate calibrators, individual efficiency rates and qPCR technical repeats.

Then the normalised Ct values obtained were used to determine the $2^{-\Delta Ct}$ values of the different-sized segments of the same saliva specific mRNA markers. The $2^{-\Delta Ct}$ values were determined from the ΔCt value, calculated by subtracting the Ct value of the more stable amplicon from the less stable amplicon. The ΔCt value was then converted into $2^{-\Delta Ct}$ values for statistical analysis [78, 143]. All statistical data analyses and graphs were prepared using Minitab® 16 statistical software package (version 16, Minitab® Inc., State College, PA, USA) and the Microsoft Excel statistics program (Microsoft, USA). The level of significance (α) for any hypothesis test was 0.05. Normality was determined by the Anderson-Darling normality test. Depending on the normality test, the appropriate correlation test was used to determine the correlation between the $2^{-\Delta Ct}$ values and the age of the stains. Nonparametric methods were used because they could effectively deal with small sample sizes.

7.3.2 Results and discussion

7.3.2.1 Evaluation of KRT4 mRNA 76 bp - 61 bp amplicon combination

In this study a multiplex of a pair of TGEAs, namely assay ID Hs00361610_g1 and assay ID Hs00361611_m1, was used to simultaneously confirm the type of saliva stain and determine its age. This TGEA pair represents different-sized segments of KRT4 mRNA (76 bp and 61 bp amplicon length) (Figure 7-6).

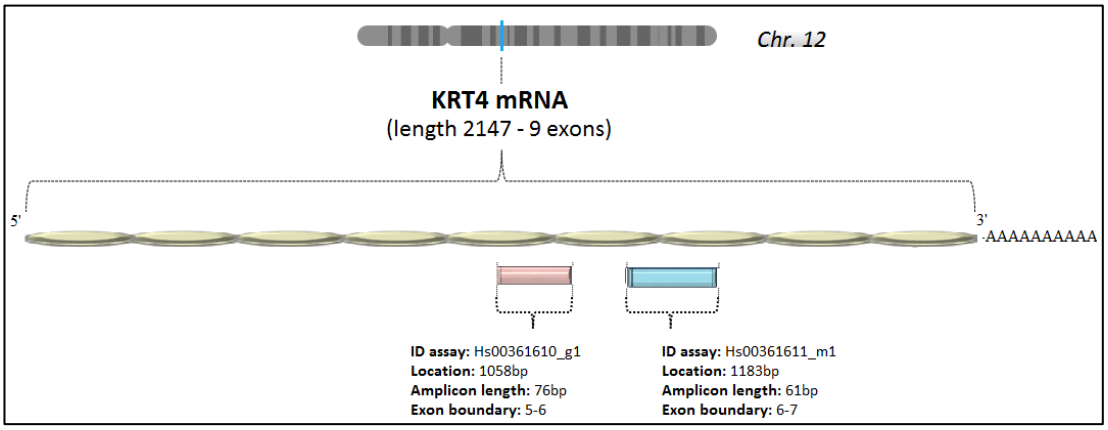

Figure 7-6: Locations of the TGEAs on the KRT4 mRNA. Figure shows the locations and amplicon lengths of the TGEA pair (assay ID Hs00361610_g1 and assay ID Hs00361611_m1) on the KRT4 mRNA.

7.3.2.1.1 The $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp and trend analysis

Table 7-6 shows the mean raw fluorescent Ct values of a TGEA pair of the KRT4 (assay ID Hs00361610_g1 labelled with FAM dye, and assay ID Hs00361611_m1 labelled with VIC dye). The raw Ct values were measured using real-time PCR at 0, 7, 14, 35, and 60 days. They were then corrected with the efficiency of the reaction using GenEx statistical software (Table 7-7). Table 7-8 illustrates the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp that were calculated from the corrected Ct values for saliva samples aged for up to 60 days.

Table 7-6: Mean raw fluorescent Ct values of KRT4 mRNA 76 bp – 61 bp amplicon combination at 0, 7, 14, 35, and 63 days before efficiency correction for 12 samples from five donors on three occasions. The mean Ct value is calculated by the software of Stratagene Mx3005P™ where each sample is run in duplicate. The Ct value of each individual sample is shown in Appendix 11.

Donors *n*=4	Occasion	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	26.94	26.26	37.05	36.88	25.00	24.78	34.44	34.75	33.42	32.78
	2	32.33	32.17	28.29	28.57	28.14	28.52	30.95	31.07	34.61	33.31
	3	33.56	34.04	28.46	28.93	30.52	30.35	25.49	24.38	38.46	36.19
2	1	30.07	29.45	28.43	26.90	36.28	34.84	27.95	26.68	33.86	31.67
	2	34.12	33.45	34.86	34.11	27.69	26.45	35.08	33.86	No Ct	No Ct
	3	25.96	24.59	38.50	38.04	37.55	38.13	32.17	30.19	36.77	35.92
3	1	31.81	31.28	33.76	33.42	35.17	34.64	36.46	34.60	28.61	27.41
	2	33.26	33.09	32.70	32.40	33.06	32.42	32.67	30.25	31.56	29.44
	3	28.69	26.88	31.81	31.02	30.77	30.46	30.42	29.03	32.89	31.41
4	1	30.27	30.73	31.12	31.85	31.50	31.43	31.73	31.99	28.14	27.96
	2	27.36	27.34	28.26	28.60	31.64	31.42	26.01	25.04	32.87	31.86
	3	31.91	32.43	33.89	34.88	29.03	29.20	32.10	31.62	32.71	32.48

Table 7-7: Corrected Ct values of KRT4 mRNA 76 bp – 61 bp amplicon combination at 0, 7, 14, 35, and 63 days after efficiency correction using GenEx software for 12 samples from five donors on three occasions. The data in this table are obtained from correcting the mean raw Ct values in Table 7-6.

.Donors *n*=4	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	26.60	24.33	35.89	33.47	24.82	23.05	33.49	31.63	32.55	29.94
	2	31.56	29.41	27.84	26.32	27.71	26.27	30.28	28.46	33.65	30.39
	3	32.68	31.02	28.00	26.63	29.89	27.85	25.27	22.71	36.76	32.87
2	1	28.56	26.21	27.05	24.02	34.26	30.85	26.61	23.83	32.04	28.12
	2	32.28	29.65	32.96	30.22	26.37	23.63	33.17	30.01	No Ct	No Ct
	3	24.78	22.02	36.31	33.60	35.43	33.68	30.49	26.85	34.72	31.78
3	1	29.24	26.93	31.03	28.77	32.33	29.82	30.06	27.89	26.30	23.59
	2	30.57	28.49	33.51	29.78	30.39	27.90	30.03	26.04	29.01	25.34
	3	25.43	24.14	29.24	26.70	28.28	26.22	27.96	24.99	30.23	27.04
4	1	25.98	24.73	26.77	25.70	27.11	25.33	27.33	25.82	24.02	22.35
	2	23.31	21.81	24.14	22.90	27.25	25.33	22.07	19.84	28.38	25.71
	3	27.50	26.20	29.31	28.30	24.84	23.41	27.66	25.50	28.22	26.24

Table 7-8: The $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp for 12 samples at 0, 7, 14, 35, and 63 days. The $2^{-\Delta Ct}$ values were calculated from the corrected Ct values in Table 7-7 by subtracting the Ct values obtained for the short amplicon from those of the long amplicon.

Donors *n*=4	Occasions	$2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp				
		day 0	day 7	day 14	day 35	day 63
1	1	0.21	0.19	0.29	0.28	0.16
	2	0.23	0.35	0.37	0.28	0.10
	3	0.32	0.39	0.24	0.17	0.07
2	1	0.20	0.12	0.09	0.15	0.07
	2	0.16	0.15	0.15	0.11	ND
	3	0.15	0.15	0.30	0.08	0.13
3	1	0.20	0.21	0.18	0.22	0.15
	2	0.24	0.08	0.18	0.06	0.08
	3	0.41	0.17	0.24	0.13	0.11
4	1	0.42	0.48	0.29	0.35	0.31
	2	0.35	0.42	0.26	0.21	0.16
	3	0.41	0.50	0.37	0.22	0.25

The results of this study show the relationship between the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp extracted from a saliva sample and the age of the sample. Table 7-9 shows that the means of the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp decrease with an increase in the sample age. Figure 7-7 shows this decreasing trend in the $2^{-\Delta Ct}$ values with increasing sample age. In addition, Figure 7-7also shows a wide range of $2^{-\Delta Ct}$ values at each time period.

Table 7-9: Statistical analysis of the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp of samples at 0, 7, 14, 35, and 63 days. Statistical calculations were carried out using Minitab® software.

	Mean $2^{-\Delta Ct}$ values	SD[a]	CV%[b]	Variance
day 0	0.28	0.10	36.81	0.010
day 7	0.27	0.15	56.09	0.023
day 14	0.25	0.09	34.59	0.007
day 35	0.19	0.09	46.67	0.008
day 63	0.14	0.08	52.54	0.006

[a] Standard deviation [b] Coefficient of variation

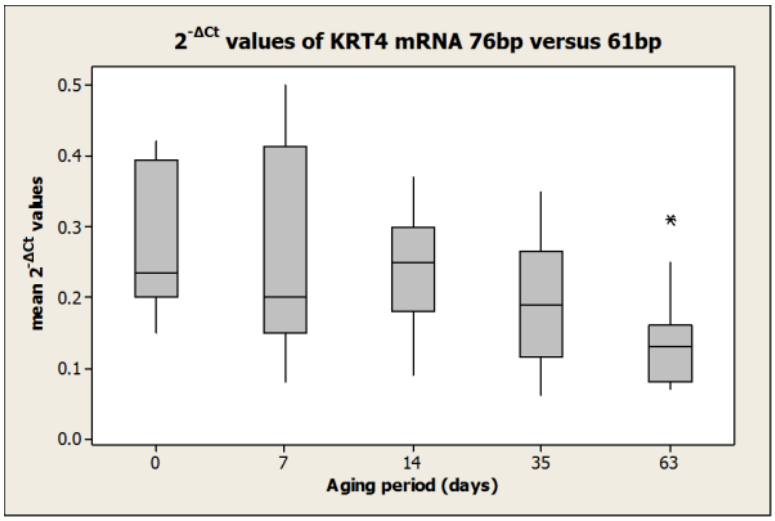

Figure 7-7: A boxplot showing $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp by age of an *ex vivo* saliva stains. Boxplot was obtained from the data shown in Table 7-8 using Minitab® 16 software.

Plotting the mean $2^{-\Delta Ct}$ values against the sample age also indicates that mean $2^{-\Delta Ct}$ values decrease with time, which can be exploited to estimate the age of a bloodstain (Figure 7-8). This indicates that the two examined amplicons differ in their degradation rate where the larger one, i.e. the 76 bp length amplicon, shows a higher tendency to degrade than the smaller one, i.e. the 61 bp length amplicon.

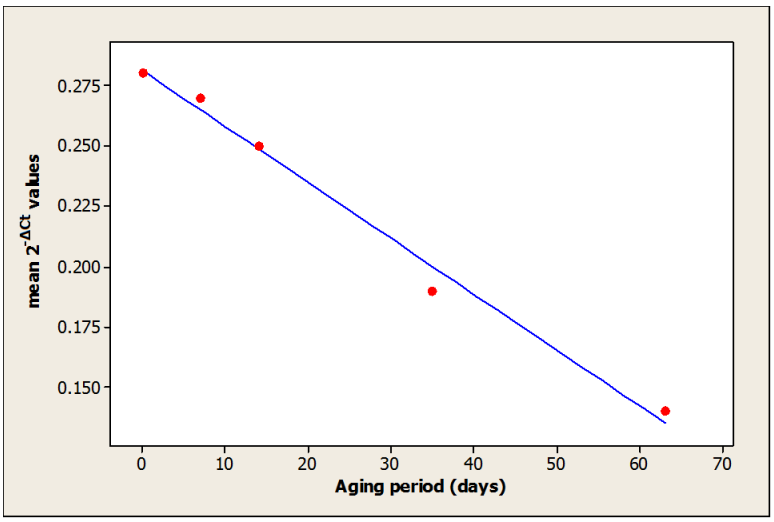

Figure 7-8: Scatterplot of the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp against the age of the saliva stain. Figures show that $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp decrease with the increasing age of saliva stains**.**

The relationship between the age of the saliva stain and the obtained mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp was statistically calculated. The Anderson-Darling normality test was carried out on the mean $2^{-\Delta Ct}$ values and on the aging period to test the normality of the data. The test shows that the variables were normally distributed. Therefore, parametric analysis was adopted for statistical analysis. Pearson's correlation indicates that there is a strong linear negative correlation between the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp and the age of saliva stains (Pearson's r = 0.99; p-value= 0.000). However, this correlation does not specify which value affects the other.

Regression analysis was applied on the data obtained by plotting the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp against the age of saliva in order to estimate the relationship between the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp and the age of the saliva stains. Fitting the data on a simple linear model demonstrates the decrease in mean $2^{-\Delta Ct}$ values with the increase in the stain's age (R^2= 98.6%) (Figure 7-9). The age of the saliva stain can be estimated based on the mean $2^{-\Delta Ct}$ values using a linear model (R^2= 98.6 %):

$$y = 120.4 - 427.3\,x \qquad \text{(Equation 7-1)}$$

where y is the age of the saliva stain in days and x is the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp.

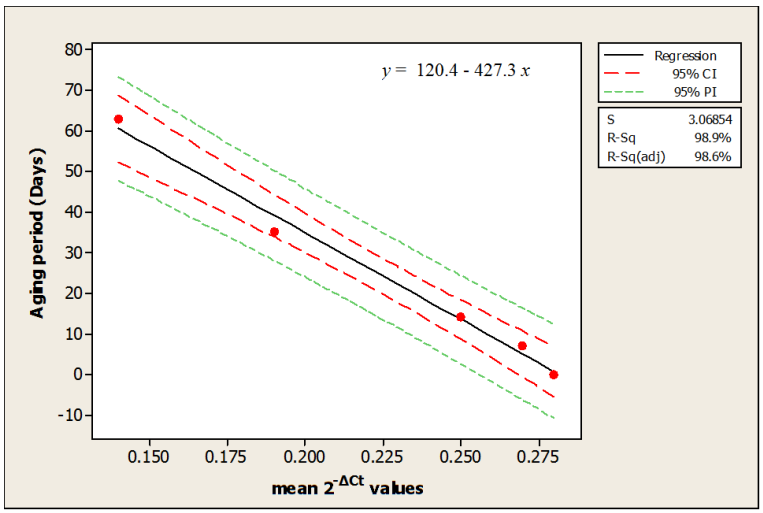

Figure 7-9: Time-wise trend in the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 bp of saliva samples over time. CI means confidence interval and prediction interval. The curve was obtained from the data shown in Table 7-9 using Minitab® 16 software.

To evaluation the effect of the ages of saliva and the individuals on the mean RQR values at specific two-way analysis of variance (ANOVA) test was carried. The results of the two-way ANOVA showed that the difference in the means of different sample ages was highly significant (*p value* <0.01). The results also showed that the difference in means between individuals at specific ages of saliva stains is also significant (*p value* <0.01) (results of ANOVA test are shown in Appendix 12 and Appendix 13).

The nested analysis of variance results showed that the variability in the $2^{-\Delta Ct}$ values accounting for the inter-person variability was 9.4%, and for the intra-person variability was 0.2%. In addition, the gender effect on the $2^{-\Delta Ct}$ values that was found was 7.8%.

7.3.2.1.2 The inter- and intra-person variations

Statistical analysis of the inter-person variation at each age period, using the $2^{-\Delta Ct}$ values, indicated that the inter-person variation differs at each age period. The inter-person variation ranged from 34.59% (14 days) to 56.09% (seven days), with a mean value of 45.34% across all aging times (Table 7-10 and Figure 7-10). This high inter-

person variation may be due to the natural biological variance *in vivo* and the constituent components of saliva, as discussed in previous chapters.

Table 7-10: Inter-person variations over time. Mean $2^{-\Delta Ct}$ values calculated from results by day given in Table 7-8 using Minitab® software. (Equation used to calculate coefficient of variation (CV) is CV= standard deviation / mean × 100).

	Mean RQR ***n*=12**	**Inter-person variation (Coefficient of variation, %)**
Day 0	0.28	36.81
Day 7	0.27	56.09
Day 14	0.25	34.59
Day 35	0.19	46.67
Day 60	0.14	52.54

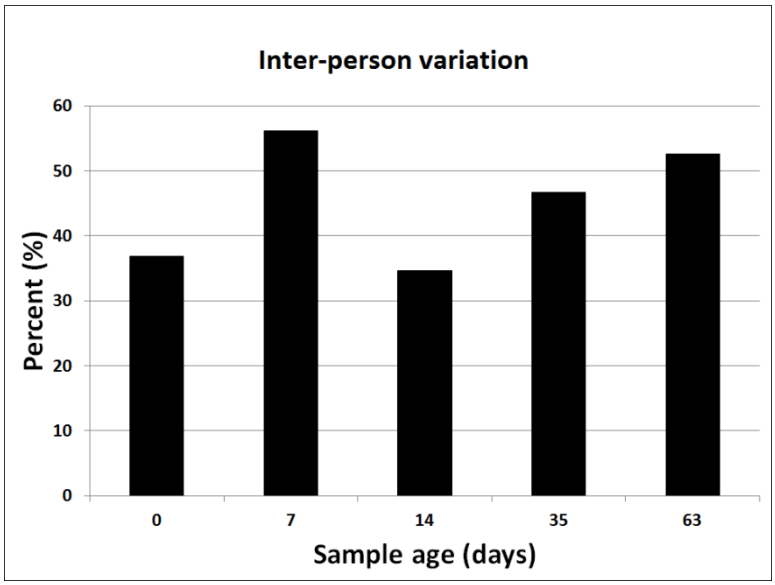

Figure 7-10: Inter-person variation samples over time. The bar chart was obtained from the data shown in Table 7-8 using the Microsoft Excel statistics program (Microsoft, USA).

