INCREASING THE EVIDENTIAL VALUE OF BIOLOGICAL EVIDENCE

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DECLARATION

This thesis is submitted in fulfilment of the requirements for the Doctorate of Philosophy (PhD) Degree from the School of Pharmacy and Biomolecular Sciences at Liverpool John Moores University. Except where otherwise stated, the work presented is that of the author. Sources of other information have been appropriately acknowledged.

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ABSTRACT

With current scientific technologies, a significant amount of genetic information can be obtained from biological evidence found at a crime scene. Not only is it possible to identify the donor of the evidence through routine DNA profiling techniques, but new RNA based methods are being developed to determine the tissue type as well as the physical characteristics of the donor. Despite the information that can be obtained, the ability to determine the age or time the biological material was deposited at the crime scene has eluded the forensic community thus far. Timing is critically important as it could help police determine when the crime was committed. In this body of work an investigation was conducted into whether the degradation rates of nucleic acid macromolecules could serve as molecular clocks for age estimations. An attempt was made to gain a better understanding of the degradation products produced from an internal urban environment and to develop an optimal assay accordingly. A number of different RNA based techniques for ageing both hair and blood samples were also examined.

Degradation assays have been traditionally designed around amplicon size however, it was established that testing loci stability is an essential requirement in the optimisation process. The results presented in this thesis suggest the reliability of the data can be increased when the two competing target species are selected from the same loci, which minimised the effect of loci susceptibility to degradation. It was determined that blood stains aged up to 60 days in an internal urban environment were best distinguished (in terms of age estimations) by using targets that differed in size by 170 to 240 base pairs, with one of the targets being between 200 and 300 base pairs in length. Despite using a robust TH01 qPCR assay it was established that an internal “urban” environment was not as stable as predicted and that seasonal temperature variation had a large effect on degradation rates. Interpretation of the results was therefore limited suggesting these optimised target sizes may only be relevant to the winter months.

Using a carefully designed hermetically sealed dry swab we were able to remove moisture and inhibit the growth of DNA consuming micro-organisms. It was determined that bacteria alone can cause a 2-fold increase in the degradation rate of a sample aged at room temperature. In terms of integrity, storing samples at room temperature in a moisture free environment was equivalent to storing standard samples (exposed to normal humidity levels) in refrigerated conditions. It was also determined that the effect of bacterial degradation can be halved by lowering the storage temperature from room temperature to 4°C.

RNA was examined in an attempt to reduce the large variations that had inhibited previous DNA methodologies. IL-6 and TNF-α were initially selected due to their rapid post-extraction change in expression levels. However, their levels were highly variable, unpredictable and therefore not suitable for this type of analysis even on samples that had been aged for only ten days. It is thought that their dynamic roles in a number of haemopoietic processes could be responsible for the poor results.

A new RNA methodology, as described by Nolan *et al* (2008) was used to analyse samples that had been aged over 80 days. Four targets, AMICA1, MNDA, CASP1 and GAPDH were chosen based on their cell lineage as it was hypothesised that inter-donor variation could be reduced by using targets confined to the granulocytic cell lineage. Using the novel 3'/5' assay, AMICA1, MNDA and CASP1 all performed poorly and no correlation could be determined between the 3'/5' ratio and sample age. GAPDH showed some encouraging results with a correlation of 0.912 (age to 3'/5' ratio) although initial stability over the first 20 days and the inter-donor variation were still limiting factors. It was also thought that the various mRNA degradation processes, in particular the 5'/3' exonuclease activity, contributed to the poor results generally.

A large inter-donor variation was a common aspect to all the blood based methodologies trialled. This meant that none of the methods had any practical value. As a result, an alternative RNA method was used to determine if it was possible to age another forensically important type of biological evidence; hair. Using a Reverse Transcription Quantitative PCR (RT-qPCR) assay, we monitored the Relative Expression Ratio (RER) of two different RNA species (18S rRNA and B-actin mRNA) in hair samples that were aged naturally over a period of three months. Overall the results presented here suggest that the age of hair samples containing follicular tags can be approximated using a second order polynomial ($Age = 3.31RER^2 - 2.85RER - 0.54$), although with limitations.

LIST OF ABBREVIATIONS

cDNA- Complementary DNA

DEPC- Diethyl pyrocarbonate

DNA-Deoxyribonucleic acid

DNase- Deoxyribonuclease

dNTP- Deoxynucleotriphophate

DTT- Dithiothreitol

eIF-4F- Eukaryotic initiating factor

FDP-D – Fibrinogen degradation product – D dimer

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

LCN- Low copy number

microRNA – Micro Ribonucleic Acid

mRNA- Messenger Ribonucleic Acid

mt - mitochondrial

PABP- Poly (A) binding protein

PCR- Polymerase chain reaction

qPCR – Real time (quantitative) Polymerase Chain Reaction

qRT-PCR – Real Time (quantitative) Reverse Transcription Polymerase Chain Reaction

RQ – Relative Quantity

RQR – Relative Quantity Ratio

RE – Relative Expression

RER – Relative Expression Ratio

rRNA- Ribosomal ribonucleic acid

RNA- Ribonucleic acid

tRNA – Transfer RNA

snRNA – small nuclear RNA

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CHAPTER 1: INTRODUCTION

1.1 The Value of Physical Evidence

Forensic science has played an important role in the criminal investigation process for more than a century now (Schiro, 2000) but its contribution has never been as significant as it is today. There are a number of reasons for this including; enhanced technology (Shelton *et al,* 2009), an increase in the training and education of crime scene officers and scientists (Pyrek, 2007) and the increased awareness and understanding of this type of science by the jury (Shelton *et al,* 2009). Forensic science is the recognition, identification, comparison and interpretation of physical evidence and the application of science and medicine for the purpose of our legal system (Lee *et al,* 2001). Although forensic science is a multidisciplinary area covering specialisms such as biology, entomology, chemistry, pathology and anthropology to name just a few, each of these areas have a common dependency on physical evidence. Physical or trace evidence has been the cornerstone of forensic investigations even before Professor Edward Locard first postulated his exchange principle:

"Wherever he steps, whatever he touches, whatever he leaves, even unconsciously, will serve as a silent witness against him. Not only his fingerprints or his footprints, but his hair, the fibres from his clothes, the glass he breaks, the tool mark he leaves, the paint he scratches, the blood or semen he deposits or collects…" (Horswelland, 2004).

Its value lies in the fact that it is unbiased and factual, unlike witnesses, who are often unreliable and have the ability to deceive the jury (Spellman and Tenney, 2010). Even honest witnesses can mislead the jury as their perception of events may be distorted if the situation is stressful or emotionally damaging (Wells *et al,* 2003). Physical evidence is not affected in the same way and can be used to corroborate or refute a witness's version of events. Juries also tend to favour physical evidence over witness accounts and when managed correctly, this type of evidence can be very influential in their decision making process (Tyler, 2006).

Physical evidence is examined for the purpose of identification and comparison (Saferstein, 2006). Connecting a person or an object to a specific crime scene is often essential to establishing the facts of a case (Saferstein, 2006). Establishing this link is dependent on identifying the material in question and comparing it to known reference samples. This process

can often be difficult when comparing similar samples with only a small number of unique characteristics (Curran *et al*, 1998). Less specific techniques employed as recently as forty or fifty years ago have often failed to discriminate between two like objects, thus reducing the value of the evidence. For example the examination of different types of glass based on their refractive index alone often results in samples being indistinguishable (Saferstein, 2006). Today many of these obstacles have been overcome as we can now extract more information from most forms of evidence than at any time in the past. There is often a drawback to this progression as more often than not new techniques require complicated statistical evaluation making interpretation more difficult (Gill, 2013). Examples include the evolution of DNA profiling from random fragment length polymorphism (RFLP) to short tandem repeats (STR) and low copy number (LCN) methods (Gill, 2013). The evolvement of glass analysis which now routinely uses particle composition techniques such as induced-coupled plasma mass spectroscopy (ICP-MS) as opposed to traditional refractive index or density measurements is another example (Schmidt *et al*, 2002).

On the 9th February 1988, Helen McCourt, mysteriously disappeared on her way home from a Liverpool Insurance company. After a lengthy investigation, Ian Simms, the landlord of a Lancashire pub was convicted and jailed for her murder (Saferstein, 2006). This case is one of many that have relied on the examination of physical evidence and provides a good illustration of its strength when compared to a witness or suspect's testimony. The physical evidence in this case challenged certain inaccuracies and omissions made by the accused who denied that clothes found on a public walkway belonged to him. Police were, however, able to cross-match fibres found on these clothes with carpets from inside Simms's flat, which resulted in a reluctant admission of ownership. Police were also able to match fibres recovered from Ms McCourt's trousers with the same carpets. More conclusive evidence came later with the discovery of an earring and blood that matched the victim, found in Simm's flat. On this evidence the jury found Simms guilty of murder despite the fact the body was never found (Saferstein, 2006). The conviction of one of the most notorious serial killers in the US history was based on accumulation of the physical evidence. Within a six month period in 1982, the bodies of five young females were discovered along the banks of the Green River in Seattle, Washington. The victims were known prostitutes who had been strangled and raped. By the end of 1986 the body count had risen to forty and police began focussing their investigation on one particular suspect, Gary Ridgeway (Saferstein, 2006). Despite suspecting Ridgeway for more than a decade, police were unable to make an arrest due to the lack of physical evidence.

The case remained unsolved for more than ten years before DNA profiling was introduced into the justice process. Semen samples collected from three of the victims were compared to Ridgeway's saliva and a match was obtained. Further trace evidence was later discovered as minute amounts of paint particles found on the clothing of six victims were indistinguishable from paint collected from Ridgeway's workplace. Despite passing a lie detector test in 1984, Ridgeway when presented with this evidence confessed to the murders of 48 women (Saferstein, 2006).

Physical evidence exists in many different forms (Jackson and Jackson, 2011). The type of evidence will generally determine its value in court although this can be influenced by the specifics of the case including the evidence handling procedures (Jackson and Jackson, 2011), the skills of the respective legal teams and its relevance to any issues at trial. Forensic scientists around the world continue to engage in research to increase the value of physical evidence by developing techniques to increase the reliability of the information obtained or by developing techniques to establish new information. The body of work in this thesis focuses on the latter.

1.1.1 Blood Evidence

Traditionally blood was collected from crime scenes for serological testing, which allowed investigators to establish the blood type of the donor (Saferstein, 2006). Though helpful in terms of eliminating potential suspects, ABO blood grouping was unable to positively identify an individual therefore limiting its evidential value (Butler, 2009). Its ability to exclude a particular individual depended on the blood type found. For example in the UK approximately 37% of the population have blood group O^+ (NHS) therefore a O^+ crime scene sample could only eliminate 63% of the population. AB^- blood group exists in only 1% of the population, meaning 99% of the population could be excluded. An AB^- blood group therefore has a greater evidential weight in terms of identifying a suspect. Even so, these numbers pale in significance to the values obtained through modern DNA profiling techniques where the discrimination ability is in the billions (Butler, 2009). Blood evidence can also be used for purposes other than identification. By examining the shape, size and distribution of individual bloodstains a trained investigator is, *inter alia*, able to determine the events that lead to the deposition of blood at a crime scene (James *et al*, 2005). This can in certain circumstances increase the evidential value of blood evidence as it can corroborate or refute witness accounts or assist in determining the culpability of potential suspects (James *et al*, 2005).

1.1.2 DNA Evidence

Despite what evidence could be adduced from blood samples, its value increased exponentially with the introduction of DNA profiling in 1984. Sir Alec Jeffreys established a technique that could positively identify the donor of an individual blood sample (Zagorski, 2006). This technique targeted multiple small areas of repeating sequences, which when looked at collectively were unique to each individual (Jeffreys *et al*, 1984), with the only exception being identical twins (Butler, 2009). This technique allowed for the positive identification of the donor as the area of DNA examined does not significantly change over time and does not vary between cell types (Saferstein, 2006). This means a perpetrator's DNA can be recovered from numerous different sources at a crime scene including blood, saliva, semen and skin cells (Butler, 2009).

DNA analysis was successfully used for the first time in a criminal trial in 1987 to convict Colin Pitchfork for the rape and murder of two girls in Leicester (Saferstein, 2006). Initially large volumes of high quality DNA were needed for conclusive results (Rudin and Inman, 2001), but over time the profiling technique has evolved. This was largely due to the introduction of the Polymerase Chain Reaction (PCR) which is a highly sensitive and specific process for amplifying small quantities of DNA (Bartlett and Stirling, 2003). As a result profiles can now be routinely obtained from microscopic amounts of blood with unprecedented levels of accuracy (Shewale and Liu, 2013). Common DNA profiling techniques used in the UK, USA and Australia have discriminating power in the magnitudes of billions (Lucy, 2005). These values are calculated by multiplying together the frequencies of each of the individual alleles that make up the DNA profile (Balding and Nichols, 1994). The number of alleles varies depending on the technique used however most commercially available products use between 10 and 16 loci (target regions) on the DNA for identification (Butler, 2006). The recovery, amplification and purification techniques along with optimised protocols have greatly increased the sensitivity of DNA analysis. Degraded samples that once posed problems can now be analysed using miniFiler, which is a commercial profiling technique designed to amplify smaller repeating DNA fragments (Mulero *et al*, 2008). The introduction of the low copy number (LCN) technique has meant that profiles can now be obtained from a single cell sample (Carsten *et al*, 2008) suggesting the sensitivity of this technique has reached its maximum. Whilst the examination of a greater number of loci could increase the

discriminating power of these techniques it is hard to imagine the need given the values that can already be obtained from a 16 loci system.

The advances in DNA profiling over the past 30 years have largely focused on the laboratory aspects of this process (Norrgard, 2008), and as such most modern forensic laboratories have abandoned serology based testing of blood evidence in favour of DNA testing (Houck and Siegel, 2010). Techniques regarding the preservation of samples from the crime scene have been investigated, but not to the same extent (Lee *et al*, 1994). More recently, issues regarding the presentation of the scientific results in court have been raised, stressing that results as compelling as they may be, will be useless unless they are fully comprehendible by the jury (Coles, 2006). This issue is of particular relevance today given the sophisticated equipment and complex techniques that are now routinely employed in forensic laboratories.

DNA material is routinely collected from a wide range of offences including property crime, such as burglary and motor vehicle theft, as well as personal serious crime such as assaults, sexual offences and murders (Bond and Hammond, 2008). The conversion of biological evidence into DNA profiles and then into matches with offender profiles held on DNA databases or to a suspect's reference sample, is a process that today, is carried out rapidly and with relative ease (Bond and Hammond, 2007). This timely processing is a key element to its contribution in solving crime. Like fingerprints, there is a comprehensive DNA database which has the potential to increase its evidential value (Butler, 2011). Millions of DNA samples are stored on a searchable database providing investigators and scientists with a power investigative tool (Johnson and Williams, 2004). Profile matches can be obtained directly (suspect to crime scene sample) or indirectly (crime scene sample to unknown samples) from the National DNA database. The size of the DNA database is an important consideration. As it increases so does the probability of obtaining a "hit" (Doleac, 2011). According to the Home Office (UK) 2012/2013 annual statistical report on the National DNA database, the UK database contains 7.16 million profiles. At the time of writing the Home Office (UK) estimates that 5% of the UK's population is criminally active and in January 2014, 8.8% of the UK's population was on the National DNA database (Home Office, 2014). This is significantly greater than the US where according to the Genewatch website it is estimated that 10% of the population is criminally active, but only 1% is currently on their National Database. The size of the DNA database increases the likelihood of obtaining a match from a crime scene profile (Doleac, 2011). According to the Home office statistical report the UK national DNA database

match rate on loading a crime scene profile in 2012/2013 was 61.4% (Home Office, 2014), which is exceptionally high and provides law enforcement agencies with a powerful crime fighting tool. The importance of a national database was highlighted in the Joseph Kappen case. In 1973 in South Wales, three 16-year-old girls were found raped and murdered (Williams and Johnson, 2005). At the time DNA analysis did not exist and there was no further evidence to identify the killer. In 2001, a sample was taken from an item of clothing and using the highly sensitive LCN (low copy number) DNA technique a profile was obtained. A novel search (looking for similarities rather than an exact match) on the DNA database was performed and it identified potential relatives of the murderer. Using existing intelligence police obtained a suspect, Joseph Kappen who had died of lung cancer in 1991. The body was exhumed in 2002 and the DNA from the body matched that of the evidence from the victims, bringing an end to a 30 year old unsolved mystery (Williams and Johnson, 2005). Furthermore a number of old cases that were subject to much criticism at the time have been laid to rest by DNA testing. James Hanratty was hung in 1962 for the murder of Michael Gregsten and the rape and attempted murder of Valerie Stone (Saferstein, 2006). Various pieces of evidence lead to his arrest, but Hanratty protested his innocence to the very end. The case attracted great scrutiny from legal experts, journalists and politicians as potential alibis and the conduct of the police investigation was brought into question. Hanratty's family maintained that the case was a miscarriage of justice and embarked on a 40 year campaign to clear his name. On appeal in 1999, DNA profiling was carried out on underwear recovered from the female victim and on a handkerchief that was found wrapped around the gun. In March 2001, DNA was extracted from the teeth pulp of his exhumed body and it was shown to match the evidence profiles. The court of appeal concluded that the original conviction was safe (Saferstein, 2006).

DNA evidence is also a powerful tool for exonerating the innocent, which in some eyes is more important than convicting the guilty (Dwyer *et al*, 2003). The Innocence project in the US, which is a non-profit organisation created by Barry Scheck and Peter Neufeld in 1992 (Dwyer *et al*, 2003) is dedicated to exonerating the wrongfully convicted through DNA testing. According to the Innocence project website, as of January 2014, there have been 312 post-conviction DNA exonerations in the US.

DNA analysis has revolutionised the investigation process and for the reasons discussed, it is and will remain a powerful tool in solving crime. However, DNA and blood evidence are not without their limitations and it is these limitations that continue to frustrate police, forensic

scientists and the judicial system (Lieberman *et al*, 2008). These limitations, which are discussed on page 33, cause uncertainty in the courtroom and can reduce the reliability and evidential value of such evidence (Lieberman *et al*, 2008).

1.1.3 Hair Evidence

One of the most common types of trace evidence found at crime scenes is hair, owing to the fact that each human loses on average 100 hairs a day (Houck and Siegel, 2010) it's generally very persistent especially on rough fabrics and beneath fingernails (Dachs *et al*, 2003) and is easily removed in a struggle. Forensic Access, a leading forensic provider in the UK, suggests hair evidence is recovered in 24% of all assault cases in the UK (www.forensic-access.com). The significance of hair evidence is dependent on the method of evidence collection, the evidence processing techniques, the methodology used, and the experience of the scientist (Sen, 2010). A physical and visual examination can provide corroborating evidence as hair, like glass, is characterised as "class" evidence, meaning that it cannot be definitively linked to a single source. This means it cannot be used to positively identify an individual (Stafford Smith and Goodman, 1996). The value of "class" evidence lies in its ability to corroborate events with other information obtained. Trying to determine the significance of class evidence is often difficult if not impossible. Despite not being unique, experience tells us that, significant items of physical evidence such as hair are very diverse in our environment (Kiely, 2011). A physical examination of hair samples also involves the comparison of a number of different chemical and physical features therefore scientists are still able to reach a conclusion that the chances of encountering two indistinguishable items of "class" evidence at a crime scene that actually originated from different sources are considerably low, thus giving this type of evidence its value (Buscaglia, 1994).

Traditionally hair examination involved the use of a variety of microscopic techniques (Saferstein, 2006). Features such as colour, thickness, cortex, cuticle and medulla characteristics are all compared to determined species and potential donors (Robertson, 2002). At times the physical condition of the hair could provide investigators with information regarding the possible nature of transfer and whether the hair was forcibly removed or whether it was shed naturally (Robertson, 2002).

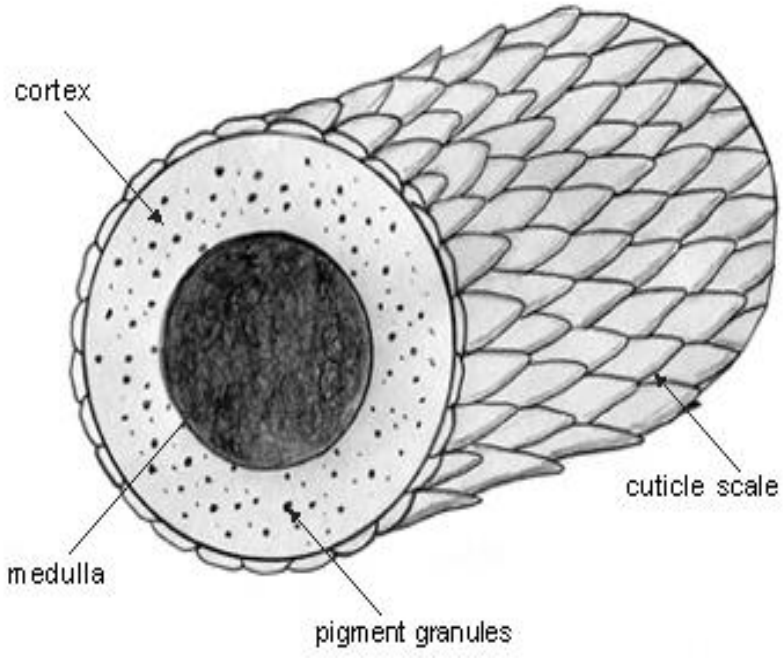


Figure 1: The Gross Anatomy of a Human Hair: The medulla, cortex and cuticle are commonly used to distinguish between different species of hair.

Hair samples have been used to convict numerous offenders from as early as the beginning of the 20th century. In 1924 a murder case in Glasgow, Scotland, was solved using a single sample of hair (Glaister, 1931). Alexander Gibson who was later dubbed the "Cat Killer" was arrested and charged with the death of a young 10-year old boy. The only evidence recovered was hair from the victim's jacket. The sample consisted primarily of small white hairs, with the odd one containing a light browning pigment. A longer darker hair was found on the inside of the victim's jacket. The first sample was consistent with the suspect's cat, and the longer, darker hair was consistent with the suspect himself. This evidence alone led to his conviction.

Although hair analysis does not generally enable the donor to be identified, it can now provide a wide range of other information that may be beneficial to the investigation, increasing its overall evidential value. Hair samples can now be examined for illicit drugs and alcohol using gas chromatography-mass spectrometry (Romolo *et al*, 2003) and liquid chromatography-mass spectrometry (Musshoff and Madea, 2007). These techniques separate the components of the sample based on their affinity between liquid and gas mediums. Once separated the components are ionized into mass fragments which are detected and identified (Adlard and Handley, 2001). The drug analysis of hair is based upon the assumption that drugs become

entrapped in the keratin of the hair (Romolo *et al,* 2003). Although the concentration of drugs in hair tends to be very low, this type of analysis in hair has the advantage over blood, urine and saliva samples in that the drugs persist in hair for much longer periods of time (Kintz, 2004). Finding drugs in hair is therefore useful in establishing a drug history rather than recent drug use (Kintz, 2004). Ion Mass Spectrometry has recently been used to distinguish hair samples based on the identification of consumer chemicals within the hair (Ingram, 1998). These chemicals arise from shampoos, conditions and various other hair products and provide a distinctive chemical signature (Groenewold *et al,* 2000). This technique also has the capacity to detect environmental contaminants such as insecticides as well as water, air and chemical isotopes which can help establish the person's place of inhabitants or whereabouts (Ingram, 1998). Hair has the ability to trap pollen and other microbial substances that are location specific. Forensic scientists have used this to establish links between suspects and particular locations or crime scenes (Wiltshire, 2006). If the hair sample contains a root (or follicular tag) then the hair sample can be used to identify the donor as the follicular tag is rich in cellular (DNA) material (Bertino, 2011).

1.1.4 Other forms of Physical Evidence

Glass is another common type of physical evidence found at particular types of crime scenes such as burglaries and those involving vehicles (Saferstein, 2006). Traditionally, physical examination techniques such as density, refractive index and melting points were used to link a person or item of clothing, to a particular scene (Jackson and Jackson, 2011). When using these techniques to conduct comparison examinations, experts often concede the possibility that a particular glass fragment could have originated from a source other than the one being proposed (Dodds *et al,* 2010). Like hair evidence, glass was and often still is, considered a "class" type of evidence meaning that it cannot be definitively linked to a particular source (Buscaglia, 1994). This limitation meant that glass was often ignored unless all other avenues were exhausted. The recent developments in laboratory techniques and instrumentation have significantly increased the amount of information retrievable from this type of evidence (Montero *et al,* 2003). Glass is made from a variety of metal and silicon oxides and as such, each piece of glass contains its own characteristic composition which can be detected using X-ray diffraction and Inductively coupled- mass spectrometry (ICP-MS) (Montero *et al,* 2003). These techniques bombard the sample with electrons effectively ionizing the sample to create charged molecules or fragments (Sparkman, 2000). The mass to charge ratio of each of these

fragments is calculated to identify the molecules present in the sample, thus allowing for identification of the sample (Sparkman, 2000). According to Forensic Access, glass evidence now provides reliable and meaningful evidence in 60% of cases where it is recovered, because of the information that can be obtained. Glass also has a unique advantage in that unlike biological samples, it is relatively stable and will not degrade over time. Furthermore, damage to glass can provide information regarding the sequence of events that caused the damage (Saferstein, 2006). For example, a visual examination of the radial and concentric cracks that have been created by projectiles such as bullets, allow an examiner to determine the order in which the holes were made (Saferstein, 2006). This timing may be important to an investigation.

Fibres originate from a number of synthetic and natural sources including clothing, bedding, towels, carpets and plant materials. Fibres are commonly found at crime scenes, predominately due to their high shedding and transfer ability (Saferstein, 2006). They are one of the largest categories of polymer evidence examined in forensic chemistry laboratories and, like glass evidence their evidential value has increased dramatically in recent years (Robertson *et al,* 2002). Prior to electron microscopes and x-ray diffraction systems fibre analysis consisted of visual examination using light microscopy (Robertson *et al,* 2002). The information extracted from this type of analysis was limited to colour, size, weave as well as primitive burn and solvent tests to determined physical characteristics (Robertson *et al,* 2002). Today fibres are amenable to analysis with nearly every tool available to the forensic scientist, ranging from optical microscopes to microspectrophotometry, chromatography, and X-ray diffraction methods (Kobus, 2001). These techniques not only allow for the determination of physical characteristics of the fibre but dye analysis can also be performed routinely. Like hairs, fibres can provide information relating to the transfer activity. In a case already described (Helen McCourt), the accused tried to explain the presence of carpet fibres on Ms McCourt's coat by claiming she had draped her coat over bar stools in his pub (Saferstein, 2006). However, the number and distribution of fibres on the back of the coat indicated forcible contact between the coat and the carpets in Simms's flat (Saferstein, 2006).

Fingerprints have been used for identification and conviction since the 19th century (Saferstein, 2006). Fingerprints are formed during the foetal stage of development and do not change as a person ages (Hawthorne, 2008). The unique patterns are believed to be caused by a variety of localised factors, both environmentally and genetically, during embryonic growth (Kucken and

Newell, 2005). As a result no two people have ever been identified with the same fingerprint pattern (Jackson and Jackson, 2011), which is unique from DNA (identical twins have the same genetic profiles). This arguably makes fingerprinting a more superior means of discriminating individuals than DNA. With the implementation of a national database and automated fingerprint identification system, fingerprint evidence is being used to help solve thousands of cases in the UK every year. The techniques employed for identifying and recovering latent (invisible) fingerprints from crime scenes are effective, yet relatively simple (Saferstein, 2006). The application of recovering fingerprint evidence using powders and chemicals such as ninhydrin, cyanoacrylate and physical developer, do not require any sophisticated laboratory equipment (Jackson and Jackson, 2011) and as such this area of forensic science has not experienced the same development as other forensic disciplines such as DNA. In striving to increase the evidential value of fingerprint evidence there has been recent efforts to incorporate the latest technology into this discipline. The Forensic Research Centre at the Leicester University (UK) developed a new method of recovering fingerprints from metallic surfaces such as firearms and bullets (Bond, 2008). This method uses X-rays to detect microscopic corrosion caused by the chemicals in fingerprint residue and is effective regardless of whether the object has been wiped clean. A team at the University of East Anglia (UK) have developed a fingerprint powder using nano-particles which can visualise an individual's fingerprints, whilst simultaneously revealing lifestyle information about the donor such as their drug history by analysing sweat secretions in the fingerprint (Legget *et al,* 2007). With the increasing awareness and developments in nanotechnology it may not be long before information other than individual identification is being routinely recovered from fingerprint evidence. Although its value in discriminating and identifying a donor is unquestionable, fingerprints evidence is limited. The inability to age a fingerprint is one such limitation (Jackson and Jackson, 2011).

1.2 Limitations of Physical Evidence

Despite what information can be obtained or determined, every type of physical evidence maintains some form of limitation. As such forensic scientists are still unable to obtain "the complete picture". Limitations come in a variety of different forms (Saferstein, 2006). Some are specific whilst others are common to all evidence types. These limitations can exist in any phase of the evidence handling process from the crime scene to the laboratory (Swanson *et al,*

1977). Whatever the limitation may be, it can impact on the evidential weight of the evidence presented at trial (Saferstein, 2006).

1.2.1 Limitations of Blood Evidence

There are a number of limitations to blood evidence. Firstly its biological nature means that it is easily degraded by environmental factors such as UV light and heat (Thacker *et al*, 2006). This places time limits on the collection and preservation of blood related evidence. The application of earlier forensic methods such as ABO blood grouping and early generation DNA profiling techniques were dependent on relatively large quantities of good quality DNA (Collins *et al*, 2000). Even the latest DNA profiling techniques are affected by the degradation levels of the samples (Chung *et al*, 2004). Generally speaking the more degraded the sample, the less information (alleles). This effectively reduces the evidential weight and confidence of any identification made. Although improved methodologies, such as minifiler and LCN have resulted in the ability to analyse samples of poorer quality, there is a limit that if exceeded, prevents any usable results from being obtained (Chung *et al*, 2004). Blood evidence can also be easily removed or altered, which will affect any subsequent interpretation of the blood pattern (James *et al*, 2005). The most significant limitation of blood evidence involves the inability to age a bloodstain, which is the basis for the work in this thesis.

1.2.2 Limitations of DNA Evidence

Despite its prevalence, popularity and ability in the field of Forensic Science, DNA is not perfect. DNA alone cannot, or should not, be able to bring about a conviction, though in reality it does (Hindmarsh and Prainsack, 2010). The information that DNA can provide is limited to the identification of the donor but the presence of DNA at a crime scene does not necessarily indicate guilt of the donor therefore further corroborating evidence should always be required for a safe conviction (Gabriel *et al*, 2010). One of the most significant limitations of DNA evidence is that it requires a suspect's sample for comparison, whether it is being used for identification or exclusionary purposes. Without this comparison sample, the evidence becomes no more than a series of numbers on a database. This limitation is a real practical concern as police constabularies across the world have thousands of crime scene profiles held on databases that have not been identified.

The ability to determine the physical characteristics of a donor from a DNA crime scene sample would be an invaluable investigative tool. The most obvious and useful descriptors of an individual's appearance from a forensic perspective would be skin, hair and eye colour, as well as height, ethnicity and facial features. There has been a substantial amount of research into the genetic disposition of human pigmentation. Many of the genes associated with hair [Bleicher, (1996); Van Daal, (2008); Branicki *et al*, (2006)], skin (Goldschmidt and Raymond, (1972); Tully, 2007) and eye colour [Branicki *et al*, (2008); Duffy *et al*, (2007)] are already well understood. Unfortunately many of these traits are complex and controlled by multiple genes making the process more difficult. Height has been used for more than a century as a model by which to understand quantitative genetic variation in humans (Deng *et al*, 2007). The success of identifying genetic markers specific for height has not been as successful as identifying those for pigmentation. It is thought that height is influenced by complex additive heritable factors across numerous chromosomes as well as environmental factors. Deng *et al*, (2006) stated that notwithstanding the proposed architecture of complex traits that involves widespread gene-gene and gene-environment interactions, their results suggest that variation in height in humans can be explained by many loci distributed over all autosomes, with an additive mode of gene action. There has also been some degree of success in determining ethnicity (Hughes *et al*, 2008) using methods that identify particular sequences of DNA that are found more frequently in certain ethnic groups (Lowe *et al*, 2000). The accuracy of this methodology may be affected by interracial breeding, which is becoming more common in our society (Sampson *et al*, 2011). Despite all this research and the developments that are being made, there is still no validated and reliable technique that is routinely used in a forensic setting to determine the physical characteristics of a donor.

The value of DNA is limited in other ways. Like blood, the level of DNA degradation can have a profound effect on the statistical strength of the evidence (Bar *et al*, 1988). DNA is degraded by numerous various different processes which are discussed on page 67. Although there have been recent improvements to help analyse degraded samples, for example the use of low copy number DNA and minifiler (Mulero *et al*, 2008), current DNA profiling techniques still require a certain level of integrity for analysis (Budowle *et al*, 2009). Degradation results in the loss of genotype data (alleles) which can occur at any loci or position on the DNA strand that is examined (Butler, 2009). This loss is often described as allelic dropout (Butler, 2009) and when it occurs it reduces the discriminating power or ability to distinguish one DNA sample from another (Balding and Nichols, 1994). Although degradation is a major cause of allelic

dropout, it is not the only cause. A small starting amount of DNA or sequence variation that results in primer incompatibility can also cause allelic dropout (Boutrand *et al*, 2001).

DNA profiling techniques are extremely sensitive due to the application of PCR. The high sensitivity allows for the analysis of smaller quantities of DNA material but it also increases the chance of detecting contaminating DNA (Kayser and Knijff, 2011). This poses a particular problem with forensic samples as the integrity of the sample could be compromised. It also raises uncertainty as to how the evidence came into existence. It would be difficult if not impossible to determine whether the detected DNA resulted from primary or secondary transfer or from some form of contamination during the evidence handling process. The sensitivity of the LCN technique, which is capable of determining a DNA profile from a single cell source (Budowle *et al*, 2009), was recently questioned by the judicial system in the UK and Australia (Caddy *et al*, 2008). Sean Hoey who was charged with 29 counts of murder in the Omagh Bombings in Belfast in 1998, was cleared in a case that made international headlines and prompted a review of the LCN technique. The prosecution's case was heavily dependent on LCN DNA evidence, which the judge in summarising concluded was "unreliable" (see *R v Hoey* [2007] NICC 49). This decision resulted in numerous appeals in a number of countries where suspects had been convicted on this type of evidence. This included the conviction of Terence and David Reed in North Yorkshire in 2009 and the conviction of Bradley Murdoch in Australia in 2008 (see *R v Reed* [2009] EWCA Crim 2698; *The Queen v Murdoch* 2005 NTSC 80). The sensitivity of the LCN technique was being challenged and those opposing the technology argued it was not possible to determine whether the results obtain originated from the actual evidence or from contamination (Lawless, 2012). A subsequent independent review was conducted by Professor Brian Caddy in 2008, who came to the conclusion the technique was fundamentally safe, but not being used as effectively as it might be (Caddy *et al*, 2008). The main area of concern was the evidence collection procedures at the crime scene, rather than the laboratory process or issues relating to the presentation of this evidence in the court room (Caddy *et al*, 2008).

1.2.3 Limitations of Hair Evidence

Unfortunately hair evidence when used for comparison purposes rarely adopts such significance as it did in the Glasgow case discussed on page 28 and its evidential value is for most cases, considerably less. In 1990 a sexual assault case in the United States sparked

worldwide criticism of hair evidence (Dwyer, 2002). A group of Harlem teenagers were accused of beating and raping a jogger in Central Park, New York. Hairs attributed to the victim were recovered from the clothing of two of the suspects. These hairs were the only physical evidence presented by the prosecution (Dwyer, 2002). Nicholas Petraco, a detective who examined the hairs testified that the techniques for hair examination at the time were not discriminative enough to link anyone to the crime with certainty and at best could only be described as "consistent with and similar to" those of the victim (Dwyer, 2002). The criticism continued from legal scholars who went on record by stating that historically, the value of hair evidence has often been overstated (Dwyer, 2002). Petraco outlined the problems associated with this type of evidence. He stated that results varied depending on the individual examiner and that the results could not be reliably replicated. In a proficiency test of crime laboratories across the US at the time, some of the worst results came from hair analysis, with error rates as high as 68 percent (Dwyer, 2002). In an Oklahoma death penalty case in 1982, a hair examiner misidentified 17 out of 17 hairs (Innocence Project 2008). However, this evidence put Ronald Williamson and Dennis Fritz, in prison for life. DNA tests later proved both men were innocent (Innocence Project 2008). The lack of precision and accuracy with comparison analysis changed the way hair evidence was perceived and today is often delivered in court with caution. New techniques such as ICP-MS have strengthened this type of evidence for comparison purposes (Goulle *et al*, 2005) but the underlying precautions still exist.

Drug testing using hair samples can provide information regarding the drug history of the donor. However, due to differences in hair growth rates (depending on anatomical region), gender, age, ethnicity and inter-donor variability (which is the variation observed between individuals), interpretation of hair analysis results are often difficult (Wennig, 1999). Furthermore, the mechanism of drug incorporation into the hair matrix is not fully understood, and it is thought that the rate, time and dosage levels are specific to the donor, thus making any method of interpretation difficult to standardise (Wennig, 1999). Cosmetic treatments such as hair colouring and dyes may also introduce bias that could affect interpretation (Wennig, 1999). These are some of the issues that limit drug analysis from hair samples. With the introduction of DNA profiling, the evidential value of hair evidence had the potential to increase. Hair samples that contained a root or follicular tag were commonly used, via DNA profiling, to identify the donor and where possible, spatially link a person to a crime scene (Norrgard, 2008). This increased the evidential value of hair evidence significantly. Hair could now be examined for identification rather than comparison purposes. The types of hairs that

contain roots or follicular tags are called anagen or active phase hairs and are still growing prior to being forcibly removed (Robertson, 1999). Unfortunately anagen hairs are not the most commonly encountered type of hair found at crime scenes (Saferstein, 2006).

Figure 2: Hair Growth Cycle: The human hair cycle consists of three phases. The anagen or growing phase contains hair follicular tags rich in DNA material whilst the telogen phase hairs, which are the most common type of hair found at a crime scene, do not. Image adopted from http://www.nanogenhairlossrestoration.ie/documents/hair-growth-phases.html.

Anagen hairs are generally only found in assault cases, or where some form of physical struggle has taken place (Saferstein, 2006). The most common type of hair found at crime scenes are telogen phase (keratinised) hairs, which are those that shed naturally and do not contain a follicular tag. Extracting genomic DNA from telogen hairs is often difficult and consistent results are rarely achieved (Barbaro, 2005). Given the difficulties, mitochondrial DNA (mtDNA) is generally targeted when dealing with telogen hair samples (McNevin *et al*, 2005). Mitochondrial DNA is present in greater amounts (higher copy numbers) within a cell when compared to genomic DNA (Linch *et al*, 2001). It is therefore more likely that mtDNA will be successfully extracted from keratinised hair (McNevin *et al*, 2005). The difference in the required amount of DNA for successful PCR amplification is consistent with the copy number difference per cell between genomic DNA (one copy per cell) and mt DNA ($10^3 – 10^4$

per cell) (Allen *et al*, 1998). There are however, a number of disadvantages when compared to genomic DNA analysis (Allen *et al*, 1998). mtDNA profiling is still not routinely used in the forensic investigation because: there are no mtDNA databases for criminal matters, therefore profiles cannot be compared at a national and international level; mtDNA is inherited through the maternal lineage, therefore profiles cannot individualise between maternal relatives; mtDNA profiles do not have the same discriminating power as genomic STR profiles (magnitude difference of up to 10^6); and there is a significant occurrence of heteroplasmy in mtDNA genotypes which is the presence of more than one type of mtDNA in an individual (Allen *et al*, 1998). The reason for the lower discriminating power is that mtDNA is inherited as a single locus from only one lineage and it contains less variation (Buckleton *et al*, 2005). mtDNA is also notorious for producing unsuccessful profiling results. In an exhaustive survey by Prieto *et al*, (2003) on the results of the Spanish and Portuguese working group of the international Society of Forensic Genetics Collaborative exercise (GEP-ISFG), they found that only three of the twenty three laboratories were able to obtain a consensus sequence for control hair samples and ten laboratories did not obtain or report any result at all.

1.2.4 Limitations of other types of Physical Evidence

Glass, fibres and fingerprints are all limited in one way or another. Although the advances in technology allow scientists to obtain some individual linkage evidence for glass, the majority of information that is recoverable is still "class characteristic" data (Kiely, 2011), therefore source identification is not possible. Glass evidence is still predominately used as corroborating, rather than conclusive evidence. The stability or persistence of glass means that it is impossible to determine when it was broken or how long it has been in a certain environment (Caddy, 2001). It is also very difficult to determine the method of transfer (Caddy, 2001). Knowing this type of information would allow prosecutors to counteract defence arguments of how and when glass fragments where transferred or deposited on certain items of interest. Defence lawyers often raise plausible explanations, which creates doubt in the minds of the jury and subsequently the evidence loses its value. Fibres, although to a lesser extent, are affected in the same manner.

Whilst fingerprints are a much more cost-effective method of identification than DNA (Jackson and Jackson, 2011), the interpretation of the evidence is largely based on the experts opinion. Fingerprint examiners can be influenced by external factors when making judgements which

can affect the accuracy and reliability of results (Guy, 2007). A study from Southampton University, UK, suggested that subjective bias can creep into situations in which a match between two prints is ambiguous (Guy, 2007). This bias has led to experts contradicting their own results previously given in court (Cole, 2006). There are many cases of fingerprint experts making misidentifications. In June 2002, a corpse turned up near Las Vegas. A fingerprint taken from the body was matched to Kathleen Hatfield of California. Hatfield's mother was notified, funeral arrangements were made and just prior to the burial, Hatfield turned up alive and well (Cole, 2006). In the UK, Neville Lee was arrested and convicted in 1991 for allegedly raping an 11-year-old girl. It was only after another person confessed to the crime that Lee was released (Cole, 2006). One of the more publicised cases involved Brandon Mayfield, a lawyer in the United States. Mayfield was identified as a participant in the 2004 bombings of commuter trains in Madrid, Spain (Girard, 2011). Mayfield was identified by a fingerprint recovered from one of the detonating devices. A fingerprint expert from the FBI analysed the print and reported a match against one of the candidates returned by the "Integrated Automated Fingerprint Identification System". This system searches for any potential match on the national fingerprint database (Girard, 2011). The comparison was initially described by the FBI as "100 percent positive match" and as an "absolutely incontrovertible match" (US Department of Justice, 2006). However the Spanish national police fingerprint examiners concluded the prints did not match Mayfield, and identified another man, an Algerian who they believed to be the offender. Upon re-examination the FBI acknowledged the error, and a judge released Mayfield in May 2004 (see *Mayfield v United States,* 599 F.3d 964). Fingerprinting is undoubtedly a valuable tool for identifying criminals but nobody knows how often fingerprint examiners make a wrong call. The majority of misidentifications arise from human error and yet fingerprint experts are still willing to testify in court that a particular fingerprint can be identified with 100% certainty. Forensic Biologists refrain from using terminology such as, "a certain match", "no doubt", "100 percent" when presenting DNA evidence. Instead language such as "the suspect cannot be excluded" or "the results of the DNA analysis are approximately 1 billion time more likely if the DNA came from the suspect than if it had come from some other unrelated male" is used (Goodwin *et al,* 2007). It has been recently suggested that fingerprint experts should provide more scientifically defensible-and legally palatable-testimony (Kaye, 2010). There are certain standards that scientific evidence must meet if it is to be admitted as evidence in a court of law. In the US the Daubert Standard, which provides a rule of evidence regarding the admissibility of expert witnesses' testimony, is applied. Similar standards are used in other jurisdictions such as the UK (House of Commons Science and

Technology Committee, 2004) and Australia (see for example *R v Bonython*, (1984) 38 SASR 45), which require the error rates of scientific techniques to be included (Williams, 1994). For some reason, fingerprint identification has not been required to meet this standard (Cole, 2006). Fingerprint examiners make a distinction between "practitioner error" and "methodological error" with the latter encompassing the notion that if an examination is done correctly the error will always be zero. History tells us that fingerprint experts are not perfect and they make mistakes. Examiners' decisions are influenced by what they are told before they examine a fingerprint (Guy, 2007) and such biases will remain so long as examiners refuse to acknowledge them (Cole, 2006). According to Cole (2006) two-thirds of the mistakes emerged only in exceptional circumstances, such as an unexpected confession. These mistakes should not be ignored despite what difficulties lay in understanding and calculating the error rates in fingerprint identification (Kaye, 2010). Ignoring the existence of error prevents fingerprint analysis from being improved (Cole, 2006). Fingerprint evidence is also limited in a number of other ways. Crime scene prints are often transient and are easily removed by human or environmental factors (Barnum and Klasey, 1998). Recovered prints are often only partial prints reducing the amount of material available for comparison. Furthermore the laboratory techniques used for identifying latent fingerprints all have limitations. Ninhydrin and DFO will not work on non-porous surfaces, such as glass or polished wood, nor will they work on surfaces that are wet (Home Office Scientific Development Branch, 2005). DFO cannot be applied to papers or inks that fluoresce. Powders do not work well on aged prints or surfaces that are rough, dirty or porous, whilst cyanoacrylate (superglue) treatment requires optimal developing conditions (temperature, humidity and time), can rarely be performed at the scene and is a health and safety concern (Home Office Scientific Development Branch, 2005). Like DNA, fingerprints lose their evidential value if there is no reference sample to compare with and even though this type of evidence can link a person to a crime scene, it often falls short of proving innocence or guilt without further corroborating evidence.

1.3 Age Determination of Physical Evidence

The ability to determine the age or more specifically the time the evidence was deposited at a crime scene is a limitation common to almost all forms of physical evidence and is something that continues to elude forensic investigators today. Determining the time of deposition is often

crucial to an investigation and without it the value of the evidence can be decreased or completely lost. The power of CCTV (and to a lesser extent the eye witness) lies in the fact that a time frame can be established and therefore particular events or evidence can be attributed to a particular scene at a specified time. Unfortunately the majority of crimes do not occur in the presence of CCTV or witnesses therefore investigators rely on scientific methods to provide this information. Unfortunately there are very few methods that exist with any real accuracy or precision, thus supporting the applicability of this research.

1.3.1 Ageing Non Biological Evidence

Evidence is often classified as either chemical or biological. Biological evidence includes blood, semen, hairs, saliva, urine, faeces and tissue whilst chemical evidence deals with paints, inks and drugs to list a few (Saferstein, 2006). These two groups of evidence are fundamentally different in their composition and therefore age differently. Materials such as paper and ink tend to age in chemically predicable ways (Brunelle and Crawford, 2003). The initial phase of the ageing process of ink involves solvent evaporation (Bugler *et al,* 2008). Oxidation, polymerisation and cross linkage processes then act to create a protective film with which materials can diffuse across into the substrate (Bugler *et al,* 2008). Environmental degradation factors such as Ultra Violet (UV) light, temperature and moisture then act on the protective film causing a loss of colour, and in the case of paint, the outer primer layers can crack and peel away. Whilst this process is consistent under controlled conditions (Bugler *et al,* 2008) most forensic samples will be aged in uncontrolled and unknown environmental conditions. This will create uncertainty in any age estimation. The constituents of inks have changed over time, which also has an effect on the ageing process. Inks manufactured post 1970's contained fluorescent taggants which are easily identified and can be matched to reference collections making the ageing process simpler. Brunnell and Lee (1989) used a single solvent extraction mass independent process to age ballpoint ink whilst techniques such as High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), UV and Infra-red (IR) spectroscopy have all been used to target specific components across the drying process (Bell, 2006) with moderate success.

1.3.2 Ageing Biological Evidence

The most common types of biological material recovered from crime scenes include blood, semen, hair, fingerprints, saliva, bones, teeth and human tissue (Jackson and Jackson, 2011) however, ageing this type of evidence has proved difficult. There are three key elements in ascertaining an accurate method for ageing biological material. Firstly, the technique must measure a component that degrades at a consistent rate, independent of environmental factors. Secondly the levels of the component being measured must not vary significantly between individuals (small inter-donor variation) and thirdly, the technique must be sensitive enough to measure changes in the components levels as the biological material ages. Unfortunately developing methods that satisfy these criteria has been relatively unsuccessful so far.

1.3.2 (i) Ageing Decomposing Body

In a murder investigation, the most important piece of evidence is often the victim. Determining the time since death or post mortem interval (PMI) is the most common form of ageing evidence used in the forensic arena today. Establishing the PMI is vitally important as it can help establish a timeline of events as well as corroborate or refute witness and suspect's alibis or stories (Catts, 1992). Determining the PMI is a complex problem involving a number of intrinsic factors that may alter the normal course of post-mortem change, which can affect the accuracy of age estimations (McClay, 1996). These include the age, sex, constitution and previous physiological and pathological states of the subject, as well as numerous external factors (Prieto-Castello, 2007). It is often debated whether pathological or entomological techniques for determining PMI are more precise (Joseph *et al,* 2011). Pathology methods include measuring body temperature and determining the state of rigor mortis and lividity, whereas entomology is based on the succession rate of various insects on the decomposing corpse (Saferstein, 2006). Both approaches are routinely used and generally provide sufficient information regarding the time of death to be an important investigative tool. Other methods have also been tested, though none are routinely used as they lack precision, accuracy and robustness. For example single-cell gel electrophoresis (SCGE), also known as the comet assay, has been used to evaluate post-mortem cell death processes, specifically nuclear DNA fragmentation (Caglar *et al,* 2007). Following the death of an organism, intra-cellular nucleases cause chromosomal DNA to degrade into increasingly smaller fragments over time (Johnson and Ferris, 2002). These fragments can be separated and visualised but the assessment is rather

subjective and inaccurate. The level of precision of this technique falls short of what is required from a forensic application.

Figure 3: Comet Assay: The degraded DNA (fluorescent green material) is separated using gel electrophoresis however accurately quantifying the fragments and hence calculating the rate of degradation is difficult using this methodology. Image adopted from Amsbio Research (http://www.amsbio.com/Comet-Assays.aspx).

Determining the age of a bruise is another pathological based technique which has been used in assault cases. Age determination can help to corroborate or dispute the version of events provided by witnesses or suspects. Though the research in this field is limited, there has been a moderate amount of success in analysing the colour changes that occur in the skin with time. Langlois and Gresham (1991) noted that bruising underwent a number of different colour changes (red followed by blue and finally yellow) but unfortunately it was only during the "yellow" phase that an accurate estimation could be obtained. Trujiilo *et al,* (1996) who used a "tri-stimulus colourimeter" to measure the colour density of bruising concluded that although visual examination may be the easiest method for estimating the age of a bruise, it is unreliable and inconsistent. Hayashi *et al,* (2004) used an immunohistochemical method to age skin wounds. Wound healing is a complicated but well organised biological response consisting of three phases, inflammation, proliferation and maturation (Mori *et al,* 2002). Various biological substances such as growth factors, cytokines and adhesion molecules are known to be closely associated with each phase (Mori *et al,* 2002). Hayashi *et al,* (2004) looked at the levels of one

of these molecules, vascular endothelial growth factor (VEGF), in wounds aged up to 21 days. They suggested that VEGF levels could possibly be used to predict the age of a wound that was older than seven days. This technique could not distinguish between wounds that were less than seven days old limiting its value as a forensic tool.

1.3.2 (ii) Ageing Skeletal and Teeth Remains

Determining the age of a person at the time of their death can be achieved by examining the bone morphology (Dorandeu, 2008), or by measuring the levels of aspartic acid racemization in dentin, which is the rate of transformation of one aspartic acid stereoisomer to another (Ritz *et al*, 2003). Alternatively the accumulation of D-aspartic acid in bone osteocalcin, one of the most abundant noncollagenous proteins of the organic bone matrix, can also be used (Ritz *et al*, 1996). Using these techniques a person's age at death can be estimated within a certain age range [Curate *et al*, (2013); Crowder *et al*, (2012)]. This type of examination is different to determining the time since death or post mortem interval. This requires the application of different techniques. Over time degradation processes can alter the molecular integrity of bone and thus provide an indicator of age. As bone decays, the organic matter undergoes a complex process of disintegration, which leads to the formation of simple chemical substances which can be analysed (Prieto-Castello, 2007). Prieto-Castello (2007) used X-ray diffraction techniques to measure potassium, sulphur, nitrogen, urea, total protein, phosphorus, and certain other parameters related to the degree of crystallinity of the mineral component in medullar and cortical bone. They concluded that a combination of both crystallinity and biochemical analyses (especially of urea, potassium and sulphur) in tandem could be an alternative method for dating bone remains (Prieto-Castello, 2007). Nagy *et al*, (2008) used Fourier transform infrared spectroscopy (FT-IR) to chemically analyse various bone samples. Infrared spectroscopy involves passing infrared radiation through a sample (Griffiths and De Hasseth, 2007). Some of the radiation is absorbed whilst some is transmitted through the sample. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum (Griffiths and De Hasseth, 2007). Nagy *et al*, (2008) used the amount of crystals and carbonate–phosphate as a means to distinguish between recent and archaeological bone samples but stopped short of providing an accurate age of the samples. Ageing archaeological samples (more than 500 years old) can be achieved by measuring the carbon ratio of different species in much the same way as fossils are dated (Prieto-Castello,

2007). Unfortunately this technique is not suitable for distinguishing between younger bone samples (Prieto-Castello, 2007).

Many other forms of analysis have been trialled ranging from the determination of radionuclide and trace elements (Swift *et al*, 2001) to biological analysis using blood typing methods (Kamenev, 1983). Though some of the results have been encouraging there are a number of factors that affect techniques based on the analysis of chemical degradation products. The bone structure, density and composition are affected by the age and health of the person at the time of death (Johnston *et al*, 1992). Pathological conditions such as osteoporosis and leukaemia as well as non-pathological factors such as diet will also affect bone structure (Wohl *et al*, 1998). If the remains are discovered buried, the acidity of the soil will also affect the degradation rate of bone (Trueman *et al*, 2004). With so many potential variables the task of developing a robust reliable forensic method for ageing bone samples remains a difficult one.

1.3.2 (iii) Ageing Semen

The conviction rate for sexual assault offences tend to be very low for a number of reasons including shame, fear and deep-seated cultural notions that the woman is somehow to blame (Cannold, 2011). In those cases that are reported and make it to court the issue of consent is often argued, which can be very difficult to prove given these types of crimes generally lack independent witnesses (Cannold, 2011).

There are three routinely used methods for identifying the presence of semen. The first is a cytological examination for the presence of sperm using microscopy techniques, the second involves the detection of the enzyme, acid phosphatase, and the third method is an immunoassay to detect an enzyme specific to seminal fluid; prostate specific antigen (PSA) (Allery *et al*, 2003). Other methods exist such as measuring free choline levels in the vagina (Davies and Wilson, 1974) or levels of zinc contained within the sperm [Hooft (1990); Suzuki (1983)] however, they are rarely used. The three routinely used methods can provide some indication of when the semen was deposited. The enzymatic activity of acid phosphatase generally only lasts for 48 hours therefore a positive result not only indicates the presence of semen, but also suggests the sample is less than 48 hours old (Allery *et al*, 2003). A microscopic examination of the quality of the sperm cells can also provide some indication regarding the age of the sample (Romero-Montoya *et al,* 2011). As sperm age they degrade and their tails begin to drop off (Noureddine, 2011). Although the proportion of complete intact

sperm to tailless sperm can be used to estimate the age of the sample, the environmental conditions in which these samples age, can affect the degradation rate (Saferstein, 2006) and accuracy of age estimations. The longevity of sperm varies from six hours in the mouth to 7-10 days in the cervix (Zinaman *et al*, 1989) and this is primarily due to the acidity of the environment and the presence of degradation enzymes in the different locations (Zinaman *et al*, 1989). PSA tends to be an unreliable age indicator due to both natural and pathological changes in its concentration. For example, elevated levels are seen in prostate cancer as well as other prostate related diseases such as prostatitis and benign prostatic hyperplasia (Zorn and Nueber, 2012). Although estimations can be made based on these techniques, there is still no specific method for accurately determining the age of a semen sample.

1.3.2 (iv) Ageing Fingerprints

Fingerprints are a powerful tool for identification purposes but like DNA they have always been susceptible to the issue of "when" rather than "who". Defence lawyers manipulate this deficiency and as a result the value of fingerprint evidence is often lost. There has been a significant amount of research into solving this problem but unfortunately it has been met with limited success. Darymple *et al,* (1977) first noticed differences between fresh and older fingerprints. Fresh prints displayed a yellow green fluorescence when exposed to a high intensity argon laser light, whilst older fingerprints fluoresced orange. These findings suggested there was a definite change in the molecular architecture of the components found in fingerprint residue but this colour change was variable, inconsistent with time and was heavily dependent on both the environmental conditions as well as the individual donor (Darymple *et al*, 1977). Dikshitulu *et al,* (1986) examined changes in the chemical substances found in the perspiration of the fingerprint residue over time using chromatography methods. They found that the residue from different individuals contained essentially the same components (such as amino acids, glucose, proteins and fatty acids) but on ageing, some of the components disappeared and the relative proportion of the individual components essentially changed (Dikshitulu *et al*, 1986). They concluded that ageing fingerprints using this approach may be possible if one knows the person who has committed the crime and can compare the pattern for different components on ageing (Dikshitulu *et al*, 1986). This is obviously a major limitation given the offender will be unknown in a large percentage of criminal cases. Another major concern with this study concerned the sample preparation. All samples were aged under

controlled conditions and there was no apparent consideration given to the many environmental factors that forensic samples would be exposed to. The size of the population tested by Dikshitulu *et al*, (1986) was small (four males and four females) and therefore caution must be taken with any data interpretation involving such a small sample size.

Even with today's technology, researchers are still no closer to solving the problem of ageing fingerprints. Archer *et al*, (2005) looked at changes in the lipid composition of latent fingerprint residue using gas chromatography – mass spectroscopy, a technique often described as the gold standard in analytical chemistry. They observed a loss of squalene (skin) cells from fingerprints over time. The concentration of saturated fatty acids, including tetradecanoic, palmitic and stearic acid, followed an unusual time wise pattern. There was an initial increase in levels over the first 20 days, followed by a decrease back to or below original levels. The authors stressed these were only trends and that large intra and inter donor variations were seen throughout the study (Archer *et al*, 2005). These variations and the weak correlations meant that the measured variables could not provide an accurate age estimation of latent fingerprints. Baniuk (1990) highlighted a number of issues that need to be considered when attempting to age fingerprints. The rate of the ageing process depends on the qualitative and quantitative composition of the sweat (eg sodium, potassium, calcium and chloride ions) and grease (eg lipids, fatty acids and triglycerides) components in fingerprints, the type of surface containing the print and the environmental conditions experienced during ageing. Baniuk, (1990) claims the content of the grease components plays a major role in the ageing process. The amount of grease will be donor dependant and as such difficult to standardise. The physical and chemical properties of the surface also have an effect with smoother, non-absorptive surfaces being conducive to good preservation of fingerprint traces for a long time, as opposed to rough and absorptive surfaces that are not (Baniuk, 1990). It was suggested that the change in the rate of ageing process can occur at any stage. Temperatures above 37°C, low humidity, precipitation, light and air pollution will all increase the rate of ageing. Alternatively temperatures below 0°C have a delaying effect (Baniuk, 1990). Reconstructing the exact conditions in which the fingerprints were aged at a crime scene is difficult if not impossible to replicate therefore related age estimations will always contain a degree of uncertainty (Baniuk, 1990). Baniuk, (1990) comprehensive analysis highlights the complexity of ageing fingerprints and with so many variables it is not surprising that an accurate method does not exist.

1.3.3 Ageing Bloodstains

In the past 40 years there has been a significant amount of research into developing methods for ageing bloodstains. The most likely explanation for this is the prevalence of blood at crime scenes, especially serious crime, and the ability to identify the donor of the blood. There have been numerous different approaches to achieve this common goal however the techniques trialled so far have had limited success. As such there are no routinely used methods for ageing bloodstains.

1.3.3 (i) Colourmetric Methods

It has been known for some time that bloodstains change colour with age. This colour change from red to brown is predominately caused by the oxidation of haemoglobin to methaemoglobin and many of the early methods were based on this phenomenon. Patterson (1960) measured the colour change using a reflectance colorimeter and although he was able to produce an objective method for colour assessment, the reproducibility and reliability of the colour assay lacked the accuracy and precision required for forensic purposes (Patterson, 1960). The oxidation rate of haemoglobin (and therefore colour change) is thought to be highly variable and susceptible to many intracellular and extracellular factors, such as humidity, temperature, direct sunlight and even the surface the bloodstain is deposited on (Kind *et al,* 1972). There are also a number of intracellular inorganic and organic reducing agents, such as ferrous ion, that can affect the conversion rate of haemoglobin to methaemoglobin (Castro *et al,* 1978). These can result from both pathological (eg methemoglobinemia, pyruvate kinase and Glucose-6-phosphate dehydrogenase (G6PD) deficiency) and natural conditions within the body (Ash-Bernal *et al,* 2004). It is also suggested that the initial ratio or amount of methaemoglobin can be affected by factors such as a person's dietary and social habits, such as smoking, anaemia and vitamin D deficiency (Rehman, 2001). With so many variables to consider, all of which have the potential to effect age estimations, it is understandable why this method was imprecise and unreliable. Most of these variables will be unknown for any given crime scene sample which makes this technique an estimation based on a number of assumptions.

Research that followed involved non-destructive spectrophotometric methods, which analysed the light absorbing properties of blood extracts. Kind *et al,* (1972) developed an analogous procedure that had a similar level of accuracy to that achieved by Patterson (1960), which was

again inadequate for forensic applications. Ballantyne (2009) revisited old colourmetric techniques with newer technology. He looked at the visible spectral profile of haemoglobin and observed a hypochromic shift of the soret band. By determining the extent of the shift, it was found that a distinction could be made between blood stains that were deposited hours, days and months prior to analysis (Ballantyne, 2009). According to Ballantyne (2009) this method requires as little as 20-40 nanolitres of blood, which is beneficial for forensic applications where sample quantity may be an issue. Bremmer *et al,* (2011) successfully applied visible reflectance spectroscopy for the non-destructive identification and age estimations of blood stains. When blood exits the human body, oxyhemoglobin auto-oxidizes into methemoglobin, which in turn denatures into hemichrome (Bremmer *et al,* 2011) causing a colour change. Visible reflectance spectroscopy can be used to measure this colour change quantitatively (Bremmer *et al,* 2011). In this particular study, blood stains were distinguished from other blood resembling substances (e.g. ketchup, red wine, lip gloss) deposited on white cotton. Bremmer *et al,* (2011) demonstrated that under controlled circumstances the presence and accumulation of the three haemoglobin derivatives showed a distinct time-dependent behaviour in samples aged over 60 days. Sample age could be estimated with an uncertainty margin of 14 days (Bremmer *et al,* 2011). Edelman *et al,* (2012) conducted a feasibility study into the use of near-infrared (NIR) spectroscopy for age estimations of blood stains. Near-infrared spectroscopy (NIRS) is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum (from about 800 nm to 2500 nm). Molecules are distinguished by their absorption and vibrational characteristics (Heinz *et al,* 2008). Edelman *et al,* (2012) concluded that the age of blood stains up to 1 month old could be estimated successfully with an 8.9% error rate. Botonjic-Sehic *et al*, (2009) also explored the potential use of NIRS for ageing blood stains. They demonstrated that spectral changes in a broad wavelength band from 1460 to 1860 nm were useful to estimate the age of a blood stain on glass and gauze. The disadvantage of using spectroscopy, as demonstrated by these authors, is that point measurements of all suspected stains at the crime scene can be time consuming and labour intensive (Edelman *et al,* 2012). Furthermore careful development of a set of calibration samples and application of multivariate calibration techniques is essential for near-infrared analytical methods (Roman *et al,* 2007). As such Edelman *et al,* (2012) evaluated the feasibility of using hyperspectral imaging for aging bloodstains. Hyperspectral imaging integrates conventional spectroscopy and imaging, thereby obtaining both spatial and spectral information from all objects in the field of view (Edelman *et al,* 2012). The spectral properties of all objects can be recorded together with information about their location and distribution

within the scene, without the need for further documentation. Current hyperspectral imaging systems are also fast and portable, therefore they can be transported to the crime scene where traces can be analysed and interpreted in the original context (Edelman *et al,* 2012). Edelman *et al,* (2012) determined the relative amounts of oxyhemoglobin, methemoglobin and hemichrome within the blood stains from the spectra. These values were compared to a reference dataset which allowed age estimations on stains aged for up to 200 days. The absolute error of the age estimations increased with age, with a median relative error of 13.4%. By clustering the stains according to their haemoglobin derivative fractions Edelman *et al,* (2012) was able to determine the order of formation of blood stains aged in unknown environmental circumstances and without the use of a proficient reference dataset.

Figure 4: Near-infrared spectroscopy: Hypercube of a simulated crime scene, with two spatial (x,y) and one wavelength (l) dimension (left). From the hypercube a reflectance spectrum was obtained from each pixel (right). Image taken from Edelman et al, (2012).

Guo *et al,* (2012) adopted a fluorescent based technique that measured the fluorescence lifetime of solid-state blood. There are many different fluorescent molecules in blood and their fluorescence lifetime is strongly dependent on the environment in which they exist (Guo *et al,* 2012. The fluorescent lifetime is the average time the molecule remains in its excited state before decaying back to its ground state by emitting light. Guo *et al,* (2012) found that as the environment changes and the proteins age, the fluorescence lifetime decreases. The molecule

that was targeted was the amino acid, tryptophan. Although this study showed promise the authors agreed that more work was required to determine precisely how external environmental factors influence the lifetime of the fluorescence signal.

Whilst all these findings are an important step toward the practical implementation of blood stain age estimation in forensic casework, to date none of the methods described have been validated or implemented in forensic practice.

1.3.3 (ii) Enzymatic Methods

Brinkmann *et al,* (1979) analysed acid phosphatase, phosphoglucomutase, adenylate kinase, adenosine deaminase and phosphoglucomutase dehydrogenase from stains that had been aged under differing environmental (temperature) conditions. They noted that the rate of decline in enzyme activity correlated to the type of enzyme, the phenotype within the enzyme system and the temperature and storage times. Unfortunately the correlations were relatively weak and their conclusions were no more than generalisations. Furthermore the stains and enzyme cocktails were artificially produced. Initial enzyme concentrations were known and standardised to ensure the same amount of enzyme was present in each stain. This limits its relevance to real forensic case samples unless these enzyme concentrations were consistent with levels seen *in vivo*. Tsutsumi *et al,* (1983) examined forensic like samples and found that enzyme analysis provided results that were neither precise nor consistent enough to be applied to criminal case work. Ballantyne (2009) examined over thirty enzyme candidates for ageing studies. They found that six enzymes, lactate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glycerate dehydrogenase, alcohol dehydrogenase and phosphogluconate dehydrogenase, had differing levels of activity that correlated with stain age. Unfortunately the resolution was limited to months-years rather than days, limiting its value (Ballantyne, 2009). It is thought that the enzymatic decomposition process requires an aqueous environment (Tsutsumi *et al,* 1983), which is an environment that is rarely present in dried, ageing bloodstains. This could explain why enzymatic techniques for ageing bloodstains have not been successful.

1.3.3 (iii) Chromatographic Methods

In the past researchers have utilised reverse phase high performance liquid chromatography (HPLC) to examine the degradation changes in haemoglobin within a bloodstain [Andrasko,

(1997); Inoue *et al* (1992)]. HPLC an analytical technique used for the separation of compounds (Meyer, 2010). The separation of a sample into its constituent parts occurs because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation column (Meyer, 2010). This separation allows constituents to be identified and quantified by a detector.

Figure 5: Components of HPLC system (Image adopted from http://www.shodex.net)

Andrasko, (1997) examined three (which they designated as peaks, "X", "Y" and "Z") of the many compounds formed when blood degrades and measured these using HPLC over time. Though he concluded a lineal correlation could be obtained between one of the decomposition peaks (designated "X") and time, the lack of precision was an issue. Inoue *et al,* (1992) used HPLC to measure the globin chains of the red blood cell. They found a general decline in the number of alpha chains in relation to the heme molecule with increasing age of the bloodstain. Kumagai, (1993) used cation-exchange HPLC to measure haemoglobin fractions. He found that the amount of HbA1d fraction increased linearly as the age of the bloodstains increased, although the amount of HbA1c remained virtually unchanged. Despite these findings the data

contained significant variation between individuals, limiting the use of this technique as a forensic tool.