Intra-person variation ranged from 9.63% (donor 3) to 39.35% (donor 4) with an overall average of 21.92%. These results also show the high intra-person variation that may arise, from time to time, from a change in saliva composition due to physiological and chemical causes (these causes were discussed in previous chapters).

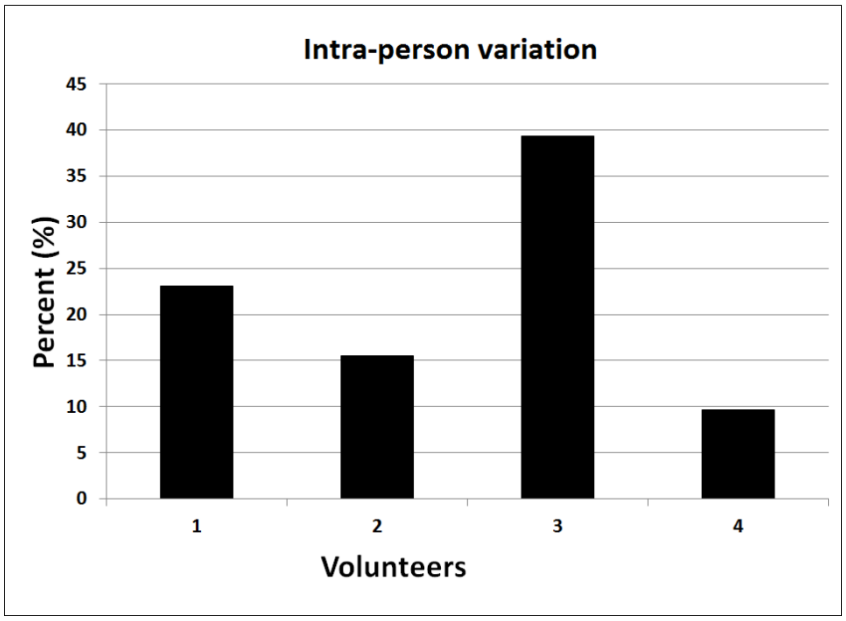

Figure 7-11: Intra-person variation of the four volunteers. The bar chart was obtained using the Microsoft Excel statistics program (Microsoft, USA).

7.3.2.2 Evaluation of KRT4 mRNA 80 bp - 61 bp amplicon combination

In this study, a multiplex of a pair of TGEAs, namely assay ID Hs00970607_g1 and assay ID Hs00361611_m1, was used to simultaneously confirm the type of saliva stain and determine its age. This TGEA pair represents different-sized segments of KRT4 mRNA (80 bp and 61 bp amplicon length) (Figure 7-12).

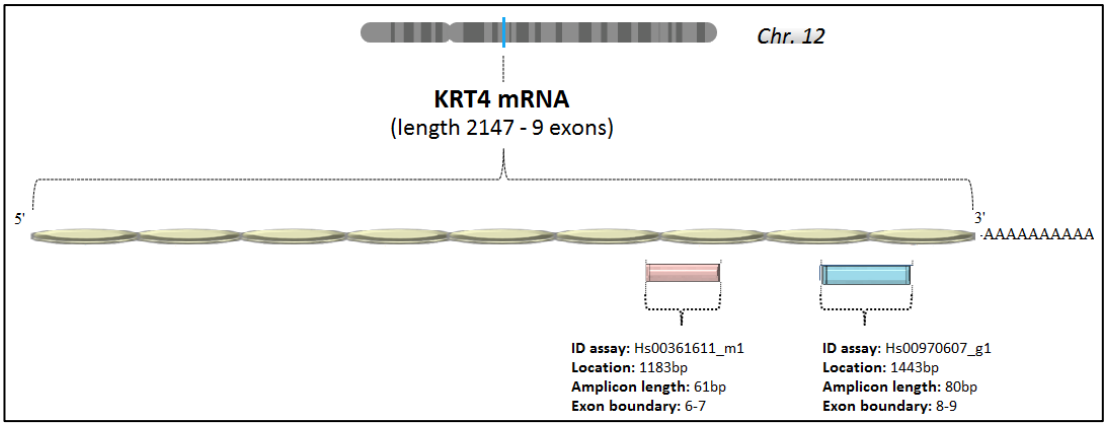

Figure 7-12: Locations of the TGEAs on the KRT4 mRNA. Figure shows the locations and amplicon lengths of the TGEA pair (assay ID Hs00970607_g1 and assay ID Hs00361611_m1) on the KRT4 mRNA.

7.3.2.2.1 The $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp and trend analysis

Table 7-11 shows the mean raw fluorescent Ct values of a TGEA pair of the KRT4 (assay ID Hs00970607_g1 labelled with FAM dye, and assay ID Hs00361611_m1 labelled with VIC dye). The raw Ct values were measured using real-time PCR at 0, 7, 14, 35, and 63 days. They were then corrected with the efficiency of the reaction using GenEx statistical software (Table 7-12). Table 7-13 illustrates the $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp that were calculated from the corrected Ct values for saliva samples aged for up to 60 days.

Table 7-11: Mean raw fluorescent Ct values of KRT4 mRNA 80 bp – 61 bp amplicon combination at 0, 7, 14, 35, and 63 days before efficiency correction for 15 samples from five donors on three occasions. The mean Ct value is calculated by the software of Stratagene Mx3005P™ where each sample is run in duplicate. The Ct value of each individual sample is shown in Appendix 14.

Donors *n*=5	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	26.88	25.65	38.03	35.89	25.15	23.92	34.77	33.48	33.84	32.76
	2	32.52	31.45	28.28	27.34	28.69	27.86	31.45	30.61	33.92	32.93
	3	33.85	33.16	28.67	27.93	30.90	29.82	24.90	23.67	36.88	36.80
2	1	29.48	29.44	27.76	27.50	35.05	35.58	27.48	27.32	32.31	32.73
	2	33.07	33.57	33.80	33.57	26.71	26.76	34.46	34.30	No Ct	No Ct
	3	24.86	24.78	37.76	38.40	38.12	38.30	30.97	30.96	35.88	36.60
3	1	31.18	31.18	33.02	33.15	34.17	34.91	31.88	32.16	27.69	27.71
	2	32.61	33.09	34.27	34.43	31.74	32.00	31.46	31.03	29.88	29.93
	3	27.75	27.34	30.76	30.82	30.24	30.76	29.43	29.50	31.84	31.53
4	1	26.24	25.08	32.58	31.93	25.70	24.42	30.31	29.57	27.90	26.82
	2	31.96	31.19	32.69	31.44	31.72	30.53	32.41	31.39	30.81	29.38
	3	30.95	29.85	29.82	28.80	36.18	35.74	28.11	26.93	28.34	27.31
5	1	32.28	31.16	32.88	31.70	32.70	31.25	33.01	31.88	29.19	27.90
	2	28.76	27.33	29.25	28.32	32.89	31.80	26.63	25.50	33.32	31.78
	3	33.10	32.01	35.77	34.57	30.40	29.27	33.65	31.78	33.85	32.43

Table 7-12: Corrected Ct values of KRT4 mRNA 80 bp – 61 bp amplicon combination at 0, 7, 14, 35, and 63 days after efficiency correction using GenEx software for 15 samples from five donors on three occasions. The data in this table are obtained from correcting the mean raw Ct values in Table 7-11

Donors *n*=5	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	24.60	23.25	34.43	32.19	23.07	21.75	31.55	30.09	30.73	29.46
	2	29.57	28.31	25.83	24.73	26.19	25.19	28.62	27.58	30.80	29.61
	3	30.74	29.81	26.17	25.25	28.13	26.89	22.85	21.53	33.42	32.98
2	1	26.00	25.69	24.49	24.00	31.41	31.05	24.23	23.84	29.00	28.56
	2	29.67	29.29	29.82	29.30	23.56	23.35	30.39	29.93	No Ct	No Ct
	3	21.93	21.63	33.80	33.51	33.62	33.42	27.32	27.02	32.15	31.94
3	1	27.50	27.21	29.13	28.93	30.64	30.47	28.12	28.06	24.42	24.18
	2	29.26	28.88	30.23	30.05	28.00	27.92	27.75	27.08	26.36	26.12
	3	24.48	23.86	27.13	26.89	27.17	26.84	25.96	25.74	28.08	27.52
4	1	22.27	21.01	27.85	26.99	21.79	20.44	25.85	24.93	23.73	22.53
	2	27.31	26.35	27.95	26.56	27.10	25.77	27.71	26.52	26.30	24.77
	3	25.45	25.17	25.42	24.26	31.03	30.31	23.91	22.63	24.11	22.96
5	1	27.59	26.31	28.12	26.79	27.96	26.40	28.24	26.95	24.87	23.48
	2	23.83	22.98	24.92	23.84	28.13	26.88	22.61	21.38	28.51	26.86
	3	28.31	27.07	30.67	29.30	25.93	24.67	28.80	26.86	28.98	27.43

Table 7-13: The $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp for 15 samples on 0, 7, 14, 35, and 63 days. The $2^{-\Delta Ct}$ values were calculated from the corrected Ct values in Table 7-12 by subtracting the Ct values obtained for the short amplicon from those of the long amplicon.

Donors *n*=5	Occasions	$2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp				
		day 0	day 7	day 14	day 35	day 63
1	1	0.39	0.21	0.40	0.36	0.41
	2	0.42	0.47	0.50	0.49	0.44
	3	0.52	0.53	0.42	0.40	0.74
2	1	0.80	0.71	0.78	0.76	0.73
	2	0.77	0.70	0.87	0.73	ND
	3	0.81	0.82	0.87	0.81	0.86
3	1	0.82	0.87	0.89	0.96	0.85
	2	0.77	0.88	0.95	0.63	0.84
	3	0.65	0.85	0.80	0.86	0.68
4	1	0.42	0.55	0.39	0.53	0.44
	2	0.51	0.38	0.40	0.44	0.35
	3	0.82	0.45	0.61	0.41	0.45
5	1	0.41	0.40	0.34	0.41	0.38
	2	0.55	0.47	0.42	0.43	0.32
	3	0.42	0.39	0.42	0.26	0.34

The results of this study show the relationship between the $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp extracted from a saliva sample and the age of the sample. Table 7-14 shows that the means of the $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp slightly decrease with an increase in the sample age. Figure 7-13 also shows the decreasing trend in the $2^{-\Delta Ct}$ values with increasing sample age. The decreasing trend indicates that the two examined amplicons differ in their degradation rate where the larger one, i.e. the 80 bp length amplicon shows a higher tendency to degrade than the smaller one, i.e. the 61 bp length amplicon. In addition, a wide range of $2^{-\Delta Ct}$ values was noticed at each time period.

Table 7-14: Statistical analysis of the $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp of samples at 0, 7, 14, 35, and 63 days. Statistical calculations were carried out using Minitab® software.

	Mean $2^{-\Delta Ct}$ values	SD[a]	CV%[b]	Variance
day 0	0.61	0.18	29.13	0.031
day 7	0.58	0.21	36.54	0.045
day 14	0.60	0.23	37.64	0.052
day 35	0.57	0.21	37.33	0.045
day 63	0.56	0.21	37.57	0.044

[a] Standard deviation [b] Coefficient of variation

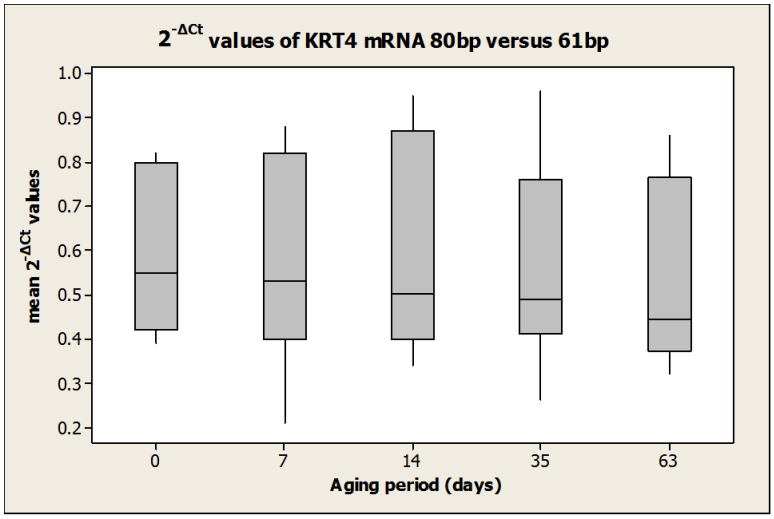

Figure 7-13: A boxplot showing $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp by age of an *ex vivo* saliva stains. Boxplot was obtained from the data shown in Table 7-13 using Minitab® 16 software.

The relationship between the age of the saliva stain and the obtained mean $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp was evaluated using the following statistical methods. The Anderson-Darling normality test was carried out on the mean $2^{-\Delta Ct}$ values and on the aging period to test the normality of the data. The test showed that the variables were normally distributed. Therefore, parametric analysis was adopted for statistical analysis. Pearson's correlation indicates that there is no significant correlation between the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp and the age of the saliva stains (Pearson's r = -0.820; p-value= 0.089).

In addition, regression analysis indicated that there is no significant relationship between the data obtained by plotting the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp (*p*-value= 0.089). This means that the mean $2^{-\Delta Ct}$ values of KRT4 mRNA 80 bp versus 61 bp cannot be used to estimate the age of the saliva stains.

7.3.2.2.2 The inter- and intra-person variations

High inter- and intra- person variations were also noticed. Statistical analysis of the inter-person variation at each age period using the $2^{-\Delta Ct}$ values indicated that the inter-person-variation differs at each age period. The inter-person variation ranged from 29.13% (0 days) to 37.64% (14 days) with a mean value of 35.64% across all aging times (Table 7-15 and Figure 7-14). This high inter-person variation may be due to the natural biological variance *in vivo* and the constituent components of saliva discussed in previous chapters.

Table 7-15: Inter-person variations over time. Mean $2^{-\Delta Ct}$ values calculated from results by day given in Table 7-8 using Minitab® software. (Equation used to calculate coefficient of variation (CV) is CV= standard deviation / mean × 100).

	Mean RQR *n*=15	**Inter-person variation (Coefficient of variation, %)**
Day 0	0.61	29.13
Day 7	0.58	36.54
Day 14	0.60	37.64
Day 35	0.57	37.33
Day 60	0.56	37.57

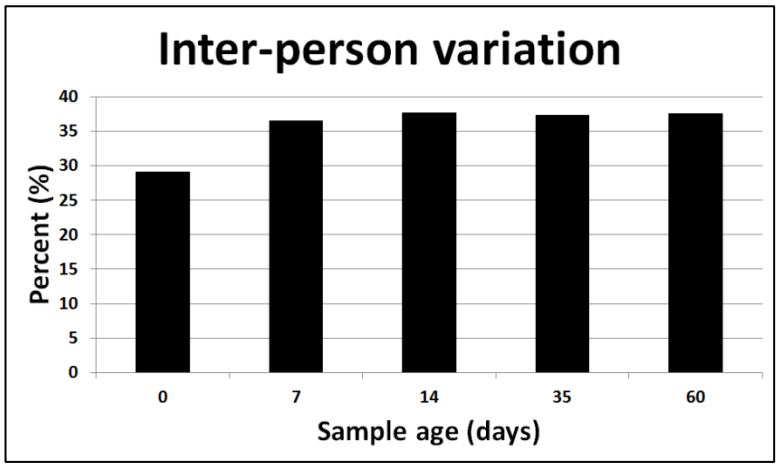

Figure 7-14: Inter-person variation samples over time. The bar chart was obtained from the data shown in Table 7-8 using the Microsoft Excel statistics program (Microsoft, USA).

Statistical analysis of the intra-person variation indicated that it varied from 2.62% (donor 2) to 35.97% (donor 4), with an overall average of 16.52% (Figure 7-15). These high intra-person variation results may result from changes in saliva composition, from time to time, due to physiological and chemical causes. These causes were discussed in previous chapters.

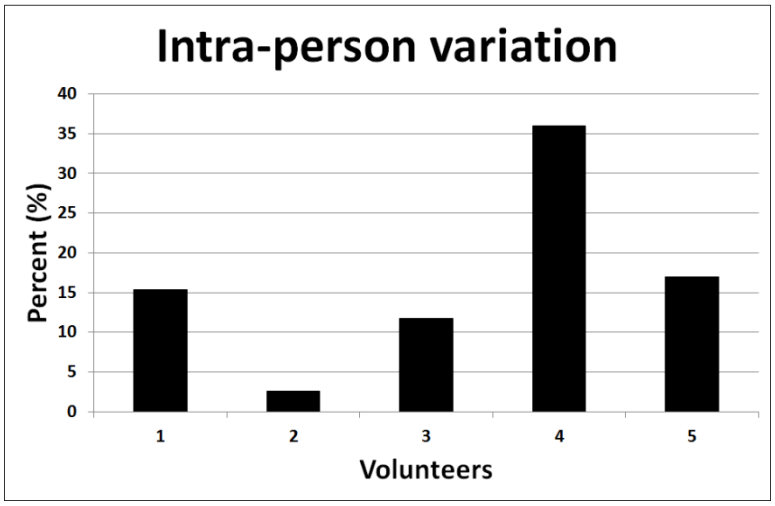

Figure 7-15: Intra-person variation of the five volunteers. The bar chart was obtained using the Microsoft Excel statistics program (Microsoft, USA).

7.3.2.3 Evaluation of HTN3 mRNA 136 bp -79 bp amplicon combination

In this study, a multiplex of a pair of TGEAs, namely assay ID Hs04194749_g1 and assay ID Hs00264790_m1, was used to simultaneously confirm the type of saliva stain and determine its age. This TGEA pair represents different-sized segments of HTN3 mRNA (136 bp and 79 bp amplicon length) (Figure 7-16).