1.3.3 (iv) Electron Paramagnetic Resonance Spectroscopy

As technology evolved, new techniques were tried on old theories. In 2005, a group from the Department of Forensic Medicine at the University of Tokushima used Electron Paramagnetic Resonance (EPR) Spectroscopy for estimating the age of human bloodstains (Fujita *et al*, 2005). This EPR dating technique was based on measuring the denaturation of haemoglobin and hemoproteins. Samples were prepared without anticoagulant making them more akin to crime scene samples but unfortunately the samples were then aged under controlled conditions, distinguishing them from forensic samples. Fujita *et al*, (2005) established that at a certain resonance (77K), human blood gave four definitive signals, which was due to the presence of four different species of iron, namely ferric high spin, ferric non heme, ferric low spin and free radical species. By plotting double logarithms of the EPR intensity ratio of one of these signals against the number of days since bleeding, they identified a correlation, albeit under controlled conditions (Fujita *et al*, 2005). This method was non-destructive in that it did not consume any of the evidence, which is a significant advantage given many forensic samples exist in small quantities and the sample will often require subsequent DNA profiling (Fujita *et al*, 2005). There were a number of limitations relating to this study, one of those being the volume or amount of blood required for such analysis. Ten milligrams of dried blood was required to estimate the age of a bloodstain using EPR, which is often far greater than the volumes recovered from crime scene samples. DNA profiling require as little as 10 picograms (1×10^9 fold difference) of starting material (Rudin and Inman, 2001) whilst presumptive blood screening tests such as Kastle Meyer and Leucomalachite Green require significantly less blood to obtain a positive reaction (Webb *et al*, 2006). Fujita *et al*, (2005) observed that the designated peaks or signals varied in intensity, depending on the material the bloodstain was deposited on. Although this suggests the adsorbates will affect the age estimations it would not prevent this technique from being a useful forensic tool because the material the bloodstain was deposited on, will be known. Fujita *et al*, (2005) concluded by stating that there was a good lineal correlation under controlled conditions however outside these conditions the calibration curve was distorted and significantly affected by temperature and light exposure. Furthermore whilst the lineal correlation was useful for samples aged up to 432 days, any predicted value had an error range of 25%, even under controlled conditions. An error rate of this magnitude is

unlikely to be acceptable for a forensic application and will have no real benefit in a criminal court.

Haemoglobin based techniques for ageing bloodstains have lacked precision and accuracy. It has been suggested this is due to the number of different and uncontrollable chemical and physio-chemical processes that degrade haemoglobin, including proteolysis and degradation by endopeptidases (Pontremoli *et al*, 1984). Ageing blood cells can also rupture releasing the haemoglobin, which is subsequently ingested by macrophages (Hendil, 1980). The globin chains are broken down into individual amino acids and are either metabolised or used to build new proteins. The remaining structure is converted to bilirubin as shown in Figure 6 (Hendil, 1980). Various parasites such as *Plasmodium Falciparum* uses host erythrocyte haemoglobin as a major nutrient source resulting in its degradation (Goldberg *et al*, 1990). This process is mediated by the concerted action of nearly a dozen proteases (Goldberg, 2013). With some many potential variables it is not unexpected that techniques concerned with measuring haemoglobin levels are susceptible to large variation and error rates.

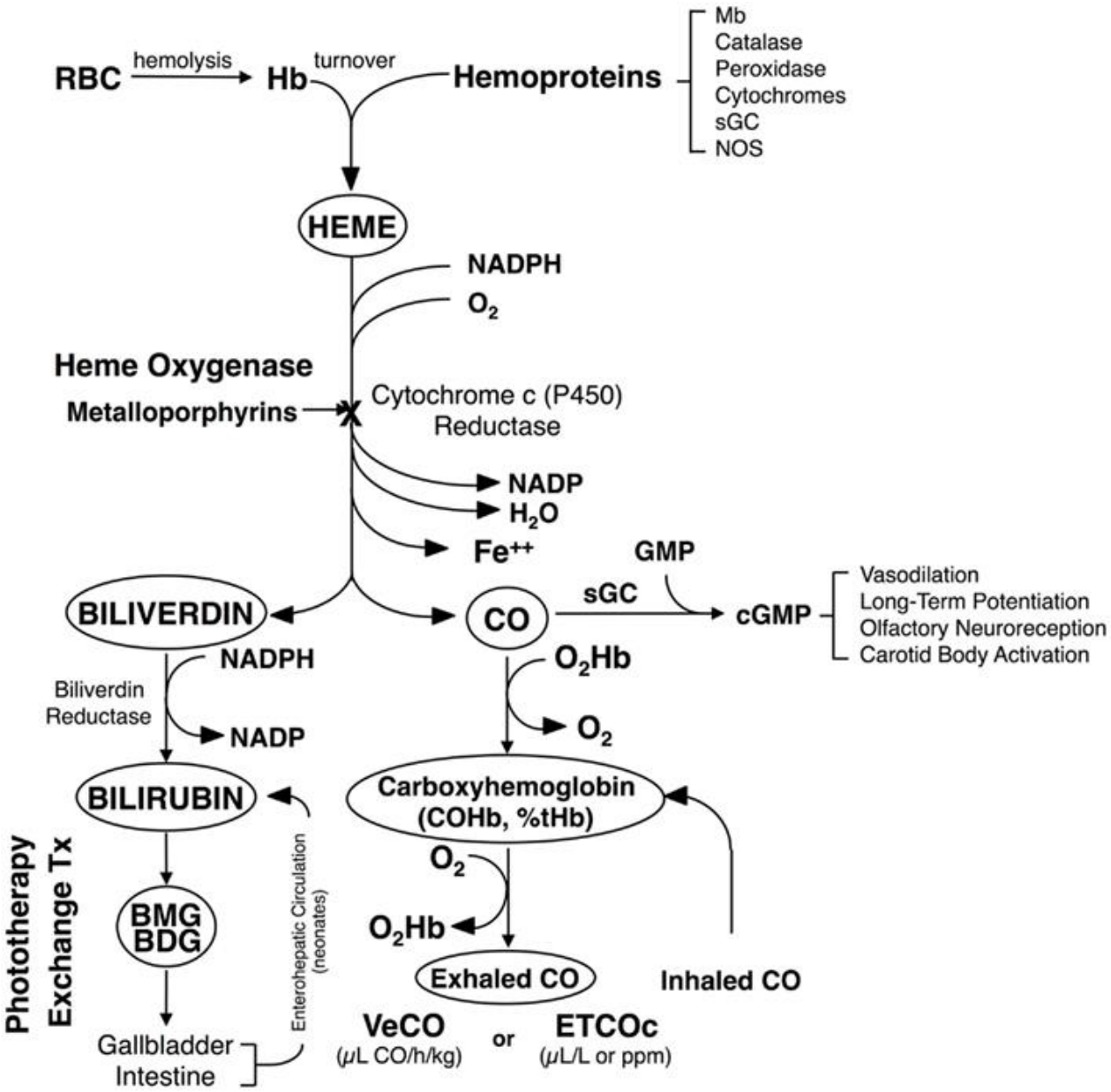


Figure 6: Heme Degradation Pathway. The turnover of haemoglobin (Hb) and other hemoproteins yields heme. This heme is metabolized to equimolar quantities of carbon monoxide (CO), iron (Fe2+), and biliverdin. Biliverdin is subsequently reduced to form bilirubin. Image modified from Vreman et al, (2001).

1.3.3 (v) Atomic Force Microscopy

Strasser *et al,* (2006) adopted a different approach to age determination focussing on the physical properties of blood cells, in particular the cell walls. Strasser *et al,* (2006) used Atomic Force Microscopy (AFM) to determine whether there were any physical and morphological changes to the cell shape over time. AFM has a very high lateral and vertical resolution, which can reach 0.01nm and a force resolution capability that can reach ranges of 10^{-4} nm. It is therefore an ideal tool for producing high resolution images and measuring the elasticity properties of blood cells.

Figure 7: Schematic diagram of the workings of an Atomic Force Microscope (AFM). The AFM is a high-resolution scanning probe microscope, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The information is gathered by "feeling" the surface with a mechanical probe (Lang et al, 2004).

Strasser *et al*, (2006) made blood smears on glass slides which were then left to age for varying lengths of time. They demonstrated that the appearance of red blood cell was consistent and indistinguishable in blood stains aged up to four weeks. By calculating the force-distance curves they determined that the elasticity of the blood cells decreased with the age of the sample. This finding was based on averages and when individual results were examined there was a high level of standard deviation and hence error rate, limiting the discriminating ability of this technique (Strasser *et al*, 2006). Variation between blood cells from the same individual (intra-donor variation) was also observed. It was found that the degree of variation was dependent on where the cell was probed and therefore influenced by the operator. If points were chosen too close to the edge of the cell, any "stiffness" could be due to the solid support (usually glass slide) that housed the cells. To obtain some level of consistency measurements were carried out on the thick centre, but the technique was still susceptible to operator variation. A further limitation of this study was that samples were standardised against healthy individuals. Prescribed or illicit drugs, blood pathologies such as spherocytosis, anisopoichilocytosis, sickle cell anaemia (Nader *et al*, 1999), certain leukaemias (Petitou *et al*, 1978), and chronological age of the donor (Chabenal *et al*, 1987) are all conditions that affect the shape and/or the elasticity of the blood cell membrane/wall. A healthy 60 year old could present with similar cell wall characteristics as an unhealthy 20 year old. It would be difficult to standardise this technique, thus limiting its potential for ageing forensic blood samples.

1.3.3 (vi) Aspartic Acid Racemization Rates

Traditionally, the aspartic acid racemization (AAR) rate in slow turnover tissues, such as tooth tissue, has been used to determine the age of an individual (Ritz *et al*, 1996) but recently it has been applied to determining the age of bloodstains (Arany and Ohtani, 2011). Arany and Ohtani, (2011) suggested that non-enzymatic, spontaneous reactions, including deamidation, isomerization and racemization, lead to the accumulation of D-aspartyl derivatives in proteins. They found the soluble protein fraction of a bloodstain produced a stronger correlation between elapsed time and D-aspartic acid content than total amino acid fractions. They also established that the time lapse after the creation of a bloodstain can be determined *ex vivo* by measuring the extent of aspartic acid racemization. The accumulation of D-aspartyl derivatives are however affected by certain haematological conditions such as chronic haemolytic anaemia, hereditary spherocytosis and chronic renal failure (Ingrosso and Perna, 1998). Brunauer and Clarke (1986) observed variable *in vivo* rates of accumulation of D-aspartyl derivative in different

tissue types. Lowenson and Clarke (1988) made similar findings suggesting the accumulation rate is 10-100 times greater in blood cell proteins than in some slow metabolism tissue. This variation makes standardising a technique difficult. The discriminating ability of the technique proposed by Arany and Ohtani, (2011) was also limited suggesting that aged bloodstains could only be distinguished by years, rather than days. This would significantly reduce the value of this technique as a routine forensic tool.

1.3.3 (vii) Degradation Rates of Nucleic Acids (DNA and RNA)

DNA degradation rates have not been used to age bloodstains but they have been used to determine the degradation levels of DNA within blood samples. Swango *et al,* (2006) examined the levels of degradation in an attempt to find a defining limit that would result in unsuccessful DNA profiling. They used a multiplex quantitative Real Time PCR (qPCR) assay that amplified two human genomic DNA target sequences of different length. The first target region, tyrosine hydroxylase gene (TH01), is a tetrameric short tandem repeat locus located on chromosome 11 (11p15.5). Tyrosine hydroxylase catalyses the hydroxylation of l-tyrosine to L-DOPA and is the rate limiting enzyme in the synthesis of catecholamines including noradrenaline and adrenaline (Connor *et al,* 2011). Swango *et al,* (2006) designed primers to produce an amplicon of approximately 170-190 base pairs in length. The second region, Colony Stimulating Factor gene (CSF), located on Chromosome 5 (5q33.3-34), codes for a secreted cytokine that influences hematopoietic stem cells to differentiate into macrophages and other related cell types (Stanley *et al,* 1997). Primers were designed to create an amplicon of approximately 70 base pairs in length. Both target regions are commonly amplified in SGM+ and Identifiler, which are commercially available DNA profiling kits used by forensic laboratories in the UK. The approach adopted by Swango *et al,* (2006) was based on the assumption that natural degradation processes predominately cleave DNA in a random manner. If the DNA strand is cleaved at random locations, larger targets will be more susceptible to degradational cleavage than smaller ones. In theory this creates a difference in degradation rates (ratio) between the two different sized amplicons over time. It was suggested by Swango *et al,* (2006) that if this change (ratio) occurs in a consistent and reproducible fashion, it could be used to determine the degradation level of the sample. This approach has many advantages including its extremely high specificity which allows for the simultaneous amplification of both products in a single reaction and is independent of the initial and often variable amounts of template DNA (Swango *et al,* 2006). The technique also saves time, labour, reagents and

the original sample which is important in the forensic context. Furthermore it is highly sensitive, being able to detect a single copy of target DNA (Swango *et al,* 2006). The samples used by Swango *et al,* (2006) were degraded using artificial UV light or digestive enzymes, which had the benefit of speeding up the degradation process. But by using these methods, time course studies relevant to the natural degradation process could not be carried out. If samples were left to age and degrade naturally over time, these studies could have been linked to the age of the blood samples. This particular aspect will be explored later in this thesis.

Attempts have been made to age bloodstains using RNA. Methodologies have utilised the fact that different types of RNA decay at different rates resulting in a change in the ratios of RNA species as time progresses [Anderson *et al,* (2005); Bauer *et al,* (2003)]. Anderson *et al,* (2005) measured the quantity of 18S rRNA and B-actin mRNA using a reverse transcription duplex real-time PCR method. 18S rRNA is a component of the small eukaryotic ribosomal subunit (40S) (Gonzalez and Schmickel, 1986), whilst B-actin mRNA is one of the two non-muscle cytoskeletal actins found in humans (Hanukoglu *et al,* 1983). Ct values, which are the number of PCR cycles required to reach a pre-determined fluorescence intensity, were used to determine the degradation level of the blood stain. The more degraded a sample, the greater number of PCR cycles required to reach the settled fluorescence. Anderson *et al,* (2005) successfully obtained a moderate lineal relationship between the relative ratio of 18S rRNA and B-actin mRNA, and time (up to 150 days), when the bloodstains were subject to controlled conditions (refer to Figure 8). It was thought that the 18S rRNA and B-actin mRNA degraded at different rates because of their location within the cell. 18S rRNA is almost exclusively complexed with the small subunit of the ribosome which is thought to provide a protective environment for the rRNA, shielding it from enzymatic and chemical attack. In contrast B-actin mRNA exists in the cytoplasm and is not afforded any specific protection (Anderson *et al,* 2005). RNA is thought to be a good candidate for this type of study as they are highly abundant, relatively labile and differences between species can be exploited to develop species-specific tests (Anderson *et al,* (2005).

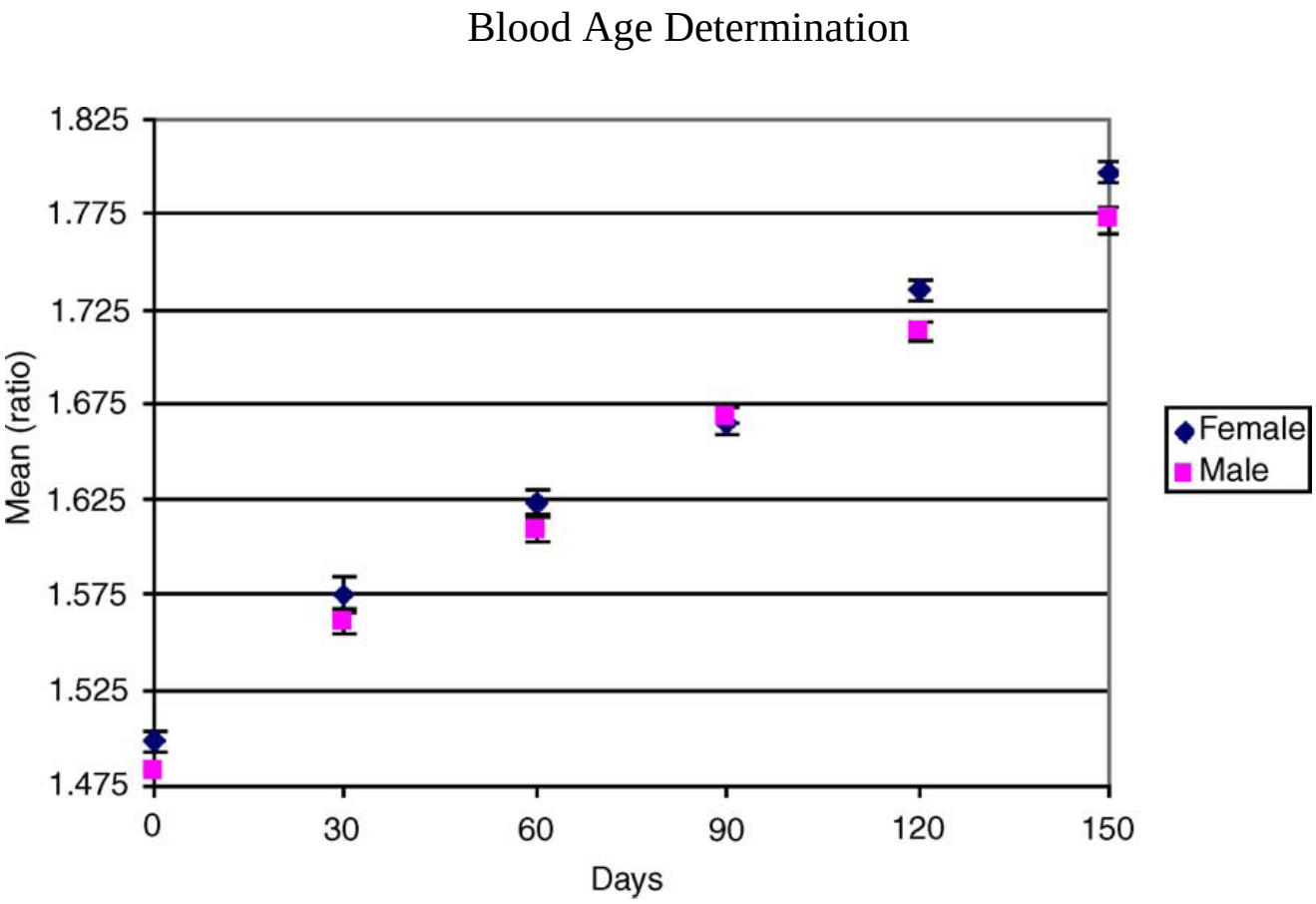


Figure 8: Correlation between 18S/B-actin ratio and Age. The Figure presents a one way analysis of the mean ratio (18S rRNA to β-Actin mRNA) and age, of ex vivo blood samples. Blood was collected from eight individuals ranged between 21 and 55 years of age. The blood was deposited onto individual pieces of fabric and left to age for up to 150 days. The DNA was then extracted from the samples and the quantities of 18S rRNA and B-Actin mRNA were determined using real-time reverse transcription PCR. A lineal correlation was observed between the mean RNA ratio and the age of the bloodstain. The trend was observed in both sets of samples obtained from male and female donors. Image extracted from Anderson et al (2005).

Bauer *et al,* (2003) conducted a similar study using the degradation rates of two housekeeping mRNAs, Cyclophilin protein and B-actin mRNA, which differed in size. Blood aliquots from each donor were deposited onto cotton fabric and stored up to five years at room temperature and protected from sunlight. RNA was extracted and reverse transcribed into complementary DNA (cDNA). Complementary DNA is DNA synthesized from a messenger RNA (mRNA) template in a reaction catalysed by the enzymes reverse transcriptase and DNA polymerase

(McNaught and Wilkinson, 1997). The quantity of each target was measured using a quantitative PCR method. Bauer *et al,* (2003) concluded that the change in expression levels of each target when expressed as a ratio, correlated with the age of the sample.

Although Anderson *et al,* (2005) and Bauer *et al,* (2003) approached it differently, both studies relied on two target species that degraded at different yet consistent rates. Anderson *et al* (2005) chose 18S rRNA and B-actin mRNA because of their different locations within the cell (cytoplasm compared with the nucleus) which imparted different degrees of susceptibility to degradation factors. Bauer *et al,* (2003) chose two RNA species that were present in the cytosol and therefore affected by the same degradation factors. However Bauer *et al,* (2003) relied on the theory adopted by Swango *et al,* (2006). The two targets differed in size and it is thought that the smaller target is less prone to being randomly cleaved. Bauer *et al,* (2003) demonstrated it was possible to estimate the age of a bloodstain due to the fact that the ratio of degradation between the long and short RNA decreased dependently with time. Though both of these studies produced encouraging results there were still areas of uncertainty. Anderson *et al,* (2005) did not address a number of key issues with regards to the Reverse Transcriptase Real time PCR assay, which meant that data interpretation was potentially unreliable and therefore not suitable for forensic type samples. Samples were aged under controlled conditions and significant inter and intra-donor variation was observed limiting the precision of any age estimation. The technique employed by Bauer *et al,* (2003) had large error rates (up to 74%) and therefore an age range, rather than an exact estimate could only be described. Bauer *et al,* (2003) did not address whether there were optimal sized targets for this type of analysis which may have provided a more accurate gauge of the levels of DNA degradation.

1.3.4 Ageing Hair Samples

Whilst there has been an intense focus on developing methods for ageing bloodstains using a variety of different approaches, there has been no attempt to age hair samples. This aspect was explored in this body of work.

1.4 Current and Potential Forensic Applications of Nucleic Acids

The introduction of DNA profiling has been arguably the most significant and influential technique in the last century and today it has become a discipline in its own right having a substantial impact on the criminal justice system (Walsh and Buckleton, 2005). Since establishing the initial operating platforms, scientists have continued to expand and develop new aspects of DNA analysis. Some of the relevant developments have included the introduction of mini STR's (Pizzamiglio *et al,* 2006); modifications to the run cycle number as well as pre and post PCR purification and processing (Smith and Ballantyne, 2007). Testing was originally limited to relatively high quality samples of blood, semen, saliva or hair. This progression has meant DNA can now be successfully analysed from items of clothing (Webb *et al*, 2001), touched surfaces, fingernail scrapings (Harbison *et al*, 2003), dandruff (Lorente *et al*, 1998) as well as partially degraded DNA samples (Pizzamiglio *et al,* 2006). Routine STR profiling is now a high-throughput, largely automated technique and computer assisted data analysis has streamlined the entire analytical process (Gill *et al*, 2007). Next generation instrumentation is continually being developed with the aim to improve sensitivity, reproducibility, reliability, whilst reducing processing time and cost. This includes the development of smaller portable units that can be taken to the crime scene (Hopwood *et al*, 2006).

Y-STR and mtDNA profiling are now routinely used when traditional STR methods fail to produce results, when difficult samples are analysed or when familial studies are required (Rapley and Whitehouse, 2007). Whilst mtDNA uses a different form of typing technology (sequencing), Y chromosomal analysis is based on traditional STR methods, with the obvious difference being the target chromosome (Rapley and Whitehouse, 2007). Another area of interest is the application of single nucleotide polymorphisms (SNPs). SNPs are the most common type of genetic variation among people. Each SNP represents a difference in a single nucleotide. SNPs occur normally throughout a person's DNA (Nachman, 2001). According to the National Human Genome Research Institute SNPs occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. Whilst SNPs act as biological markers, helping scientists locate genes that are associated with disease, they can potentially be used in novel areas such as identification and ethnic determination (Cortellini *et al,* 2013). The benefits of SNPs is that they have a low mutation rate, amenability to high throughput and automated data analysis, a shorter PCR amplicon size (which assists in

their ability to be multiplexed making them a suitable target for highly degraded samples) and they are in vast abundance in the genome (Sobrino *et al*, 2005). However SNP analysis as a routine method faces obstacles (Butler *et al*, 2007). SNP analysis for example, has a lower discriminating power when compared to STR profiling as there are only two alleles per marker (Butler *et al*, 2007). Approximately 50 SNPs are needed to achieve the same discriminating power as a 10 loci STR assay. Furthermore vast infrastructure, in terms of DNA databases, exists for STR typing but is currently lacking for SNP analysis (Butler *et al*, 2007). Whilst it is unlikely SNP analysis will replace STR analysis anytime soon, SNPs may play a useful role in niche applications such as ancestry informative markers (AIMs), predicting phenotypic traits, and other potential forensic case work applications (Butler *et al*, 2007).

Although DNA profiling has become a highly sophisticated technique scientists are now at the stage where the amount of information recoverable has begun to plateau (Butler *et al*, 2007). This has spawned a new generation of research which has included the analysis of RNA (Bauer, 2007). The shift towards RNA has been possible because of the application of Reverse Transcription Real-time PCR. This method has become the benchmark for the detection and quantification of RNA targets (Bustin and Mueller, 2005). Bauer (2007) outlined the potential forensic applications for RNA analysis, which included the identification of body fluids, wound age determination, post-mortem interval estimations and the potential application for determining the age of bloodstains. The identification of body fluids involves the detection of specific mRNA markers for different tissue types such as systemic blood, saliva, semen and vaginal secretions (Bauer, 2007). Previous tests for body fluid identification, which are still largely used, include serological, protein, chemical, chemiluminescent, immunological and protein catalytic activity tests, as well as spectroscopic methods and microscopy (An *et al*, 2012).

Although these tests are simple and expedient there are a number of problems that exist such as cross reactivity between species and the lack of specificity for particular fluids (Noreault and Buel, 2007). These methods may be superseded by RNA based techniques provided stable RNA markers can be identified (Zubakov *et al*, 2008). Zubakov *et al* (2008) performed whole genome gene expression analyses on a series of time wise degraded blood and saliva samples in order to identify stable mRNA markers within these tissue types. They identified nine blood and five saliva markers that were stable in stains aged for six months. Bauer and Palzet (2002) suggested that using mRNA markers, MMP-7 and MMP-11, which are expressed to a large

extent in menstrual blood but not in vascular blood, could lead to the correct identification of menstrual blood as distinguished peripheral blood from ruptures caused during sexual assaults. This research was further supported by Ferri *et al,* (2004) who successfully identified the MMP-11 mRNA marker in two year old menstrual bloodstains. The difficulty with using MMP-7 and MMP-11 is that they are common cancer markers (Bar-Or *et al*, 2003). Any person with a cancerous condition will produce artificially high results, which may affect any age estimation based on analysing these particular markers. Complementary testing may then be required (to test for the pathological condition) in order to obtain reliable results.

Juusola and Ballantyne (2003) successfully managed to isolate mRNAs for the housekeeping genes S18 rRNA, B-actin mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA from blood, saliva and semen. GAPDH mRNA is an enzyme that is involved in the glycolysis process (Sikand *et al*, 2012) and is also thought to be involved in several non-metabolic processes, including transcription activation, initiation of apoptosis (Tarze *et al,* 2007). These mRNAs are characteristically expressed at different levels within the three different tissue types allowing identification of each of the three biological fluids. Tissue or stain identification using RNA based methods is possible because each tissue type plays a unique role in the body and hence requires a unique and characteristic genetic expression which can be determined or visualised by examining the RNA profiles present Bauer (2007). DNA cannot be used for the same purpose given it is the same in every cell.

Preliminary studies involving the analysis of mRNA patterns have also been used to estimate the post mortem interval although this type of research is still in its infancy. Early research has shown that these patterns change with the functional status of the cell (Bauer, 2007). Inoue *et al,* (2002) successfully identified the degradation profile of GAPDH mRNA in rat brains as a function of time using real-time PCR and were able to use this to indicate the post-mortem interval. They also successfully identified degradation profiles for interleukin-1β in lung cells up to three days after death. Bauer, (2007) hypothesised that the semi-quantification of the 3' poly-A tail of mRNA, which is naturally degraded by ribonucleases upon cell death, could lead to the estimation of the post-mortem interval. This was based on the theory that the poly-A tail gets progressively shorter as time increases. However, it was discovered that the stability of RNA was longer than expected. It was initially thought that RNA had a relatively short *in vitro* life span due to its exposure to various internal and external RNA degradation factors. Its unexpected stability has been supported by various authors [Zubakov *et al,* (2008);

Nussbaumer *et al*, (2006)]. Setzer *et al*, (2008) examined the stability and recoverability of RNA from forensic samples, which included blood, semen, saliva and vaginal secretions that had been exposed to a range of environmental conditions for up to one and a half years. They used similar quantitative PCR techniques employed by Anderson *et al*, (2005) and Bauer *et al*, (2003) to detect eight different mRNA transcripts. Some of their specific findings included the recovery of several hundred nanograms of total RNA from 50 µl blood stains aged up to 365 days at room temperature and 1µg of total RNA from a 50 µl semen stain stored for 90 days under luminescent light. mRNA was also detected in samples stored at room temperature for at least 547 days. These results demonstrate that RNA is significantly more robust than initially thought and can be recovered from biological samples in sufficient quantity and quality for subsequent mRNA analysis. Zubakov *et al*, (2008) identified blood and saliva mRNA specific markers that were stable in extremely old stains. Nine blood-specific and five saliva-specific mRNA markers were amplified successfully and reliably in stains aged up to 16 and six years respectively. They suggest that forensic RNA testing can be reliable and robust if degraded samples are considered in the marker ascertainment procedure, with promising expectations beyond tissue identification purposes (Zubakov, 2008). One of the expectations may be the age determination of these types of samples.

1.5 Nucleic Acid Degradation

The experiments conducted as part of this thesis involved examining the DNA and RNA degradation characteristics in forensic like samples for the purpose of obtaining a better understanding of the kinetics involved. The ultimate aim was to develop a method for ageing biological samples. Factors that cause of influence DNA and RNA degradation need to be understood and considered when carrying out expression analysis and ageing studies. In living organisms, DNA damage is repaired by various enzymatic mechanisms such as direct chemical reversal (Sancar, 2003), excision repair in cases of single strand damage (Watson *et al*, 2004) and homologous recombination, which is a process for repairing double stranded damage (Watson *et al*, 2004). However, once the metabolic pathways of a cell cease to operate, DNA and RNA molecules begin to progressively decay. The range of DNA and RNA degradation depends on the nature of the sample source and the environment to which it is exposed (Lindahl, 1993). There are two broad categories to describe the factors involved. The first concerns external environmental factors which include heat, humidity, light and bacteria. The

second concerns the internal cellular mechanisms that degrade nucleic acids, which are largely dependent on the specific molecule and the state of the cell (Rapley and Whitehouse, 2007). Nuclear DNA is a long linear molecule making it more susceptible to cleavage than other forms of tertiary or quaternary folded structures. UV rays degrade DNA by creating cyclobutane rings that form intra-strand pyrimidine dimers (Ou *et al*, 1996) as well as pyrimidone photoproducts. Pyrimidine dimers are molecular lesions formed from thymine or cytosine bases in DNA (Goodsell, 2001). UV light is also known to induce apurinic and apyrimidinic sites, which are spontaneous lesions in the DNA molecule (Boiteux *et al,* 1985). Exposure to heat accelerates the hydrolytic and oxidative reactions in aqueous solutions causing fragmentation, nicks, abasic sites, oxidized bases, deaminated cytosine and cyclopurine lesions (Bruskov, 2002). Under physiological conditions the most labile bond in DNA is the N-glycosyl bond that attaches the base to the backbone. Hydrolysis of this bond results in the loss of a base leaving an apurinic/apyrimindinic (AP) site that eventually decomposes into a nick, which is a discontinuity in a double stranded DNA molecule where there is no phosphodiester bond between adjacent nucleotides of one strand (Iyer *et al,* 2006). The phosphodiester bond in the backbone of RNA is the least stable under the same conditions.

Figure 9: Direct effects of UV light on nucleic acids: Dimerizations between adjacent pyrimidine bases are by far the most prevalent photoreactions resulting from UV irradiation of DNA. Cyclobutyl pyrimidine dimer (CPD) is one of the major photoproducts produced. Imaged taken from http://www.orgs.muohio.edu/uvlakes/.

Another common type of DNA damage is the hydrolytic deamination of cytosine to form uracil. This type of damage is particularly important because it may result in PCR inhibition, which is an important step in the DNA profiling procedure (Kimpton *et al*, 1993). Methods that are based on amplicon length, rather than sequence (such as DNA profiling and many degradation techniques) are not as significantly affected by this mutagenic effect (Zimmermann *et al*, 2008). Exposure to oxygen can cause base modification in both DNA and RNA and this is of particular interest with forensic samples that may be exposed to such conditions for long periods of time. The most common change is guanine into 8-oxo-guanine which can also be caused by organic extraction methods (Zimmermann *et al*, 2008).

Figure 10: Differences in DNA and RNA bonds. This image depicts the difference in DNA and RNA, including the presence of uracil in RNA. Extracted from http://education-portal.com/academy/lesson/uracil

The magnitude and type of post-mortem modifications can affect the success rate of the analytical technique employed. Some changes can block the amplification process whereas others allow PCR products to be obtained, but with incorrect bases incorporated into the amplification products. These kind of miscoding lesions or PCR artefacts are commonly represented by two types of transitions: (A →G)/(T →C) (type I) and (C →T)/(G →A) (type II) (Hansen *et al*, 2001). The second type is more frequent in nuclear and mitochondrial DNA. The continuous improvement of amplification techniques has reduced the number of such artefacts, but the precise rate and pattern of miscoding lesions remains difficult to estimate (Mateiu and Rannala, 2008). Hofreiter *et al*, (2001) attempted to quantify the rate of post-mortem damage by comparing the PCR products of ancient samples (more than 10,000 years old) with a database reference sequence. They concluded that miscoding lesions are unlikely to occur in less than 0.1% of the genome.

Despite the unexpected stability of RNAs described by Zubakov *et al* (2008), intracellular degradation factors are a part of the normal cell process and vary depending on the type of RNA. According to Zubakov *et al*, (2008) the molecular reasons for differences in RNA degradation between different types of RNAs as well as between different genes are largely unknown. It is assumed that the degradation process of messenger RNA (mRNA) is influenced by various factors, including structural peculiarities (like the presence of AU-rich regions), protein binding properties and cellular location (Zubakov *et al*, 2008). Bauer (2007) also suggests that mRNA is less stable than DNA after death because it is readily degraded by ubiquitous ribonuclease activity within the cell environment. It was initially thought this type of degradation occurred rapidly after cell death, which could explain why RNA analysis has been largely over looked in forensic investigations (Heinrich *et al*, 2007). mRNA naturally degrades *in vivo* as a way to control translation or the rate of protein synthesis (Kozak, 2005). When a cell no longer requires a certain protein, specific mechanisms will act to degrade the mRNA that codes for that particular protein. This process occurs first by removing the protective elements of the mRNA, the 3' poly-A tail and the 5' cap, which are structures that are added to precursor mRNA as a signal to induce translocation of the mRNA to the ribosome (Kozak, 2005). They also act to protect the mRNA from degradation attack prior to translation, ensuring that the important exon coding regions are preserved (Kozak, 2005). There are two major natural mRNA degradation pathways, the deadenylation dependant decay pathway and the deadenylation independent decay pathway (Gopee and Howard, 2006). In the former the poly-A tail is progressively shortened due to the ribonuclease enzymes present in the cytosol,

until the tail is too short to bind the poly -A binding protein (PABP). PABP is required in order to stimulate the initiation of translation, by binding to the poly-A tail and then to eukaryotic initiating factor (eIF-4F), therefore causing the mRNA to loop as a signal for translation to begin. Without this looped structure translation of the mRNA cannot occur. Towards the end of deadenylation the 5' cap is removed through enzymatic cleavage, although little is known about the exact kinetics of this process. Removal of these protective elements frees the coding sequence for degradation from the 5'end to 3' end by exonucleases (Gopee and Howard, 2006). During the deadenylation independent decay pathway the mRNA does not need to have the poly-A tail removed in order for the 5' end to be decapped and for the coding region to be cleaved internally by endonucleases (Gopee and Howard, 2006). Another mRNA degradation method has been suggested by Dallas and Vlassov (2006), whereby small interfering double stranded molecules stimulate RNA degradation via a gene silencing pathway.

Unlike messenger RNA, ribosomal RNA is thought to be very stable due to its association with ribosomal proteins within the ribosome complex (Gonzalez and Schmickel, 1986). Given the known methods of natural RNA degradation *in vivo*, the major factors involved in post mortem degradation of RNA are most likely to be endogenous nucleases, which are released during cellular death as well as environmental factors such as light, microorganisms and heat (Gopee and Howard, 2006). Most studies have taken the necessary steps to control many of these factors, but in order to maintain a sense of realism in relation to forensic samples it is important to use natural conditions and test the robustness of any trialled methodology.

1.6 Measuring Nucleic Acid Degradation

There are a number of different approaches to measuring the DNA and RNA degradation rates of a biological sample, including gel electrophoresis (El-Harouny *et al*, 2008), southern blotting (Winter *et al,* 1998), PCR assays [Vass *et al*, 2013; Swango *et al*, 2006)], RT-PCR assays [Anderson *et al*, (2005); Bauer *et al*, (2003)] and high density oligonucleotide arrays (Yang *et al*, 2003). The preferred forensic approach is to determine and compare the quantities of two types of species, which requires an accurate relative quantification method but is independent of the amount of starting material which is often variable and difficult to determine (Bustin, 2002). Often forensic samples will exist in very small quantities therefore DNA and RNA analysis will generally require an amplification step. This can be achieved

using Real-time PCR (qPCR) and Reverse Transcription qPCR (qRT-PCR) respectively (Heinrich *et al*, 2007). These techniques are desirable because of their high sensitivity, reliability and reproducibility; a comment supported by many researchers including Inoue *et al* (2002), who suggested that qPCR is very reliable for producing a degradation mRNA profile from autopsied biological tissues. qPCR protocols are also straightforward (Sharbati *et al,* 2012) and are amenable to automation and the high through-put of samples, which will save time and money if a method was to become routinely used in a forensic laboratory (Huggett *et al*, 2005). This technique also requires very little post amplification processing (Wong and Medrano, 2005) making it an ideal candidate for forensic type work. Despite these advantages, obtaining accurate and reliable results requires a clear understanding of the key components of a real time experiment as normalisation, interpretation and statistical analysis can often be complicated (Nolan and Bustin, 2008).

qPCR, like normal end point PCR, is a technique that uses thermally stable polymerase enzymes to amplify DNA (or cDNA) via repeated thermal cycling (Logan *et al,* 2009). Real time PCR differs from end point PCR in that it measures the quantity of target product during the amplification process, effectively combining amplification and detection into a single step, whereas with end point PCR, the DNA is quantified at the end of the reaction (Nolan and Bustin, 2008). Reactions are characterised by determining the number of cycles it takes for a target amplicon to reach a desired fluorescence level (Logan *et al,* 2009). This value is referred to as the cycle threshold or Ct value and it represents the time at which fluorescence intensity is greater than the baseline fluorescence. The baseline is the average background, calculated according to the noise level in the early cycles when there is no detectable increase in fluorescence, caused by PCR products (Logan *et al,* 2009). An amplification plot of the Real Time reaction has four distinct phases: a linear ground phase (baseline), exponential phase (log-linear phase), linear phase and finally the plateau phase (see Figure 11, page 74). The linear ground phase usually occurs during the first 10 to 15 cycles and is important to identify any artificial fluorescence that may be a result of contamination or experimental error (Logan *et al,* 2009). During this phase the baseline is calculated as any fluorescence should not exceed the background noise. The exponential phase signals the point where sufficient quantities of product have been amplified to raise the fluorescence signal above the background noise (Logan *et al,* 2009). The cycle at which this occurs is called the Ct value. The Ct value is inversely proportional to the amount of target DNA (which is proportional to the amount of RNA that has been reverse transcribed or converted into DNA) in the sample (Anderson *et al,*

2005). The greater the quantity of DNA in the starting material, the faster an increase in fluorescent signal will appear, resulting in a lower Ct value (Logan *et al,* 2009). Ct values are used to calculate the expression levels. During the exponential phase when all reagents are in excess the PCR reaction experiences its optimal amplification rate, with the amount of product doubling with every cycle, if the reaction kinetics are operating at 100% efficiency (Wong and Medrano, 2005). In practice this is rarely achieved as the efficiency of amplification is not constant throughout the PCR reaction (Wong and Medrano, 2005). The linear phase represents the beginning of reagent depletion. The reaction rate slows and there is no longer an exponential growth. When the components become depleted any further amplification is inhibited and the reaction enters the final plateau phase (Logan *et al,* 2009).

There are two basic methods for detecting the PCR product. The first involves the use of probes, which can exist in many different forms. Dual labelled fluorescent probes are the most common type used (Yeung *et al*, 2004). These probes are short oligonucleotides that contain a fluorescent compound attached to the 5' end and a quencher group attached at the opposite 3' end (Yeung *et al*, 2004). Due to the probes conformational shape (folded together) the fluorescent signal is quenched when free in solution however when the probe is incorporated into the amplicon it becomes a lineal molecule and hence there is a change in the conformation of the probe (Holland *et al*, 1991). The thermostable polymerase enzyme that is responsible for the synthesis of the new DNA strand also has a 5' to 3' exonuclease activity (Logan *et al*, 2009). During the extension of the new strand the probe will be hydrolysed releasing the fluorescent compound into solution (and away from the quencher group) where it will then emit a fluorescent signal (Bustin 2000). This signal is measured by the fluorescent detector and analysed by the RT-PCR software. There are a number of other types of probes available each with their own unique benefits. Regardless of the binding mechanism or the type of probe used, there are two requirements of fluorescent labels for real time detection of PCR. First they must show an increased fluorescence when bound to double-stranded DNA and secondly they must not inhibit the PCR process (Nolan, 2008).

Figure 11: Real Time PCR amplification plot. There are four distinct phases of real time PCR that describe the kinetics of the reaction. The information obtained can be used to calculate background noise, cycle threshold and amplification efficiency. Image taken from http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechQPCR.shtml.

Dual labelled probes are useful when measuring two components in a single reaction, but where a single target is being measured, a different approach can be used. Intercalating dyes such as SYBR green can be used to label a single nucleic acid target (Logan *et al*, 2009). Intercalating dyes bind double stranded DNA proportionally (Rapley and Whitehouse, 2007). Fluorescent emission of the dye is detected following excitation (Rapley and Whitehouse, 2007). There are a number of limitations regarding intercalating dyes including; non-specific binding (therefore will label any contaminating genomic DNA) and limited dye stability which can cause dye-dependent PCR inhibition (Monis *et al*, 2005).

As previously mentioned, the number of cycles required to reach the threshold level in the qPCR process depends on the amount of starting material. When selecting the appropriate

number of cycles for the run protocol, consideration must be given to the type and quantity of the sample (Nolan *et al*, 2006). The dynamic range of the assay (which can be determined using Standard curves) must also be considered as Ct values obtained from excess or trace amounts of starting material may fall outside this range and therefore be unsuitable for interpretation (Nolan *et al*, 2006). Twenty eight amplification cycles is the standard protocol for STR profiling whilst 34 is the standard for LCN DNA samples (Kloosterman and Kersbergen, 2003). It was suggested by McNevin *et al*, (2005) that a cycle number greater than 40 is required to achieve successful amplification of nucleic acids for subsequent analysis of trace samples. However, there was no mention of whether this cycle number fell within the dynamic range of their assay. The cycle number is less important with quantitative PCR because over amplification is not as critical (excluding Tm determination) as the analysis can be digitally cut off as long as the log phase is identical (Wong and Medrano, 2005).

When analysing RNA it must initially be converted into complementary DNA (cDNA). The cDNA is then analysed like normal DNA in the qPCR process. This entire process is referred to as quantitative reverse transcriptase PCR (qRT-PCR) (Heid *et al*, 1996). cDNA differs from nuclear DNA in that it contains only coding regions (exons) and no intervening sequences (introns) which is caused by the intron splicing process that precursor mRNA and rRNA undergo before becoming mature RNA species (Clancy, 2008). This has implications when determining suitable primer and probe locations. There are three main priming mechanisms for this process: Random oligonucleotides anchor themselves randomly across the RNA strand and allow the reverse transcriptase to fill up the gaps (Filion, 2012). This type of priming tends to produce the greatest yield of cDNA so it is often used when the template is limiting (Filion, 2012). The resulting pieces of cDNA are scattered all over the mRNA. However the cDNA will not be full length. Using this method does, however, increase the chance that the 5' ends of the mRNA will be converted to cDNA (Nolan, 2008). Random hexamers are currently the method of choice for general mRNA research after the possible bias reported with oligo-dT primers (Nolan, 2008). Oligo-dT primers bind specifically to the 3' poly-A tail of the mRNA transcript and therefore transcription is carried out in a known and controlled region (Filion, 2012). The number of thymines used to bind the poly-A tail varies from 12 to 30 depending on the manufacturer. A break in the RNA chain will result in the termination of transcription therefore oligo-dT priming may not be as robust as other priming methods, especially for samples that may be degraded (Nolan, 2008). Oligo-dT priming is specific for RNA as the primer will only bind to the unique poly-A tail of the RNA (Filion, 2012). This is advantageous

as the method will not be affected by any contaminating genomic DNA. The final priming method, gene specific priming, is used when there is a need to produce cDNA from a specific subset of the total mRNA (Logan *et al*, 2009). Each of the methods is graphically presented in Figure 12.

Figure 12: RNA Priming Mechanisms: A schematic representation of the three methods of priming Reverse transcription reactions, namely oligo-dT priming, random hexamer priming and specific priming. Figure extracted from www.bio.davidson.edu

qRT-PCR can be carried out in either a one-step or two-step reaction. When using specific primers the one-step process is preferred as oligo-dT and random primers can result in specific products after the PCR process. A two-step approach uses either oligo-dT or random primers for the reverse transcription step, whilst using specific primers in the PCR step, leading to specific cDNA. qRT-PCR has become the most efficient technique for quantification of RNA in recent years with many researchers successfully achieving results from biological samples [Anderson *et al*, (2005); Gopee and Howard, (2006); Heinrich *et al*, (2007); Inoue *et al*, (2002); Juusola and Ballantyne, (2003)] therefore it was the method of choice for work in this thesis.

Figure 13: Reverse Transcription Process: The schematic diagram illustrates the process of reverse transcription using oligo–dT primers. Image extracted from www.promega.com/paguide/images.

1.7 Assay Optimisation

Prior to any sample analysis the Real Time PCR assays should be assessed to determine the working efficiency of the reaction. This optimisation process is a vital component in acquiring accurate quantification but it is often overlooked (Nolan *et al,* 2006). Many studies, including those conducted by Anderson *et al,* (2005) and Bauer (2007) do not address the various optimisation steps and in doing so decrease the reliability of the results obtained (Burns *et al,* 2005). Some of the common assumptions made include the reference gene having a consistent expression, a similar amplification efficiency rate between the test and control samples (used to

generate standard curves) and the initial target concentration having no effect on the kinetics of the amplification process.

1.7.1 Primer Concentrations

Optimal primer concentrations are essential for maximal specificity and efficiency in singleplex PCR (Nolan *et al*, 2006). Primer concentrations which are too high increase the chance of mispriming and subsequent extension of the misprimed molecules results in the amplification of nonspecific PCR products (Innis and Gelfand, 1990). In multiplex reactions, it is important to establish the desired concentrations that prevent preferential amplification of one of the targets (Logan *et al*, 2009). This is particularly important when using amplicons of differing sizes, a common feature of degraded DNA. In determining the optimal concentration a number of factors are considered. The reaction (amplification cycle) must reach completion (i.e. reach the plateau phase) and if using subsequent multiplex reactions, must have primers that start the exponential phase around the same cycle number (Logan *et al*, 2009). This prevents one species amplifying too early and consuming all the reagents in the reaction mix prior to other completing its exponential amplification. The primer concentrations with the lowest Ct values that meet these criteria will be the optimal concentration.

1.7.2 Amplification Efficiency

It is important to calculate the amplification efficiency of each target species so that accurate expression (quantification) levels can be determined (Wong and Medrano, 2005). This can be achieved by running a 10-fold serial dilution (Lifetechnologies, 2012). The slope of the resulting standard curve can then be used to calculate the amplification efficiency rate (see Figure 14). In theory, a reaction that occurs at 100% efficiency should produce a 2-fold (doubling) increase in the amount of PCR product with each PCR cycle (Lifetechnologies, 2012). It follows that a reaction operating at 100% efficiency should produce a 10-fold increase in the amount of amplified in 3.33 cycles ($2^{3.3} = 10$). To calculate the amplification efficiency rates of each target species the following formulas are used (Wong and Medrano, 2005):

$$\text{Exponential Amplification} = 10^{(-1/\text{slope})}$$

$$\text{Efficiency Rate} = [(10^{(-1/\text{slope})}) - 1] \times 100$$

The value of the slope is obtained by conducting a serial dilution assay, using high quality DNA (Lifetechnologies, 2012). These DNA samples will be pure and may produce different reaction kinetics to the actual test samples therefore the amplification rate of the actual samples should also be determined to ensure the rates are comparable (Lifetechnologies, 2012). Any variation between these values will be significant especially when dealing with trace amounts of starting material. A 5% difference in efficiency rates can result in a 2-fold difference in the amount of PCR product after 26 cycles (Lifetechnologies, 2012). This is an issue that is often overlooked when analysing and interpreting qPCR data and is particularly important with forensic samples as they will tend to be of a poorer quality with a greater chance of containing PCR inhibitors and contaminants (Nolan *et al*, 2006). Without this assurance quantity calculations which are based on the efficiency rates may be inaccurate and hence this optimisation step should be employed routinely with qPCR assays. This recommended approach was adopted in this body of work.

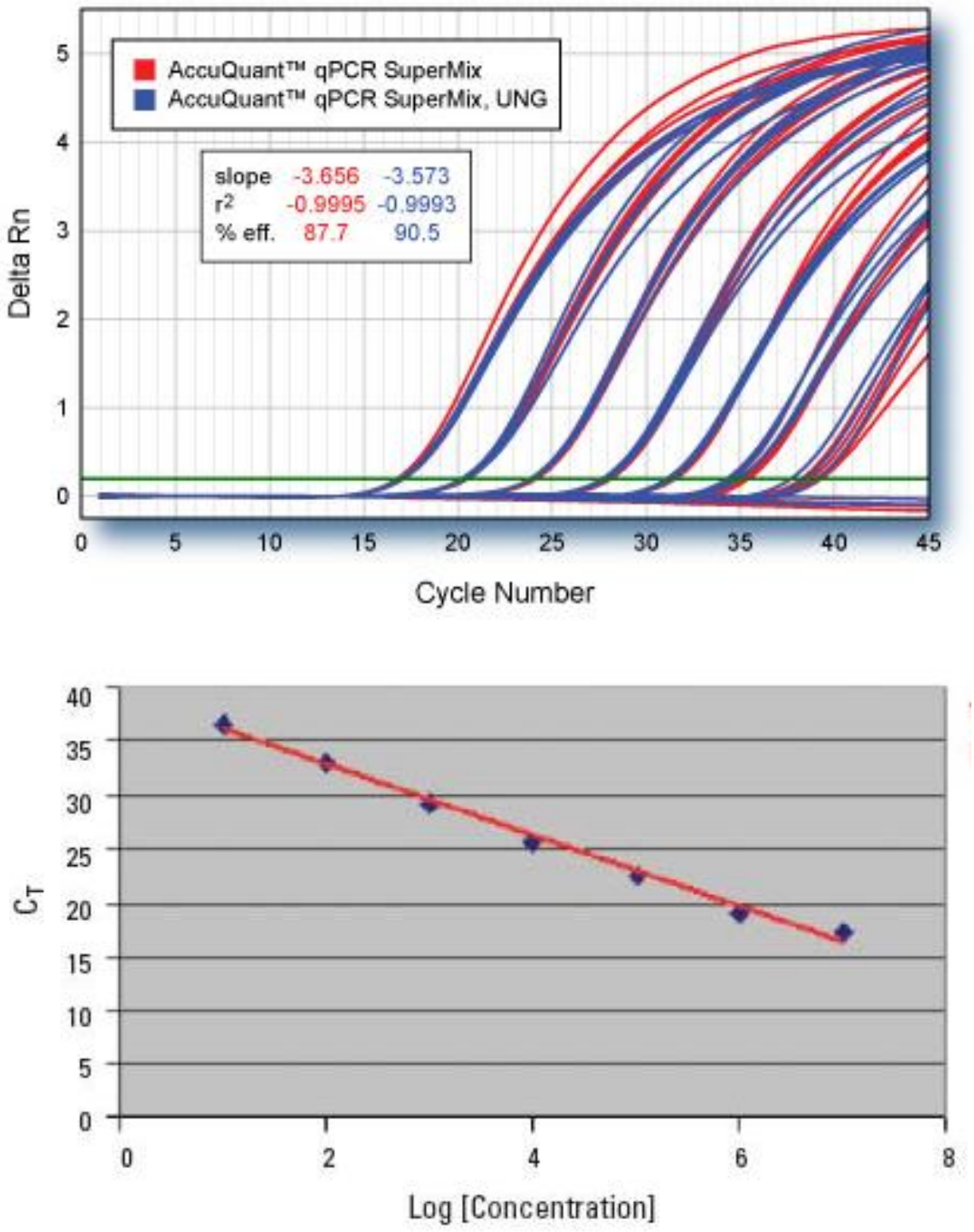


Figure 14: Amplification Efficiency rates using a Serial Dilution Technique. To calculate the rate, a 10-fold serial dilution series can be run, producing a similar plot to shown in the top graph. The log values are then used to create a standard curve seen in the lower graph from which the rate can be calculated using the equation described on page 75.

1.7.3 Quantification Strategies

There are a number of different quantification strategies that can be used with real time qPCR (Wong and Medrano, 2005). Broadly speaking there are absolute and relative methods for calculating the DNA concentration from the raw Ct values obtained from the qPCR reaction. Absolute quantification relies on standard curves, which are constructed using standards of known concentration (Wong and Medrano, 2005). This approach has several disadvantages. The standard curve is generally constructed from a purified product free of any PCR inhibitors therefore the amplification efficiency rate will be optimal. This rate may vary from the efficiency rates obtained from test samples (Bustin, 2002) and therefore using the optimal rate will be inappropriate and result in inaccurate measurements (Lifetechnologies, 2012). This can be overcome by testing for inhibitors however this will make the process labour intensive. Given a standard curve should ideally be run with every reaction plate (Lifetechnologies, 2012), this absolute quantification approach is already laborious.

The work carried out in this thesis involved comparing the relative amounts of target species therefore absolute values were not required (Wong and Medrano, 2005). Comparative or relative quantification methods were preferred. Relative methods measure the change in target (DNA) relative to another reference sample whereas comparative quantification involves the measurement of the change in expression of a target species (Wong and Medrano, 2005). These methods still require careful manipulation of endogenous reference genes and control calibrators to allow for the natural peculiarities of biological systems (Bauer *et al*, 2005), but were more suited to this work than absolute methods. There are numerous different relative quantification methods all requiring different considerations. Table 1 on page 79 lists these different methods and the relevant considerations regarding amplification efficiency.

qPCR data requires normalisation and there are a number of ways this can be achieved including: in accordance with known amounts of extracted RNA (this requires an accurate quantification method); according to mass or volume of extracted tissue; according to a single housekeeping gene; or by using an index of regulated housekeeping genes (Lifetechnologies, 2012).

Table 1: Characteristics of the Relative Quantification Methods Available for Measuring DNA (adopted from Wong and Medrano, (2005). The table provides details regarding the considerations that need to be made with regards to each quantification method.

Method	Amplification Efficiency correction	Amplification efficiency calculation	Amplification efficiency assumptions
Standard Curve	No	Standard curve	No experimental sample variation
Comparative Ct ($2^{-\Delta\Delta Ct}$)	yes	Standard curve	Reference = target
Pfaffl et al	yes	Standard curve	Sample = control
Q-gene	yes	Standard curve	Sample = control
Gentle et al	yes	Raw data	Researcher defines log – linear phase
Lui and Saint	yes	Raw data	No need for similar efficiencies
DART-PCR	yes	Raw data	Statistically defined log-linear phase

There are a number of advantages to relative quantification techniques. The Comparative Ct method ($2^{-\Delta\Delta Ct}$) can correct for amplification efficiencies that are not ideal but it is based on the assumption that the amplification rates of the two targets are similar (Livak and Schmittgen, 2001). The degree of similarity can be tested by performing serial dilution assays on both targets and plotting the results of the log concentration versus Ct difference for each dilution (Livak and Schmittgen, 2001). If the absolute value of the slope (trend line) is less than 0.1, the efficiencies are considered similar enough for this method. Compared to semi-quantitative methods such as western blots, the 0.1 threshold is acceptable but even values of less than 0.1 will incorporate a small degree of error into the analysis if the rates are not identical (Pfaffl, 2002). This error is further compounded by a common assumption that is made regarding the initial efficiency rate calculations. These rates are determined using a serial dilutions standard curve method. Mean Ct values from the replicate samples are used to calculate the rate, which simplifies the statistical process. However the standard error generated from calculating the mean is not carried forward to the rate calculation (Pfaffl, 2002). If the correct approach to

regression analysis is considered (confidence levels are stipulated), efficiency rates should be described as a range within a specified confidence level (Pfaffl, 2002) rather than an individual value. Obtaining accurate efficiency rates is vital as they are used to quantify the target species and given that PCR operates on a logarithmic scale, any variation between the calculated and actual efficiency rates will have an exponential effect on the final data analysis. Increasing the number of replicates and the number of serial dilutions used to create the standard curve can help to confidence of the rates obtained. Pfaffl (2002) proposed an alternative formula that compensates for slight variations in efficiency rates making any subsequent calculation more accurate. As such Pfaffl's moderated equation was used for all quantity calculations in this thesis. The formula described was:

$$\text{Relative Expression Ratio} = (1 + E_{\text{target}})^{\Delta Ct\,(\text{target})} / (1 + E_{\text{reference}})^{\Delta Ct\,(\text{reference})}$$

where E is the efficiency of the individual target species

ΔCt is the change in the Ct values

This compensation does not address the degree of uncertainty resulting from the standard curve analysis but it does ensure that each individual efficiency rate is treated on its own merits and not just an average. Statistical analysis of real time PCR data in biological systems can be complicated but often they do not require complex models. Before using any parametric tests (ANOVA, ANCOVA etc) the raw Ct data (log) must be transformed to a linear scale (Lifetechnologies, 2012). When using relative methods more simplified alternatives can be employed to analyse the replicate real time data as there is a primary assumption with that the additive effect of concentration, gene and replicate can be adjusted by subtracting target gene from the reference gene to give ΔCt. Simple t-tests can then be applied to estimate ΔΔCt if it is assumed that a Gaussian distribution exists and that both groups of data have equal variances (Hollander and Wolfe, 1973). In cases where sample size is small, there assumptions will not be valid and therefore non-parametric tests such as a Wilcoxon Rank or Mann Whitney tests are a more robust and appropriate alternative (Hollander and Wolfe, 1973). These considerations were made before selecting the appropriate statistical tests in this thesis.

1.8 Aims and Objectives

The initial aim of this body of work was to critique and examine a common DNA methodology used to measure the degradation levels and thus age of biological samples. Past research was based on a common methodology, which in itself had not been fully tested or optimised for specific environmental conditions. With this in mind the aim was to gain a better understanding of the degradation kinetics of in internal "urban" environment, a setting common to forensic case work.

A further aim of this work was to develop a method for ageing blood and hair samples that was more accurate and precise than any current approach. This involved using the findings from the initial studies and combining them with novel techniques, such as those proposed by Nolan (2008) that targeted specifically selected RNA molecules. Different ageing lengths were used to determine the accuracy and limitations of the proposed methodologies as well as to identify any trends in the degradation patterns. The sensitivity and robustness of the assays were tested using samples that had aged over different seasonal periods and various targets were examined to determine the best results.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample and Consumable Preparation

Plastic consumables were initially treated overnight with Diethyl pyrocarbonate (DEPC), and then autoclaved. Sterilised filtered pipette tips (Anachem, UK) were used, all bench spaces were cleaned using RNAzap (Ambion) and equipment was exposed to UV light treatment for an hour prior to any laboratory work. All samples were obtained from healthy human male and female volunteers following ethical approval from John Moores University. Further details regarding the samples and the specific collection and ageing conditions are stipulated in each of the relevant sections throughout this thesis.

2.1.1 UV Induced DNA Degradation Series

A degradation series was prepared in chapter three to test the validity of using different sized amplicons for estimating the age of blood samples. Two microliters (2 µl) of stock solution (200 µg/ml, Bioline, UK) was diluted 1:100 with nuclease free water buffer so that each sample consisted of 200 µl at a concentration of 2 ng/µl. Nine samples were prepared and exposed to specified amounts of UV light using a CL-1000 Ultraviolet crosslinker (Ultraviolet Products, UK). The time points used to create the degradation series were 0, 0.5, 1, 2, 5, 10, 15, 20 and 30 minutes. All samples were placed directly beneath a UV tube at a spatial distance of five centimetres. Five microlitres (5 µl) aliquots were then extracted and used for each of the subsequent Real time PCR reactions.

2.1.2 DNase I DNA Degradation Series

An alternative degradation series was prepared in chapter three to confirm the findings from the UV induced DNA degradation series. The time periods (treatment time) used to create the degradation series were 0, 1, 2, 5, 10, 15, 20, 30, 60 and 90 minutes. A master mix was prepared by adding 10 µg of DNA (high molecular weight) with 10 µl of 10x DNase I reaction buffer (Invitrogen, UK). The final volume was made up to 100 µl with DEPC-treated water (Sigma, UK). Ten microliters (10 µl) was immediately removed and used as the 0 minute sample. A small volume (2.5 µl) of DNase I (1U/µl) was then added to the master mix and incubated at room temperature (22°-24°C), removing 10 µl aliquots at each of the specified

time points. The DNase activity was stopped by adding 2 µl of 25 mM EDTA and heating at 65°C for 15 minutes. Despite the fact that the 0 minute sample contained no DNase I, it was subjected to the same stopping process to ensure consistency amongst all the samples. Each degraded sample was diluted 1:100 with DEPC treated water (total of 150 µl at a concentration of 1ng/µl) from which 5 µl aliquots in triplicate were used for the real time PCR assay.

2.1.3 Naturally Aged Blood Samples

Five millilitres (5ml) of whole blood was initially extracted from each donor in chapter three. Before the clotting process could begin, 10 µl aliquots were immediately dispensed onto laminate flooring which was previously sterilized (autoclaved) and UV treated for 60 minutes. Each donor contributed three, 10 µl aliquot samples for each time point making a total of 150 samples. The samples were left on the floor of the laboratory in the presence of ambient light (that consisted of natural and fluorescent artificial light) to simulate a likely internal urban forensic environment. Samples were at no stage exposed to any direct sunlight. The site selected was based on the prior knowledge of the number or people that normally occupy the laboratory and in terms of a normal academic institution was considered to be "very low traffic level" but moderate for a typical suburban household. The area was cordoned off and the samples were left to age for the desired lengths of time (0 to 90 days and measured at ten day increments).

2.1.4 Preparation and Ageing Blood Samples to Test Loci Susceptibility

In chapter four, samples were obtained from six donors, selected randomly from a group of students aged between 20 and 30-years-old. All donors were male Caucasians with English or Irish background. Whole blood aliquots of 100 µl were pipetted into sterile microcentrifuge tubes and left to age for 0 to 50 days under elevated room temperature (26°C) to speed up the degradation process. DNA was extracted at ten day intervals.

2.1.5 Preparation of Samples for Dry and Standard Swabs

The blood samples used in chapter five were drawn from a single individual of Indian ancestry. Before the clotting process could begin, 20 µl aliquots were immediately dispensed onto both "Standard" and "Dry" cotton swabs (see section 5.2.2 on page 174). Swabs were set up as either "clean" or "soiled" to examine the effects of microbial degradation. All swabs used were new, clean and sterile and this satisfied the "clean" conditions. Soiled swabs were created by rubbing the tip of the swab in ordinary dry garden soil prior to sample set up. Samples were aged under different temperature conditions, namely, 30ºC, room temperature (RT), 4ºC and -20°C (frozen) for periods of 0, 10, 20, 30 and 60 days. Room temperature samples were aged on a bench in the laboratory and the 30°C swabs were incubated and aged in an incubator, which maintained a temperature range of 30°C to 32°C. Samples designated as day 0 were processed within an hour of being taken from the volunteer. Triplicate samples were used for each ageing condition and for each time point (excluding 0 day samples where only 20 samples (ten dry and ten standard samples) were analysed) resulting in 196 samples in total.

2.1.6 Preparation of Blood Samples for the RNA Assay Measuring Components of the Inflammatory Response

Five millilitre (5ml) samples were extracted from eight volunteers (four male and four females) in chapter six. All donors were between twenty and thirty years of age and of Caucasian decent. One hundred and twenty five microliters (125 µl) aliquots were immediately distributed into each of the sterilised microcentrifuge tubes that were labelled according to the specified ageing period (0-days, 1-days, 2-days, 3-days, 4-days, 5-days, 6-days, 7-days, 8-days, 9-days and 10-days), making a total of 88 samples for this study. Before RNA extraction and further analysis, samples were aged at room temperature (22°-24°C) for the respective times.

2.1.7 Sample Preparation of Blood Samples used to Test the Validity of the 3'/5' RNA Assay

Fifty microlitres (50 µl) of whole blood was extracted from each of the five donors in chapter seven, using a BD vacutainer system (Becton, US). This system consists of a small needle connected to a vacuum tube. All donors were aged between twenty and thirty years. Blood samples were pipetted into sterile microcentrifuge tubes which were then left to age at room temperature and humidity for up to 80 days.

2.1.8 Hair Samples

Hairs were removed using forceps and a plucking action, from the head of each volunteer and examined microscopically to ensure that a hair root (follicular tag) was attached. The final one centimetre of each hair (containing the follicular tag) was cut and stored in sterile 1.5 ml micro centrifuge tubes. This was repeated so that each sample contained ten hair roots in one tube.
A total of twenty hairs were collected from each person for each time point (0-days [control], 1-day, 5-days, 15-days, 30-days, 45-days, 60-days, 75-days and 90-days), ensuring there were duplicate samples for each time frame. This created a total of 180 samples for all time points. The samples were then left to age at room temperature for the specified time frames.

2.2 RNA extraction

2.2.1 Organic Extraction of RNA from Blood Samples

In chapter six, RNA was extracted using an organic extraction method. For each microcentrifuge tube 750 µl TRIzol ®LS Reagent (Invitrogen, UK) was mixed with the blood samples (125 µl). Each tube was vigorously vortexed for 30 seconds to ensure that any clot that had formed had been completely dispersed. Samples were then incubated at room temperature (22°-24°C) for five minutes. Two hundred microliters (200 µl) of chloroform was added and mixed well by vortexing for 15 seconds. The solutions were then incubated at room temperature (22°-24°C) for 15 minutes. Samples were then centrifuged for 15 minutes at 12,000 x g and at 4ºC. Following centrifugation the upper aqueous layer was transferred into a new sterilised centrifuge tube containing 500 µl of 2-propanol (Sigma, US). Samples were inverted several times and then incubated at room temperature for ten minutes followed by centrifugation for ten minutes at 12,000 x g (4°C). The liquid supernatant was removed and 1 ml of 75% ethanol (Sigma, US) was added to wash the RNA pellet. The RNA pellet was gently dispersed back into solution and then centrifuged for ten minutes under conditions previously described. The ethanol was removed and the wash step was repeated. Following the second wash, the ethanol was completely removed and the RNA pellet was allowed to air dry for approximately five minutes. Air drying the pellet did not exceed the five minutes as any longer would reduce the solubility of the RNA. The RNA pellet was resuspended in 40 µl of RNase free water (Eppendorf, US) and then frozen (-20°C) until required.

2.2.2 Extraction of RNA from Blood using a Commercial Kit

In chapter seven, RNA was extracted using the QIAamp RNA Blood Mini kit (Qiagen, UK) according to manufacturer's instructions but extended incubation times were used as aged samples provided additional difficulties. For each sample 250 µl of Buffer EL was added to each sample (50 µl of whole blood) and then incubated on ice for 20 minutes. Samples were vortexed on several occasions during the incubation period and were then centrifuged at 500 x g for ten minutes at 4°C. The supernatant was removed and 150 µl of EL buffer was added to the pellet. The pellet was resuspended before being centrifuged at 500 x g for ten minutes at 4°C. After removal of the supernatant 350 µl of Buffer RLT (containing 10% β-Mercaptoethanol) was added to each sample and mixed vigorously to ensure the pellet was resuspended. The lysate was then transferred to a QIAshredder spin column in a 2 ml collection tube and centrifuged for two minutes at 16,000 x g. The QIAshredder column was then discarded and 350 µl of 70% ethanol was added to the homogenised lysate. The lysate was then mixed by gentle pipetting before being transferred to a QIAamp spin column in a 2 ml collection tube. The column was then spun for 15 seconds at 8,000 x g. The flow through was discarded and the QIAamp spin column was then transferred to a new 2 ml collection tube. Seven hundred microliters (700 µl) of Buffer RW1 was added to the column and centrifuged for 15 seconds at 8,000 x g. The QIAamp spin column was again transferred to a new 2 ml collection tube. 500 µl of Buffer RPE was added to the column and centrifuged for 15 seconds at 8,000 x g. The flow through was discarded another 500 µl of RPE was added to the spin column before being centrifuged for three minutes at 16,000 x g (full speed). The QIAamp spin column was then removed and placed into a 1.5 ml microcentrifuge tube. Forty microliters (40 µl) of RNase-free water was added directly onto the QIAamp membrane. The column was then spun for two minutes at 16,000 x g. The eluted RNA was then stored at -20°C until required.