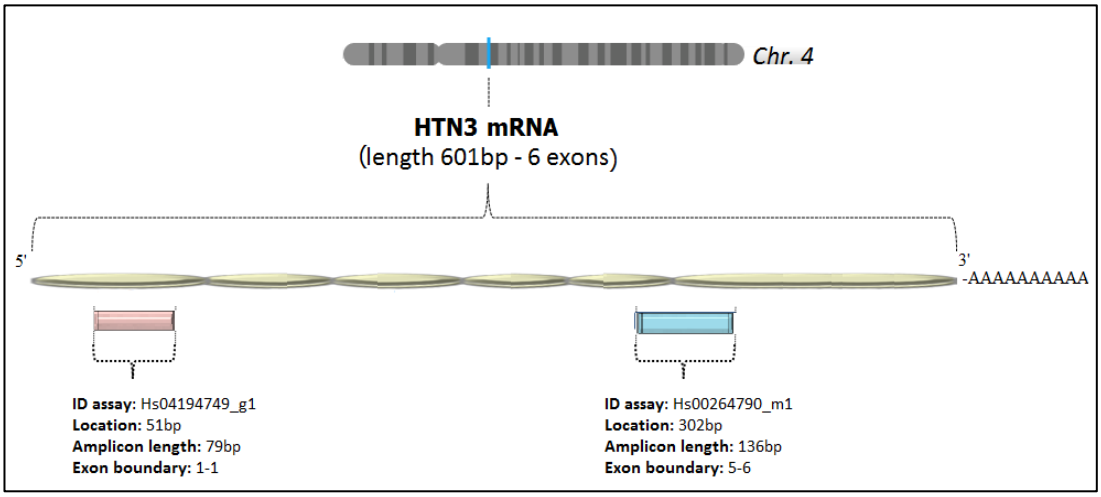

Figure 7-16: Locations of the TGEAs on the HTN3 mRNA. Figure shows the locations and amplicon lengths of the TGEA pair (assay ID Hs04194749_g1 and assay ID Hs00264790_m1) on the HTN3 mRNA.

7.3.2.3.1 The $2^{-\Delta Ct}$ values of HTN3 mRNA 136 bp versus 79 bp and trend analysis

Table 7-16 shows the mean raw fluorescent Ct values of a TGEA pair of the HTN3 (assay ID Hs04194749_g1 labelled with FAM dye, and assay ID Hs00264790_m1 labelled with VIC dye). The raw Ct values were measured using real-time PCR at 0, 7, 14, 35, and 63 days. They were then corrected with the efficiency of the reaction using GenEx statistical software (Table 7-17).

Table 7-18 illustrates the $2^{-\Delta Ct}$ values of HTN3 mRNA 136 bp versus 79 bp calculated from the corrected Ct values for saliva samples that were aged for up to 60 days.

Table 7-16: Mean raw fluorescent Ct values of HTN3 mRNA 136 bp – 79 bp amplicon combination at 0, 7, 14, 35, and 63 days before efficiency correction for 15 samples from five donors on three occasions. The mean Ct value is calculated by the software of Stratagene Mx3005P™ where each sample is run in duplicate. The Ct value of each individual sample is shown in Appendix 15.

Donors *n*=5	Occasion	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	32.27	28.16	38.13	33.89	38.32	34.74	38.02	33.31	38.37	33.55
	2	28.39	26.55	30.29	27.83	32.80	28.19	32.61	28.34	31.44	27.92
	3	33.37	29.26	34.38	29.85	26.58	37.93	26.51	38.40	34.07	30.84
2	1	35.99	33.97	26.55	30.90	34.59	30.85	25.96	25.21	34.22	30.64
	2	32.66	30.95	36.78	32.98	33.88	32.37	36.66	31.81	33.87	33.22
	3	37.09	35.07	35.97	34.23	33.87	32.77	34.31	33.11	38.59	35.08
3	1	35.63	31.74	34.39	31.12	34.35	30.58	31.31	28.12	31.79	28.08
	2	31.51	29.23	26.44	27.70	32.41	30.71	28.65	29.70	29.26	30.69
	3	25.53	28.03	25.87	27.71	28.02	27.61	29.74	28.50	32.99	30.10
4	1	28.38	No Ct	29.25	27.67	32.96	31.89	25.92	39.42	28.52	No Ct
	2	27.00	31.69	28.32	30.93	28.09	29.76	35.36	33.69	26.38	No Ct
	3	23.82	No Ct	25.03	38.59	36.59	32.64	36.54	32.64	35.54	32.12
5	1	39.24	34.78	39.69	35.67	38.30	34.03	38.53	35.09	38.59	37.79
	2	39.31	35.52	37.46	34.76	36.83	34.15	30.36	33.62	28.78	38.75
	3	31.61	32.22	32.67	30.53	32.88	30.54	32.94	31.17	33.38	31.97

Table 7-17: Corrected Ct values of HTN3 mRNA 136 bp – 79 bp amplicon combination at 0, 7, 14, 35, and 63 days after efficiency correction using GenEx software for 15 samples from five donors on three occasions. The data in this table are obtained from correcting the mean raw Ct values in Table 7-16.

Donors *n*=5	Occasion	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	25.96	21.60	30.81	26.17	30.96	26.81	30.73	25.74	31.00	25.92
	2	23.14	20.67	24.64	21.62	26.62	21.89	26.47	22.01	25.54	21.70
	3	27.06	22.70	27.86	23.14	21.72	29.20	21.66	28.32	27.62	23.89
2	1	28.89	25.96	21.69	23.93	28.02	23.89	21.23	26.53	27.73	23.74
	2	26.50	23.97	29.75	25.49	27.46	25.03	29.65	24.61	27.46	25.67
	3	29.99	27.06	29.11	26.43	27.46	25.33	27.80	25.58	31.17	27.07
3	1	28.05	23.81	27.08	23.35	27.04	22.94	24.65	21.09	25.03	21.06
	2	24.81	21.92	20.82	20.80	25.52	23.03	22.56	22.28	23.04	23.02
	3	20.10	21.03	20.37	20.58	22.06	20.71	23.41	21.38	25.98	22.58
4	1	22.35	No Ct	23.03	21.12	25.95	23.92	20.41	29.57	22.46	No Ct
	2	21.26	23.77	22.30	23.20	22.12	22.32	27.84	25.27	20.77	No Ct
	3	18.76	No Ct	19.71	28.95	29.02	24.49	28.77	24.49	27.99	24.09
5	1	30.11	25.34	30.47	26.00	29.37	24.78	29.55	25.57	30.03	26.22
	2	30.17	25.90	28.71	25.32	28.21	24.86	23.12	23.44	21.87	29.29
	3	24.10	23.42	24.93	22.15	25.10	22.16	25.15	22.63	25.50	23.23

Interestingly, in most of the analysed samples, smaller amplicons (79 bp) resulted in Ct values smaller than the larger amplicons (136 bp). This finding was contrary to expectations, and indicates that smaller amplicons tend to be more degradable than larger ones, while larger ones are more stable. This higher degradation tendency of the smaller amplicon and greater stability of the larger amplicon may be due to the location of the two used TGEAs on the mRNA, where the smaller one is near to the 5' end of the mRNA, while the larger one is in the middle of the mRNA (Figure 7-16).

There are many suggested explanations for the higher stability of the large amplicons. Firstly, the activity of exonucleases in the sample may be higher than that of the endonucleases, resulting in a higher degradation rate of the RNA ends than the middle of the RNA. Secondly, HTN3 mRNA may undergo a stochastic degradation mechanism, resulting in random degradation of its parts. In fact, the latter explanation is favoured in this study, where many samples showed a higher degradation tendency of the large amplicon. This means HTN3 mRNA may randomly degrade *ex vivo,* i.e. stochastic process mechanism, where the degradation of the mRNA occurs in random order. Therefore, in some samples, amplicons near 5' end of the mRNA were more abundant than those near the middle of the mRNA while in other samples they were lesser.

Table 7-18 shows that ΔCt values calculated by subtracting Ct values obtained for the long amplicon show more stability than those of the short amplicon.

Although the results of this study suggested the stochastic mechanism as the degradation mechanism of the HTN3, more research (beyond the scope of this thesis) is needed to confirm this. In general, there is a gap in knowledge about the *ex vivo* degradation mechanisms of RNAs in the literature. The finding here suggests the degradation mechanism happens in HTN3 mRNA in the saliva stain stored under specific conditions.

Table 7-18: The ΔCt values of HTN3 mRNA 136 -79 bp for 15 samples at 0, 7, 14, 35, and 63 days. The ΔCt values were calculated from the corrected Ct values in Table 7-17 by subtracting the Ct values obtained for the long amplicon from those of the short amplicon. Minus values indicate that the long amplicon is more stable than the short amplicon.

Donors *n*=5	**Occasions**	**ΔCt values of HTN3 mRNA 136 bp versus 79 bp**				
		day 0	**day 7**	**day 14**	**day 35**	**day 63**
1	1	4.36	4.64	4.15	4.99	5.08
	2	2.47	3.01	4.72	4.46	3.85
	3	4.36	4.71	-7.48	-6.65	3.73
2	1	2.94	-2.24	4.14	-5.31	3.99
	2	2.53	4.26	2.43	5.04	1.79
	3	2.94	2.68	2.13	2.22	4.10
3	1	4.24	3.73	4.11	3.56	3.97
	2	2.88	0.02	2.48	0.28	0.02
	3	-0.92	-0.21	1.35	2.03	3.40
4	1	ND	1.91	2.03	-9.16	ND
	2	-2.51	-0.90	-0.20	2.57	ND
	3	ND	-9.24	4.53	4.28	3.89
5	1	4.77	4.46	4.59	3.98	3.81
	2	4.27	3.39	3.35	-0.32	-7.42
	3	0.68	2.79	2.94	2.52	2.27

Table 7-19: The $2^{-\Delta Ct}$ values of HTN3 mRNA 136 bp versus 79 bp for 15 samples at 0, 7, 14, 35, and 63 days. The $2^{-\Delta Ct}$ values were calculated from the corrected ΔCt values in Table 7-18 Values greater than one mean the long amplicon shows more stability than the short amplicon.

Donors *n*=5	Occasions	$2^{-\Delta Ct}$ values of HTN3 mRNA 136 bp versus 79 bp				
		day 0	day 7	day 14	day 35	day 63
1	1	0.05	0.04	0.06	0.03	0.03
	2	0.18	0.12	0.04	0.05	0.07
	3	0.05	0.04	178.53	100.43	0.08
2	1	0.13	4.72	0.06	39.67	0.06
	2	0.17	0.05	0.19	0.03	0.29
	3	0.13	0.16	0.23	0.21	0.06
3	1	0.05	0.08	0.06	0.08	0.06
	2	0.14	0.99	0.18	0.82	0.99
	3	1.89	1.16	0.39	0.24	0.09
4	1	ND	0.27	0.24	572.05	ND
	2	5.70	1.87	1.15	0.17	ND
	3	ND	604.67	0.04	0.05	0.07
5	1	0.04	0.05	0.04	0.06	0.07
	2	0.05	0.10	0.10	1.25	171.25
	3	0.62	0.14	0.13	0.17	0.21

In this study, most of the samples showed higher stability of the long amplicons than the short amplicons, which may be because of their location on the mRNA. However, because some of the samples demonstrated a different tendency, despite being the same age, the two used TGEAs are not suitable for the aging study of saliva as shown in Figure 7-17 and Figure 7-18.

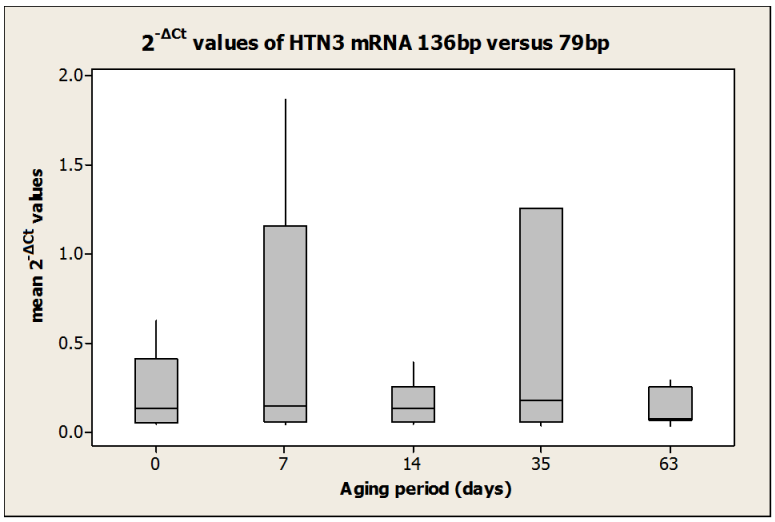

Figure 7-17: A boxplot showing $2^{-\Delta Ct}$ values of HTN3 mRNA 136 versus 79 bp by age of an *ex vivo* saliva stain. Boxplot was obtained from the data shown in Table 7-18 using Minitab® 16 software. Outliers were excluded from the figure.

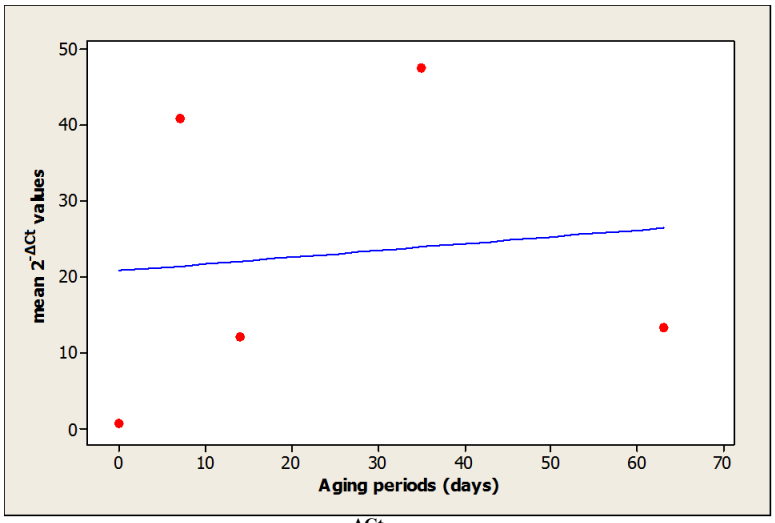

Figure 7-18: Scatterplot of the mean $2^{-\Delta Ct}$ values of HTN3 mRNA 136 versus 79 bp against the age of the saliva stain. Figure shows no correlation between the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 79 bp and the age of the saliva stains.

7.3.3 Conclusion

In this study, saliva specific mRNA markers were used to determine the age of the saliva stains. As the different-sized segments of RNA have different stability tendencies, this study was carried out to determine the relationship between the age of the saliva stains and the relative stability of different-sized segments of the same

saliva specific mRNA markers. A multiplex of TGEA pairs of the same saliva specific mRNA markers were used to determine the relative quantity between them using the $2^{-\Delta Ct}$ method. Three pairs of TGEAs for two saliva specific mRNA markers (KRT4 mRNA 76 bp - 61 bp, KRT4 mRNA 80 bp - 61 bp and HTN3 mRNA 136 -79 bp amplicon combinations) were chosen, based on specific criteria that were set to choose the most appropriate TGEAs for this study.

The results of this study showed a relationship between the $2^{-\Delta Ct}$ values of only one pair of the examined TGEA pairs and the age of saliva stains. This was the TGEA pair of the KRT4 mRNA 76 bp - 61 bp (assay ID Hs00361611_m1 and assay ID Hs00361610_g1). It was found that the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp - 61 bp were inversely proportional with age, i.e. the values decreased with an increase in the age of the stain. This finding indicates that the examined TGEA pair of KRT4 mRNA 76 bp - 61 bp can be used to estimate the age of saliva stains. On the other hand, no relationship was found between the $2^{-\Delta Ct}$ values of the other two TGEA pairs (KRT4 mRNA 80 bp - 61 bp and HTN3 mRNA 136 -79 bp amplicon combinations).

However, high inter- and intra-person variations were noticed, despite setting controlled conditions for collection in order to decrease the effect of external materials, such as food and smoke. This high level of variation could be due to the variation between individuals in the degradation status of RNA molecules in saliva, and due to the presence of various types of ribonucleases in saliva. This high variation resulted in low precision and accuracy in determining saliva age, which can be considered a limitation to applying this approach in casework.

Interestingly, the results of the HTN3 mRNA 136 -79 bp amplicon combinations showed that, in most of the samples, the large amplicon exhibited a higher stability than the short amplicon, which was contrary to expectations. This was attributed to the effect of the location of each assay on the HTN3 mRNA. In addition, a random degradation mechanism including endonucleases activity was suggested as the mechanism that leads to *ex vivo* degradation of the HTN3 mRNA. However, more study is needed to confirm this finding.

Moreover, a complete DNA profile was obtained for all samples amplified using investigator Decaplex SE Kit. No evidence of contamination was noticed in any of the samples.

As blood shows a lower level of variation, this method will be applied on bloodstains to evaluate its applicability to simultaneous estimation of the age of blood and identification and confirmation the type. This will be discussed in the next chapter.

8 Analysing two different sized TaqMan® Gene Expression Assays of blood mRNA markers for simultaneous determination of bloodstain age and type

8.1 Introduction

In Chapter 7 a novel method of simultaneous determination of saliva type and age was described. In this chapter, the applicability of using two different-sized segments of the same saliva-specific mRNA markers, quantified using TaqMan® Gene Expression Assays (TGEA), is evaluated further.

This evaluation will be carried out by studying the correlation between the age of the bloodstains and the relative quantity of two different-sized segments of the same blood-specific mRNA markers, quantified using off-the-shelf TGEA. As in Chapter 7 the $2^{-\Delta Ct}$ method [78, 143] will be used to determine the relative quantity of the segments. In addition, the available TGEAs will be assessed using the specific criteria described in section 7.1.3, the criteria having been set in order to select suitable off-the-shelf TGEAs for the study.

8.1.1 Blood-specific RNA markers

Seventeen transcripts have been nominated in literature as suitable markers for blood samples for forensic applications. These transcripts are listed in Table 8-1, shown below. Some of these markers were used with other body fluid-specific transcripts in multiplex assays in order to simultaneously test several of the mRNA markers in a single sample extract [45, 57, 58, 61].