2.2.3 RNA Extraction from Hair Samples

In chapter eight, RNA was isolated from each sample at the various time periods using an organic extraction method based on guanidium isothiocyanate (www.sigma.com). Each sample (Eppendorf tube) contained ten hair follicles, 500 µl of TRI reagent BD (Sigma-Aldrich, St.Louis, US) and 200 µl of PCR grade water (Eppendorf, Germany). The solution was then vortexed for 15 seconds and incubated for ten minutes at 50^{o}C. One hundred microlitres (100 µl) of 1-bromo-3-chloropropane (Sigma-Aldrich, St.Louis, US) was then added to each sample

and vortexed for 15 seconds. The samples were then incubated for five minutes at room temperature before being centrifuged at 12,000 x g for 15 minutes at 4^{o}C.

The aqueous layer was transferred to a new sterile 1.5 ml Eppendorf tube where 500 µl of cold isopropanol (Sigma-Aldrich, St.Louis, US) and 3 µl of a polyacryl carrier (Bioline, UK) was added. The samples were mixed gently and incubated at room temperature for ten minutes. The samples were then centrifuged at 12,000 x g for ten minutes at 4°C. The supernatant was removed and 1 ml of 75% ethanol (Sigma-Aldrich, St.Louis, US) was added to wash the RNA pellet. The samples were then vortexed briefly and centrifuged at 12,000 x g for another five minutes at 4°C and after removal of the supernatant the wash step was repeated. Following the completion of the second wash, the RNA pellet was allowed to air dry for five minutes. The pellet was then resuspended in 20 µl of RNase free water (sterile water treated with 1% DEPC and 20 minutes of UV light (240nm) exposure). Two negative control samples (containing no hair) were carried out at each time point and processed in the same manner.

2.2.4 Ambion Kit RNA Extraction from Hair Samples

In chapter eight, a sensitivity assay was performed. RNA was extracted from hair samples using Ambion's RNAqueous Micro-kit and according to manufacturer's instructions (Ambion, 2014). Three wash solutions were initially prepared using the recommended quantities of 100% ethanol. One hundred microlitres (100 µl) of Lysis solution was added to each sample and vortexed vigorously for 15 seconds followed by the addition of 50 µl of 100% ethanol. Samples were mixed thoroughly and then the solution was then passed through a Micro Filter Cartridge Assembly. The cartridge assembly was centrifuged for ten seconds at 20,000 rpm. The assembly was then washed using 180 µl of wash solution-1 and re-centrifuged for another ten seconds at 20,000 rpm. This was repeated twice using wash solution-2/3. The flow through was then discarded and the cartridge assembly was centrifuged at maximum speed for one minute to remove any residual moisture in the assembly. The cartridge assembly was then transferred to a clean 1.5 ml elution tube. Twenty microliters (20 µl) of pre-warmed (75°C) Elution solution was added to the cartridge assembly and allowed to incubate for one minute. The cartridge was then centrifuged for 30 seconds as maximum speed to elute the RNA. The elution step was repeated. Samples were then frozen at -20°C until required.

2.3 Deoxyribonuclease Treatment (DNase I)

2.3.1 DNase I Treatment of Blood Samples

The RNA extracted from samples in chapter six was processed to ensure there was no contaminating DNA. One microliter (1 µl) of DNase Amplification Grade (Invitrogen, UK) and 4 µl of 10 x DNase Reaction Buffer (Invitrogen, UK) were added directly to the 40 µl of RNA sampled obtained during the extraction step. Samples were then incubated for 15 minutes at room temp (23ºC). The digestion process was inactivated by adding 1 µl of 25 mM EDTA (Invitrogen, UK) to each sample and heating the samples to 65ºC for ten minutes.

2.3.2 DNase I Treatment of Blood and Hair Samples

The RNA extracted from the blood and hair samples in chapters seven and eight respectively were processed to ensure there was no contaminating DNA. The 20 µl samples of RNA acquired from the RNA extraction step were treated with 2 µl of 10X DNase I Reaction Buffer (Invitrogen Corporation, US), (composed of 200 mM Tris-HCL (pH 8.4), 20 mM magnesium chloride and 500 mM of potassium chloride), and 1 µl of DNase I (Amplification Grade: Invitrogen Corporation, US). This solution was then incubated at room temperature for 15 minutes. One microliter (1 µl) of 25 mM EDTA, pH 8.0 (Invitrogen Corporation, US) was then added to the solution, which was incubated at 65^{o}C for ten minutes to stop the reaction. DNase treatment was carried out on all control samples.

2.4 DNA Extraction

2.4.1 DNA Extraction from Naturally Aged Blood Samples

In chapter three blood samples were extracted from donors and deposited immediately onto pieces of laminate flooring, a common covering in many houses in the UK. A scalpel was used to cut the dried blood stain from the laminate flooring and any dust that had settled was removed using a sterile Zephyr brush (WA Products, UK). The dried blood stains were then placed in a nuclease free 1.7 ml microcentrifuge tube. Two hundred and fifty microliters (250 µl) of DNAzol BD (Invitrogen, UK), 20 µl of 20 mg/ml Proteinase K and 10 µl of 10% sodium

dodecyl sulphate (SDS) was added and vortexed for 15 seconds followed by a 60 minute incubation period at 50°C (occasional vortexing) and then left overnight at room temperature. One hundred microliters (100 µl) of cold (4°C) 2-propanol (Sigma-Aldrich, US) was added to each sample and shaken vigorously for 15 seconds followed by a five minute incubation period at room temperature (22°-24°C). Samples were then centrifuged at 6,000 x g for six minutes at room temperature. The supernatant was then removed and a further 250 µl of DNAzol BD was added to the DNA pellet. The pellet was dispersed before being centrifuged at 6,000 x g for five minutes at room temperature. The supernatant was again removed and 1ml of 75% ethanol was added to wash the DNA pellet. Samples were then centrifuged at 6,000 x g for five minutes at room temperature and the wash step was repeated. Following the last spin, the ethanol was removed by decanting and the tubes were stored vertically for five minutes in a PCR clean hood. The remaining residual ethanol was then removed with a micropipette. The DNA pellet was then resolubilised with 20 µl of nuclease free PCR grade water and frozen at -20°C until required.

2.4.2 DNA Extraction from Human Blood Samples

In chapter four, DNA was extracted from blood samples using an organic method (www.invitrogen.co.uk). Two hundred and fifty microliters (250 µl) of DNazol BD (Invitrogen, UK), 20 µl of 20 mg/ml Proteinase K and 10 µl of 10% SDS were added to a 1.7ml centrifuge tube. The solution was vortexed for 15 seconds followed by a 60 minute incubation period at 50°C with occasional vortexing and then left overnight at room temperature. One hundred (100) µl of cold (4°C) isopropanol was added to each sample and shaken vigorously for 15 seconds followed by a five minute incubation period at room temperature. Samples were then centrifuged at 6,000 x g for six minutes at room temperature. The supernatant was then removed and a further 250 µl of DNazol BD was added to the DNA pellet. The pellet was completely dispersed before being centrifuged at 6,000 x g for five minutes at room temperature. The supernatant was again removed and 1 ml of 75% ethanol was added to wash the DNA pellet. Samples were then centrifuged at 6,000 x g for five minutes at room temperature and the wash step was repeated. Following the last spin, the ethanol was removed by decanting and the tubes were stored vertically for five minutes in a PCR clean hood. The remaining residual ethanol was then removed with a micropipette. The DNA pellet was then resolubilised with 20 µl of nuclease free PCR grade water and frozen at -20°C until required.

2.4.3 DNA Extraction from Dry and Standard Swabs

In chapter five, DNA extraction was performed using an organic extraction method (www.invitrogen.co.uk). The blood stained cotton tips were placed into sterile 1.5 ml microcentrifuged tubes and left to soak in 1 ml of DNazol BD (Invitrogen, UK) for four hours at room temperature with continual mixing throughout the incubation period. The cotton tips were removed and 10 µl of 10% SDS and 20 µl of 20 mg/ml of proteinase K were added to each tube and incubated for 48 to 72 hours at room temperature with the occasional vortexing. The samples were then centrifuged at 12,000 x g for ten minutes at 4°C. The supernatant was removed and discarded. Three microliters (3 µl) of linear polyarcylamide carrier) and 500 µl of DNazol BD were added until the pellet was completely dissolved. Samples were then centrifuged at 12,000 x g and 4°C for ten minutes. The supernatant was removed and 500 µl of 75% ethanol was added to each sample and inverted several times. Samples were then incubated for five minute at room temperature. Samples were then centrifuged at 8,000 x g for eight minutes at room temperature and washed with 500 µl of 75% ethanol. The wash cycle was repeated. Following the last wash, the supernatant was removed and discarded and the DNA pellet was allowed to air dry for five minutes at room temperature. The pellet was resuspended in 40 µl of nuclease free water and frozen at -20ºC until required.

2.5 DNA and RNA quantification

The concentration of DNA and RNA in all test and control samples was determined individually using Implen's NanoPhotometer and LabelGuard™ Microliter Cell (Implen, Germany) and according to their instructions. Using the pre-set menu function, sample concentrations were calculated from a combination of absorbance readings at four different wavelengths (230nm, 260nm, 280nm and 320nm).

2.6 Reverse Transcription

2.6.1 Random Hexamer Priming – Blood Samples

In chapter six, the reverse transcription was performed using random hexamers (Invitrogen, UK). To each sterilised Eppendorf tubes containing 10 µl of the RNA sample, 1 µl of random primer (Invitrogen, UK) and 1 µl of 10 mM dNTP mix (Invitrogen, UK) were added. Samples were centrifuged briefly (ten seconds) before being heated at 65ºC for five minutes. Samples

were then quickly chilled on ice before being centrifuged for a further 15 seconds. Four microlitres (4 µl) of 5X Strand Buffer (Invitrogen, UK) and 2 µl of 0.1 M DTT (Invitrogen, UK) were added to each sample and vortexed for ten seconds and then incubated at 25ºC for two minutes. One microliter (1 µl) of 200ul/U Superscript II Reverse Transcription Enzyme (Invitrogen, UK) was added to each sample and mixed by gently pipetting up and down. The samples were then heat treated at 42ºC for 50 minutes, which was then increased to 70ºC for 15 minutes. Samples were then frozen until required. Both positive [2 µl of control RNA (Applied Biosystems, US) and 8 µl of RNase free water (Eppendorf, UK)] and negative [10 ul of RNase free water (Eppendorf, UK)] control samples were used in the RT process.

2.6.2 Oligo dTs Priming – Blood Samples

In chapter seven the reverse transcription was performed using oligo-dT primers (Invitrogen, UK). The total RNA samples extracted from the blood stains were reverse transcribed using oligo-dT priming. One microliter (1 µl) of deoxynucleotriphophate (dNTP) mix (containing 10 mM of each dNTP) (Sigma-Aldrich, St.Louis, Missouri), 0.2 µl (2500 µg/ml) of Anchored Oligo-dT20 primers (Invitrogen, UK), 5.8 µl of sterile water and 5 µl of the RNA sample were placed in a sterile Eppendorf tube. The solution was incubated for five minutes at 65^{o}C followed by a 30 second chill on ice. The samples were then briefly centrifuged for 30 seconds at 12,000 rpm at room temperature to collect all the contents to the bottom of the tube. Four microliters (4 µl) of 5X strand buffer (containing 250 mM of Tris-HCL, pH 8.3, 375 mM of potassium chloride and 15 mM of magnesium chloride) and 2 µl of 0.1M DTT (Invitrogen Corporation, California) were added. Samples were briefly mixed and then incubated at 42°C for two minutes. One microliter (1 µl) of Superscript II Reverse Transcriptase (Invitrogen Corporation, California) was then added to each tube and mixed by gently pipetting up and down. The samples were then heated at 42^{o}C for 50 minutes, followed by an increase to 70^{o}C for 15 minutes to stop the enzymatic reaction. The samples were then frozen at -20˚C until required for the real-time PCR analysis. Positive (containing control human RNA at a concentration of 5 ng/µl (Applied Biosystems, California) and negative controls (containing sterile water) were run with each batch of samples.

2.6.3 Random Hexamer Priming – Hair Samples

In chapter eight, reverse transcription was carried out using random priming and Superscript II Reverse transcriptase (Invitrogen, US). One microliter (1 µl) of random hexamers (Invitrogen, USA), 1 µl of deoxynucleotriphophate (dNTP) mix (containing 10 mM of each dNTP) (Sigma-Aldrich, St.Louis, USA), 5µl of sterile water and 5 µl of RNA sample were placed in a sterile Eppendorf tube and incubated for five minutes at 65^{o}C. Samples were immediately chilled on ice before and being briefly spun. Four microliters (4 µl) of 5X strand buffer (containing 250 mM of Tris-HCL, pH 8.3, 375mM of potassium chloride and 15 mM of magnesium chloride) and 2 µl of 0.1M DTT (Invitrogen, USA) were added and the samples vortexed for five seconds before being incubated at room temperature for two minutes. One microliter (1 ul) of Superscript II Reverse Transcriptase (Invitrogen, USA) was then added to each tube and gently mixed by inverting the samples twice. The samples were incubated for ten minutes at room temperature and then incubated for 50 minutes at 42^{o}C. To stop the enzymatic reaction the samples were heated for to 70^{o}C for 15 minutes. The cDNA produced was then frozen at -20˚C until required for the real-time PCR reaction. Both positive (containing control human RNA at a concentration of 5 ng/µl (Applied Biosystems, California) and negative controls (containing sterile water) were run with each batch of samples.

2.7 Real-time PCR

2.7.1 Protocol for Hair Analysis

A real time PCR master mix was prepared using Applied Biosystem's (US) chemistry and consisted of B-actin primers and probes (B-actin control reagent kit), 18S primers and probes (Ribosomal RNA control kit), 2x TaqMan Universal PCR Master Mix (composed of AmpliTaq gold DNA polymerase, Amperase UNG, dNTP's and optimised buffer components) and nuclease free water. Optimised primer concentrations of 300 nM for both the forward and the reverse B-actin primers and 100 nM for both the forward and the reverse 18s rRNA primers were used. The final probe concentrations were 200 nM for both the B-actin probe (FAM dye) and 18s rRNA probe (VIC dye).

Each well of the MicroAmp® Optical 96-well reaction plate (Applied Biosystems, California) contained 23 µl of PCR master mix and 2 µl of the each cDNA sample to make a final reaction

volume of 25 µl. The reaction plate was centrifuged for 15 seconds prior to real-time analysis. Triplicate samples were run for each cDNA sample. Negative control samples were run with every batch to ensure contamination had not occurred. A positive PCR control was run with each batch using control human genomic DNA (200ng/µl - Bioline, UK).

The Real-time analysis was performed using an ABI 7500 Real time PCR machine. The run protocol consisted of one cycle at 50^{o}C for two minutes followed by a further cycle at 95^{o}C for ten minutes. The amplification stage consisted of 35 cycles of 95^{o}C for 15 seconds followed by 60^{o}C for 60 seconds. The final holding stage was set at 4°C.

The data were analysed using Applied Biosystems 7500 Real-time PCR SDS software (version 1.4.0). The fluorescent threshold bar was set at 0.200 RFU (relative fluorescent units), which blocked out any background noise and yet fell within the exponential amplification phase of all the samples. A passive reference dye (ROX) was included in the master mix to account for any subtle differences in PCR master mix volumes. An exogenous control (interplate calibrator - cDNA) was used to adjust any instrumental variation between runs.

2.7.2 Real Time PCR Protocol for Blood Analysis

All Real-time analyses were performed on the ABI 7500 Real time PCR machine, using a singleplex SYBR Green detection system. The Real time run conditions were as follows unless otherwise stated in the chapter content.

The first stage consisted of one cycle at 50^{o}C for two minutes. This was followed by one cycle at 95^{o}C for ten minutes to activate the polymerase enzyme. The amplification stage consisted of 35 cycles at 95^{o}C for 15 seconds and 60^{o}C for 60 seconds. A final dissociation stage was also added which consisted of one cycle of 95°C for 15 seconds, then 60°C for 60 seconds and finally 95°C for 15 seconds.

A dissociation stage was included to confirm the absence of non-specific amplification. The data was analysed using Applied Biosystems 7500 Real-time PCR SDS software (version 1.4.0).

2.8 Equations

The following equations are referred to throughout the thesis.

Equation 1:

Amplification Efficiency rate = $[(10^{-1/slope}) -1] \times 100$.

Equation 2:

Relative Quantity = $(1+\text{Efficiency Rate})^{-Ct}$

Ct = Real time PCR Cycle Number

Equation 3:

Relative Expression (RE) = $(1+E)^{-\Delta Ct}$

$\Delta Ct = Ct\ (\text{time X}) - Ct\ (\text{time 0})$

E = efficiency rate

Equation 4:

Relative Expression Ratio = $(1 + E_{\text{target}})^{-\Delta Ct\ (\text{target})} / (1 + E_{\text{reference}})^{-\Delta Ct\ (\text{reference})}$

E is the efficiency of the individual target species

ΔCt is the change in the Ct values (Ct X – Ct 0)

2.9 Chemicals, Enzymes, Kits and Reagents

A list of the chemicals, enzymes, reagents, kits, primers and probes used in this thesis can be found in Appendix I. All primers and probes used in this thesis were checked to identify any potential regions of similarity with other areas of the human genome. This was done using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (www.ncbi.nlm.nih.gov).

CHAPTER 3: OPTIMAL SIZED AMPLICONS FOR CALCULATING THE DEGREDATION LEVEL OF A BLOOD STAIN

3.1 Introduction

Human identification is the primary objective of most forensic DNA casework but the introduction of qPCR and qRT-PCR (for RNA analysis) has opened the door to a variety of other aspects of forensic biology including stain identification, ageing biological samples and determining the degradation levels of a sample (Nussbaumer *et al,* 2006). The majority of DNA degradation studies have concentrated on determining the integrity of the sample rather than the chronological age of the sample [Hudlow *et al,* (2008); Swango *et al,* (2007); Timken *et al,* (2005)]. Swango *et al,* (2006) suggested the cleavage of nucleic acids by environmental degradation processes occurs randomly along the DNA strand and as such larger fragments will generally degrade at a greater rate than smaller ones. Comparing the quantity of each of these different sized targets can provide an indication of the degradation level of a sample (Bauer *et al,* 2003). Although this approach has been adopted by a number of authors [Zubakov *et al,* (2007); Juusola and Ballantyne (2005)] the methodology has not been optimised for forensic samples.

Swango *et al,* (2006) developed a multiplex qPCR assay specifically designed to amplify target sequences of different lengths, which allowed for the assessment of DNA degradation in samples of forensic interest. This particular study targeted the TH01 gene and primers were designed to produce an amplicon of 170-190 bp in length. The second target was the human CSF gene, where primers were designed to create an amplicon size of 67 bp. Their assay was validated and rigorously tested in accordance with the current SWGDAM guidelines, for precision, sensitivity, accuracy, reproducibility, species specificity and stability, therefore satisfying the relevant quality assurance issues. Swango *et al,* (2006) tested their methodology using artificially degraded DNA, which meant that time course studies and therefore age determinations could not be performed. Niederstatter *et al* (2007) developed a modular qPCR method for determining the quality of human nuclear. Degraded DNA was prepared using DNase I digestion and three different sized amplicons were targeted. The target sizes were 79, 156 and 246 base pairs. Absolute quantification methods were used and they concluded that as the target size increased, the amount of amplifiable DNA decreased, which was largely expected. This was consistent with up to 180 seconds of DNase I treatment, at which point it

became impossible to amplify any target species. With the limited data published (Ct values were not converted to relative quantities and quantification ratios were not calculated) it was difficult to assess not only the level of degradation, but also the effect the different target sizes had on the accuracy of the methodology. Kaiser *et al,* (2008) examined samples that had been degraded naturally over time, which would enable an assessment to be made between degradation levels and sample age. Kaiser *et al,* (2008) tested human bones with post-mortem intervals times varying from one to more than 200 years. The human specific B-actin gene was the gene of interest and primers were designed to amplify three PCR products with defined lengths of 150, 507 and 763 base pairs. They concluded that post-mortem degradation into increasingly smaller fragments is a time dependent process but again they failed to directly correlate their findings with the chronological age despite having the appropriate samples. Opel *et al,* (2007) used DNA amplicon targets of 82, 124 and 201 base pairs to determine the degradation levels of genomic DNA (gDNA) extracted from telogen hair, whilst Alonso *et al,* (2004) examined the gDNA degradation levels of 1500 year old human remains using target sizes of 113 and 287 base pairs. Bauer *et al,* (2003) applied the same size principle to RNA in an attempt to age blood stains. They targeted two different regions of the B-actin gene producing amplicons of 157 bp and 300 bp respectively.

As demonstrated by these studies, there is a large variation in the size of the targets used for measuring degradation levels. No author offered a reason for selecting the respective amplicon sizes, nor did they investigate whether there were optimal sizes or size differences for determining the degradation rates of their particular samples under their defined operating conditions. qPCR was the method of choice adopted by all these authors. Although qPCR is considered the gold standard for nucleic acid quantification and the protocols are relatively straightforward, experimental design is not standard and optimisation of all relevant aspects of the experiment should be an essential requirement (Bustin, 2002). It is arguable that the target size is the most important variable in this type of qPCR analysis (degradation studies) and yet optimisation of this particular variable is rarely considered. Had the appropriate optimisation been carried out in these instances, greater precision and accuracy may have been achieved, but as it stands, these studies were prone to significant variation and relatively weak correlations.

The importance of size has recently been recognised with DNA (STR) profiling techniques with the development of the new generation of STR profiling platforms. Minifiler (Applied Biosystems, US) targets the same ten regions as the conventional SGM+ Kit but the primers

have been redesigned to create smaller amplicons. Target areas are therefore more likely to be in tact in degraded samples. This was recently supported by Opel *et al,* (2007) who validated a set of Miniplex Primers in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM). Butler *et al,* (2008) also carried out extensive work in developing new, shorter STR targets that were less susceptible to degradation. They identified and developed 26 non-CODIS STR markers suitable for forensic investigations. This was achieved by designing primers that were as close as possible and at times, directly next to the repeat sequence. The subsequent amplicons were all between 50 and 150 base pairs in length which is significantly smaller than the traditional CODIS markers that range from 140 to 450 base pairs in length (Krenke *et al,* 2002). With traditional profiling techniques the concept of downsizing STR markers is relatively straight forward as the smaller the amplicon size, the greater the chance of a successful amplification. Traditional profiling techniques are only concerned with the defining limit (cut off point) that distinguishes between successful or failed amplification. When applied to age or degradation level estimations, the issue becomes more complex as the entire degradation range for the specified sample set must be considered. The optimal target sizes will be dependent on the fragment sizes present, which in turn is largely determined by the conditions and length of time in which the samples were aged or degraded.

The aim of this experiment was to determine the optimal size amplicon sizes for samples that had aged for different lengths of time in an environment that was representative of a typical interior urban dwelling, a scene commonly encountered in real forensic case work. It was thought that the particular size of the amplicons chosen will have an effect on the quality of the results obtained and will be dependant and unique to the sample type, level of degradation and the specific environmental conditions.

3.2 Methodology

3.2.1 Primer Design and Assay Approach

Five sets of primers were designed to amplify approximately the same region of the TH01 gene with the main criteria being that the amplified products were all distinguishable by size. A further two sets (CSF and TH01) were adopted from Swango *et al,* (2006) and used as a comparative tool. The TH01 gene was chosen because it has been well characterised with respect to cross-species reactivity, robustness and low mutation rate as well as its general use and relevance in DNA profiling platforms (Oorschot *et al,* 1994). The primers were designed using Beacon designer Version 7 (Premier Biosoft, US). There were a number of key elements that were addressed during the design process. Where possible runs of four or more bases were avoided (as this reduced annealing efficiency) and the G/C content of the primers was kept between 40 and 60% (as high G/C content promotes non-specific binding). Furthermore complementary runs in both the sense and anti-sense primers were avoided where possible (which reduced the formation of secondary structures such as hairpins and primer dimers that decrease the efficiency of the PCR reaction) and a G/C clamp at the 3' end was used to increase binding strength. The designed primers were checked using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (www.ncbi.nlm.nih.gov) to confirm that the primer sequences avoided inadvertent homology to non-target sequences within the human genome. Primer details can be found in Table 2.

It was necessary to use a singleplex approach to the qPCR assay because the primers were designed to amplify the same region of DNA. There are inherent advantages and disadvantages to singleplex assays. PCR efficiencies tend to be considerably higher in singleplex reactions due to the lack of competition for available reagents (Wong and Medrano, 2005). Optimisations of multiplex assays are tedious and labour intensive and the reaction usually requires a rate limiting primer concentration making singleplex an attractive alternative (Logan *et al,* 2009). Singleplex reactions can also utilise SYBR Green dye, which is highly sensitive and unlike Taqman (and to a lesser extent, MGB) probes, does not show a decrease in binding efficiency with larger amplicon sizes (Logan *et al,* 2009). There are also a number of disadvantages, the main one being an increase in susceptibility to experimental variations such as pipetting differences and instrument drift/shift as well as the non-specific binding of SYBR green to DNA contaminants (Wong and Medrano, 2005). However, any technical or

experimental "disadvantages" did not outweigh the value or importance of ensuring that the same target area was amplified, as this would exclude the possibility of introducing an integrity bias between targets of interest (i.e. targets were equally susceptible to degradation).

Table 2: Primer sequence details for the five designed TH01 primers (Set 1-5) plus details from the two published sequences from Swango et al, (2006) (CSF and TH01)

Primer Name	Primer Type	Primer Sequence
CSF (64bp)	Forward	5' – GGGCAGTGTTCCAACCTGAG – 3'
	Reverse	5' – GAAAACTGAGACACAGGGTGGTTA – 3'
TH01 (190bp)	Forward	5' – AGGGTATCTGGGCTCTGG – 3'
	Reverse	5' – GGCTGAAAAGCTCCCGATTAT – 3'
Set 1 (64bp)	Forward	5' – GCCTGTTCCTCCCTTATTTCCC – 3'
	Reverse	5'- TGGTGAATGAATGAATGAATGAATGAATG– 3'
Set 2 (136bp)	Forward	5' – GGGGCAAAATTCAAAGGGTATCTG– 3'
	Reverse	5' – GCAGGTCACAGGGAACACAG– 3'
Set 3 (198bp)	Forward	5' – ATGGAGTCTGTGTTCCCTGTG– 3'
	Reverse	5' – CTACAACTCACACCACATTTCAATC– 3'
Set 4 (239bp)	Forward	5' - TCATTCATTCATTCATTCATTCATTC– 3'
	Reverse	5' – ACTCACACCACATTTCAATCAAGG– 3'
Set 5 (308bp)	Forward	5' – GGGGCAAAATTCAAAGGGTATCTG– 3'
	Reverse	5' – AACTCACACCACATTTCAATCAAGG– 3'

Figure 15: Genetic details for TH01. Primer sequences were designed to amplify across the same region of the TH01 gene, which is located on chromosome 11 at position 11p15.5. The position of the primer sequences were carefully designed to ensure that each set produced an amplicon that was clearly distinguishable by size and that would be appropriate to cover the range of fragment sizes expected from this study. Set 1 through to Set 5 produced amplicon sizes of 64, 136, 198, 239 and 308bp respectively. The figure above is taken from www.ncbi.nlm.nih.gov and shows the genomic data for the target (TH01) area.

The proposed methodology was designed for forensic samples which are often degraded. It was therefore necessary to test the validity of the proposed assay and assess each target's ability to provide accurate and precise results. A stability study was performed using an artificially prepared DNA degradation series as it was hypothesis that the degradation patterns of naturally aged blood samples would be unpredictable and variable. Therefore it would not be possible to make an accurate assessment of the validity of the technique using naturally degraded samples. The post mortem degradation of biological samples is complicated and not fully understood (Kaiser *et al*, 2008). It is known that oxidative processes, cellular nucleases and other hydrolytic enzymes, which are released when cellular membranes breakdown as a result of autolysis, cause DNA degradation (Kaiser *et al*, 2008). Given that autolysis is highly dependent on environmental conditions, these factors are difficult if not impossible to control so a decision was made to initially test the methodology using artificially degraded DNA.

The most common methods for generating degraded DNA include the use of digestion enzymes (deoxyribonucleases), incubating samples in high humidity or elevated temperatures, or the use of UV light (Pang and Cheung, 2006). UV light is generally cheap, easy to use and relatively quick. Pang and Cheung (2006) developed a one-step method for generating degraded DNA by UV irradiation. They created a DNA degradation series using a variety of UV exposure times and intensities at a wavelength of 254nm. The optimal energy rating for this process was 10mJ as the DNA degradation was mild under these conditions and PCR products of a range of sizes could be easily prepared with varying exposure times (Pang and Cheung, 2006). They also concluded that unlike DNase I treatment, this method could produce fragments in a full range of sizes in a short period of time (less than two minutes), whereas incubation times as long as 30 minutes with DNase I only produced fragments as small as 200 base pairs (Tamariz *et al*, 2006). However, they did note that fragment sizes were clearly depended on a number of factors including the quantity of the DNA used, the volume of the buffer (the smaller the volume, the greater the degree of degradation) and the more obvious variables such as the exposure time and energy of the UV light. An artificial degradation series was produced using Ultra violet light using a stringent protocol to minimise the potential effects of these factors.

Given the use of UV light for producing a uniform degradation series can be problematic a second DNA degradation series was prepared using a time dependant treatment of a DNA digestion enzyme, DNase I. Bender *et al* (2003), developed a method for preparing artificially

degraded DNA under controlled conditions using DNase I and sonication. Sonication involves the application of sound energy to the DNA solution, which breaks the hydrogen bonds causing single strand and double strand ruptures of the DNA helix (Elsner and Lindblad, 1989). They found that DNase I treatment could consistently break the DNA into fragment sizes in the range of 220 to 300 base pairs in length. This is just one of many studies regarding DNase I digestion and of interest is the large degree of variability in the methods describing the digestion procedure. It was unclear whether these studies were amplifying comparable sized targets, which could account for the variation in times. The method employed by Swango *et al,* (2007) used an enzyme incubation time of up to 180 minutes, whilst Bender *et al* (2003) required only twenty minutes. Niederstatter *et al,* (2007) reported that no DNA was amplifiable after as little as two minutes. Given the variation it was considered best practice to test the assay dynamics and target stability on both UV and enzymatic prepared DNA.

3.2.2 Sample Preparation of the Various DNA Degradation Series

The UV degradation series was generated using genomic DNA supplied by Bioline (UK) as described in section 2.1.1 on page 82. Five (5) µl aliquots were used for the subsequent Real Time PCR reactions. A second degradation series was prepared digesting aliquots of the same untreated DNA (Bioline, UK) with the enzyme, DNase I (Invitrogen, UK) for progressive lengths of time and according to manufacturer's instructions. This procedure is described in section 2.1.2 on page 85. Each sample was diluted to produce a working concentration of 1 ng/µl of DNA from which 5 µl aliquots were used in the qPCR assay. All samples were run in triplicate. The details describing the preparation of the naturally aged blood samples can be found in section 2.1.3 on page 83.

3.2.3 Sample Processing

Samples that had aged for longer than 20 days were contaminated with dust. These samples were cleaned using a careful brushing procedure and a sterile Zephyr brush (designed for fingerprint analysis) prior to being recovered. DNA for the naturally aged samples was recovered from the laminate flooring using a sterile scalpel and placed directly into a sterile microcentrifuge tube for extraction. An organic extraction technique was used which simultaneously removed any RNA contaminants. A detailed protocol of the extraction step can be found in section 2.4.1 on page 88. The concentration of DNA in each of the extracts was

determined using a nanophotometer (Implen's Nanodrop). The purpose of this measurement was to ensure suitable quantities of DNA were added to the subsequent Real Time PCR process. A detailed description of the run protocol can be found in section 2.5 on page 90.

The Real time amplification was carried out using Applied Biosystems ABI 7500 thermocycler. The run parameters were as stipulated in section 2.7.2 on page 93. Each sample type required slightly different protocols which are outlined in the following section.

3.2.3.1 Run Protocol for UV Degraded Samples

Samples were loaded into MicroAmp® Optical 96-well reaction plates (Applied aBiosystems, California, USA). Each well contained 10 µl of Applied Biosystem's 2 x Power SYBR Green PCR Master Mix and the appropriate volumes of forward and reverse primers so that the final optimised primer concentrations were obtained (see section 3.3.1.1 on page 104). Five (5) µl of the template (UV degraded) DNA was added to the respective wells and the total reaction volume was made up to 20 µl by the addition of nuclease free water. The reaction plate was gently mixed and then centrifuged for 15 seconds prior to real-time analysis. The reaction plates were then loaded and run according to the conditions stipulated in section 2.7.2 on page 93. The resulting data was analysed using the automatic baseline feature. The threshold was set just above the baseline value to ensure any back ground noise was blocked but within the log-linear phase of the qPCR. A passive reference dye (ROX) was used to correct any well to well variation in the background fluorescence. An exogenous standard control (DNA) was used to adjust for any instrumental variation between runs. Human genomic control DNA (Bioline, UK) was used as the positive control whilst negative controls using PCR grade water were run with every assay. All samples were run in duplicate.

3.2.3.2 Run Protocol for DNase I Degraded Samples

The samples were prepared and run in a similar manner as previously described in section 3.3.2.1 with one modification. The template DNA was obtained from the DNase I degradation series as opposed to the UV degradation series. The data was again analysed using the automatic baseline feature with the same threshold criteria as previously described. The appropriate negative and positive control samples were run with each batch of samples.

3.2.3.3 Run Protocol for Naturally Aged Blood Samples

The run protocol was the same as previously described in 3.3.2.1 with the exception of the template DNA, which was obtained from the aged blood samples. These samples were more akin to those recovered from a forensic environment and as such were expected to show a greater variability. All samples were therefore run in triplicate. All the necessary controls were included as previously described.

3.3 Results

3.3.1 Assay Optimisation

The importance of assay optimisation with qPCR has previously been described (see section 1.7 on page 74) and as such, a stringent testing regime was again carried out for this particular study.

3.3.1.1 Primer Optimisation

A full Primer optimisation (300-600 nM) was performed on all seven sets of primers. The optimisation was carried out using a singleplex SYBR green approach. All samples were run in duplicate. The optimal primer concentrations are listed in the table below. The complete set of details can be found in Appendix II.