Table 8-1: Blood-specific mRNA markers. This table shows transcripts that were identified as specific mRNA markers for blood, their symbols and the location of their genes.

Transcript Name	Symbol	Gene Location	Ref
Spectrin, beta, erythrocytic	SPTB	Chr. 14	[45, 57, 61, 111, 192, 193]
Porphobilinogen deaminase	PBGD	Chr. 11	[45, 61, 111, 193]
Ankyrin 1, erythrocytic	ANK1	Chr. 8	[61, 183]
Aminolevulinate, delta-, synthase 2	ALAS2	Chr. X	[57, 61]
Alpha subunit of haemoglobin1	HBA1	Chr. 16	[46, 61]
Beta subunit of haemoglobin	HBB	Chr. 11	[61, 111]
CD3 gamma molecule	CD3G	Chr. 11	[61]
Aquaporin 9	AQP9	Chr. 15	[58, 98, 180]
Glycophorin A	GYPA	Chr. 4	[98, 180]
Neutrophil cytosolic factor 2	NCF2	Chr. 1	[98, 180, 192]
Caspase 1, apoptosis-related cysteine peptidase	CASP1	Chr. 11	[98, 180]
Adhesion molecule, interacts with CXADR antigen 1	AMICA1	Chr. 11	[98, 180]
CD93 molecule	C1QR1	Chr. 20	[98, 180]
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	Chr. 13	[98, 180]
Complement component 5a receptor 1	C5AR1	Chr. 19	[98, 180]
Myeloid cell nuclear differentiation antigen	MNDA	Chr. 1	[98, 180]
Rho GTPase activating protein 26	ARHGAP26	Chr. 5	[98, 180]

8.1.2 The predesigned off-the-shelf TGEAs chosen for bloodstain age determination

The Applied Biosystems predesigned off-the-shelf TGEAs were evaluated depending on the previously adopted five criteria that were described in section 7.1.3 in Chapter 7 to choose the most appropriate TGEA pairs for aging of bloodstains. These criteria take into account some of the characteristics of the available off-the-shelf TGEAs, such as amplicon length, spanning the exon-exon junction and assay location.

The transcripts listed in Table 8-1 meet the first criterion, as specified in section 7.1.3 in Chapter 7, i.e. the use of tissue-specific RNA markers instead of housekeeping gene RNA.

The available TGEAs for these mRNA were evaluated further to choose those that fulfilled the other criteria given in Chapter 7, i.e. to identify appropriate TGEA pairs for aging of biological stains. Specifically, the fulfilment of criterion 4, as described in section 7.1.3, concerning the selection of TGEA pairs that show the largest difference between amplicon sizes. This information is collated in Table 8-2, which also shows the exon boundaries, of each of the available Applied Biosystems TGEAs for blood-specific mRNA.

Table 8-2: Available Applied Biosystems TGEAs for blood-specific mRNA. This table shows the number of available TGEAs and the length of each assay product. Number between brackets shows exon boundaries of the TGEAs. Asterisk indicates assay does not span exon-exon junction.

blood-specific mRNA	No. of available TGEA	Amplicon product length of each TGEA arranged from the shortest to the longest (bp)			
		Shortest	2nd shortest	2nd longest	Longest
SPTB	26	58 (16-17)*	60 (18-19)	129 (28-29)	169 (35-35)*
PBGD	4	62 (10-11)*	64 (1-2)	69 (13-14)*	125 (4-5)*
ANK1	39	60 (11-12)*	62 (7-8)	119 (31-32)	137 (37-38)
ALAS2	10	61 (10-11)	62 (1-2)	85 (4-5)	128 (7-8)
HBA1	1	156 (1-2)*	---	---	---
HBB	2	106 (1-2)*	---	---	166 (1-2)
CD3G	6	60 (4-5)	65 (5-6)	89 (6-7)	105 (2-3)
AQP9	5	55(1-2)	61 (3-4)	98 (5-6)	117 (4-5)
GYPA	4	68 (4-5)	71 (6-7)	114 (5-6)	170 (7-7)*
NCF2	11	69 (11-12)*	72 (4-5)	121 (2-3)	131 (6-7)
CASP1	8	68 (5-6)	76 (6-7)	126 (6-7)*	140 (2-3)
AMICA1	9	69 (4-5)	80 (9-10)	131 (1-2)	152 (8-9)
C1QR1	1	79 (1-2)	---	---	---
ALOX5AP	3	73 (3-4)	80 (5-6)	---	90 (4-5)
C5AR1	5	68 (2-2)*	75 (2-2)*	108 (1-2)	122 (2-2)*
MNDA	6	68 (3-4)*	101 (4-5)	124 (1-2)	138 (2-3)
ARHGAP26	21	63 (20-21)	65 (1-2)	138 (5-6)	142 (4-5)

Figure 8-1 shows the base pair size difference between each of the two longest amplicon products and each of the two shortest amplicon products of each Applied Biosystems TGEA for each blood-specific marker. Note that the differences in amplicon sizes are shown for different combinations of TGEA's for each marker (see the annotation for Figure 8-1).

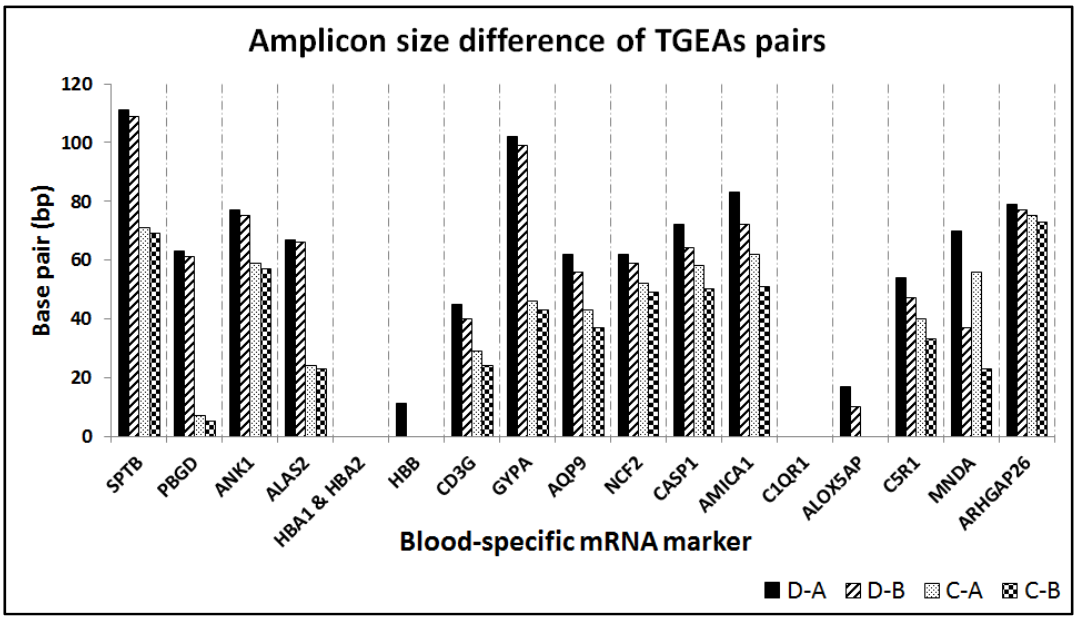

Figure 8-1: Amplicon size difference of the Applied Biosystems predesigned TGEAs pairs for blood-specific RNA markers. This figure shows the base pair size difference between the two longest amplicon products and the two shortest amplicon products of each Applied Biosystems TGEA for each blood-specific marker. (A) shortest amplicon. (B) second shortest amplicon. (C) second longest amplicon. (D) longest amplicon.

Figure 8-2 shows the distance between the locations of Applied Biosystems predesigned TGEAs on the blood-specific RNA markers. The distance between the two TGEAs was presented as a percentage of the distance between the locations of each of the two assays with the longest amplicon products and each of the two assays with the shortest amplicon products to the whole size of the blood-specific mRNA.

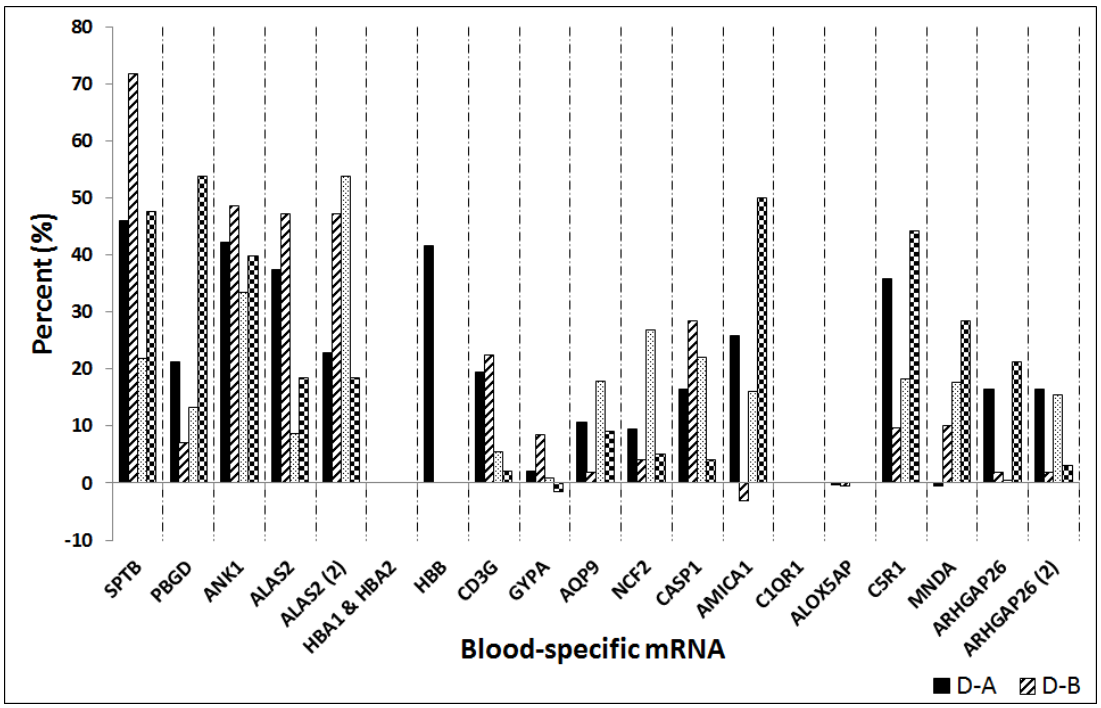

Figure 8-2: Percentage of the distance between the locations of two Applied Biosystems predesigned TGEAs to the whole size of the transcript. This figure shows the distance between the locations of Applied Biosystems predesigned TGEAs on the blood-specific mRNA as a percentage of the distance between each of the two assays with the longest amplicon products, and each of the two assays with the shortest amplicon products to the whole size of the blood-specific mRNA. Positive results indicate the locations of the assays differ from one another and they are far from each other; negative results indicate the locations of the two assays overlapped while zero is because of unavailability of the assay. (A) shortest amplicon. (B) second shortest amplicon. (C) second longest amplicon. (D) longest amplicon.

The results of the evaluation are summarised in Table 8-3. The results of the evaluation show that some TGEAs fully meet some of the criteria, while they partially meet other criteria. In addition, some TGEAs fail to meet some of the criteria.

Table 8-3: Evaluation of Applied Biosystems predesigned TGEAs for blood age determination. This table shows the summary of the evaluation of the available predesigned TGEAs for this study. (✓) indicates that the TGEA pair definitely meets criterion, (×) indicates that it does not meet criterion and (p) indicates it partially meets criterion.

Blood-specific mRNA	Criteria				
	1	2	3	4	5
SPTB	✓	✓	p	✓	p
PBGD	✓	✓	p	p	p
ANK1	✓	✓	p	✓	✓
ALAS2	✓	✓	✓	p	p
HBA1	✓	×	×	×	×
HBB	✓	✓	p	×	p
CD3G	✓	✓	✓	×	×
AQP9	✓	✓	✓	p	×
GYPA	✓	✓	p	p	×
NCF2	✓	✓	p	p	p
CASP1	✓	✓	p	✓	p
AMICA1	✓	✓	✓	✓	p
C1QR1	✓	×	✓	×	×
ALOX5AP	✓	✓	✓	×	×
C5AR1	✓	✓	p	p	p
MNDA	✓	✓	p	p	p
ARHGAP26	✓	✓	✓	✓	×

Indeed, according to the criteria set, there are many TGEA pairs suitable for this study. However, only two transcripts of these TGEAs pairs were chosen for use in this study. These chosen TGEA pairs are:

- **TGEA pair for ANK1 mRNA:** the TGEA with the longest amplicon product (assay ID Hs00986655_m1, occupying the location 796 bp of the transcript) and the TGEA with the shortest amplicon product (assay ID Hs00986664_m1, occupying the location 137 bp of the transcript). The distance between the locations of these TGEAs is approximately 49%. In addition, the length difference between the amplicon products of the TGEA pair is 75 bp.

- **TGEA pair for ALAS2:** the TGEA with the longest amplicon product (assay ID Hs01085693_m1, occupying the location 119 bp of the transcript) and the TGEA with the shortest amplicon product (assay ID Hs01085699_m1, occupying the location 1144 bp of the transcript). The distance between the locations of these TGEAs is approximately 47%. In addition, the length difference between the amplicon products of the TGEA pair is 66 bp.

Table 8-4 shows the TGEA pairs that will be used in this study to determine the age of the bloodstain.

Table 8-4: Chosen TGEA pairs of ANK1 mRNA and ALAS2 mRNA to determine the age of the bloodstain. This table shows pairs of TGEA for KRT4 mRNA and HTN3 mRNA. Each pair consists of two different labelled dyes.

TGEA pair	mRNA	Assay ID	Refseq	Assay location	Amplicon Length	Labelled dye
1	ANK1	Hs00986655_m1	NM_000037.3	796	62	VIC
		Hs00986664_m1	NM_020477.2	4624	137	FAM
2	ALAS2	Hs01085693_m1	NM_000032.4	119	62	VIC
		Hs01085699_m1	NM_000032.4	1144	128	FAM

8.2 Age of bloodstain using blood-specific mRNA marker

In this section, the relationship between the age of the bloodstain and the $2^{-\Delta Ct}$ values of the two chosen pairs of TGEAs for ANK1 mRNA and for ALAS2 will be assessed (Table 8-4). The two TGEA pairs that will be used in the examination are ANK1 and ALAS2, i.e. ANK1 mRNA 137 bp – 62 bp and ALAS2 mRNA 128 bp-62 bp amplicon combinations.

8.2.1 Materials and method

8.2.1.1 Preparation of samples

Blood was collected by finger stick from six volunteers with no history of malignancy. About 50 µL of blood was dropped on clean sterile cotton swabs and allowed to dry at room temperature in a sterile hood. The stains were then stored at

room temperature in a dry place to simulate natural aging (0, 7, 14, 21, and 30 days), and they were immediately extracted on the reaching the desired age. As samples were collected from all volunteers at the same time, samples from all volunteers at each age period were extracted in the same batch.

8.2.1.2 RNA extraction and reverse transcription

RNA extraction was carried out using the TRI reagent as described in section 2.3.1. All samples were treated with TURBO DNase I (Ambion) as described in section 2.3.3. The reverse transcription was carried out using SuperScript® III (Invitrogen) as described in section 2.5.2.

8.2.1.3 Real-time PCR

Real time PCRs were carried out in a multiplex of each pair of TGEA using 0.5 µL of each TGEA where the procedure described in section 2.8 was followed.

8.2.1.4 DNA extraction, quantification and profiling

DNA extraction was carried out using the TRI reagent as described in section 2.9.1. The DNA quantification was carried out using the Investigator Quantiplex kit as described in section 2.10. DNA profiling was carried out using Investigator Decaplex SE Kit as described in section 2.11.

8.2.1.5 Data analysis

The same method of data analysis used in the previous chapter (section 7.3.1.5) was employed.

8.2.2 Results and discussion

8.2.2.1 Evaluation of ANK1 mRNA 137 bp versus 62 bp amplicon combination

In this study a multiplex of a pair of TGEAs, namely assay ID Hs00986664_m1 and assay ID Hs00986655_m1, was used to simultaneously confirm the type of bloodstain and determine its age. This TGEA pair represents different-sized segments of ANK1 mRNA (137 bp and 62 bp amplicon length) (Figure 8-3).

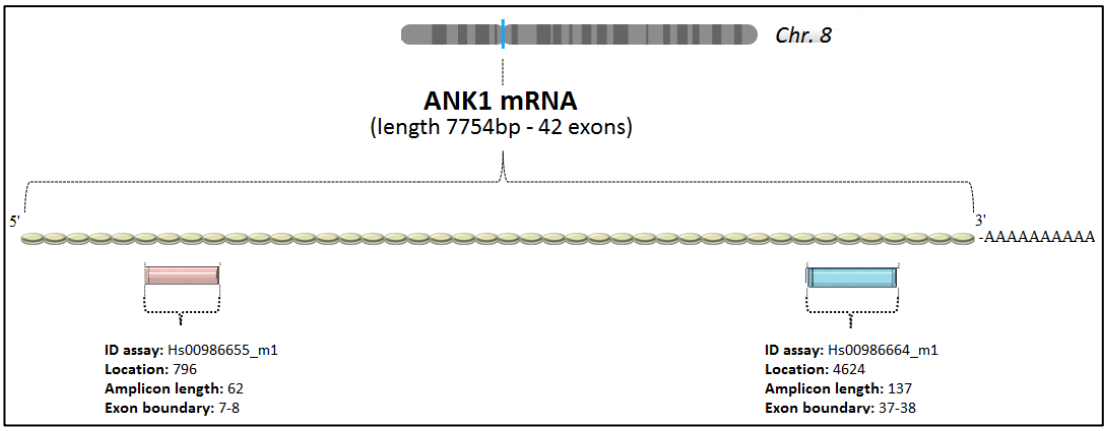

Figure 8-3: Locations of the TGEAs on the ANK1 mRNA. Figure shows the locations and amplicon lengths of the TGEA pair (assay ID Hs00986664_m1 and assay ID Hs00986655_m1) on the ANK1 mRNA.