Table 3: Optimal primer concentrations of CSF and TH01 and Set 1 -5.

Primer	***Forward Concentration (nM)***	***Reverse Concentration (nM)***
CSF (67bp)	400	400
TH01 (170-190bp)	600	600
Set 1 (64bp)	500	500
Set 2 (136bp)	400	400
Set 3 (192bp)	500	500
Set 4 (239bp)	400	400
Set 5 (308bp)	500	500

3.3.1.2 Efficiency Assays for all Sets of Primers

To ensure accurate quantification, the amplification efficiency rates for each target were determined using a serial dilution method. The serial dilution was created using control DNA (Bioline, UK). To ensure the assay was carried out using DNA concentrations similar to those expected in the test samples, an initial neat concentration of 20 ng/µl was used. The Ct values were obtained using a singleplex SYBR green approach and all samples were run in triplicate. The mean Ct values are shown in Table 4. These mean values were graphed against the log DNA concentration to determine the slope of the regression line, details of which can be found in Figure 16. The amplification efficiency rates where then calculated from the slope values using Equation 1 described in section 2.8 on page 94. The results are summarised in Table 6.

Table 4: Serial Dilution Assay. To determine the amplification efficiency rates of CSF, TH01 and Set 1-5 a 10-fold serial assay was prepared by diluting the neat DNA template DNA. Each dilution was amplified until a set fluorescence value was obtained. The number of cycles taken to reach this value (Ct value) can be used to calculate the reaction efficiency using the formula described in 2.8 at page 94. The table below shows the mean Ct values for the replicate samples run for each dilution and each target species

Serial Dilution	**CSF Mean Ct value** (StDev)	**TH01 Mean Ct value** (StDev)	**Set 1 Mean Ct value** (Std Dev)	**Set 2 Mean Ct value** (St Dev)	**Set 3 Mean Ct value** (StDev)	**Set 4 Mean Ct value** (StDev)	**Set 5 Mean Ct value** (StDev)
Neat	**23.83** (0.02)	**22.51** (0.22)	**21.33** (0.67)	**22.43** (0.36)	**22.65** (0.34)	**22.71** (0.46)	**23.58** (0.27)
1/10	**26.95** (0.14)	**26.01** (0.21)	**24.58** (0.42)	**25.43** (0.31)	**25.84** (0.13)	**25.81** (0.15)	**27.05** (0.38)
1/100	**30.35** (0.04)	**29.10** (0.05)	**28.38** (0.38)	**28.78** (0.05)	**28.97** (0.14)	**29.17** (0.10)	**30.27** (0.04)
1/1000	**33.37** (0.12)	**32.25** (0.87)	**31.40** (0.45)	**31.92** (0.13)	**32.54** (1.64)	**32.21** (0.40)	**33.73** (1.11)

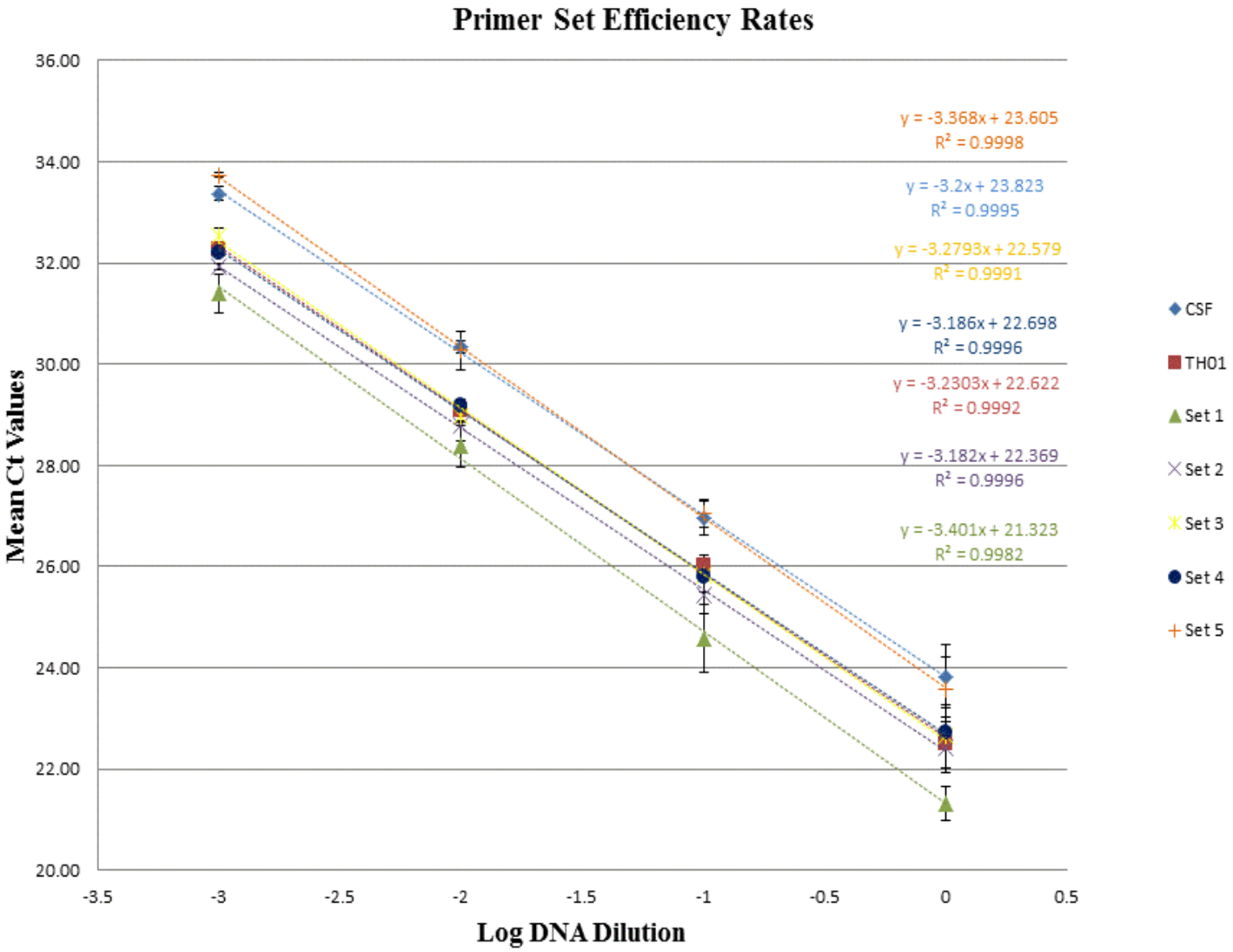


Figure 16: Log Concentrations of CSF, TH01 and Sets 1 to 5 from the Serial Dilution Assay: The slope of the lineal regression between the logarithmic transformed DNA concentration and mean Ct values is used to calculate the amplification efficiency rates. The errors bars represent ± 1 Standard Deviation and the variation was quite small throughout.

Table 5: Amplification Efficiency rates for each set of Primers. Using the Ct values for each of the serial dilutions the efficiency rate of the PCR amplification process can be calculated using Equation 1 described in section 2.8.

Primer Set	***Slope***	***Efficiency Rate (%)***
CSF	-3.200	**105.3**
TH01	-3.230	**103.9**
Set 1	-3.401	**96.8**
Set 2	-3.182	**106.2**
Set 3	-3.279	**101.8**
Set 4	-3.186	**106.0**
Set 5	-3.368	**98.1**

3.3.2 UV Degradation Series

The UV DNA degradation series was produced using a stringent protocol that ensured the quantity of the DNA. The volume of the buffer, energy of the UV light and the distance between the samples and UV source were all kept constant. This was to ensure that the only variable component was the exposure time. The raw logarithmic Ct values (shown in Table 6) were converted to a lineal measurement [Relative Quantity (RQ)] before any subsequent statistical analysis was carried out. This was done using Equation 2, described in section 2.8. The RQ values are presented in Table 7. The quantity ratios between the different targets were calculated and are shown in Table 8.

Table 6: The mean Ct values from UV degraded samples are presented with the variation expressed as standard deviation. Each sample was run in triplicate.

Target Amplicon	Mean Ct and StDev	UV Exposure Time (min)								
		0	0.5	1	2	5	10	15	20	30
CSF (67bp)	Ct Value	25.17	25.35	25.19	25.50	26.16	26.73	28.46	28.61	ND
	Std Dev	0.17	0.08	0.01	0.11	0.08	0.04	0.04	0.14	n/a
TH01 (170-190bp)	Ct Value	23.78	24.46	24.60	25.40	26.27	28.56	29.30	30.05	ND
	Std Dev	0.09	0.07	0.10	0.01	0.15	1.26	0.04	0.12	n/a
Set 1 (64bp)	Ct Value	23.47	23.59	23.77	24.64	24.54	25.26	25.20	25.25	25.34
	Std Dev	0.21	0.13	0.35	0.69	0.31	0.15	0.02	0.10	0.11
Set 2 (136bp)	Ct Value	23.52	24.07	25.06	24.60	25.54	26.51	27.41	27.63	ND
	Std Dev	0.01	0.01	1.35	0.07	0.14	0.18	0.18	0.06	n/a
Set 3 (198bp)	Ct Value	23.77	24.95	25.06	26.44	29.31	31.97	31.97	32.98	33.66
	Std Dev	0.07	0.33	0.06	0.11	0.98	0.21	0.21	0.81	0.88
Set 4 (239bp)	Ct Value	24.00	25.26	25.39	26.57	29.06	34.83	34.83	35.00	ND
	Std Dev	0.02	0.22	0.13	0.00	0.03	0.76	0.76	1.21	n/a
Set 5 (308bp)	Ct Value	24.98	26.25	26.45	28.12	30.96	37.36	37.36	ND	ND
	Std Dev	0.29	n/a	0.25	0.11	n/a	n/a	n/a	n/a	n/a

Table 7: Relative Quantities for UV Degraded Samples

Exposure Time (min)	Relative Quantities (RQ)						
	CSF	TH01	Set 1	Set 2	Set 3	Set 4	Set 5
0	1.37E-08	4.40E-08	1.26E-07	4.05E-08	5.65E-08	2.94E-08	3.85E-08
0.5	1.25E-08	2.73E-08	1.16E-07	2.73E-08	2.47E-08	1.18E-08	1.61E-08
1	1.36E-08	2.45E-08	1.03E-07	1.34E-08	2.28E-08	1.08E-08	1.42E-08
2	1.09E-08	1.39E-08	5.69E-08	1.86E-08	8.67E-09	4.58E-09	4.59E-09
5	6.75E-09	7.47E-09	6.09E-08	9.40E-09	1.16E-09	7.57E-10	6.43E-10
10	4.47E-09	1.46E-09	3.75E-08	4.66E-09	1.75E-10	1.04E-10	9.78E-11
15	1.29E-09	8.62E-10	3.91E-08	2.44E-09	1.79E-10	1.17E-11	8.19E-12
20	1.15E-09	5.05E-10	3.77E-08	2.07E-09	8.78E-11	1.04E-11	n/a
30	n/a	n/a	3.56E-08	n/a	5.45E-11	n/a	n/a

The Relative Quantity ratios for all combination of targets were calculated and are presented in the following table.

Table 8: Relative Quantity Ratios (RQR) for all target combinations. Where the targets failed to amplify no result was obtained (n/a)

Exposure Time (min)	Relative Quantity Ratio (RQR)									
	Set1 / Set 2	Set 1 / Set 3	Set 1 / Set 4	Set 1 / Set 5	Set 2 / Set 3	Set 2 / Set 4	Set 2 / Set 5	Set 3 / Set 4	Set 3 / Set 5	Set 4 / Set 5
0	3.110	2.231	4.284	3.276	0.717	1.377	1.053	1.919	1.468	0.764
0.5	4.253	4.712	9.820	7.221	1.107	2.308	1.697	2.084	1.532	0.735
1	4.682	4.491	9.517	7.305	0.584	1.238	0.950	2.119	1.626	0.767
2	3.067	6.566	12.43	12.65	2.141	4.054	4.124	1.893	1.926	1.017
5	6.479	52.52	80.46	94.66	8.105	12.41	14.60	1.532	1.802	1.176
10	8.057	213.9	360.5	383.5	26.55	44.74	47.60	1.685	1.792	1.063
15	16.036	218.1	3342.2	4826.6	13.60	208.4	300.9	15.31	n/a	n/a
20	18.182	428.7	3627.8	n/a	23.57	199.5	n/a	n/a	n/a	n/a
30	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

3.3.3 DNase I Degradation Series

A DNA degradation series was prepared using a time dependant treatment of DNase I. DNase I is a single, glycosylated polypeptide that degrades both single and double stranded DNA (Samejima and Earnshaw, 2005). DNase I preferentially cleaves DNA at the phosphodiester linkages adjacent to a pyrimidine nucleotide, yielding 5'-phosphate-terminated polynucleotides with a free hydroxyl group at the 3' end (Samejima and Earnshaw, 2005). Each time treated sample was tested in duplicate with each of the five primer sets and the two comparative targets, CSF and TH01 used by Swango *et al,* (2006). The mean Ct values for each target at each treatment time are shown in Table 9.

Table 9: The mean raw Ct values from the DNase I degraded samples are presented with the variation expressed as standard deviation. Each sample was run in triplicate.

Target Amplicon	Mean Ct and StDev	DNase I Treatment Time (min)									
		0	1	2	5	10	15	20	30	60	90
CSF	**Ct Value**	**27.99**	**30.69**	**32.34**	**31.96**	**34.73**	**36.89**	**36.30**	**38.41**	**38.09**	**42.33**
(67 bp)	**Std Dev**	0.49	0.41	0.27	0.43	0.25	0.64	0.85	0.56	0.96	3.31
TH01	**Ct Value**	**26.18**	**30.00**	**32.47**	**34.14**	**34.78**	**35.93**	**37.80**	**41.40**	**40.68**	**42.98**
(170-190 bp)	**Std Dev**	0.03	0.15	0.12	0.90	0.85	0.91	0.43	4.26	2.30	n/a
Set 1	**Ct Value**	**24.16**	**25.02**	**25.40**	**26.22**	**26.98**	**26.16**	**27.85**	**26.81**	**27.75**	**28.63**
(64 bp)	**Std Dev**	0.08	0.61	0.43	0.16	0.53	0.48	0.97	0.68	0.39	0.24
Set 2	**Ct Value**	**24.50**	**27.72**	**28.73**	**30.90**	**31.92**	**33.52**	**34.64**	**35.67**	**35.94**	**36.04**
(136 bp)	**Std Dev**	0.22	0.12	0.49	1.41	0.23	0.91	0.97	1.10	0.85	0.49
Set 3	**Ct Value**	**24.95**	**29.04**	**32.42**	**33.12**	**35.78**	**36.78**	**38.76**	**42.84**	**41.45**	**43.12**
(192 bp)	**Std Dev**	0.06	0.08	0.23	0.69	0.96	0.72	0.56	1.33	n/a	2.04
Set 4	**Ct Value**	**25.31**	**30.63**	**33.53**	**35.09**	**37.85**	**39.00**	**41.02**	**40.42**	**ND**	**ND**
(239 bp)	**Std Dev**	0.13	0.38	0.94	0.36	1.01	0.62	1.22	n/a	n/a	n/a
Set 5	**Ct Value**	**25.90**	**32.06**	**35.22**	**40.15**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
(308 bp)	**Std Dev**	0.15	0.27	0.04	0.79	n/a	n/a	n/a	n/a	n/a	n/a

The raw logarithmic Ct values were converted to relative quantities using Equation 2 described in section 2.8 on page 94. The relative quantities are shown in Table 10.

Table 10: Relative quantities of each target digested by DNase I.

DNase I Exposure Time (min)	Relative Quantity (RQ)						
	CSF (67 bp)	TH01 (170-190 bp)	Set 1 (64 bp)	Set 2 (136 bp)	Set 3 (192 bp)	Set 4 (239 bp)	Set 5 (308 bp)
0	1.803E-09	5.064E-09	7.876E-08	1.994E-08	2.46E-08	1.137E-08	2.04E-08
1	2.593E-10	5.230E-10	4.410E-08	1.935E-09	1.39E-09	2.427E-10	3.02E-10
2	7.894E-11	8.978E-11	3.394E-08	9.343E-10	1.30E-10	2.992E-11	3.50E-11
5	1.040E-10	2.725E-11	1.948E-08	1.943E-10	7.98E-11	9.668E-12	1.20E-12
10	1.418E-11	1.731E-11	1.167E-08	9.287E-11	1.23E-11	1.315E-12	9.71E-14
15	2.992E-12	7.632E-12	2.038E-08	2.917E-11	6.05E-12	5.743E-13	
20	4.573E-12	2.009E-12	6.492E-09	1.294E-11	1.51E-12	1.334E-13	
30	1.002E-12	1.549E-13	1.312E-08	6.171E-12	8.67E-14	2.05E-13	
60	1.262E-12	2.593E-13	6.915E-09	5.063E-12	2.29E-13		
90	5.978E-14	5.026E-14	3.828E-09	4.721E-12	7.10E-14		

The relative expression ratios (RER) were calculated according to Equation 4 described in section 2.8 on page 94. The results are presented in the following table.

Table 11: The Relative Expression Ratios (RER) for all target combinations

DNase 1 Exposure Time (min)	Relative Expression Ratio (RER)									
	Set1 / Set 2	Set 1 / Set 3	Set 1 / Set 4	Set 1 / Set 5	Set 2 / Set 3	Set 2 / Set 4	Set 2 / Set 5	Set 3 / Set 4	Set 3 / Set 5	Set 4 / Set 5
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
1	5.770	9.891	26.23	37.83	1.714	4.547	6.558	2.653	3.825	1.442
2	9.202	81.69	163.83	251.13	8.878	17.80	27.29	2.006	3.074	1.533
5	25.39	76.47	291.06	4206.6	3.011	11.46	165.6	3.806	55.00	14.45
10	31.83	297.2	1281.7	31188.0	9.338	40.26	979.7	4.312	104.9	24.33
15	176.9	1054.8	5125.9	NR	5.962	28.97	NR	4.859	NR	NR
20	127.0	1339.6	7028.8	NR	10.545	55.32	NR	5.247	NR	NR
30	538.7	47406.3	9211.8	NR	87.99	17.09	NR	0.194	NR	NR
60	345.8	9433.4	NR	NR	27.27	NR	NR	NR	NR	NR
90	205.3	16869.6	NR	NR	82.14	NR	NR	NR	NR	NR

3.3.4 Naturally Aged Blood Samples

The five primer sets (sets 1 to 5), which targeted the same region of the TH01 gene were used to determine the optimal sized targets for the naturally aged blood samples. In order to make an accurate assessment of the degradation that occurs during the ageing process, the relative expression (RE) was calculated. The relative expression values are corrected according to a reference or calibrator samples. In this case the calibrator samples were those obtained at time 0. This process normalises the data and provides a measure of the degradation that occurs once the blood is deposited.

The mean logarithmic Ct values of each of the five primer sets from all five donors across the entire 90 day ageing period can be found in Appendix III. The relative expression (RE) values can be found in Appendix IV and discussed in section 3.4.3 on page 130. The relative expression ratios (RER) (calculated from the RE values) were used to calculate the level of degradation and hence blood sample age. RERs values for all donors across the 90 day trial are presented in Table 12.

Table 12: The mean Relative Expression Ratio of the naturally aged blood samples

Donor 1	Relative Expression Ratio (RER)									
Ageing Time (Days)	Set1 / Set 2	Set 1 / Set 3	Set 1 / Set 4	Set 1 / Set 5	Set 2 / Set 3	Set 2 / Set 4	Set 2 / Set 5	Set 3 / Set 4	Set 3 / Set 5	Set 4 / Set 5
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
10	1.280	1.410	1.972	5.364	1.102	1.541	4.192	1.399	3.805	2.720
20	1.120	1.807	7.272	8.333	1.613	6.492	7.439	4.025	4.612	1.146
30	2.041	2.335	12.675	17.934	1.144	6.211	8.788	5.429	7.682	1.415
40	1.879	7.915	24.217	24.434	4.211	12.885	13.001	3.060	3.087	1.009
50	2.169	10.897	34.437	32.209	5.025	15.881	14.853	3.160	2.956	0.935
60	7.571	45.810	30.837	119.196	6.051	4.073	15.743	0.673	2.602	3.865
70	7.504	31.839	52.065	179.888	4.243	6.938	23.973	1.635	5.650	3.455
80	12.737	15.574	63.616	232.725	1.223	4.994	18.271	4.085	14.943	3.658
90	7.352	63.281	58.256	102.561	8.607	7.924	13.950	0.921	1.621	1.761

Donor 2	Relative Expression Ratio (RER)									
Ageing Time (Days)	Set1 / Set 2	Set 1 / Set 3	Set 1 / Set 4	Set 1 / Set 5	Set 2 / Set 3	Set 2 / Set 4	Set 2 / Set 5	Set 3 / Set 4	Set 3 / Set 5	Set 4 / Set 5
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
10	1.591	2.029	3.065	6.079	1.276	1.927	3.822	1.511	2.996	1.983
20	1.266	1.310	6.793	10.499	1.035	5.365	8.293	5.186	8.016	1.546
30	1.820	3.803	8.386	14.553	2.090	4.609	7.998	2.205	3.827	1.735
40	2.007	2.991	20.654	26.868	1.491	10.293	13.390	6.906	8.983	1.301
50	5.377	6.332	25.506	71.152	1.177	4.743	13.232	4.028	11.237	2.790
60	1.827	11.941	52.423	94.266	6.538	28.701	51.609	4.390	7.894	1.798
70	2.317	29.763	55.948	89.490	12.843	24.142	38.615	1.880	3.007	1.600
80	5.449	16.569	58.620	57.031	3.041	10.757	10.466	3.538	3.442	0.973
90	1.923	7.916	79.284	70.863	4.117	41.232	36.853	10.016	8.952	0.894

Table 12 continued:

Ageing Time (Days)	Relative Expression Ratio (RER)									
Donor 3	**Set1 / Set 2**	**Set 1 / Set 3**	**Set 1 / Set 4**	**Set 1 / Set 5**	**Set 2 / Set 3**	**Set 2 / Set 4**	**Set 2 / Set 5**	**Set 3 / Set 4**	**Set 3 / Set 5**	**Set 4 / Set 5**
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
10	0.716	1.700	2.078	3.381	2.375	2.903	4.724	1.222	1.989	1.627
20	1.278	5.434	11.842	15.979	4.252	9.266	12.503	2.179	2.941	1.349
30	1.089	2.050	10.687	22.273	1.882	9.815	20.455	5.214	10.866	2.084
40	1.347	6.733	20.183	36.909	4.999	14.985	27.404	2.998	5.482	1.829
50	1.333	7.226	51.806	58.283	5.422	38.871	43.731	7.169	8.065	1.125
60	2.186	28.229	41.470	65.958	12.916	18.974	30.179	1.469	2.337	1.591
70	6.683	45.583	88.855	216.794	6.820	13.295	32.438	1.949	4.756	2.440
80	5.857	51.304	86.546	185.381	8.760	14.777	31.652	1.687	3.613	2.142
90	7.266	61.714	94.292	118.114	8.493	12.977	16.255	1.528	1.914	1.253
Donor 4	**Set1 / Set 2**	**Set 1 / Set 3**	**Set 1 / Set 4**	**Set 1 / Set 5**	**Set 2 / Set 3**	**Set 2 / Set 4**	**Set 2 / Set 5**	**Set 3 / Set 4**	**Set 3 / Set 5**	**Set 4 / Set 5**
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
10	1.036	1.250	3.271	1.976	1.206	3.156	1.906	2.616	1.580	0.604
20	1.112	1.833	9.959	5.839	1.648	8.957	5.252	5.435	3.187	0.586
30	1.538	2.257	8.668	10.249	1.467	5.634	6.662	3.841	4.541	1.182
40	1.446	3.620	33.671	24.226	2.503	23.283	16.752	9.302	6.693	0.719
50	1.755	12.606	54.154	37.696	7.182	30.856	21.479	4.296	2.990	0.696
60	9.079	57.727	92.470	101.895	6.358	10.185	11.223	1.602	1.765	1.102
70	4.824	15.492	67.566	41.080	3.212	14.007	8.516	4.361	2.652	0.608
80	4.780	20.475	18.397	82.453	4.283	3.849	17.249	0.899	4.027	4.482
90	9.513	23.829	90.475	94.639	2.505	9.511	9.948	3.797	3.972	1.046
Donor 5	**Set1 / Set 2**	**Set 1 / Set 3**	**Set 1 / Set 4**	**Set 1 / Set 5**	**Set 2 / Set 3**	**Set 2 / Set 4**	**Set 2 / Set 5**	**Set 3 / Set 4**	**Set 3 / Set 5**	**Set 4 / Set 5**
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
10	1.744	2.892	5.100	5.851	1.658	2.925	3.355	1.764	2.023	1.147
20	1.288	1.603	9.245	10.283	1.245	7.179	7.985	5.767	6.415	1.112
30	3.170	2.581	13.802	29.918	0.814	4.354	9.437	5.348	11.592	2.168
40	1.161	2.159	23.510	32.893	1.860	20.258	28.344	10.891	15.239	1.399
50	2.036	6.260	31.997	54.063	3.074	15.714	26.550	5.112	8.637	1.690
60	6.679	18.814	96.965	71.455	2.817	14.518	10.699	5.154	3.798	0.737
70	4.612	6.955	63.370	87.578	1.508	13.740	18.988	9.112	12.593	1.382
80	9.042	21.532	89.809	172.075	2.381	9.933	19.031	4.171	7.991	1.916
90	9.809	51.960	93.878	245.975	5.297	9.571	25.077	1.807	4.734	2.620

3.3.5 Variation in the Ct data

3.3.5.1 Variation between Primer Sets

The data was examined to determine whether there were any observable trends in the variation between the primer sets. The mean variation (%CV) from each primer set was similar ranging from 1.45 (set 2) to 1.75 (set 3). To determine whether this variation was significant a Repeated Measures ANOVA with post-test was performed, the results of which are presented in Table 15. The data indicates that the variation was not significant (ANOVA; p = >0.1).

Table 13: Statistical analysis of the reproducibility of the Primer Set 1-5. Each primer set was tested using a standardised DNA sample. Triplicate samples were run to determine the variation in the Ct values obtained.

Statistical Parameter	Set 1	Set 2	Set 3	Set 4	Set 5
Mean	1.562	1.454	1.746	1.689	1.702
Std Deviation	0.748	0.749	1.113	1.007	1.088
P value	>0.1	>0.1	0.015	0.022	>0.1

3.3.5.2 Variation between Aged Samples

A Repeated Measures ANOVA with post-test was performed to test the variation observed between different age periods. All samples were paired according to donor and primer set type to ensure the only variable being measured was the ageing period. The mean variation for each age period ranged from 1.2% (50 day samples) to 2.2% (60 day samples). The complete statistical data can be found in Table 16. The data suggests the variation observed was statistically significant (ANOVA; p = 0.01), which suggests the age of the samples did have an effect on the size of the variation observed.

Table 14: Statistical data comparing the variation between ageing periods. The Ct values obtained for all donors were grouped examined to determine whether the age of the sample effected the variation observed in the Ct values. The variation in the Ct values increased as the samples aged.

	Ageing Period (days)									
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Mean	1.636	1.696	1.491	1.356	1.594	1.203	2.192	1.389	1.921	1.830
Std Deviation	0.891	0.664	0.882	0.878	0.908	0.761	1.183	0.717	1.058	1.181
P value	0.164	>0.10	0.037	>0.10	>0.10	>0.10	>0.10	>0.10	0.055	0.007

3.3.5.3 Variation between donors

A Repeated Measures ANOVA with post-test was performed to determine whether the variation observed between donors (inter-donor variation) was significant. The results are shown in Table 17 and suggest that inter-donor variation was not significantly greater than expected by chance (ANOVA; p = > 0.1).

Table 15: Statistical analysis of the reproducibility between donors. All samples were paired according to primer set and ageing time to ensure the only variable being measured was the inter-donor variation. The mean variation for each donor ranged from 0.88% (donor 5) to 1.04% (donor 4).

Statistical Parameter	Set 1	Set 2	Set 3	Set 4	Set 5
Mean	1.791	1.666	1.410	1.658	1.629
Std Deviation	1.018	0.905	0.915	1.037	0.878
SEM	0.144	0.128	0.129	0.147	0.124
P value	0.001	0.004	0.007	>0.1	>0.1

3.4 Discussion

When DNA is degraded, successful amplification using the standard STR profiling platforms can be limited (Goodwin *et al,* 2007). The level of degradation is dependent on a number of factors including the sample type, the environmental ageing conditions and the influence of various physical and biological factors such as bacteria, intra-cellular nucleases and oxidative processes (McCord *et al,* 2011). To increase the chance of obtaining a full profile from degraded samples, new profiling platforms have been introduced with carefully designed primers that reduce the size of the target being amplified (Opel *et al,* 2007*).* Whilst this concept is relatively straight forward with STR profiling the issue becomes more complicated with techniques concerned with evaluating the degradation level or determining the age of a sample.

The size of target amplicons has been used to gauge the level of degradation of a sample (Swango *et al,* 2006). The most common method involves the amplification of two targets of varying size and to perform a comparative analysis of the relative copy numbers. Studies that have adopted this approach have been diverse in terms of sample type, ageing conditions and experimental parameters. However, there has been no attempt to optimise the size of the amplified targets or to determine whether there is an optimal size difference between the targets chosen. The amplicons used by Niederstatter *et al*, (2007), Alonso *et al*, (2003), Bauer *et al* (2003) and Swango *et al,* (2006) ranged in size from 67 to 763 base pairs (bp) and the samples analysed ranged from artificially synthesised degradation products (Niederstatter *et al*, 2007) to 1500 year old human remains (Alonso *et al*, 2003). It was thought that optimal sizes are likely to exist and by determining what they are, may result in more precise and accurate ageing methodologies. This theory was tested using the set of five primers (described as Primer Set 1-5) that had been designed to produce amplicons of 64, 136, 198, 239 and 308 base pairs respectively. Based on a thorough literature search it was thought that this size range would be sufficient to discriminate between blood samples aged for up to 90 days in an internal urban environment.

3.4.1 Stability and Functionality of the Assay

Prior to assessing the degradation levels of the aged blood samples, it was necessary to test the stability and functionality of the five primer assay on a uniform degradation series. In order to do so, DNA was degraded using UV light and DNase I.

3.4.1.1 UV Degradation Series

A UV degradation series was created and tested on the five different sets of primers as well as the two sets (CSF and TH01) described by Swango *et al,* (2006). These were included for comparison purposes. Given the amount of starting template DNA was identical for each sample (there will be minor variations due to experimental error but these are generally accounted for by the normalisation procedures so reliable interpretation can still be made) the Ct values and the relative quantities (RQ) were used to evaluate degradation levels.

The results from this experiment suggest that the target region of the five primer sets (TH01) is relatively robust as all amplicons performed well on the UV degraded samples. After 15 minutes of UV exposure all five targets amplified. The largest amplicon (308bp) was the only target that failed following a 20 minute exposure time and even after the maximum period of UV treatment (30 minutes), two of the targets [Set 1 (64bp and Set 3 (198bp)] still produced a result. The CSF target adopted as a comparative tool from Swango *et al,* (2006) had an amplicon size of 67bp and this could be directly compared (size wise) to Set 1 (64bp) however where Set 1 succeeded, CSF failed to amplify after 30 minutes of UV exposure. This suggests the TH01 target is less susceptible to UV degradation than the CSF target.

The mean Ct values are shown in Figure 17 and trends can be observed, albeit on a logarithmic scale. In terms of discrimination power, the largest amplicon (Set 5) was the most effective for samples exposed to UV light for less than 15 minutes (the 308bp amplicon was not detected in samples exposed for 20 minutes or more). Sets 3, 4 and TH01 (which produced amplicons of 198bp, 239bp and 190bp respectively) showed a moderate ability to distinguish between samples whereas CSF and Set 1 (69bp and 64bp respectively) had a very poor discriminating power. These smaller amplicons were slightly more effective as the exposure time increased (and fragment sizes became smaller) which was expected. The relative quantity of CSF was consistently lower than Set 1 (TH01). This difference was statistically significant (ANOVA; $p = 0.0005$). Such difference could not be explained by experimental variation alone (including efficiency rates). Given the same amount of template DNA was used and the amplicon sizes were virtually the same, the results suggest there is a difference in the susceptibility to degradation between these two loci. This aspect is explored further in chapter four but highlights the need for a detailed investigation into the stability characteristics of the loci being examined.