8.2.2.1.1 The $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp and trend analysis

Table 8-5 shows the mean raw fluorescent Ct values of a TGEA pair of the ANK1 (assay ID Hs00986664_m1, labelled with FAM dye, and assay ID Hs00986655_m1, labelled with VIC dye). The raw Ct values were measured using real-time PCR at 0, 7, 14, 21, and 30 days. They were then corrected with the efficiency of the reaction using GenEx statistical software (Table 8-6). Table 8-7 illustrates the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp, calculated from the corrected Ct values for blood samples aged for up to 30 days.

Table 8-5: Mean raw fluorescent Ct values of ANK1 mRNA 137 bp – 62 bp amplicon combination from six donors at 0, 7, 14, 21, and 30 days before efficiency correction. The mean Ct value is calculated by the software of Stratagene Mx3005P™, where each sample is run in duplicate. The Ct value of each individual sample is shown in Appendix 16.

Donors n=6	Mean Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	36.10	32.28	38.49	36.03	36.64	34.18	No Ct	37.98	38.14	34.66
2	No Ct	37.56	37.63	34.40	36.83	32.61	37.82	35.96	37.45	33.07
3	35.79	31.88	37.11	32.05	34.64	30.64	37.39	35.22	37.62	36.08
4	35.35	31.55	36.39	32.28	37.80	33.62	34.66	32.05	36.39	34.39
5	32.75	29.59	36.51	31.79	38.21	32.83	36.01	33.44	36.24	32.36
6	31.94	28.58	37.46	32.27	36.65	33.45	37.51	33.88	37.08	32.72

Table 8-6: Corrected Ct values of ANK1 mRNA 137 bp – 62 bp amplicon combination from six donors at 0, 7, 14, 21, and 30 days after efficiency correction using GenEx software. The data in this table are obtained from correcting the mean raw Ct values in Table 8-5.

Donors n=6	Mean corrected Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	26.73	31.79	27.44	33.48	26.12	33.66	No Ct	37.40	27.19	34.13
2	No Ct	36.99	26.82	32.88	26.25	32.11	26.96	34.41	26.69	33.57
3	25.51	31.39	26.45	31.56	24.69	30.17	26.65	33.68	26.82	34.53
4	25.20	31.07	25.94	31.79	26.94	33.11	24.71	31.56	25.94	33.87
5	23.34	29.14	26.02	32.31	27.24	33.33	25.67	32.93	25.83	32.87
6	22.77	28.14	26.70	31.78	26.12	32.94	26.74	33.36	26.43	33.22

Table 8-7: The $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp for samples at 0, 7, 14, 21, and 30 days. The $2^{-\Delta Ct}$ values were calculated from the corrected Ct values in Table 8-6 by subtracting the Ct values obtained for the short amplicon from those of the long amplicon.

Donors *n*=6	$2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp				
	day 0	day 7	day 14	day 21	day 30
1	0.0301	0.0151	0.0054	ND	0.0081
2	ND	0.0151	0.0172	0.0057	0.0085
3	0.0169	0.0290	0.0224	0.0076	0.0048
4	0.0171	0.0173	0.0140	0.0086	0.0041
5	0.0180	0.0129	0.0146	0.0065	0.0076
6	0.0241	0.0296	0.0089	0.0101	0.0090

The results of this study show the relationship between the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp extracted from a blood sample, and the age of the sample. Table 8-8 shows that the means of the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp decrease with an increase in the sample age. Figure 8-4 also shows this decreasing trend in the $2^{-\Delta Ct}$ values with increasing sample age.

Table 8-8: Statistical analysis of the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp of samples at 0, 7, 14, 21, and 30 days. Statistical calculations were carried out using Minitab® software.

	Mean $2^{-\Delta Ct}$ values	SD[a]	CV%[b]	Variance
day 0	0.0212	0.0057	27.05	0.0000
day 7	0.0198	0.0075	37.66	0.0001
day 14	0.0137	0.0060	43.84	0.0000
day 21	0.0077	0.0017	22.55	0.0000
day 30	0.0070	0.0021	29.50	0.0000

[a] Standard deviation [b] Coefficient of variation

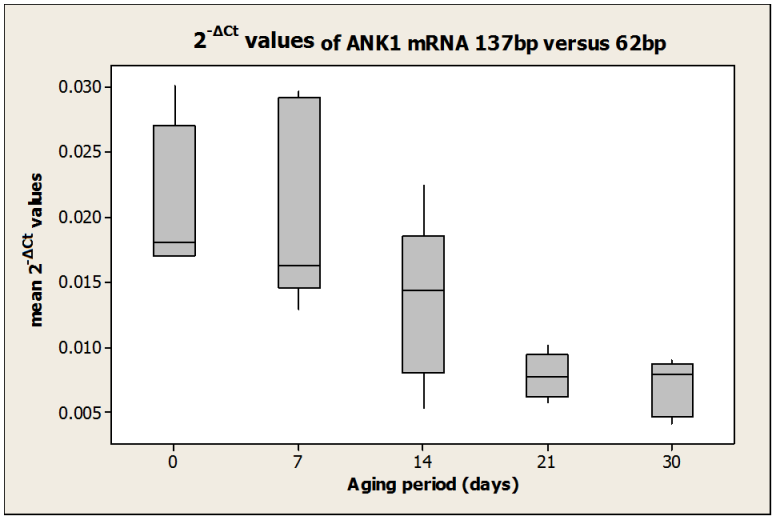

Figure 8-4: A boxplot showing $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp by age of an *ex vivo* bloodstain. Boxplot was obtained from the data shown in **Table 8-7** using Minitab® 16 software.

Figure 8-5 shows that plotting the mean $2^{-\Delta Ct}$ values against the sample age also indicates that mean $2^{-\Delta Ct}$ values decrease with time, and can therefore be used to predict the age of a bloodstain. This indicates that the two examined amplicons differ in their degradation rate where the larger one, i.e. the 137 bp length amplicon shows a higher tendency to degrade than the smaller one, i.e. the 61 bp length amplicon.

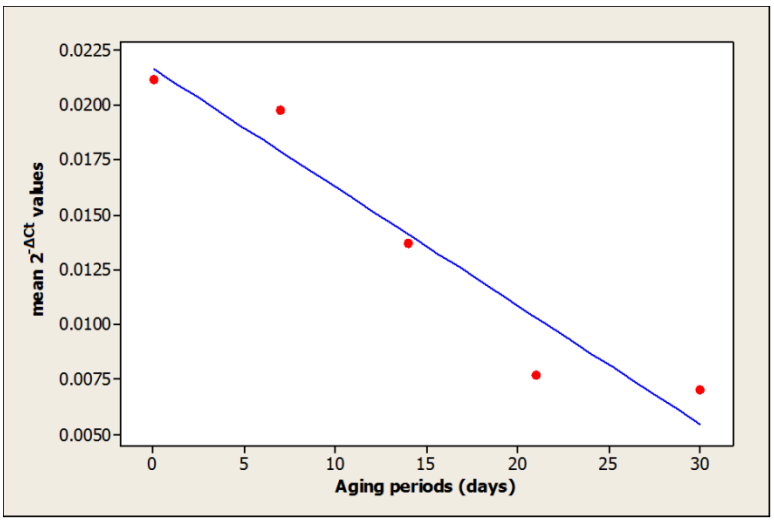

Figure 8-5: Scatterplot of the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp against the age of the bloodstain. Figures show that $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp decrease with the bloodstains' age increase**.**

The relationship between the age of the bloodstain and the obtained mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp was statistically calculated. The Anderson-Darling normality test was carried out on the mean $2^{-\Delta Ct}$ values and on the aging period to test the normality of the data. The test shows that the variables were not normally distributed. Therefore, nonparametric analysis was adopted for statistical analysis. Spearman's correlation indicates that there is a strong linear negative correlation between the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp and the age of bloodstains (Spearman's r = 0.961; p-value= 0.009). However, this correlation does not specify which value affects the other.

Regression analysis was applied on the data obtained by plotting the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp against the age of blood in order to estimate the relationship between the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp and the bloodstain's age. Fitting the data on a simple linear model demonstrates the decrease in mean $2^{-\Delta Ct}$ values with the increase in the stain's age (R^2= 89.8%) (Figure 8-6). The age of the bloodstain can be estimated based on the mean $2^{-\Delta Ct}$ values using a linear model (R^2= 89.8 %):

$$y = 38.09 - 1707\ x \qquad \text{(Equation 8-1)}$$

where y is the age of the bloodstain in days and x is the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp.

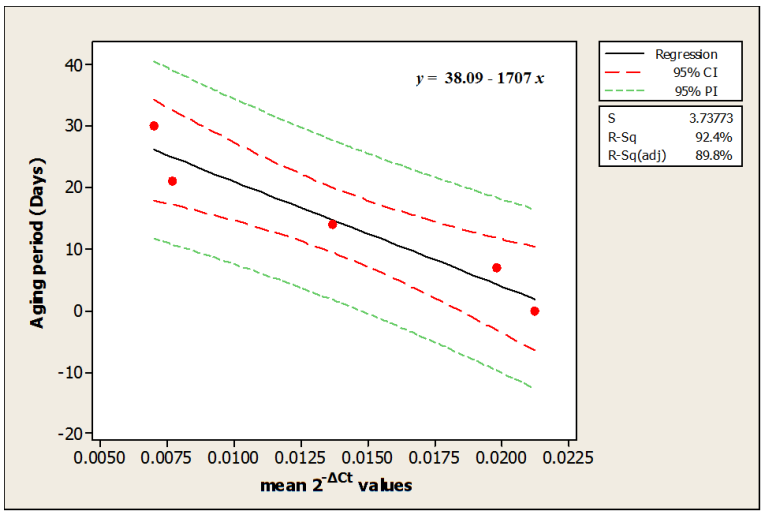

Figure 8-6: Time-wise trend in the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp of blood samples over time. The curve was obtained from the data shown in Table 8-8 using Minitab® 16 software.

The results of the analysis of variance (ANOVA) test performed on the mean $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 bp showed that the difference in the means of different sample ages was highly significant (*p value* <0.001). The results also showed that the difference in means between individuals at specific ages of bloodstains is not significant (*p value* >0.05). The results of ANOVA test are shown in Appendix 17 and Appendix 18. These results illustrated that the only significant factor contributing to the differences in the $2^{-\Delta Ct}$ values of samples was the age of the bloodstain.

8.2.2.2 Evaluation of ALAS2 mRNA 128 bp - 62 bp amplicon combination

In this study a multiplex of a pair of TGEAs, namely assay ID Hs01085693_m1 and assay ID Hs01085699_m1, was used to simultaneously confirm the type of bloodstain and determine its age. This TGEA pair represents different-sized segments of ALAS2 mRNA (128 bp and 62 bp amplicon length) (Figure 8-7).

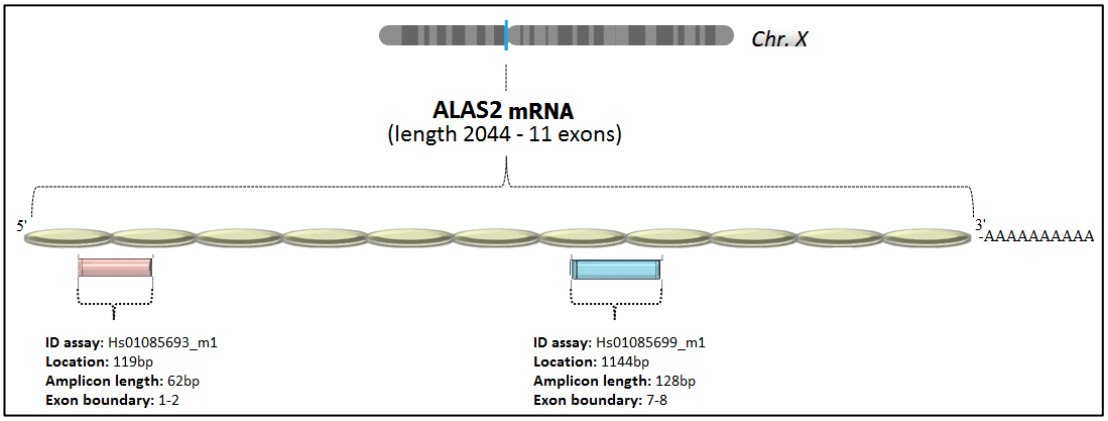

Figure 8-7: Locations of the TGEAs on the ALAS2 mRNA. Figure shows the locations and amplicon lengths of the TGEA pair (assay ID Hs01085693_m1 and assay ID Hs01085699_m1) on the ALAS2 mRNA.

8.2.2.2.1 The $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp - 62 bp and trend analysis

Table 8-9 shows the mean raw fluorescent Ct values of a TGEA pair of the ALAS2 (assay ID Hs01085693_m1, labelled with VIC dye, and assay ID Hs01085699_m1, labelled with FAM dye). The raw Ct values were measured using real-time PCR at 0, 7, 14, 21, and 30 days. They were then corrected with the efficiency of the reaction using GenEx statistical software (Table 8-10). Table 8-11 illustrates the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp that were calculated from the corrected Ct values for blood samples aged for up to 60 days.

Table 8-9: Mean raw fluorescent Ct values of ALAS2 mRNA 128 bp – 62 bp amplicon combination from six donors at 0, 7, 14, 21, and 30 days before efficiency correction. The mean Ct value is calculated by the software of Stratagene Mx3005P™, where each sample is run in duplicate. The Ct values of each individual sample shown in Appendix 19.

Donors n=6	Mean Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	29.03	29.15	28.91	29.44	31.42	31.47	30.1	29.9	31.81	31.43
2	35.33	34.88	33.57	32.68	32.97	31.96	35.86	34.65	31.68	31.58
3	25.61	25.88	30.48	30.24	32.16	31.22	32.91	32.51	31.93	31.43
4	31.98	31.71	36.04	35.81	33.11	31.96	37.24	36.85	34.46	34.08
5	33.57	33.37	31.42	30.23	36.04	35.15	32.91	32.01	37.24	36.33
6	32.97	32.76	30.70	30.45	35.86	34.97	30.48	30.51	32.97	32.01

Table 8-10: Corrected Ct values of ALAS2 mRNA 128 bp – 62 bp amplicon combination from six donors at 0, 7, 14, 21, and 30 days after efficiency correction using GenEx software. The data in this table are obtained from correcting the mean raw Ct values in Table 8-9.

Donors n=6	Mean corrected Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	29.13	28.75	29.01	29.03	31.53	31.04	30.81	29.49	31.92	30.00
2	35.46	34.40	33.69	32.83	33.09	31.52	35.99	34.17	31.99	30.55
3	25.70	25.52	30.59	29.82	32.28	30.79	33.03	32.06	32.05	31.00
4	32.10	31.27	36.17	35.32	33.23	31.52	37.37	36.34	34.58	33.61
5	33.69	33.08	31.53	29.97	36.17	34.85	30.59	29.25	33.09	31.74
6	33.09	32.48	30.81	30.19	35.99	34.67	33.03	31.74	37.37	36.02

Table 8-11: The $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp for samples at 0, 7, 14, 21, and 30 days. The $2^{-\Delta Ct}$ values were calculated from the corrected Ct values in **Table 8-10** by subtracting the Ct values obtained for the short amplicon from those of the long amplicon.

Donors *n*=6	$2^{-\Delta Ct}$ values of ALAS2 mRNA 137 bp versus 62 bp				
	day 0	day 7	day 14	day 21	day 30
1	0.77	1.01	0.71	0.40	0.26
2	0.48	0.55	0.34	0.28	0.37
3	0.88	0.59	0.36	0.51	0.48
4	0.57	0.55	0.31	0.49	0.51
5	0.65	0.34	0.40	0.39	0.39
6	0.65	0.65	0.40	0.41	0.39

The results of this study show the relationship between the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp extracted from a blood sample and the age of the sample. Table 8-12 shows that the means of the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp decrease with an increase in the sample age. Figure 8-8 also shows this decreasing trend in the $2^{-\Delta Ct}$ values with increasing sample age.

Table 8-12: Statistical analysis of the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp of samples at 0, 7, 14, 21, and 30 days. Statistical calculations were carried out using Minitab® software.

	Mean $2^{-\Delta Ct}$ values	SD[a]	CV%[b]	Variance
day 0	0.67	0.14	21.39	0.02
day 7	0.62	0.22	35.93	0.05
day 14	0.42	0.15	35.09	0.02
day 21	0.42	0.08	19.47	0.01
day 30	0.40	0.09	22.01	0.01

[a] Standard deviation [b] Coefficient of variation

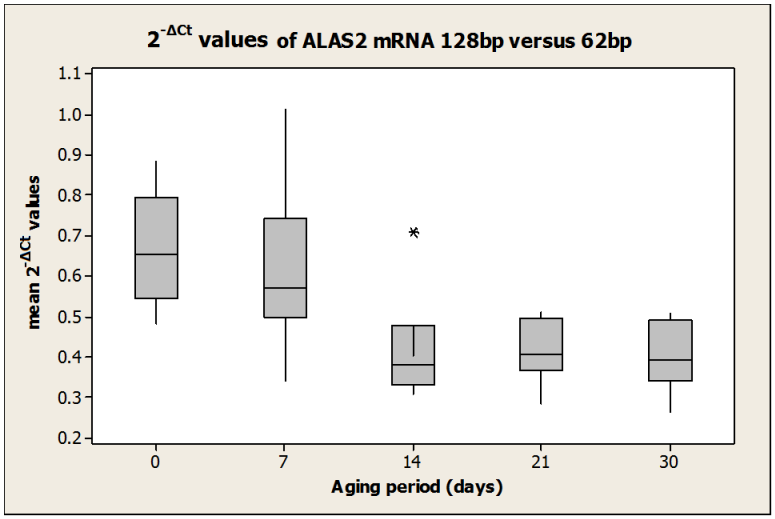

Figure 8-8: A boxplot showing $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp by age of an *ex vivo* bloodstain. Boxplot was obtained from the data shown in Table 8-11 using Minitab® 16 software.

Figure 8-9 shows that plotting the mean $2^{-\Delta Ct}$ values against the sample age also indicates that mean $2^{-\Delta Ct}$ values decrease with time, and can therefore be used to predict the age of a bloodstain. This indicates that the two examined amplicons differ in their degradation rate where the larger one, i.e. the 128 bp length amplicon, shows a higher tendency to degrade than the smaller one, i.e. the 61 bp length amplicon. As shown in Figure 8-9, the mean $2^{-\Delta Ct}$ values decrease sharply in the first two weeks and decrease gradually therefore for the rest of the month.

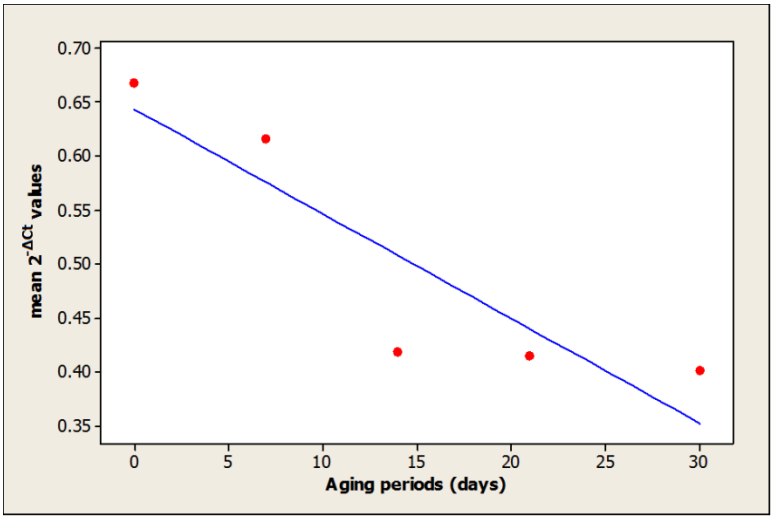

Figure 8-9: Scatterplot of the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp against the age of the bloodstain. Figures show that $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp decrease with an increase in the bloodstains' age**.**

The relationship between the age of the bloodstain and the obtained mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp was statistically calculated. Spearman's correlation indicates that there is a strong linear negative correlation between the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp and the age of bloodstains (Spearman's r = 0.89; p-value= 0.041). however, this correlation does not specify which value affects the other.

Regression analysis was applied on the data obtained by plotting the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp against the age of blood in order to estimate the relationship between the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp and the bloodstain's age. Fitting the data on a simple linear model demonstrates the decrease in mean $2^{-\Delta Ct}$ values with the increase in stain's age (R^2= 73.1 %) (Figure 8-10). The age of the bloodstain can be estimated based on the mean $2^{-\Delta Ct}$ values using a linear model (R^2= 73.1 %):

$$y = 55.75 - 82.07\,x \qquad \text{(Equation 8-2)}$$

where y is the age of the bloodstain in days and x is the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp.

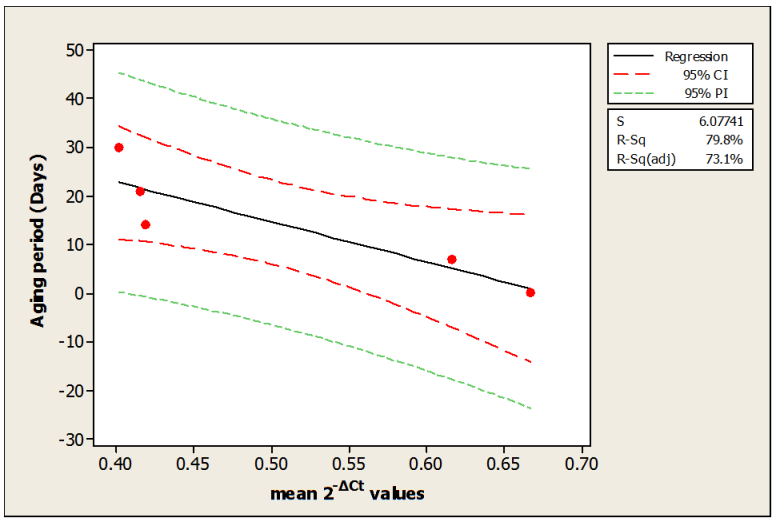

Figure 8-10: Time-wise trend in the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp of blood samples over time. The curve was obtained from the data shown in Table 8-12 using Minitab® 16 software.

The results of the analysis of variance (ANOVA) test performed on the mean $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 bp showed that the difference in the means of different sample ages was highly significant (*p value* <0.01). The results also showed that the difference in means between individuals at specific ages of bloodstains is not significant (*p value* >0.05). The results of the ANOVA test are shown in Appendix 20 and Appendix 21. These results illustrated that the only significant factor contributing to the differences in the $2^{-\Delta Ct}$ values of samples was the age of the bloodstain.

8.2.3 Conclusion

In this study, blood specific RNA markers were used to determine the age of the bloodstains. As the different-sized segments of RNA have different stability tendencies, this study was carried out to find the relationship between the age of the bloodstains and the relative stability of different-sized segments of the same blood-specific mRNA markers. A multiplex of TGEAs of the same blood specific mRNA markers were used to determine the relative quantity between them using the $2^{-\Delta Ct}$ method. Two pairs of TGEAs for two blood specific mRNA markers (ANK1 mRNA 137 bp - 62 bp, and ALAS2 mRNA 128 bp - 62 bp amplicon combinations) were

chosen based on the specific criteria that were set to identify the most appropriate TGEAs for this study.

The results show that there is a relationship between the $2^{-\Delta Ct}$ values of both chosen TGEA pairs and the age of bloodstains. It was found that the $2^{-\Delta Ct}$ values of both ANK1 mRNA 137 bp - 62 bp, and ALAS2 mRNA 128 bp – 62bp decrease with an increase in the age of the stain. These values can therefore be used to estimate the age of the stain. This finding indicates that the examined TGEA pair of ANK1 mRNA 137 bp - 62 bp and TGEA pair of ALAS2 mRNA 128 bp - 62 bp can be used to estimate the age of bloodstains. However, the $2^{-\Delta Ct}$ values of the ANK1 mRNA 137 bp - 62 bp showed stronger correlation with the age of bloodstains than the TGEA pair of ALAS2 mRNA 128 bp – 62 bp. This may have resulted from the difference in the amplicon product size of the chosen TGEAs.

To conclude, these results confirm the applicability of using two different-sized segments of the same tissue-specific mRNA markers for simultaneous identification of body fluid type and determination of its age.

9 General discussion and conclusion

9.1 General discussion

The main purpose of the work in this thesis is concerned with certain factors affecting the determination of the age of biological stains by means of the analysis of ribonucleic acids. The body fluids blood and saliva were chosen to be analysed because they are frequently encountered at crime scenes with an emphasis on saliva stains as they have not been tested for this purpose before.

The thesis encompasses two distinct bodies of work in determining the age of biological stains. The first part the work focused on finding a relationship between their age and the ratio between two species of the housekeeping gene RNA i.e. β-actin mRNA and 18S rRNA, as used in a previous study on bloodstains. The second part is a novel work concerning simultaneous determination of the age of a saliva stain and the identification and confirmation of its type. The age of the saliva stain was determined using the relative quantity of two different-sized segments of the same saliva-specific mRNA markers quantified using a pair of TGEAs. The latter was also applied to a bloodstain as a novel method of determining the age of bloodstains. The adoption of mRNA species that are also specific to the body fluid type might also be a means of concerning the cross contamination of housekeeping mRNA from different fluids, and which might be expected to occur initially at different relative levels in different fluids. This has been explored in more detail in subsequent sections of the work.

The results of the studies carried out in this thesis confirm some of the hypotheses proposed in the thesis.

9.1.1 Age determination using the RQRs of β-actin mRNA and 18S rRNA

The work in this part demonstrates many hypotheses proposed in the thesis and the findings are directly related to the forensic field and contribute to it.

Firstly, the results of the replicating Anderson *et al.* [49] work confirm the possibility of replicating the work. In addition, many discrepancies were found in the original

published work article between the results reported and the calculation method described. It is believed that these discrepancies may affect the replication of the work using the calculation described. Thus the comments made in this thesis about the discrepancies in Anderson *et al.* [49] work are intended to amend the calculation method used in calculating the RQR values. This will result in correctly calculating the correlation between the age of biological stain and RQR values. (This study is thoroughly discussed in section 3.3.3.4).

Secondly, the results of the study carried out on saliva stains confirm the relationship between the age of saliva stains and the RQR values of β-actin mRNA to 18S rRNA. They are also confirm the hypothesis proposed in this thesis about possibility of extending of the work carried out by Anderson *et al.* [49] on saliva stains. In addition, same finding were found using either stabilised- or unstabilised saliva stains.

Thirdly, the changes to the original method carried out by Anderson *et al.* [49], such as the use of column-based extraction techniques and the adoption of a reverse transcription kit, SuperScript® III First-Strand Synthesis System, were found to offer many advantages. The use of the column-based extraction techniques exhibit many advantages with respect to time, safety and the facility for automation. The column-based extraction method was also found to be able to extract RNA from very small amounts of starting material with a high purity and quality. However, it suffered from a poorer efficiency of extraction being recovered compared to the phenol/chloroform methods.

The SuperScript® III First-Strand Synthesis System was found to offer greater sensitivity, enabling the detection of template from as little as 1.0 pg.

Fourthly, studies were carried out to evaluate the effect of the RNA*later*® reagent on the stability of the RNA molecules in dried saliva stains. The results demonstrate that RNA*later*® reagent is unable to stabilise all of the salivary transcriptome effectively in dried saliva stains. However, it has been found that RNA*later*® has a positive effect on the peak heights of the STATH profile and may be considered as a possible application for RNA*later*® in the forensic field.

Fifthly, the study of the effects of mixtures of saliva and blood on the RQR values demonstrated that RQR values changed significantly. The change in the RQR values may result in under- or over-estimating the age of the stains. These results reveal the difficulties of using of the housekeeping gene RNA for the purpose of establishing the age of either bloodstains or saliva stains. This is particularly so in cases of mixtures with visually unseen stains such as saliva. In addition, these findings illustrate the importance of carrying out an independent body fluid identification test using the housekeeping gene to determine the age of bloodstains or other body fluids. This is particularly important in forensic case work where the source of the stains will, by definition, be unknown.

9.1.2 Simultaneous determination of the age and tissue type

In this part of the thesis many findings were demonstrated. Firstly, the work in this part of thesis confirms the relationship between the age of saliva stains and the $2^{-\Delta Ct}$ values of the TGEAs pair consisting of a combination of assays ID Hs00361611_m1 (76 bp amplicon) with ID Hs00361610_g1 (61 bp amplicon). This finding shows the possibility of simultaneous determination of the age of the type of saliva stain depending on the relative quantity of two different-sized segments of the same saliva-specific mRNA markers, quantified using TaqMan® Gene Expression Assays (TGEA).

Secondly, it was found that this novel method of simultaneous determination of the age of the type of biological stain can be applied out bloodstains. It was found that there is a relationship between the bloodstain's age and the $2^{-\Delta Ct}$ values of both chosen TGEA pairs. The first TGEA pair consists of assay ID Hs00986664_m1 and assay ID Hs00986655_m1, forming the ANK1 mRNA 137 bp - 62 bp amplicon combination. The second TGEA pair consists of assay ID Hs01085693_m1 and assay ID Hs01085699_m1 forming the ALAS2 mRNA 128 bp - 62 bp amplicon combination. The ANK1 mRNA 137 bp - 62 bp amplicon combination showed a stronger correlation with the age of bloodstains than the TGEA pair of ALAS2 mRNA 128 bp – 62 bp amplicon combination. The difference in the size of amplicon products may contribute to this difference in the strength in the correlation between the bloodstain's age and the chosen TGEAs.

Thirdly, the results of the analysis of TGEAs pair consisting of assay ID Hs00264790_m1 and assay ID Hs04194749_g1, forming the HTN3 mRNA 136 -79 bp amplicon combination, indicated that the most likely degradation mechanism for the HTN3 mRNA is the stochastic degradation mechanism. However, further studies are recommended to confirm this finding.

9.1.3 Further Observations

The work on saliva stains shows a high level of inter- and intra-person variation despite setting controlled conditions for collection to decrease the effect of external materials such as food and tobacco smoke. The high level of variation was noticed in both methods using housekeeping gene mRNA or saliva-specific mRNA. This high level of variation could be due to the variation between individuals, or within the same individual from time to time, affecting the degradation status of RNA molecules in saliva. The presence of various types of ribonucleases in the saliva may also contribute to such variation.

The results of quantification of the total RNA show that a continual decline was noticed in the amount of RNA extracted over the 63 days. Total RNA isolated from 0- to 63-day-old 50 µL spots of saliva sample on cotton swab, was estimated as a 6% loss of total RNA from 0 to 7 days, 28% loss from 0 to 14 days, 35% loss from 0 to 35 days and a 50% loss from 0 to 63 days. In addition, the amount of the RNA extracted from samples at 63-days-old was still for subsequent processing using qPCR. In addition the Bioanalyzer results show that the RNA extracted was degraded, or partially degraded, and no intact RNA was extracted even from freshly dried samples.

There are many challenges to be faced to implement the techniques described in this thesis to determine the age of biological stains depending on the degradation status of RNA molecules. Some of these challenges are due to the nature of the RNA molecules analysed and the constituents of the biological stains as well as others due to the techniques used.

The first challenge that may be faced is due to the nature of RNA molecules where that results of the analysis affected by the contamination of the examined stain with a different type of body fluid when housekeeping gene RNA is used in analysis.

The second challenge that may be faced is that due to the constituents of the biological stains resulting in the high levels of inter- and intra- person variation. This can be noticed when analysing saliva stains compared to bloodstains and hair samples.

The limitations of the technology used in reverse transcription and also that used in real time PCR contribute to the limitations of the technique. This is can be consider as the third challenge may be faced.

Although guidelines were suggested to overcome some of the limitations of dealing with the quantitative real-time PCR experiments such as the "minimum information for publication of quantitative real-time PCR experiments" (MIQE) guidelines, these guidelines were set to be used for clinical samples containing intact RNA not as those samples encountered at crime scenes where the RNA is mostly degraded. Therefore, guidelines for the analyses of samples stored under harsh conditions may be required to be set to overcome the limitation of using degraded RNA as well as the real time PCR limitations, in forensic case work. Such guidelines may improve the ability of replicating and confirming results, either by a different individual or in different labs.

9.2 Conclusion

This work has demonstrated that there is a relationship between the age of saliva stains and the RQR values of β-actin mRNA to 18S. In addition, contamination of the examined saliva stain with other body fluids may affect the RQR values, resulting in an over- or under-estimation of the age of the stain. Therefore, body fluid identification testing should be carried out in parallel to avoid the effect of mixtures on the RQR values. However, further validation studies are required to be used for prediction at high degree of accuracy and precision.

This work has also demonstrated the advantage of using the relative quantity of two different-sized segments of the same tissue-specific mRNA markers, in order to simultaneously determine the stain's age and type. A relationship was found between the age of saliva stains and the $2^{-\Delta Ct}$ values of TGEAs pair of KRT4 mRNA consisting of assay ID Hs00361611_m1 and assay ID Hs00361610_g1.

In addition, the later method was also applied to bloodstains where there was a relationship between the bloodstain's age and $2^{-\Delta Ct}$ values of both the chosen TGEA pair of ANK1 mRNA consisting of assay ID Hs00986664_m1 and assay ID Hs00986655_m1, and between the bloodstain's age and $2^{-\Delta Ct}$ values of the both chosen TGEA pair of ALAS2 mRNA consisting of assay ID Hs01085693_m1 and assay ID Hs01085699_m1. These pairs can be used to estimate the age of the bloodstains.

However, the main limitation of applying these two methods of determination of the age of saliva stains (i.e. using of RQR values of β-actin mRNA to 18S or using the $2^{-\Delta Ct}$ values of TGEAs pair of KRT4 mRNA) in casework is the nature of saliva itself where a high level of inter- and intra-variations were found. This high variation will result in low precision and a possible lack of accuracy in saliva age determination, which can be considered as a limitation to applying this approach in casework. In addition, the effects of unpredictable environmental conditions on the stain were not addressed in this work.

Therefore, validation studies are required for these TGEA pairs that showed a relationship between the bloodstain's age and $2^{-\Delta Ct}$ values of the chosen pair. These validation studies are recommended to be carried out on samples from larger number of volunteers and involving longer period of time. In addition, many factors need to be considered in these validation studies including specificity, accuracy, precision, robustness, prediction limit, inter- and intra-person variations and age and gender variations.

9.3 Recommendations for future work

Throughout the carrying out of this work a number of interesting questions have been raised which have opened up several paths for more research work

Firstly, the success of applying the method of determination of the stain age using the RQR values of the β-actin mRNA to18S rRNA despite the high level of inter- and intra- person variation, opens up the path for other body fluids that may characterized with lower levels of variability, such as semen. As semen is another common body fluid found at crime scenes, determination of its age may help the investigation process in sexual assault crimes. There is a need to find the time of deposition of semen in cases of rape. However, there is a possibility of a mixture effect on the semen age determination. The mixture effect could be due to mixture with vaginal secretions. Therefore, in case of using RQR method, the effect of vaginal secretions needs to be assessed.

Secondly, one might conceive of a commercial kit, in which external standards, consisting of pre-prepared cDNA from different stains stored under specific conditions and spanning different periods of time with one week intervals, is used. These would be used as standards of RQR values of the β-actin mRNA to18S rRNA to estimate the age of a stain stored under similar conditions. These standards will be used to establish the curve of the relationship between the stain age and the RQR values of the β-actin mRNA to18S rRNA that will be used to estimate the age of tested stain. Running the standards in the same run in the parallel to the examined stain will decrease the batch to batch or lab to lab variations. This standard can also be used with other methods of determining the age of the stain, depending on the $2^{-\Delta Ct}$ values of two different-sized segments of the same tissue-specific mRNA markers quantified using a pair of TGEAs. Different standards of this kind might be prepared using different storage conditions, to more closely match the different climates in different countries.