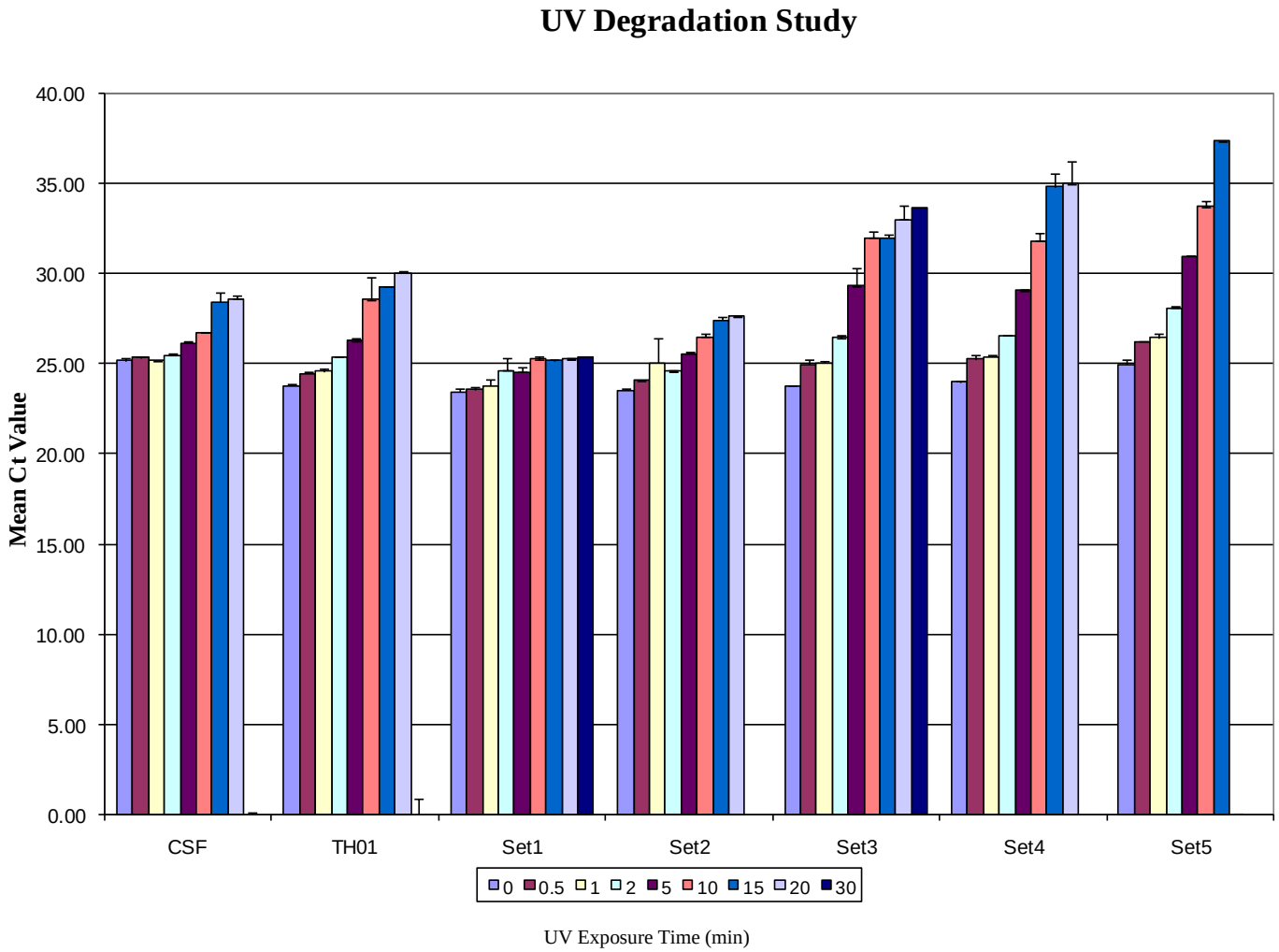
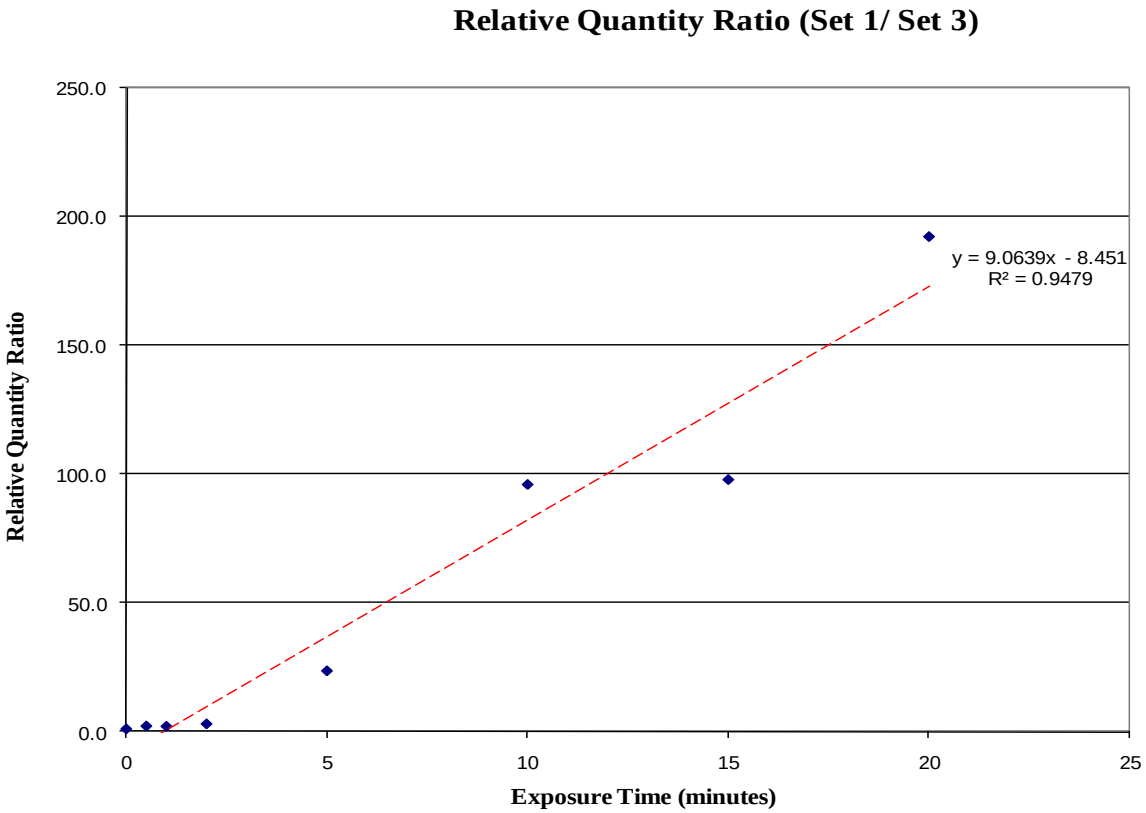


Figure 17: Mean Ct Values from the UV Degradation Study. According to this data, an amplicon of approximately 240bp (Set 4 - 239bp) is the optimal size for distinguishing between UV degraded samples treated under these specified controlled conditions, though any amplicon greater than 200 provided acceptable results. The two smallest targets, CSF and Set 1, which produced amplicons of 70 and 64 base pairs respectively, displayed very poor discriminating power. The error bars represent 1 SD and those that do not present with an error bar are simply those samples that single value from the duplicate sample.

The same size assessment was conducted between the TH01 described by Swango *et al,* (2006) and Set 3 (TH01). Although both concerned the TH01 gene, the primers were designed so that the amplicons were from different parts of the gene. The TH01 amplicon by Swango *et al* (2006) was 170-190bp in length whilst the Set 3 amplicon was 198bp, which allowed for a size comparison. Whilst the results were similar, the difference was considered statistically significant (ANOVA; p value = 0.009). This may be explained by the slight difference in amplicon length (170bp compared with 198bp).

The Ct and relative quantity values can only be used to calculate degradation levels when the same amount of starting material is used across all samples, as was the case here. This would however, be extremely difficult if not impossible to achieve with forensic samples therefore these parameters are not suitable for casework. Bauer *et al,* (2003) and Anderson *et al,* (2005) have alleviated this problem by analysing multiple targets from a single sample and using the ratio (of relative quantities) to estimate the degradation level. This approach is extremely advantageous in that the ratio is independent on the initial sample volume (Anderson *et al,* 2005). As such the relative quantity ratio (RQR) of CSF/TH01 was compared to that of Set1/Set3. Given both combinations consisted of approximately the same sized amplicons (67bp/190bp and 64bp/198bp) and had approximately the same base pair difference between the two primers (123bp and 134bp respectively) they were expected to produce similar results. However the correlation between the ratio and UV exposure time was considerably different (see Figure 18 on page 121). There was also a large difference in each of the combinations ability to discriminate between samples. The correlation for Set1/Set3 was 0.948 whilst it was 0.589 for CSF/TH01. The primers designed from the same target area clearly outperformed those designed across different loci.

The relative quantity ratios were calculated for the five primer sets (refer to Table 8 on page 109) to determine which combination of amplicons provided the best discriminating power. The RQRs proved to be a poor method of discriminating between the UV degraded samples due to the lack of precision. The discriminating power was directly influenced by not only the individual amplicon sizes but also the size difference between the two targets in question. Though not ideal, the best combination for this sample set was Set1/Set4, which consisted of amplicons that were 64bp and 239bp respectively. The size difference between these amplicons was 175 base pairs. Set 1/Set 5 ratio (64bp and 308bp with a difference of 244bp) had a relatively similar discriminating power.



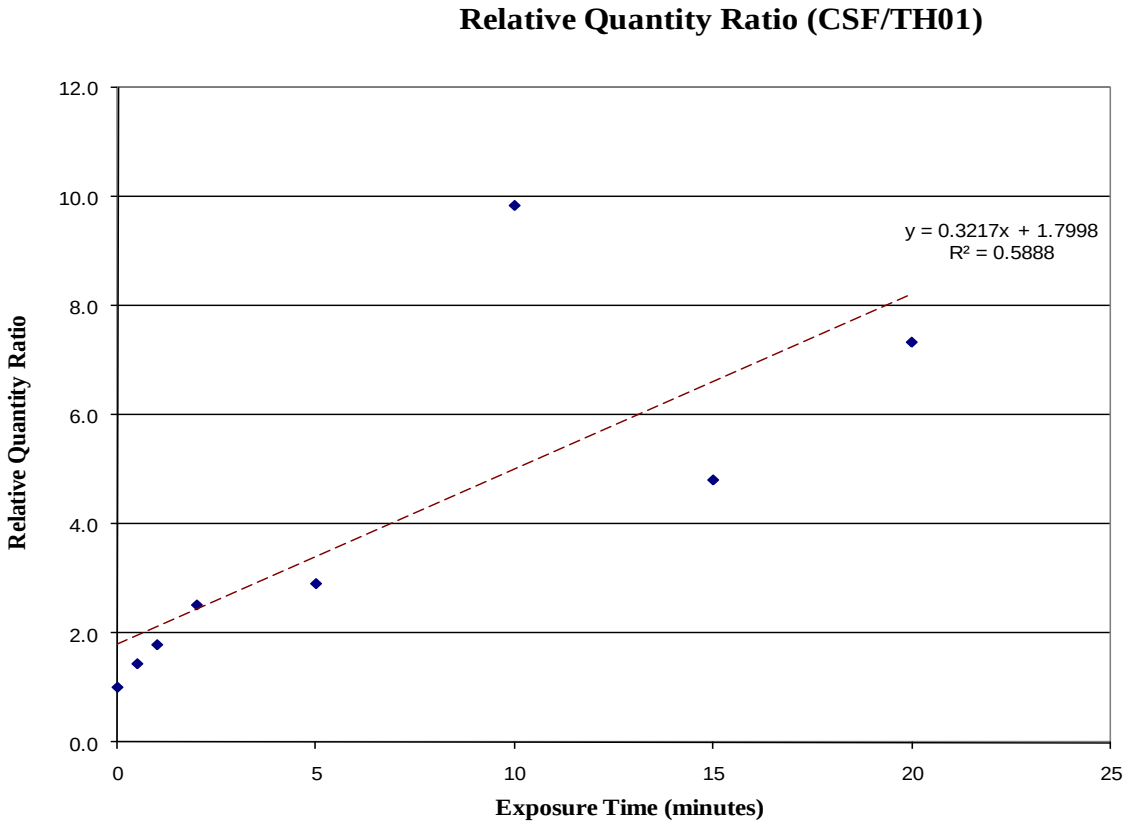


Figure 18: Comparison of Discriminating Capabilities: Amplicons designed from the same loci (Set1/Set3) outperformed those designed from different loci (CSF/TH01), independent of size.

However, the fallout (no result obtained) occurred earlier therefore limiting its ability to discriminate across the whole degradation series. The discriminating ability of sets that were similar in size (i.e. Set4/Set5 and Set1/Set2) was poor, which was expected. The results from this experiment also suggest that the discriminating ability of most combinations increased as the DNA became more degraded. Samples exposed to UV light for less than two minutes were very difficult to consistently distinguish using any combination of targets. It is suggested that a larger amplicon (greater than 308bp) would be required for such samples.

One feature of these results is that although particular combinations have an acceptable discriminating power at certain levels of degradation, there is no single combination that was appropriate across the entire degradation series. These findings suggest the use of multiple combination sets may be the best practice for obtaining the most reliable and accurate results. This could pose a problem as prior knowledge of the degradation levels may be required in order to select the optimal sized targets. This knowledge will be rarely available with forensic case samples.

The overall ratios were far from ideal in providing an accurate indicator of the degradation levels however it was unclear whether it was the methodology itself or the degradation process (UV light) that was the main compromising factor. To conclude the method was unreliable is to assume a uniform degradation series was created. UV irradiation is a common precautionary step to sterilize pre-PCR surfaces, reagents and equipment. The ways in which UV rays degrade DNA has previously been explained (Pang and Cheung, 2007). There are a number of issues regarding the ability of this method to produce a consistent, uniform range of fragments and its associated problems are well documented (Tamariz *et al,* 2006). Some of these include the direction and penetration of UV rays and the length of exposure. Tamariz *et al*, (2006) found that the length of exposure time did not always result in reproducible results. Although there was a decrease in the concentration of amplifiable DNA as exposure time increased, the time taken to successfully degrade all 500 pg of DNA (to a level not detectable by qPCR) varied significantly (Tamariz *et al,* 2006). In certain samples as little as 0.5 pg/µl was measured following 30 minutes of digestion and yet the same quantities were found in other samples after 45 minutes of treatment. Quantities pre-determined as being significant in terms of contamination levels were still found in numerous samples after 60 minutes of UV treatment (Tamariz *et al,* 2006). Inconsistent results were also observed by Thacker *et al,* (2006) who examined the effect of UV light, humidity and temperature on various different sources of

DNA including blood samples and chewing gum samples. If the suggestions proposed by Tamariz *et al* (2006) are correct, a uniform degradation series may not have been achieved. Although every measure was taken to minimise these effects, the nature of this method may still have contributed to the relatively poor results.

Degraded DNA can be separated and visualised using agarose gel electrophoresis (Aaki and Borst, 1972). The major drawback of this technique is that quantification is difficult and often inaccurate (Adkins *et al,* 2007) therefore the information obtainable is limited. Furthermore this method is not applicable when sub-nanogram amounts of DNA are present (Bourzac *et al,* 2003). Each of the UV degraded samples prepared in this study were run on an agarose gel but the expected DNA "smear" was not present and this was thought to be a result of the low quantities of DNA present in the samples.

3.4.1.2 DNase I Degradation Series

The Ct values were used to establish general trends between the level of degradation of a sample and the length of DNase I treatment, as it was assumed the initial amount of starting DNA was the same for each sample. The Ct values of both CSR and TH01 increased steadily as the time of DNase I treatment increased. This indicated that as the exposure time increased so did the level of degradation. The Ct values for Set 1 did not increase as much as CSF and this was consistent with findings from the UV study. These results suggest that when compared to CSF, TH01 target is more robust to this form of degradation. These findings are slightly unusual given Ambion Technotes (2001) suggest DNase I cleaves the DNA strand non-specifically. Herrera and Chaires, (1994) demonstrated that DNase I can show some sequence preference. For example, the enzyme is sensitive to the structure of the minor groove, and favours cleavage of purine-pyrimidine sequences (Herrera and Chaires, 1994). Hogan *et al,* (1989) suggests that DNase I cleavage rates can vary as much as 500-fold as a function of sequence. It is thought that this observed site specificity is not a result of the cleavage chemistry *per se* but arises from local variation in the physical properties of a helix at the binding site (Drew, 1984). Kowalski *et al,* (1988) and Kohwi-Shigematsu and Kohwi (1990) have also observed different degrees of stability across the DNA molecule. For example regulatory regions for replication and transcription sometimes have lower stability than gene coding regions. This has been experimentally proven by detecting regions on the DNA strand that were particularly vulnerable to single-strand specific nucleases (Huang and Kowalski,

2003). It may be possible that the CSF region targeted in this experiment had a hypersensitivity to DNase I. Specific A + T rich regions (Kowalski *et al*, 1988) as well as suppressor sites flanking immunoglobulin heavy chain enhancers (Kowhi-Shigematsu and Kohwi, 1990) have also been shown to be regions of lower stability. It is unknown how many of these types of regions exist but it can be assumed if they were to coincide with the target regions chosen for this type of analysis, the rate of degradation in these areas will be affected and may disrupt the correlation with amplicon size. Certain nucleotide positions within the human genome have been reported to mutate at rates higher than average (Aris-Brisou and Excoffier, 1996). It has been suggested there are over 25,000 of these hotspots in the human genome (Myers *et al*, 2005). It has been suggested that these sites are vulnerable to naturally occurring (un-programmed) DNA damages (Bernstein *et al*, 2011) as well as post-mortem damage and degradation (Gilbert *et al*, 2005). Although the presence of hotspots could account for the high rate of degradation observed with the CSF target, it has been suggested that mutational hotspots in the CSF gene are rare (Kosmider *et al*, 2013). Any of these factors described could have resulted in CSF's apparent increased susceptibility to DNase I degradation.

Based on the Ct results, the optimal sized amplicon for distinguishing the degradation levels of these samples were TH01 and Set 3 which were 190 and 198 base pairs respectively. Despite both having the general ability to discriminate, they lost their resolving power with samples treated for more than 30 minutes. Interestingly CSF, which was considerably smaller in size (67bp) had a similar resolving power as Set 3 and TH01, which could be explained by its apparent increase in susceptibility to DNase I degradation. The smaller amplicon sizes did not provide an accurate indication of the level of sample degradation. This is not unexpected given many of the degradation processes occur randomly along the DNA strand (Bauer *et al*, 2003). The smaller the amplicon size, the more likely they are to avoid cleavage (Bauer *et al*, 2003). The larger amplicons which had amplicon sizes of 239 bp and 308 bp (Set 4 and 5 respectively) showed signs of degradation after a minute of treatment. Set 4 could not be detected after 30 minutes whilst the larger set 5 failed to produce results following 20 minutes of treatment. Set 5 also failed to produce results after ten minutes of DNase I exposure under this particular run protocol. These results were largely expected given their size increases their vulnerability to random degradation events (Bauer *et al*, 2003).

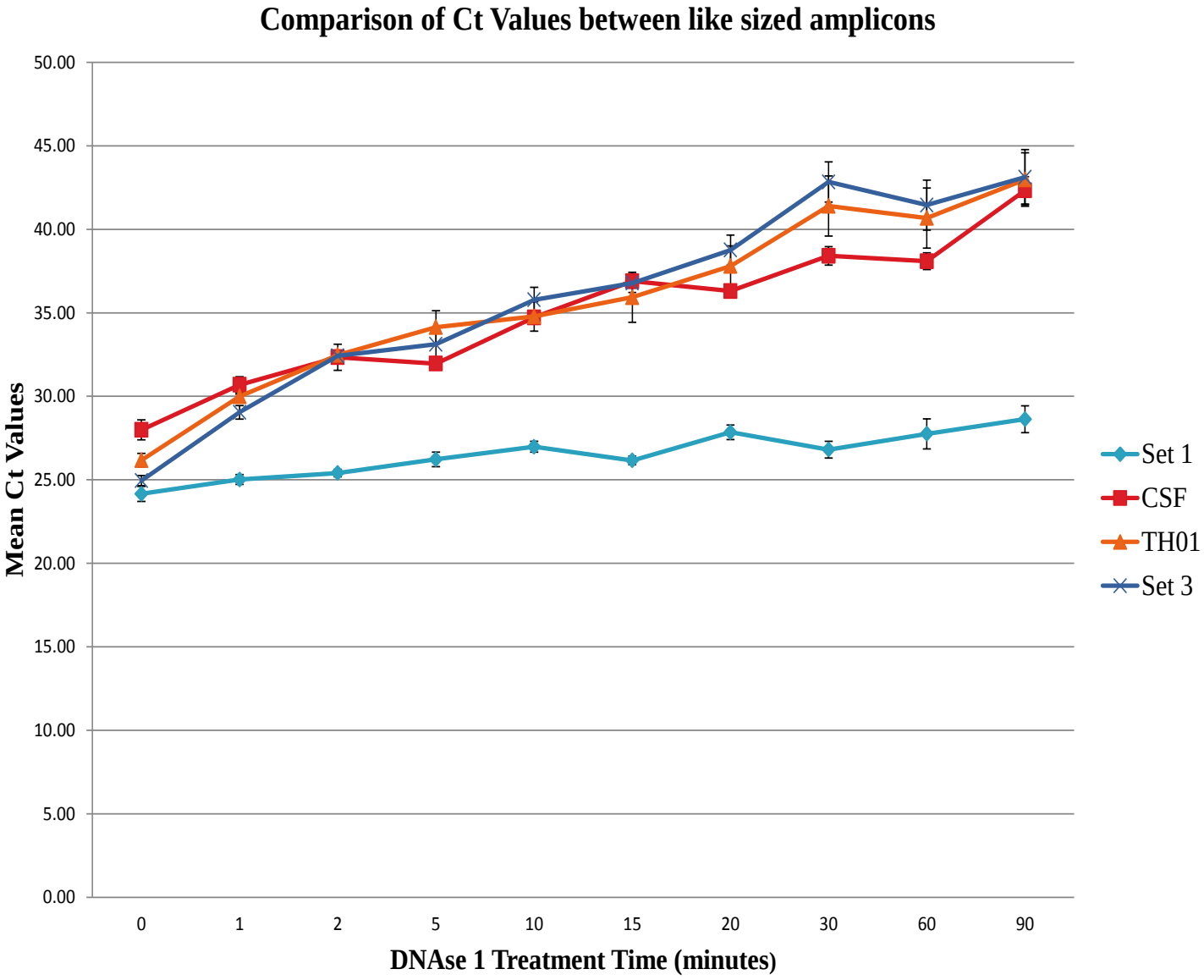


Figure 19: Comparison of Ct values between like sized targets: There is a large difference between Set 1 and CSF despite almost being identical in size. The differences observed between TH01 and Set 3 are significantly smaller. CSF degraded at similar rate to the larger targets (TH01 and Set 3) than Set 1, which was of a similar size. The error bars represent 1 standard deviation.

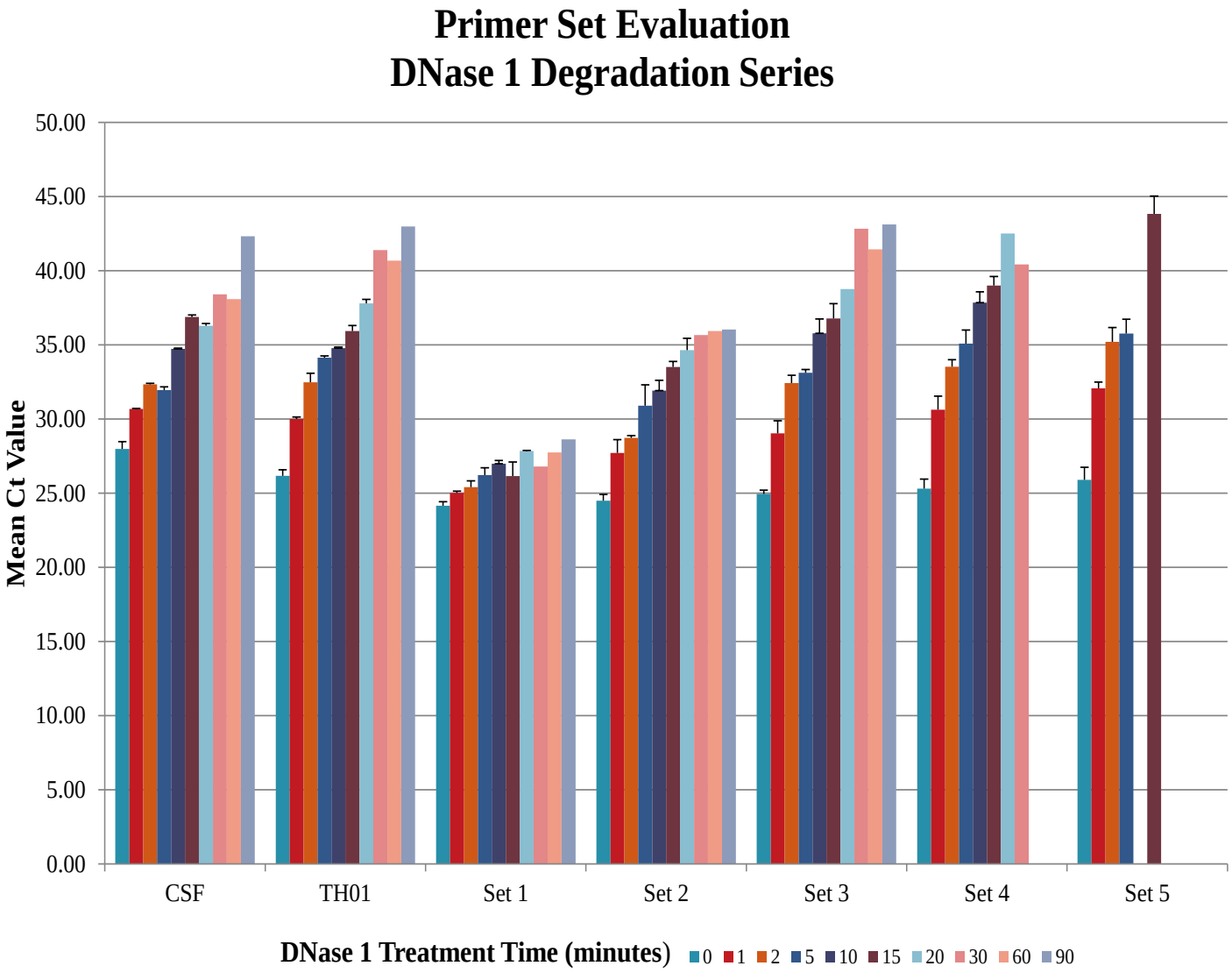


Figure 20: Mean Ct values for all Primers tested on the DNA degradation series. The above bar graph illustrates the mean Ct values of each primer set for each period of DNase I treatment. The smaller target amplicon (set 1) does not significantly change over the 90 minute treatment suggesting the digestion process did not produce fragment sizes smaller than 64 base pairs. The larger targets (239 and 308 for sets 4 and 5 respectively) show early signs of degradation early and were of little value in estimating degradation levels. The error bars represent 1 Standard deviation. Conditions that presented with only a single value (for triplicate samples) do not contain an error bar. Not all values were obtained for all treatment times with the larger amplicons (set 4 and 5), due to excessive degradation of the sample.

The DNase I degradation study confirmed the findings observed in the UV study. Different combinations experienced different sensitivities across the time dependant analysis. Certain combinations clearly outperformed others, therefore highlighting the need to address this issue (target size) during the optimisation procedure. Under these specified conditions, the 64 bp (set 1) and 239 bp (set 4) combination had the best discriminating power (although could not be applied to the longest two treatment times where dropout was observed). The difference between the amplicon sizes was 175 base pairs. A comparison of these sizes with past research is discussed on page 141. It was also observed that the five amplicons designed across the same target area behaved more consistently than the comparative amplicons, which were designed from different loci (see Figures 18). This was particularly evident in samples treated for less than ten minutes. Samples treated for longer than ten minutes produced inconsistent and unreliable results but this was largely expected because of the level of degradation from extended treatment times. This had a direct effect on the subsequent relative expression ratios which were ultimately used to assess the level of degradation.

3.4.2 Variation

The variations observed in the Ct values from the UV degraded samples were relatively small. The mean percent coefficient covariance (%CV) for the duplicate samples (across all treatment times) were 15.6, 16.6, 28.1, 22.7 and 16.7% for Sets 1 through 5 respectively. Values for CSF and TH01 were comparable at 9.5 and 14.7%. There was no correlation between variation and treatment time. Significantly smaller variations were observed from the DNase I digested samples. Mean values for CSF and TH01 were 0.8 and 1.0% respectively whilst the mean %CV values for the five primer sets were 0.5, 0.7, 0.67, 0.5 and 0.1% (sets 1 through to 5 respectively). There was no observed correlation with target size but there was a subtle trend between variation and the length of DNase I treatment. As the treatment time increased so did the variation however the correlation was not particularly strong (R^2 = 0.665). These results indicate that the DNase I degradation process was more consistent and uniform than the UV method.

More importantly the overall stability and functionality of the five primer sets and the dynamics of the assay were considered robust and suitable for subsequent use on the naturally aged samples.

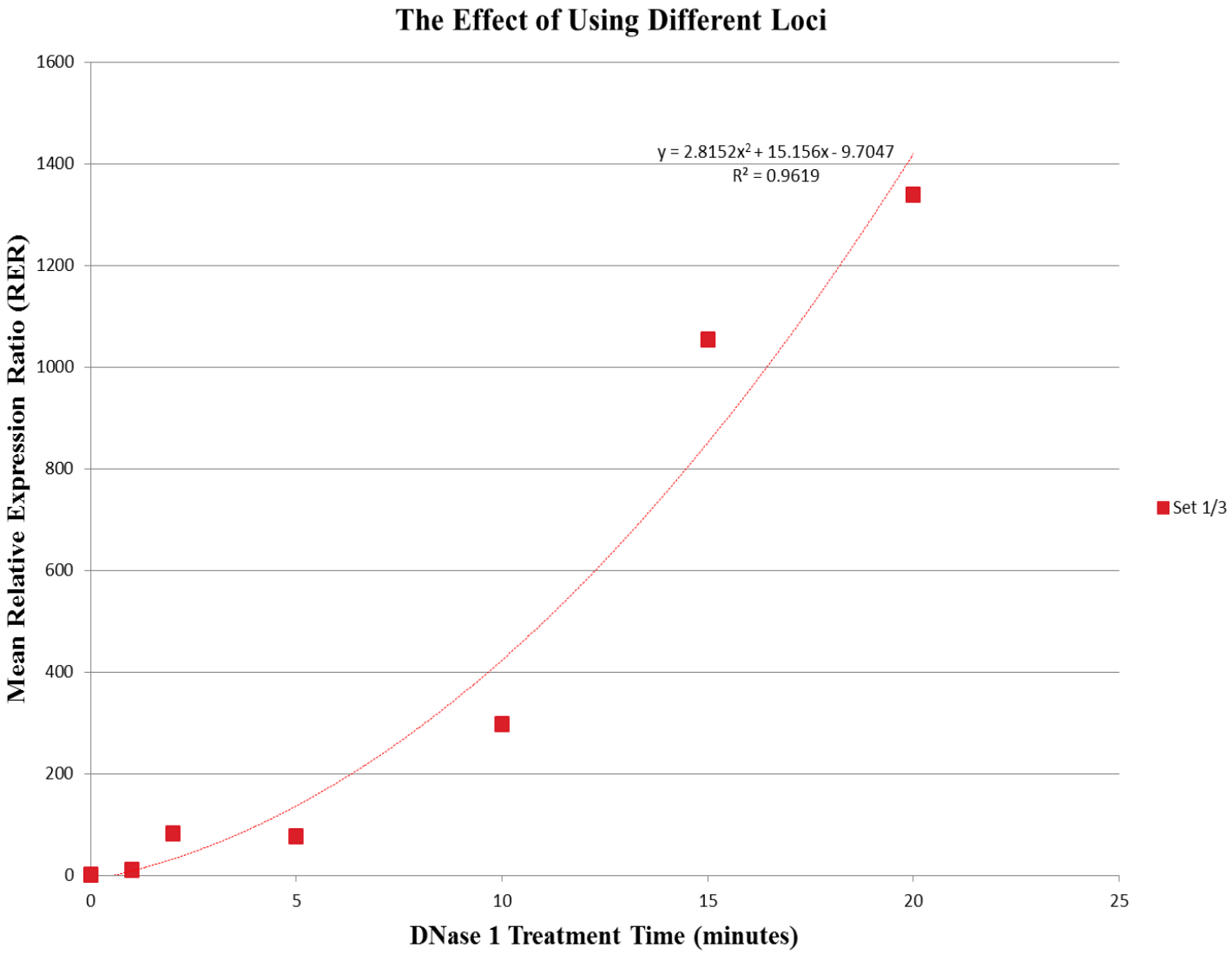


Figure 21: The effect of using same target area – Set1/3: There is a large difference in the discriminating ability between Set1/3 and CSF/TH01 shown in Figure 22. Despite both combinations of amplicons consisting of approximately the same sized targets they behaved very differently under these conditions. Though far from ideal, the targets amplified from the same region (Set1/3) had a better discriminating ability than those targets amplified from different regions (loci) (CSF/TH01).

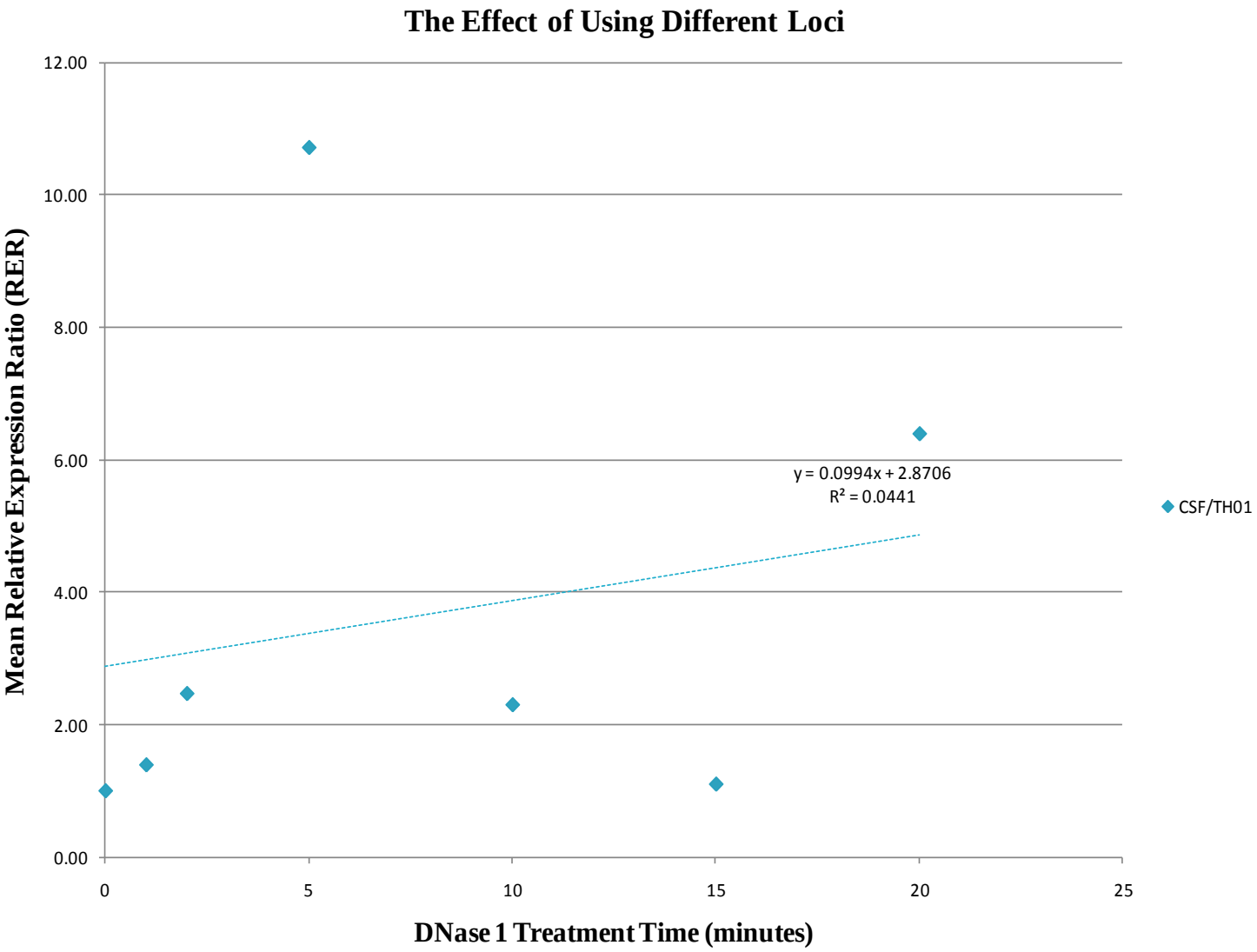


Figure 22: The effect of using different target area – CSF/TH01: The correlation observed above should be compared to that observed in from the Set1/3 data in Figure 21. The correlation for the CSF/TH01 combination was very poor (R^2 = 0.044). This is compared to 0.9215 for Set1/3 which although is not ideal is more acceptable. This demonstrates that size alone cannot be relied on for accurate degradation studies.