Thirdly, as a correlation was found between the age of a stain and the $2^{-\Delta Ct}$ values of two different-sized segments even when the off-the-shelf TGEAs pair used does not fulfil all criteria required, the design of a TGEAs pair fulfilling all the suggested

criteria, particularly a large difference in size between the tested segments, will increase the chance of obtaining a more precise age determination, especially with body fluids showing low levels of inter- and intra-person variation.

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Appendices

Well	Dye	Well Type	Threshold (dRn)	Ct (dRn)	Quantity (copies)	RSq (dRn)	Slope (dRn)
A1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
A1	VIC	Standard	0.0192	23.78	1.00E+01	0.961	-3.442
A1	FAM	Standard	0.0064	30.17	1.00E+01	0.959	-3.789
B1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
B1	VIC	Standard	0.0192	25.67	5.00E+00	0.961	-3.442
B1	FAM	Standard	0.0064	32.3	5.00E+00	0.959	-3.789
C1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
C1	VIC	Standard	0.0192	26.36	2.50E+00	0.961	-3.442
C1	FAM	Standard	0.0064	33.07	2.50E+00	0.959	-3.789
D1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
D1	VIC	Standard	0.0192	27.39	1.25E+00	0.961	-3.442
D1	FAM	Standard	0.0064	33.95	1.25E+00	0.959	-3.789
E1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
E1	VIC	Standard	0.0192	28.1	6.25E-01	0.961	-3.442
E1	FAM	Standard	0.0064	35.05	6.25E-01	0.959	-3.789
F1	ROX	Standard	Reference	Reference	Reference	Reference	Reference
F1	VIC	Standard	0.0192	No Ct	No Ct	0.961	-3.442
F1	FAM	Standard	0.0064	No Ct	No Ct	0.959	-3.789

Appendix 1: Values obtained by Stratagene Mx3005P™ to form the Standard curves for β–actin and 18S rRNA shown in Figure 3-4.

Table (A): Fluorescent Ct values of the first analysis of β-actin and 18S of six samples at age 0 to 60 days

Samples n=6	Ct Values																			
	day 0		day 1		day 2		day 3		day 7		day 14		day 21		day 28		day 52		day 60	
	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S
1	38.04	23.40	39.77	25.79	39.86	31.48	39.91	36.63	39.63	33.46	32.33	22.01	37.45	28.60	39.25	27.29	36.31	26.02	39.20	26.71
2	32.69	26.65	29.29	25.16	37.54	33.16	34.67	24.34	39.77	25.08	33.81	23.34	35.59	25.69	37.41	26.88	33.43	25.60	34.86	25.31
3	36.38	26.60	33.77	23.85	38.28	34.38	36.44	24.00	37.43	25.46	34.96	25.71	35.56	25.72	31.47	21.93	30.59	22.62	33.31	23.92
4	36.51	25.72	33.32	23.90	39.05	32.00	35.30	29.05	33.12	25.31	34.74	25.28	35.57	24.18	33.53	20.99	32.60	21.85	35.35	20.88
5	34.13	23.68	39.80	26.44	39.47	27.13	36.12	26.90	39.15	34.64	35.34	24.78	34.70	25.69	36.92	23.52	37.32	22.12	33.67	24.34
6	35.26	24.06	37.15	24.54	31.05	25.45	37.22	32.85	33.35	34.77	33.76	22.45	35.28	25.70	33.13	20.44	36.83	24.15	35.58	22.39

Table (B): Fluorescent Ct values of the second analysis of β-actin and 18S of six samples at age 0 to 60 days

Samples n=6	Ct Values																			
	day 0		day 1		day 2		day 3		day 7		day 14		day 21		day 28		day 52		day 60	
	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S
1	38.36	23.65	37.08	25.33	37.02	31.02	No Ct	34.61	38.18	31.44	32.65	26.54	38.21	26.12	37.80	27.33	34.75	23.33	35.79	24.23
2	32.94	29.41	30.11	27.92	35.31	34.62	34.99	24.66	37.29	25.90	32.25	21.89	35.91	25.94	37.16	28.34	33.68	27.06	34.40	26.77
3	34.82	27.60	36.53	23.89	36.37	32.93	34.42	27.73	35.98	28.69	35.78	23.48	32.87	24.16	32.18	22.25	30.34	23.62	37.04	21.23
4	37.51	23.49	33.64	24.15	36.82	32.25	33.85	31.81	33.16	25.63	34.99	23.83	33.34	25.00	31.51	21.24	30.37	19.62	37.65	21.13
5	35.59	24.39	36.54	24.99	39.89	28.59	33.43	27.72	43.15	36.10	33.89	22.30	38.43	27.15	37.92	22.07	39.08	27.15	33.88	25.80
6	33.81	23.81	35.59	24.29	33.81	22.97	35.77	30.83	33.21	32.75	36.99	23.27	35.03	28.00	33.34	25.47	37.83	22.13	33.67	21.93

Appendix 2: Fluorescent Ct values of β-actin and 18S for six samples at age 0 to 60 days. Samples were run in triplicate and the results of the first analysis were shown in Table (A), the results of the second analysis are shown in Table (B) and the results of the third analysis are shown in Table (C). Table (C) is in the next page.

Table (C): Fluorescent Ct values of the third analysis of β-actin and 18S of five samples at age 0 to 60 days

Samples n=6	Ct Values																			
	day 0		day 1		day 2		day 3		day 7		day 14		day 21		day 28		day 52		day 60	
	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S
1	41.71	28.64	39.76	30.24	37.21	35.93	39.95	37.33	39.76	34.16	36.00	21.47	35.37	28.38	39.38	32.74	36.55	26.01	37.12	26.49
2	37.93	22.57	33.96	21.08	38.45	30.38	38.34	28.01	39.55	29.75	34.05	23.47	39.26	30.93	41.65	24.10	38.67	22.82	39.31	22.53
3	36.62	22.90	29.69	29.30	37.49	34.51	37.14	25.76	37.56	26.22	39.63	26.62	35.55	25.96	36.25	25.60	34.83	18.92	35.07	23.91
4	32.81	26.63	36.99	29.14	39.96	37.24	35.43	24.97	38.57	28.98	39.98	25.41	36.48	28.85	34.23	26.23	33.51	22.76	30.35	26.12
5	31.35	28.46	No Ct	26.57	39.95	24.35	36.11	31.57	37.55	31.86	35.47	24.56	36.46	22.91	33.22	23.65	34.24	22.58	37.45	21.56
6	35.39	28.30	37.39	28.78	26.97	25.23	37.35	33.55	37.81	34.09	34.52	27.12	39.52	20.70	36.91	20.90	33.13	24.85	34.79	26.84

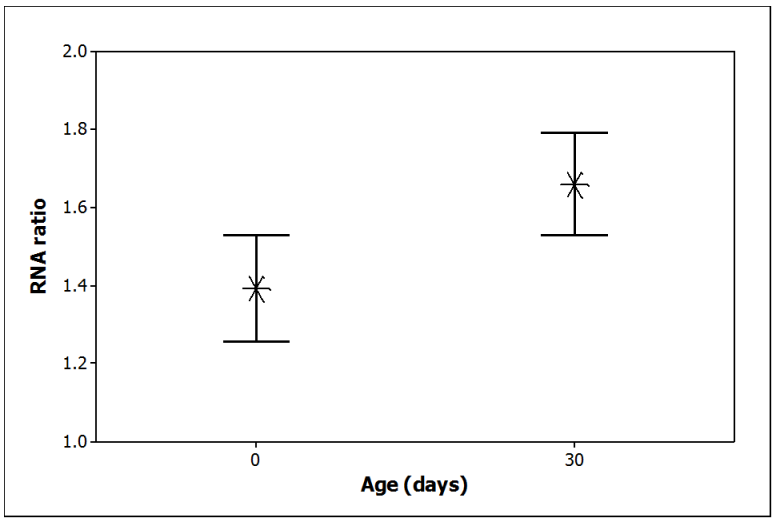
(Appendix 2: continued).

SUMMARY	Count	Sum	Average	Variance
day 0	6	8.23	1.371667	0.007177
day 1	6	8.42	1.403333	0.006947
day 2	6	8.98	1.496667	0.001387
day 3	6	9.00	1.50	0.02376
day 7	6	9.05	1.508333	0.034337
day 14	6	10.50	1.75	0.0002
day 21	6	10.01	1.668333	0.005417
day 28	6	10.56	1.76	0.0104
day 52	6	10.66	1.776667	0.003827
day 60	6	10.67	1.778333	0.001697
1	10	15.63	1.563	0.019757
2	10	16.08	1.608	0.033551
3	10	16.27	1.627	0.029379
4	10	16.01	1.601	0.031232
5	10	16.19	1.619	0.041099
6	10	15.9	1.59	0.052044

Appendix 3; summary of the data obtained by ANOVA: Two-Factor without Replication

Source of Variation	SS	df	MS	F	P-value	F crit
Sample age	1.41396	9	0.157107	15.72464	3.29E-11	2.095755
individuals	0.026133	5	0.005227	0.523132	0.757449	2.422085
Error	0.4496	45	0.009991			

Appendix 4: Results of the Analysis of variance (ANOVA) of the means RQRs shown in Table 3-3.



Appendix 5: Results of the analysis of bloodstains at age 0 and 30 days of repeating study of Anderson *et al.* [49] work. Data represents the ratio of β-actin mRNA to 18S rRNA as determined by real-time reverse transcriptase PCR. Each data point represents mean of n=6 ±1 S.D.

	Un-stabilised saliva stain				Stabilised saliva stain			
	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
	β-actin	18S	β-actin	18S	β-actin	18S	β-actin	18S
1	35.02	18.74	34.98	18.3	34.02	15.1	34.13	16.21
2	35.08	17.09	39.92	25.58	34.28	15.67	33.43	15.64
3	35.65	19.53	35.94	19.92	33.79	14.63	33.47	15.31
4	36.87	19.18	37.89	19.53	36.49	17.42	36.88	17.63
5	38.83	18.36	38.44	18.31	39.68	20.38	39.68	28.42
6	39.65	21.64	39.24	21.17	39.72	19.25	38.98	18.63
7	39.98	23.26	39.17	23.15	39.15	19.55	39.05	20.2
8	31.76	22.09	39.46	22.23	38.72	19.8	39	19.79
9	34.06	24.08	39.57	23.59	39.62	20.19	40.55	20.35
10	39.9	23	38.48	22.56	38.82	21.08	39.77	20.94
11	38.5	21.46	39.7	21.41	38.1	20.3	38.92	20.3
12	39.5	24.04	38.5	23.67	39.71	20.54	39.9	20.69
13	38.34	21.53	37.77	21.77	37.41	18.16	37.31	18.69
14	39.47	20.42	40.18	21.37	38.48	18.55	38.04	18.69
15	39.51	20.83	38.99	21.27	36.83	18.32	37.2	18.49

Appendix 6: Ct values of β-actin mRNA and 18S rRNA for stabilised and un-stabilised old saliva stains. The un-stabilised saliva stains were immediately extracted without stabilisation and the stabilised saliva stains were extracted after RNA*later*® stabilisation.

Table (A): Fluorescent Ct values of the first analysis of β-actin and 18S											
Donors n=5	**Occasions**	**Mean Ct Values**									
		day 0		**Day7**		**day 14**		**day 35**		**day 63**	
		B-actin	**18S**	**B-actin**	**18S**	**B-actin**	**18S**	**B-actin**	**18S**	**B-actin**	**18S**
1	1	25.78	20.33	No Ct	33.02	25.48	18.02	No Ct	31.18	37.69	28.86
	2	30.74	27.67	28.51	21.54	29.65	26.13	31.22	23.94	35.8	27.37
	3	35.53	28.42	29.91	23.28	33.27	26.24	30.00	20.40	No Ct	32.6
2	1	31.20	21.64	30.59	21.37	34.89	27.39	31.08	21.16	38.16	26.09
	2	34.97	26.31	38.05	27.35	28.56	18.83	38.14	27.28	No Ct	No Ct
	3	26.72	16.42	No Ct	32.79	40.8	31.07	35.34	24.11	36.82	28.64
3	1	32.84	25.36	34.73	26.53	36.25	27.41	33.8	24.86	32.78	21.06
	2	33.15	23.76	40.53	29.76	38.21	27.27	No Ct	26.42	37.52	24.76
	3	35.22	20.23	34.71	25.95	No Ct	No Ct	34.81	24.07	32.09	21.19
4	1	24.54	18.86	31.59	26.37	23.98	17.15	30.34	23.85	28.99	21.45
	2	29.06	24.21	32.11	24.97	33.92	25.79	33.56	25.67	31.84	22.87
	3	29.66	23.72	31.49	24.53	36.91	29.39	30.11	21.77	29.65	21.59
5	1	31.60	27.28	31.15	25.34	33.56	27.00	34.57	27.53	32.72	23.94
	2	27.40	20.64	28.78	23.01	34.88	27.53	33.11	22.94	35.78	27.48
	3	34.21	26.96	35.42	28.99	32.37	24.8	33.76	24.68	32.58	24.76

Appendix 7: Fluorescent Ct values of β-actin and 18S for 15 samples from five donors at three occasions at age 0 to 63 days. Samples were run in duplicate and the results of the first analysis were shown in Table (A) and the results of the second analysis are shown in Table (B). Table (B) is in the next page.

Table (B): Fluorescent Ct values of the first analysis of β-actin and 18S											
Donors n=5	Occasions	Mean Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S	B-actin	18S
1	1	25.91	20.02	No Ct	32.68	25.69	18.03	37.01	30.42	37.23	28.66
	2	30.36	27.22	27.51	21.41	29.37	25.55	30.69	23.78	35.3	27.4
	3	37.11	28.98	28.95	22.15	32.88	25.65	29.6	19.82	No Ct	33.08
2	1	30.99	21.88	30.33	21.2	34.81	27.86	30.14	20.01	37.83	25.63
	2	35.02	25.97	38.01	27.07	27.55	18.48	38.38	26.98	No Ct	37.46
	3	26.99	16.87	No Ct	32.18	No Ct	30.87	35.43	24.33	No Ct	30.39
3	1	29.6	23.16	34.24	26.12	36.58	27.73	34.15	25.11	30.92	20.34
	2	32.2	23.87	39.34	29.88	37.95	27.22	40.77	26.21	37.9	24.95
	3	33.74	20.19	34.57	26.27	No Ct	No Ct	34.49	23.7	31.81	21.63
4	1	24.84	18.64	31.17	26.22	23.97	17.26	29.3	23	28.51	20.93
	2	29.08	24.2	32.97	25.46	31.92	24.28	33.27	25.56	32.79	23.22
	3	30.14	24.45	30.85	23.83	37.2	29.12	30.66	21.92	30.3	22.17
5	1	30.35	25.96	31.14	25.08	33.53	26.92	35.23	28.65	30.63	23.06
	2	27.09	20.58	28.87	23.09	35.63	28.15	30.16	21.07	35.6	27.36
	3	34.46	26.69	35.01	29.22	30.79	24.28	33.08	24.47	32.54	24.98

(Appendix 7: continued).

SUMMARY	Count	Sum	Average	Variance
day 0	15	17.54	1.169333	0.006335
day 7	15	18.58	1.238667	0.004298
day 14	15	18.78	1.252	0.006846
day 35	15	19.67	1.311333	0.009241
day 63	15	19.81	1.320667	0.006564
1	5	6.18	1.236	0.00563
2	5	5.85	1.17	0.00765
3	5	6.29	1.258	0.00747
4	5	6.43	1.286	0.02308
5	5	6.67	1.334	0.00363
6	5	6.39	1.278	0.02252
7	5	6.47	1.294	0.00928
8	5	6.61	1.322	0.02197
9	5	6.57	1.314	0.00698
10	5	6.21	1.242	0.00577
11	5	6.19	1.238	0.00457
12	5	6.22	1.244	0.00388
13	5	5.92	1.184	0.00373
14	5	6.26	1.252	0.00517
15	5	6.12	1.224	0.00253

Appendix 8; summary of the data obtained by ANOVA: Two-Factor without Replication. The data values were calculated from the RQR values shown in Table 5-3.

Source of Variation	SS	df	MS	F	*P*-value	F crit
Sample age	0.225635	4	0.056409	10.19636	2.84E-06	2.536579
individuals	0.156168	14	0.011155	2.016337	0.032912	1.872588
Error	0.309805	56	0.005532			

Appendix 9: Results of the Analysis of variance (ANOVA) of the means RQRs shown in Table 5-3.

Table (A): Fluorescent Ct values of the first analysis of KRT4 mRNA 76 bp – 61 bp amplicon combination											
Donors n=5	**Occasion**	**Ct Values**									
		day 0		**Day7**		**day 14**		**day 35**		**day 63**	
		FAM	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**
1	1	27.01	26.25	37.04	36.6	25	24.76	34.66	35.24	33.56	33.02
	2	32.49	32.25	28.18	28.48	28.1	28.4	30.77	31.01	34.3	33.44
	3	33.98	34.09	28.43	28.88	30.44	30.42	25.33	24.29	38.94	36.63
2	1	30.06	29.44	28.52	26.9	37.07	34.95	27.84	26.62	33.84	31.68
	2	34.2	33.21	34.1	33.51	27.58	26.41	35.42	34.13	No Ct	No Ct
	3	25.9	24.55	37.57	37.11	37.18	36.94	32.28	30.51	37.02	36.12
3	1	31.67	31.34	33.59	33.55	35.19	34.48	36.58	34.51	28.49	27.33
	2	32.98	33.06	32.7	32.43	33.2	32.43	32.82	30.34	31.44	29.43
	3	28.71	26.86	31.83	31.18	30.57	30.49	30.34	29.02	32.73	31.27
4	1	30.3	31.01	31.11	31.97	31.69	31.42	31.81	32.02	28.07	27.86
	2	27.36	27.32	28.39	28.66	31.79	31.52	26.07	25.15	32.69	31.76
	3	31.99	32.53	33.58	34.66	28.77	29.15	32.56	31.9	33.01	32.7

Appendix 0-10: The raw fluorescent Ct values of KRT4 mRNA 76 bp – 61 bp amplicon combination for 15 samples from four donors on three occasions at age 0 to 63 days. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B). Table (B) is on the next page.

Table (A): Fluorescent Ct values of the first analysis of KRT4 mRNA 76 bp – 61 bp amplicon combination											
Donors n=5	**Occasion**	**Ct Values**									
		day 0		**Day7**		**day 14**		**day 35**		**day 63**	
		FAM	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**
1	1	27.01	26.25	37.04	36.6	25	24.76	34.66	35.24	33.56	33.02
	2	32.49	32.25	28.18	28.48	28.1	28.4	30.77	31.01	34.3	33.44
	3	33.98	34.09	28.43	28.88	30.44	30.42	25.33	24.29	38.94	36.63
2	1	30.06	29.44	28.52	26.9	37.07	34.95	27.84	26.62	33.84	31.68
	2	34.2	33.21	34.1	33.51	27.58	26.41	35.42	34.13	No Ct	No Ct
	3	25.9	24.55	37.57	37.11	37.18	36.94	32.28	30.51	37.02	36.12
3	1	31.67	31.34	33.59	33.55	35.19	34.48	36.58	34.51	28.49	27.33
	2	32.98	33.06	32.7	32.43	33.2	32.43	32.82	30.34	31.44	29.43
	3	28.71	26.86	31.83	31.18	30.57	30.49	30.34	29.02	32.73	31.27
4	1	30.3	31.01	31.11	31.97	31.69	31.42	31.81	32.02	28.07	27.86
	2	27.36	27.32	28.39	28.66	31.79	31.52	26.07	25.15	32.69	31.76
	3	31.99	32.53	33.58	34.66	28.77	29.15	32.56	31.9	33.01	32.7

Appendix 11: The raw fluorescent Ct values of KRT4 mRNA 76 bp – 61 bp amplicon combination for 15 samples from four donors on three occasions at age 0 to 63 days. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B). Table (B) is on the next page.

Table (B): Fluorescent Ct values of the first analysis of KRT4 mRNA 76 bp – 61 bp amplicon combination											
Donors n=5	**Occasion**	**Ct Values**									
		day 0		**Day7**		**day 14**		**day 35**		**day 63**	
		FAM	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**	**FAM**	**VIC**
1	1	26.87	26.27	37.06	37.15	25	24.79	34.21	34.26	33.27	32.54
	2	32.17	32.08	28.39	28.66	28.18	28.64	31.12	31.12	34.92	33.17
	3	33.13	33.98	28.48	28.98	30.59	30.28	25.64	24.46	37.99	35.74
2	1	30.07	29.46	28.33	26.9	35.48	34.73	28.05	26.74	33.88	31.66
	2	34.03	33.68	35.61	34.71	27.8	26.49	34.74	33.59	No Ct	No Ct
	3	26.01	24.62	39.43	38.96	37.92	39.31	32.05	29.87	36.51	35.72
3	1	31.94	31.22	33.92	33.28	35.15	34.8	36.33	34.68	28.73	27.49
	2	33.53	33.12	32.69	32.37	32.92	32.4	32.52	30.16	31.68	29.45
	3	28.66	26.9	31.79	30.86	30.96	30.43	30.49	29.04	33.05	31.55
4	1	30.23	30.44	31.12	31.73	31.3	31.43	31.65	31.96	28.2	28.06
	2	27.35	27.36	28.12	28.53	31.49	31.32	25.95	24.93	33.05	31.96
	3	31.83	32.33	34.19	35.10	29.28	29.24	31.63	31.34	32.4	32.26

(Appendix 11: continued).

SUMMARY	Count	Sum	Average	Variance
day 0	12	3.3	0.275	0.010245
day 7	12	3.21	0.2675	0.022511
day 14	12	2.96	0.246667	0.007279
day 21	12	2.26	0.188333	0.007724
day 30	12	1.74	0.145	0.005245
1	5	1.13	0.226	0.00323
2	5	1.33	0.266	0.01173
3	5	1.19	0.238	0.01567
4	5	0.63	0.126	0.00263
5	5	0.72	0.144	0.00038
6	5	0.81	0.162	0.00677
7	5	0.96	0.192	0.00077
8	5	0.64	0.128	0.00612
9	5	1.06	0.212	0.01472
10	5	1.85	0.37	0.00625
11	5	1.4	0.28	0.01105
12	5	1.75	0.35	0.01335

Appendix 12: summary of the data obtained by ANOVA: Two-Factor without Replication. The data were calculated from the $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 shown in Table 7-8.

Source of Variation	SS	df	MS	F	*p*-value	F crit
Sample age	0.150227	4	0.037557	7.495887	0.000107	2.583667
Individuals	0.362605	11	0.032964	6.579261	2.41E-06	2.014046
Error	0.220453	44	0.00501			

Appendix 13: Results of the Analysis of variance (ANOVA) of the means $2^{-\Delta Ct}$ values of KRT4 mRNA 76 bp versus 61 shown in Table 7-8.

Table (A): Fluorescent Ct values of the first analysis of KRT4 mRNA 80 bp - 61 bp amplicon combination											
Donors n=5	Occasions	Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	26.84	25.69	36.15	35.38	25.13	24	34.54	33.39	33.55	32.54
	2	32.69	31.47	28.39	27.35	28.74	27.91	31.27	30.48	33.82	32.9
	3	33.8	33.31	28.64	27.95	31.03	29.86	24.86	23.76	36.77	36.77
2	1	29.44	29.29	27.72	27.48	35.83	36.75	27.6	27.47	32.34	32.55
	2	32.91	33.46	33.31	33	26.57	26.62	33.76	33.81	No Ct	No Ct
	3	24.85	24.8	37.14	38.61	No Ct	38.3	31.02	31.01	36.12	36.44
3	1	31.29	31.23	33.02	33.22	34.5	34.62	31.72	32.14	27.78	27.73
	2	32.45	32.82	34.4	34.58	31.65	31.92	31.37	31.19	29.71	29.74
	3	27.68	27.19	30.95	30.86	30.27	31.02	29.41	29.51	31.78	31.54
4	1	26.3	25.15	32.25	31.67	25.52	24.26	30.04	29.34	27.8	26.8
	2	32.03	31.07	32.71	31.6	31.47	30.36	32.37	31.46	30.92	29.32
	3	30.94	29.72	29.73	28.67	36.37	36.14	28.01	26.9	28.47	27.39
5	1	32.24	31.12	32.54	31.45	32.59	31.33	32.98	31.86	28.85	27.79
	2	28.63	27.31	29.19	28.37	32.77	32.09	26.48	25.33	33.18	31.73
	3	33.03	32.13	35.79	34.16	30.32	29.24	33.74	31.56	34.09	32.59

Appendix 14: Fluorescent Ct values of KRT4 mRNA 80 bp – 61 bp amplicon combination for 15 samples from five donors at three occasions on age 0 to 63 days. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B). Table (B) is on the next page.

Table (B): Fluorescent Ct values of the first analysis of KRT4 mRNA 80 bp - 61 bp amplicon combination											
Donors n=5	Occasions	Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	26.92	25.6	39.9	36.39	25.17	23.83	34.99	33.56	34.12	32.98
	2	32.34	31.42	28.17	27.32	28.63	27.81	31.62	30.73	34.01	32.95
	3	33.89	33	28.7	27.91	30.76	29.77	24.94	23.58	36.99	36.82
2	1	29.51	29.58	27.8	27.52	34.26	34.4	27.35	27.16	32.28	32.9
	2	33.22	33.67	34.29	34.14	26.84	26.89	35.15	34.79	No Ct	No Ct
	3	24.87	24.76	38.37	38.18	38.12	38.3	30.92	30.91	35.64	36.76
3	1	31.06	31.13	33.01	33.07	33.83	35.2	32.04	32.17	27.6	27.69
	2	32.76	33.36	34.14	34.27	31.82	32.08	31.55	30.87	30.05	30.12
	3	27.82	27.49	30.56	30.77	30.2	30.49	29.44	29.49	31.89	31.52
4	1	26.18	25.01	32.9	32.19	25.88	24.57	30.58	29.8	27.99	26.84
	2	31.89	31.31	32.67	31.27	31.96	30.7	32.45	31.32	30.7	29.44
	3	30.96	29.97	29.91	28.92	35.99	35.33	28.2	26.96	28.2	27.23
5	1	32.31	31.19	33.21	31.95	32.81	31.16	33.04	31.9	29.53	28.01
	2	28.89	27.34	29.31	28.27	33	31.51	26.78	25.66	33.46	31.82
	3	33.16	31.89	35.74	34.98	30.47	29.3	33.55	31.99	33.6	32.26

(Appendix 14: continued).

Table (A): Fluorescent Ct values of the first analysis of HTN3 mRNA 136-79 bp amplicon											
Donors n=5	Occasions	Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	32.41	28.37	38.02	34.21	38.52	34.60	37.35	33.12	38.88	33.43
	2	28.39	26.51	30.29	27.61	33.05	28.21	32.60	28.06	31.36	28.12
	3	33.51	29.47	34.43	30.19	26.62	37.98	26.50	38.38	34.17	30.67
2	1	36.11	33.86	26.63	31.00	34.41	31.26	25.97	25.22	34.41	30.81
	2	32.80	31.30	36.61	33.05	33.55	32.55	36.45	31.93	34.18	33.25
	3	37.21	34.96	36.10	33.84	33.81	32.67	34.65	33.22	39.48	34.92
3	1	35.91	31.39	34.29	31.14	34.47	30.54	31.49	28.51	31.97	28.18
	2	31.46	29.23	26.48	27.74	32.62	30.43	28.74	29.99	29.25	30.89
	3	25.53	28.03	25.98	27.84	28.03	27.23	30.08	28.86	33.37	30.49
4	1	28.46	No Ct	29.44	27.85	32.96	32.24	25.90	39.75	28.63	No Ct
	2	27.15	31.87	28.38	31.46	27.92	29.47	35.35	33.79	26.47	No Ct
	3	23.75	No Ct	25.00	38.55	36.42	32.73	36.97	33.24	35.77	32.03
5	1	39.11	34.75	39.65	35.63	37.94	34.16	38.68	34.72	38.75	36.60
	2	39.42	35.62	37.79	35.48	36.64	34.55	30.49	34.12	28.69	38.75
	3	31.79	32.68	32.67	30.81	33.00	30.96	33.01	31.11	33.35	31.85

Appendix 15: Fluorescent Ct values of KRT4 mRNA 76 bp – 61 bp amplicon combination for 15 samples from five donors at three occasions on age 0 to 63 days. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B). Table (B) is on the next page.

Table (B): Fluorescent Ct values of the first analysis of HTN3 mRNA 136-79 bp amplicon combination											
Donors n=5	Occasions	Ct Values									
		day 0		Day7		day 14		day 35		day 63	
		FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	1	32.12	27.94	38.24	33.56	38.11	34.87	38.69	33.50	37.85	33.67
	2	28.38	26.59	30.29	28.04	32.55	28.16	32.62	28.62	31.52	27.72
	3	33.22	29.04	34.32	29.51	26.54	37.87	26.52	38.41	33.97	31.01
2	1	35.87	34.07	26.46	30.80	34.77	30.43	25.94	25.19	34.02	30.47
	2	32.51	30.60	36.94	32.90	34.20	32.18	36.86	31.68	33.56	33.18
	3	36.97	35.17	35.83	34.61	33.93	32.86	33.97	32.99	37.69	35.24
3	1	35.34	32.09	34.48	31.10	34.22	30.61	31.13	27.72	31.61	27.98
	2	31.55	29.22	26.40	27.66	32.19	30.98	28.56	29.40	29.26	30.48
	3	25.53	28.03	25.75	27.59	28.00	27.99	29.39	28.14	32.61	29.70
4	1	28.30	No Ct	29.06	27.49	32.96	31.54	25.93	39.08	28.41	No Ct
	2	26.84	31.51	28.26	30.39	28.26	30.04	35.36	33.59	26.29	No Ct
	3	23.89	No Ct	25.05	38.63	36.75	32.55	36.10	32.04	35.31	32.20
5	1	39.37	34.81	39.73	35.70	38.66	33.90	38.38	35.46	38.43	38.98
	2	39.20	35.42	37.13	34.03	37.01	33.74	30.23	33.11	28.86	No Ct
	3	31.43	31.76	32.66	30.24	32.75	30.11	32.87	31.22	33.41	32.08

(Appendix 15: continued).

Table (A)

Donors n=6	Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	36.85	32.4	38.73	35.95	36.65	33.62	No Ct	38.11	37.96	34.47
2	No Ct	37.57	38.42	34.65	35.68	32.61	37.81	36.06	36.92	33.13
3	35.55	31.74	38.85	31.82	34.44	30.55	36.65	35.2	37.61	36.77
4	34.6	31.47	36.98	32.03	36.8	33.41	34.41	32.08	36.67	34.32
5	32.91	29.52	37.31	31.75	No Ct	32.53	35.49	33.39	36.79	32.32
6	32.44	28.6	37.42	32.25	35.63	33.45	38.05	34.15	36.59	32.69

Table (B)

Donors n=6	Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	35.35	32.16	38.24	36.11	36.63	34.74	No Ct	37.84	38.31	34.84
2	No Ct	37.54	36.83	34.15	37.98	32.6	37.82	35.85	37.98	33.01
3	36.03	32.02	35.36	32.28	34.84	30.72	38.13	35.23	37.62	35.38
4	36.09	31.62	35.79	32.53	38.8	33.82	34.91	32.01	36.11	34.46
5	32.59	29.66	35.7	31.82	38.21	33.12	36.52	33.48	35.68	32.4
6	31.44	28.55	37.49	32.28	37.66	33.45	36.96	33.61	37.56	32.74

Appendix 16: The raw fluorescent Ct values of ANK1 mRNA 128 bp – 62 bp amplicon combination for samples at age 0 to 30 days from six donors. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B).

SUMMARY	Count	Sum	Average	Variance
day 0	6	0.1363	0.022717	3.97E-05
day 7	6	0.119	0.019833	5.57E-05
day 14	6	0.0825	0.01375	3.62E-05
day 21	6	0.0442	0.007367	3.07E-06
day 30	6	0.0421	0.007017	4.21E-06
1	5	0.0644	0.01288	0.000108
2	5	0.0766	0.01532	9.03E-05
3	5	0.0807	0.01614	0.000102
4	5	0.0611	0.01222	3.3E-05
5	5	0.0596	0.01192	2.33E-05
6	5	0.0817	0.01634	9.61E-05

Appendix 17: summary of the data obtained by ANOVA: Two-Factor without Replication. The data were calculated from the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 shown in Table 8-7.

Source of Variation	SS	df	MS	F	*p*-value	F crit
Sample age	0.001216	4	0.000304	10.26931	0.00011	2.866081
Individuals	0.000102	5	2.04E-05	0.690031	0.636748	2.71089
Error	0.000592	20	2.96E-05			

Appendix 18: Results of the Analysis of variance (ANOVA) of the $2^{-\Delta Ct}$ values of ANK1 mRNA 137 bp versus 62 shown in Table 8-7.

Table (A)

Donors n=6	Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	29.30	29.25	28.91	29.46	31.33	31.51	30.30	29.99	32.03	31.46
2	35.26	34.54	33.88	32.68	33.30	31.99	36.32	34.61	31.62	31.47
3	25.63	25.90	30.59	30.36	32.77	31.34	32.94	32.51	31.95	31.43
4	32.35	31.81	35.87	35.68	33.03	31.91	36.99	36.70	34.63	34.52
5	33.37	33.17	31.22	30.03	35.84	34.95	32.71	31.81	37.04	36.13
6	32.77	32.56	30.50	30.25	35.66	34.77	30.28	30.31	32.77	31.81

Table (B)

Donors n=6	Ct Values									
	day 0		Day7		day 14		day 21		day 30	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
1	28.77	29.05	28.92	29.43	31.54	31.43	29.93	29.81	31.60	31.39
2	35.39	35.30	33.20	32.68	32.72	31.94	35.38	34.69	31.74	31.69
3	25.61	25.86	30.37	30.13	31.62	31.11	32.88	32.50	31.92	31.43
4	31.73	31.61	36.22	35.95	33.19	32.01	37.58	37.00	34.34	33.73
5	33.77	33.57	31.62	30.43	36.24	35.35	33.11	32.21	37.44	36.53
6	33.17	32.96	30.90	30.65	36.06	35.17	30.68	30.71	33.17	32.21

Appendix 19: Fluorescent Ct values of ANK1 mRNA 128 bp – 62 bp amplicon combination for samples at age 0 to 30 days from six donors. Samples were run in duplicate and the results of the first analysis are shown in Table (A); the results of the second analysis are shown in Table (B).

SUMMARY	Count	Sum	Average	Variance
day 0	6	4	0.666667	0.020187
day 7	6	3.69	0.615	0.04839
day 14	6	2.52	0.42	0.0214
day 21	6	2.48	0.413333	0.006747
day 30	6	2.4	0.4	0.00784
1	5	3.15	0.63	0.09005
2	5	2.02	0.404	0.01193
3	5	2.82	0.564	0.03803
4	5	2.43	0.486	0.01068
5	5	2.17	0.434	0.01513
6	5	2.5	0.5	0.0188

Appendix 20: summary of the data obtained by ANOVA: Two-Factor without Replication. The data were calculated from the $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 shown in Table 8-11.

Source of Variation	SS	df	MS	F	*p*-value	F crit
Sample age	0.389213	4	0.097303	5.571865	0.003511	2.866081
Individuals	0.17355	5	0.03471	1.987593	0.124481	2.71089
Error	0.349267	20	0.017463			

Appendix 21: Results of the Analysis of variance (ANOVA) of the means $2^{-\Delta Ct}$ values of ALAS2 mRNA 128 bp versus 62 shown in Table 8-11.