3.4.3 Naturally Aged Blood Samples

Using artificially degraded DNA is useful for assay optimisation and stability studies however this type of analysis bears little relevance to forensic samples. This is primarily because the degradation level (and hence range of fragment sizes) of bloodstains aged in both internal and external environments will be different to those produced from artificial means. To obtain any relevant practical information, forensic type samples were required for analysis. To achieve this blood samples were aged naturally in conditions that were designed to mimic a typical urban dwelling. It was hypothesised that an internal urban dwelling is a relatively stable, controlled environment as temperature fluctuations, humidity and microbial sources will not be as diverse as those found in external settings. The work conducted by Setzer *et al*, (2008) provided some support for this hypothesis. They concluded the recovery of RNA from samples aged in internal environments was more successful than those aged outdoors. By obtaining a better understanding of the fragment sizes likely to be produced under these conditions, it was hoped that current degradation methods could be improved by identifying and using optimal sized targets for discrimination purposes.

Samples were left to age on the floor of an urban building at room temperature, humidity and exposed to general contaminants such as dust and light. Toothman *et al*, (2008) noted that environmental samples from indoor surfaces can be contaminated by dust, which is composed largely of human skin cells and has been documented to contain tens of micrograms of total DNA per gram of dust. This was consistent with Edmond Locard's findings in 1930 where he observed that dust was composed of numerous constituents including human and animal "skin peelings" (Locard, 1930). Whilst Toothman *et al* (2008) demonstrated there were measureable amounts of human DNA in indoor dust Roy *et al* (2003) quantified this finding by stating that on average there was 18.2ug of total DNA per gram of dust in urban homes. This amount is significant given the sensitivity of current DNA techniques. Although the quantity of dust that gathered during the course of these experiments was not measured, small amounts were observed on samples that had aged for longer than 20 days. The samples were processed as described in 2.4.2 on page 89, minimising the risk of any contamination.

3.4.3.1 Reproducibility of SYBR green RT-PCR assay

During the stability assays (UV and DNase I) there was evidence to suggest that the use of two different loci for this type of degradation analysis may introduce an integrity bias. As such only the five primer sets (TH01) were tested on the naturally aged blood samples. The reproducibility of the SYBR green qPCR method was good. The variability of the Ct values was low ranging from 0.05% (Donor 4, Set 4, 90 days) to 4.5% (Donor 3, Set 3, 60 days) with a mean of 1.6% across all donors, all primer sets and all time periods. Whilst these values were slightly greater than the variation observed with the DNase I degraded samples they were up to 50% lower than most reported values involving the same sample type. Swango *et al,* (2007) reported a mean variation of 7.5% for their TH01 assays whilst the CSF variability was slightly higher at 11%. Anderson *et al,* (2005) reported similar variability. The low variability is this study may be a direct result of using the same robust target region.

The variation between each individual primer set was examined. The variation observed was not significantly greater than expected by chance (ANOVA; $p = > 0.1$) suggesting the TH01 locus is suitable for this type of degradation analysis.

3.4.3.2 Optimal Sized Targets for Determining the Degradation levels of naturally aged blood samples in an Internal Urban Environment

In order to assess the level of DNA degradation that occurred from when the sample was removed from the body (which in forensic samples would be the time it was deposited at a crime scene) to the time the blood samples were processed, the change in the relative expression was examined. Relative expression values (RE) are normalised against reference samples (Livak, 1999), which in this case, were those samples measured at time 0. From a research perspective it was important to establish whether the degradation that occurs *ex vivo* was consistent and time dependant because only then can this method be applied to a practical environment.

The RE values were calculated from the Ct values as described in section 2.8 on page 94 with consideration to all normalisation factors (replicate samples, inter-run variation, efficiency rates and against a calibrator sample). A correlation between the mean relative expression and sample age was examined for each primer set, as shown in Figure 23. The mean relative expression of all target amplicons decreased over time as expected. The smaller the amplicon

the more predictable and consistent was its expression. The correlation observed with the largest amplicon (Set 5 – 308 bp) was weak ($R^2 = 0.77$) when compared to the smallest (Set 1 – 64 bp) amplicon ($R^2 = 0.98$). These values were less than those obtained by Swango *et al* (2007) who obtained correlation coefficient values of 0.99 for both the TH01 and CSF assays. This could be explained by the degradation method. Swango *et al*, (2007) used DNase I, which according to earlier stability studies conducted as part of this experiment, appears to be a more consistent degradation method. The naturally aged samples were exposed to natural conditions and therefore a greater degree of variation and subsequently weaker correlations were expected. Although the correlation for the small amplicon was strong, this assessment was based on the mean values. The variation between the five donors (expressed as %CV) ranged from 48% (set 1) to 56% (Set 4). This was significantly higher than the variation observed by Swango *et al* (2007) who observed inter-donor variations of up to 23%. Variations of the size seen in this study reduce the potential of this technique as a forensic tool. At best this level of precision and accuracy may provide police with an investigative tool but would fall well short of providing conclusive evidence.

In this experiment expression values were obtained from a single target as the initial template volume (or amount) was the same for each sample. This approach would not be possible for crime scene samples therefore it is necessary to use a comparative method adopted by Swango *et al*, (2006) that examines the ratio between two targets. This relative approach effectively addresses the problems associated with unknown and variable starting volumes. By examining the RERs the optimal size combination for discriminating between the naturally aged blood samples degraded under the conditions stipulated by this experiment were determined. The complete set of RERs can be found in Table 12 on page 113. The mean values are illustrated in Figure 24 on page 134. The results indicate a clear distinction between those combinations that had the ability to discriminate between samples aged over the 90 day period and those that could not or at best, performed poorly. The two combinations with the greatest discriminating power were set1/set4 (64/239 bp) and set1/set5 (64/308 bp). The size difference between each of the pairs was 175 and 244 bp respectively. Combinations where this difference was considerably smaller did not have the same discriminating capabilities. Set4/Set5, where the difference was 69 bp, performed poorly as did Set2/Set3 (difference of 62 bp) and Set1/Set2 (72bp difference). Set 1/3 and Set 2/5 which had differences of 134 and 172 base pairs displayed a moderate ability to distinguish between these samples.

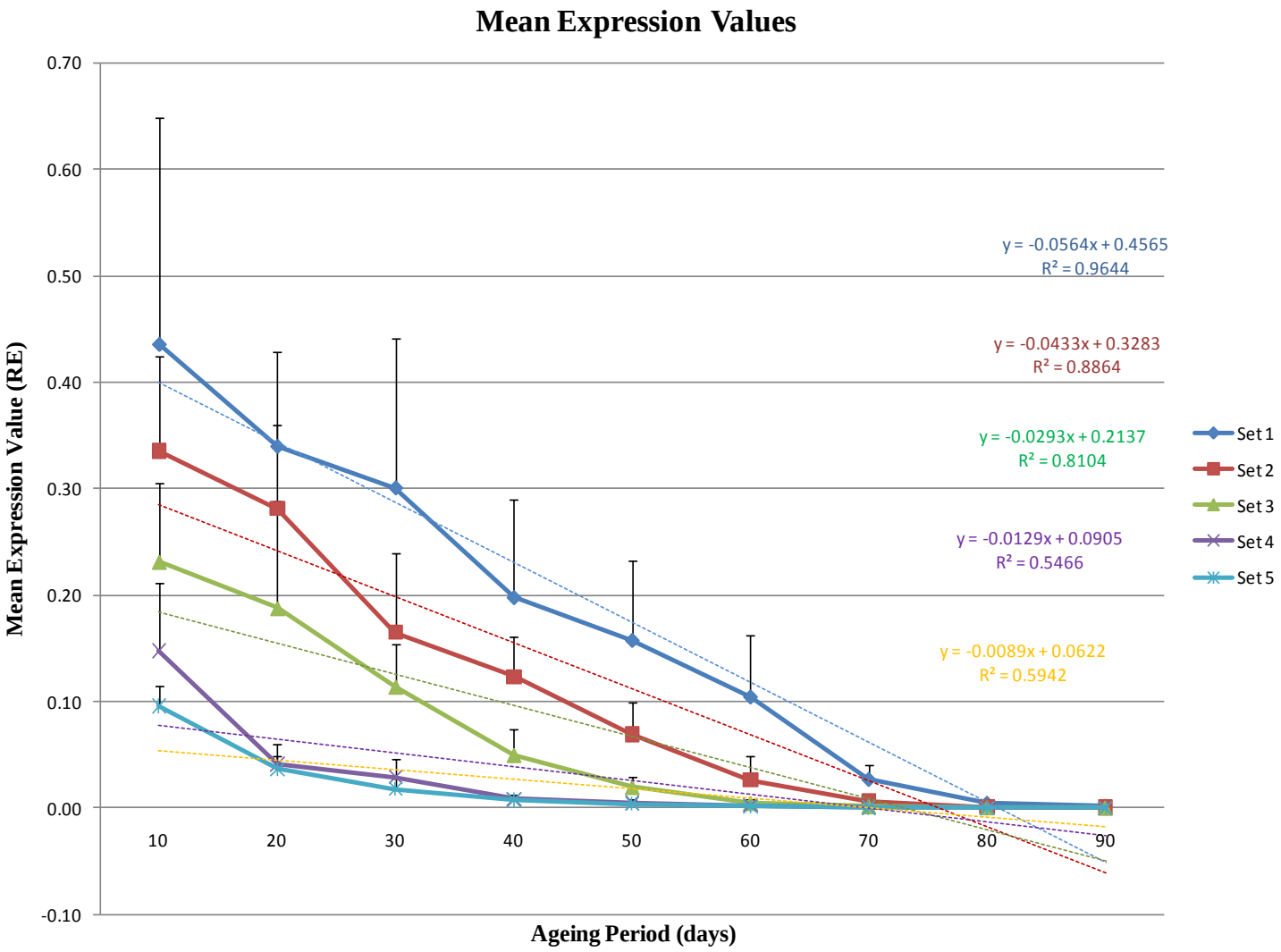


Figure 23: The mean Relative Expression values (RE) of each sized amplicon decreases with ageing time. Each sized amplicon behaves differently and it was noted that for the samples exposed to these experimental conditions, the smaller the amplicon, the more consistent and predictable was its expression. The error bars represent 1 standard deviation and the size is indicative of the large inter-donor variation that was observed.

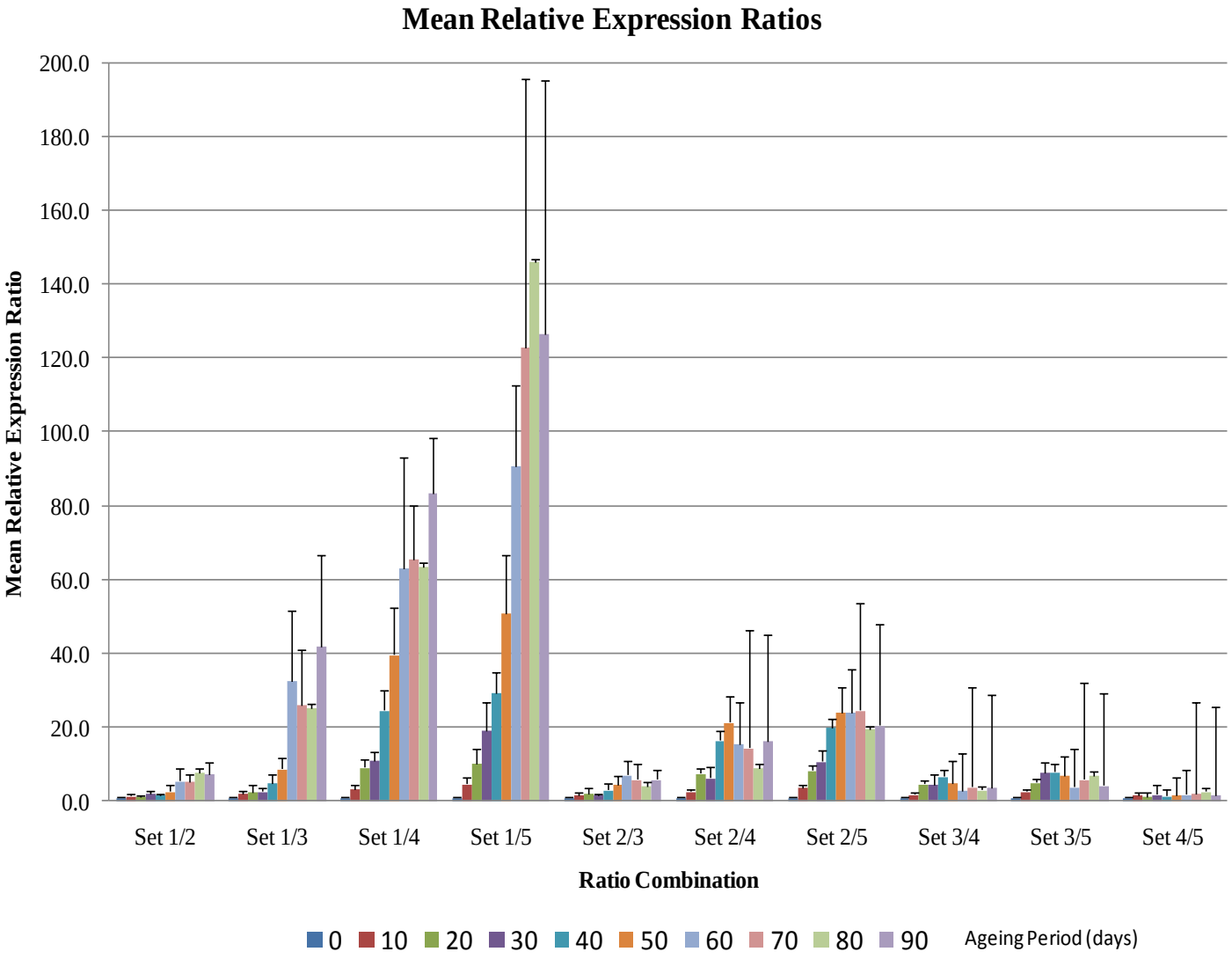


Figure 24: The mean relative expression ratios (RER) were determined for all size combinations. There is a clear distinction between those combinations that have the ability to discriminate between the aged samples and those that could not or performed poorly. For these samples aged under the given conditions of this study, the RERs from combinations 64/238 (set1/set4) and 64/308 (set 1/set5) clearly outperformed all other possible combinations. The error bars represent 1 standard deviation.

In terms of overall performance, the size difference appears to be a critical factor. Each primer set showed different degrees of sensitivity across the entirety of the study and this appears to be largely influenced by not only the actual amplicon size but also the size difference between the two targets. For samples aged for less than 30 days it appears as though the sensitivity depends on having an amplicon that is at least 200 base pairs long. The ability to discriminate was quickly diminished with combinations containing smaller amplicons even if the size difference between the two targets was approaching the perceived optimal range (175-245 bp). Even the best combinations were only consistent with samples aged for less than 60 days.

Samples older than 70 days presented with their own set of limitations. There was no particular combination that effectively distinguished between these samples and the variation observed was high and the consistency in the RERs, low. It was expected that combinations containing smaller amplicons (i.e. Set1/2) would perform better on older samples because older samples were expected to be highly degraded. Whilst some results support this proposition, the large variation associated with the older samples made analysis difficult and as such, overall results were poor. The poor results could also be explained if the smallest amplicon was not small enough. A target size in the region of 50 base pairs or less may be more appropriate however designing an assay to amplify such a small target presents its own set of difficulties (Dieffenbach and Dveksler, 2003).

As with the RE data, mean ratio (RER) values offered some promising results but again the large inter-donor variation meant that the usefulness of this technique was somewhat limited. Table 16 illustrates the levels of variation observed in the two combinations that displayed the best discriminating ability. Set1/Set4 has a %CV range of 18.2% to 47.9% with a mean variation of 30.4%, whereas Set1/Set5 ranged from 19.3% to 59.1% with a mean of 39.4%. These values are relatively high as most expression studies using biological samples observe inter-donor variation in the order of 5 to 15% [Anderson *et al* (2005); Bauer *et al,* (2003)]. The large values are likely to be attributed to the nature of the sample in that most expression research involves the analysis of fresh tissue samples, whereas the samples used here were aged, clotted and therefore problematic.

Table 16: Relative Expression Ratios and Inter-donor variation: The best correlation between the RER and age was obtained using the ratios Set1/4 and Set 1/5.The RER for each donor at each age point is shown the table below. The variation between donors was examined and is presented in this table. The results indicate that the mean RER varied significantly between donors.

Ageing Period (days)	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Mean	Std Dev	%CV
				Relative Expression Ratios				
Set ¼								
0	1.00	1.00	1.00	1.00	1.00	**1.00**	**0.00**	**0.00**
10	1.97	3.07	2.08	3.27	5.10	**3.10**	**1.26**	**40.68**
20	7.27	6.79	11.84	9.96	9.24	**9.02**	**2.06**	**22.79**
30	12.68	8.39	10.69	8.67	13.80	**10.84**	**2.39**	**22.07**
40	24.22	20.65	20.18	33.67	23.51	**24.45**	**5.44**	**22.27**
50	34.44	25.51	51.81	54.15	32.00	**39.58**	**12.69**	**32.06**
60	30.84	52.42	41.47	92.47	96.97	**62.83**	**30.13**	**47.96**
70	52.07	55.95	88.86	67.57	63.37	**65.56**	**14.37**	**21.92**
80	63.62	58.62	86.55	18.40	89.81	**63.40**	**28.64**	**45.18**
90	58.26	79.28	94.29	90.48	93.88	**83.24**	**15.23**	**18.29**
Set 1/5								
0	1.00	1.00	1.00	1.00	1.00	**1.00**	**0.00**	**0.00**
10	5.36	6.08	3.38	1.98	5.85	**4.53**	**1.78**	**39.31**
20	8.33	10.50	15.98	5.84	10.28	**10.19**	**3.74**	**36.73**
30	17.93	14.55	22.27	10.25	29.92	**18.99**	**7.54**	**39.71**
40	24.43	26.87	36.91	24.23	32.89	**29.07**	**5.61**	**19.30**
50	32.21	71.15	58.28	37.70	54.06	**50.68**	**15.80**	**31.17**
60	119.20	94.27	65.96	101.90	71.45	**90.55**	**21.98**	**24.27**
70	179.89	89.49	216.79	41.08	87.58	**122.97**	**72.67**	**59.10**
80	232.73	57.03	185.38	82.45	172.07	**145.93**	**73.66**	**50.48**
90	102.56	70.86	118.11	94.64	245.98	**126.43**	**68.97**	**54.55**

Without being able to identify the definitive fragment sizes in each sample it is difficult to accurately assess this methodology, which relies on examining the ratio of two different sized amplicon targets. Though attempts were made to prepare uniform degradation series, there has been no definitive way of checking the uniformity therefore the problem may exist with the samples and not the technique itself. Although measuring DNA fragment sizes is suitable for measuring the degradation level of a sample (Vass *et al*, 2013), these results suggest it is not suitable for predicting the age of blood samples aged in an internal environment. The size of

the variation in the naturally aged blood samples suggests the effects from degradation processes are not uniform across all samples. Environmental causes in general, such as UV light and heat, cleave DNA randomly along the entirety of the DNA molecule (Kuwahara *et al,* 1986) as does DNase I (Herrera and Chaires, 1994). Any act of randomness will limit the precision and accuracy of this technique as a particular target area may be better preserved in some samples, than others. The presence of hotspots, as previously explained, could contribute to the inter-donor variation. Age determination will be difficult if not impossible where degradation rates are not consistent, the method of analysis is based on random acts of cleavage and there is a real potential of degradation bias between loci.

The most successful studies to date have involved the analysis of different species of RNA (Anderson *et al*, 2005), which may be a more suitable macromolecule for this type of analysis as its degradation patterns may be more consistent. Bauer *et al,* (2003) produced encouraging results compared two different sized amplicons but from an RNA transcript rather than DNA. They also used the same target area of B-actin rather than the more common approach of using different loci, which may have contributed to their results.

The unfavourable and unexpectedly high variation observed effectively ruled out this approach as a potential method for accurately determining the age of a blood stain. However, this study has identified a number of important characteristics regarding the levels of degradation that are likely to be found in blood samples aged in a typical indoor urban environment. According to these results the optimal size difference between the two targets was between 170 and 240 base pairs with one of these targets being at least 200 base pairs in length. These findings are limited in that they only relate to samples aged under these experimental conditions however past experience tells us that an internal urban dwelling is a relatively stable environment (Setzer *et al,* 2008) and therefore it's possible these findings may be applied to other samples aged under similar conditions. Setzer *et al,* (2008) tested the recovery and stability of RNA from a variety of different environments. They consistently recovered RNA from blood, semen and saliva stains aged in inside environments. The recovery rate was poor in samples that were aged in outside conditions, whether directly protected from the rain or not. Luminescent light and even ultra violet light did not have a significant effect on the stability or recovery rate of the target amplicons, B-actin and GAPDH (Setzer *et al,* 2008). It was also noted that the main degradation factor appeared to relate to moisture levels. This has been theorised for some years

though it has been rarely documented or the effects quantified. These findings inspired the work in chapter five.

3.5 Limitations

One of the most difficult aspects of this study was dealing with the problematic nature of the aged and clotted blood samples. All commercial DNA extraction kits and protocols, such as those supplied by Sigma Aldrich, Applied Biosystems and Invitrogen are designed for fresh or anticoagulated samples but clotted and aged blood samples are structurally very different (Antovic and Blomback, 2013) and therefore modified steps were required. These steps involved longer incubation times, increased incubation temperatures as well as manual manipulation to dislodge and break up the clot but in doing so small amounts of sample were lost through transfer processes and this could explain the lower than expected recovery rates.

A singleplex PCR method was used for analysis in this study because the different targets overlapped the same region of DNA therefore a multiplex assay could not be used (Vanguilder *et al*, 2008). Singleplex techniques have their limitations. Any variation in the amount of starting template will affect final data interpretation and there is a greater chance of introducing experimental variation as minor differences in pipetting volumes can lead to imprecision and a lack of reproducibility (Nolan *et al*, 2006). Although this was a potential problem, the intra-donor variation observed was minor with maximum values below 5%. A multiplex assay is likely to reduce this variation further. Other benefits of multiplex reactions include the saving of time and materials once the assay is optimised. This is an important consideration for high throughput techniques. More importantly multiplex assays require less sample for analysis and this may be vital if trace amounts are recovered from the crime scene.

The nature of trace samples also presents the scientist with another problem. When extracting small quantities of DNA for qPCR (eg 5 µl) there is a better chance of obtaining a representative sample from a solution that has a higher concentration of template DNA than from one that has a low concentration of analytes. This limitation is inherent to all experiments that use minute quantities of template material. If the qPCR sample is not representative and species concentrations are likely to vary between aliquots, reproducibility will be difficult to obtain. This in turn will affect the reliability and confidence in the interpretation of the data.

This entire methodology has been based on the examination of two different sized amplicons and comparing their expression rates. One consideration that needs to be addressed is how size affects the behaviour of the PCR reaction. A decrease in PCR efficiency is commonly observed with larger amplicon sizes (Chung *et al*, 2004) and this can cause inter and intra-run variability. For this reason most qPCR designs incorporate short fragments of less than 100 base pairs (Niederstatter *et al*, 2007). This issue was considered during this study but there was no correlation between efficiency rates and amplicon size. The least efficient according to our data was Set 1 (64bp) which was the smallest of the 5 targets. The second lowest efficiency rate was observed in the largest amplicon (308bp) whilst Set 2 (136bp) and Set 4 (239bp) had the highest rates (approximately 106%). There are ways to combat any reduction seen with the larger targets. Reducing the ramp speed can result in an increase in the amplification efficiency of larger fragments, with no observed adverse effects on the shorter targets (Niederstatter *et al*, 2007) but because results did not seem to support any rate bias, these steps were not introduced into the protocol. The use of the SYBR green intercalating dye can introduce inaccurate measurements when dealing with different sized targets (Nolan, 2008). It has been shown that larger targets will take up more of the dye introducing a level of bias in the results. This consideration is more significant when absolute quantification methods are applied as opposed to the relative quantification applied in this experiment.

3.6 Conclusion

Forensic DNA samples are degraded as a result of a natural process resulting from the exposure to environmental elements such as light, humidity, temperature as well as bacterial and fungal entities, which all lead to physical, chemical and biochemical degradation (Schneider *et al*, 2004). Given sufficient time, these factors will produce DNA fragments smaller than 300 base pairs in length. Examination of degraded DNA from casework samples has shown that every sample may exhibit a different rate of degradation and therefore it is difficult to apply a systemic method to all forensic samples especially when the type and accuracy of the method is dependent on the size of the targets chosen. The aim of this particular study was to use “reference DNA” from naturally aged blood samples to determine the optimal sized targets for evaluating degradation levels and to determine the chronological age of samples aged under these conditions. Although ageing conditions play a major role in the degradation rate of a sample and these conditions can be highly diverse in a forensic

environment, the samples in this study where aged in a relatively stable and yet very common forensic environment, and that being inside a normal urban dwelling. Our findings support the notion that every experimental condition should be optimised to determine the rate of degradation and therefore the most suitable sized target amplicons. For samples aged up to 60 days (correlation becomes weaker for older samples) the optimal size difference for comparative purposes was between 170 and 240 base pairs with one of the amplicons being between 200 and 300 base pairs in length. These findings suggest that size alone cannot be used generically for these types of degradation studies and that size optimisation studies should be performed with every study with loci stability in mind. These findings may guide future design parameters when carrying out degradation studies of this nature. Furthermore the results presented here highlight the general stability of the TH01 gene for this type of analysis.

Given the large inter-donor variation that was observed in such a small population size it is unlikely this methodology of targeting two different sized DNA amplicons could have the necessary precision to become a forensic tool. Though DNA analysis of this kind can provide a general indication of the degradation level of a sample, it cannot be reliably used to determine the age of a sample. A comparison of RNA species rather than DNA sizes may be a more predictable and accurate indicator of the age of the sample. This hypothesis is tested in chapter six and seven.

CHAPTER 4: LOCI SUSCEPTIBILITY TO DEGRADATION

4.1 Introduction

In the previous chapter there was evidence to suggest a loci bias in terms of susceptibility to degradation. If true, selecting suitable targets for degradation studies based on size alone may not be a method that could be universally adopted unless the behaviour of the target regions were first established. There are hundreds of different degradation studies published to date [Bauer *et al,* (2003), Anderson *et al,* (2005), Swango *et al,* (2007), Biship (2006)] but because of the lack of optimisation and understanding concerning the target characteristics it is very difficult to compare and correlate data.

DNA is known to exhibit variable rates of degradation along its chain (Berstein *et al,* 2011). Certain sites lend to an increased susceptibility to both chemical and biological degradation factors (Sugiyama *et al,* 1994). Particular sequences act as cleavage sites for endonucleases therefore more prone to degradation as are abasic sites to high temperatures (Sugiyama *et al,* 1994). There is also the existence of chromosomal fragile sites which when exposed to certain replication stress, can form gaps and constrictions in the DNA strand, causing the strand to break (Durkin and Glover, 2007). There are more than 120 known fragile sites in the human genome and they are generally classified as "common", which are considered part of the normal structure and are present in nearly all of the population, and "rare" which are found in less than 5% of the population (Lukusa and Fryns, 2008). The instability of rare fragile sites are created by the CGG and AT-rich areas that can form hairpins (Gacy *et al,* 1995) and other structures that block replication forks which can result in strand breakage (Wells, 1996). The instability of the common sites is thought to arise from the slower replication rate that occurs in such areas. This has the potential to introduce breaks from unreplicated regions (Durkin and Glover, 2007). Whilst most fragile sites are a natural genetic abnormality they can be induced by low doses of aphidicolin (APH), an inhibitor of DNA polymerases α, δ and ε (Glover *et al,* 1984) or treatment with bromodeoxyuridine or 5-azacytidine (Dillon *et al,* 2010). El-Harouny *et al,* (2009) used a basic electrophoresis technique to show that the degradation rate of DNA differed between different tissue types. They observed a sequential and time-dependant correlation between DNA fragmentation and PMI in liver tissue. A slower rate of degradation in the brain was also noted, which was beneficial when dealing with older samples. These observations are largely expected given the different microenvironments that exist in the body

and the different effects each of these have on the degradation rate of DNA. As previously mentioned in section 3.4.1.2 on page 123, DNase I can show sequence preference (Herrera and Chaires, 1994) and rates can vary as much as 500-fold as a function of sequence (Hogan *et al,* (1989). Kowalski *et al,* (1988) and Kohwi-Shigematsu and Kohwi (1990) have also observed different degrees of stability across the DNA molecule, such that regulatory regions for replication and transcription sometimes have lower stability than gene coding regions. Specific A + T rich regions (Kowalski *et al*, 1988) as well as suppressor sites flanking immunoglobulin heavy chain enhancers (Kowhi-Shigematsu and Kohwi, 1990) have also been shown to be regions of lower stability. DNA hotspots, which are vulnerable to naturally occurring (un-programmed) DNA damages (Bernstein *et al,* 2011) as well as post-mortem damage and degradation (Gilbert *et al,* 2005) can also lead to different degrees of vulnerability across the DNA strand.

To determine whether the previous observations in chapter three were due to loci susceptibility or merely by chance four targets were chosen to ensure they were structurally similar in terms of base content but more importantly, size. A literature search was performed to identify any unusually unstable regions which may artificially increase the targets susceptibility to degradation. There was no evidence to suggest the presence of any such regions in the targets chosen. A DNA degradation series was produced using naturally aged samples and each of the targets measured. Given their size similarities it was expected that each of the targets would present with a similar relative expression unless there was a particular loci susceptibility to degradation.

4.2 Methodology

4.2.1 Sample Preparation

Blood samples were obtained from six male Caucasian donors between 20 and 23-years-of-age. Samples were prepared and left to age as described in section 2.1.4 on page 83. All samples tested were aged in uncontrolled conditions. This was done purposely to replicate crime scene-like conditions.

4.2.2 Sample Processing

DNA was extracted from each sample after it was aged for the specified period of time, which ranged from 0 to 50 days, using an organic DNAzol method (Invitrogen, US). Due to the difficult nature of the samples, manufacturer's instructions were altered slightly to increase the DNA recovery rate. A detailed description of the protocol can be found in section 2.4.2 on page 89. The extracted DNA was then measured using Implen's Nanophotometer (Munich, Germany) as concentration was important to gauge the necessary volumes required for subsequent Real Time analysis. A detailed description of the functioning parameters can be found in section 2.5 on page 90. DNA was amplified using Applied Biosystem's 7500 thermocycler. The qPCR process was carried out using a standard SYBR green detection protocol. Detailed protocol can be found in section 2.7.2 on page 93. Primers were added at an optimal concentration of 400nM which was based on the efficiency optimisation studies conducted on the four target amplicons (see page 145.)

4.2.3 Primer Design

All primers were designed to produce amplified products of between 64 and 70 base pairs. The primers were specifically designed using Beacon designer Version 7 (Sigma, UK) based on criteria previously described on page 98. The Fibrinogen alpha chain (FGA) gene and Von Willebrand (VWF) gene are two targets commonly used in DNA profiling platforms such as SGM+ and Profiler Plus (Applied Biosystems). Due to their size, the CSF amplicon (67 bp) and the Set 1 (TH01) amplicon (64 bp) used in the previous experiment were used in this study.

Table 17: Primer Sequences for VWF, FGA, CSF and Set 1

Amplicon (size - bp)	Forward Primer	Reverse Primer
VWF (70)	5' – CACCTGCCTTCCCGACAAG – 3'	5' – AGCCCTCCTCCCAGAACTG – 3'
FGA (68)	5' - GACTGGAGGTGGACATTGATATTAAG-3'	5' – CAGATCTACTTCACGAGCTAAAGC – 3'
CSF (67)	5' – GGGCAGTGTTCCAACCTGAG – 3'	5' – GAAAACTGAGACACAGGGTGGTTA – 3'
Set 1 (64)	5' – GCCTGTTCCTCCCTTATTTCCC – 3'	5'- TGGTGAATGAATGAATGAATGAATGAATG– 3'

4.3 Results

4.3.1 Assay Optimisation

The amplification efficiency of each target was calculated using a 10-fold serial dilution assay details of which can be found in Table 18.

Table 18: Amplification Efficiency rates of VWF, FGA, CSF and Set1: A 10-fold serial dilution assay was used to determine the amplification efficiency rates as described on page 75. Each dilution was measured in triplicate (R1, R2, R3).

VWF (70bp)	R1	R2	R3	Mean	Std Dev	Difference	Slope	Efficiency Rate
Neat	25.34	25.33	25.97	25.55	0.37		3.47	**94.23**
10 fold	29.09	28.94	28.89	28.97	0.10	3.42		
100 fold	32.62	32.39	32.27	32.43	0.18	3.46		
1000 fold	35.95	35.95	35.97	35.96	0.01	3.53		
FGA (68bp)	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	25.21	25.94	25.61	25.59	0.37		3.24	**103.46**
10 fold	28.89	29.21	29.01	29.04	0.16	3.45		
100 fold	31.59	32.00	31.88	31.82	0.21	2.78		
1000 fold	35.29	35.10	36.00	35.46	0.47	3.64		
CSF (67bp)	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	24.25	24.60	24.87	24.57	0.31		3.45	**94.77**
10 fold	28.33	28.04	28.05	28.14	0.16	3.57		
100 fold	31.54	31.37	31.26	31.39	0.14	3.25		
1000 fold	35.09	35.11	34.81	35.00	0.17	3.61		
SET 1 (64bp)	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	25.19	25.48	25.53	25.40	0.18		3.53	**92.03**
10 fold	28.99	28.76	29.11	28.95	0.18	3.55		
100 fold	32.74	32.31	32.10	32.38	0.33	3.43		
1000 fold	35.41	36.01	36.64	36.02	0.62	3.64		

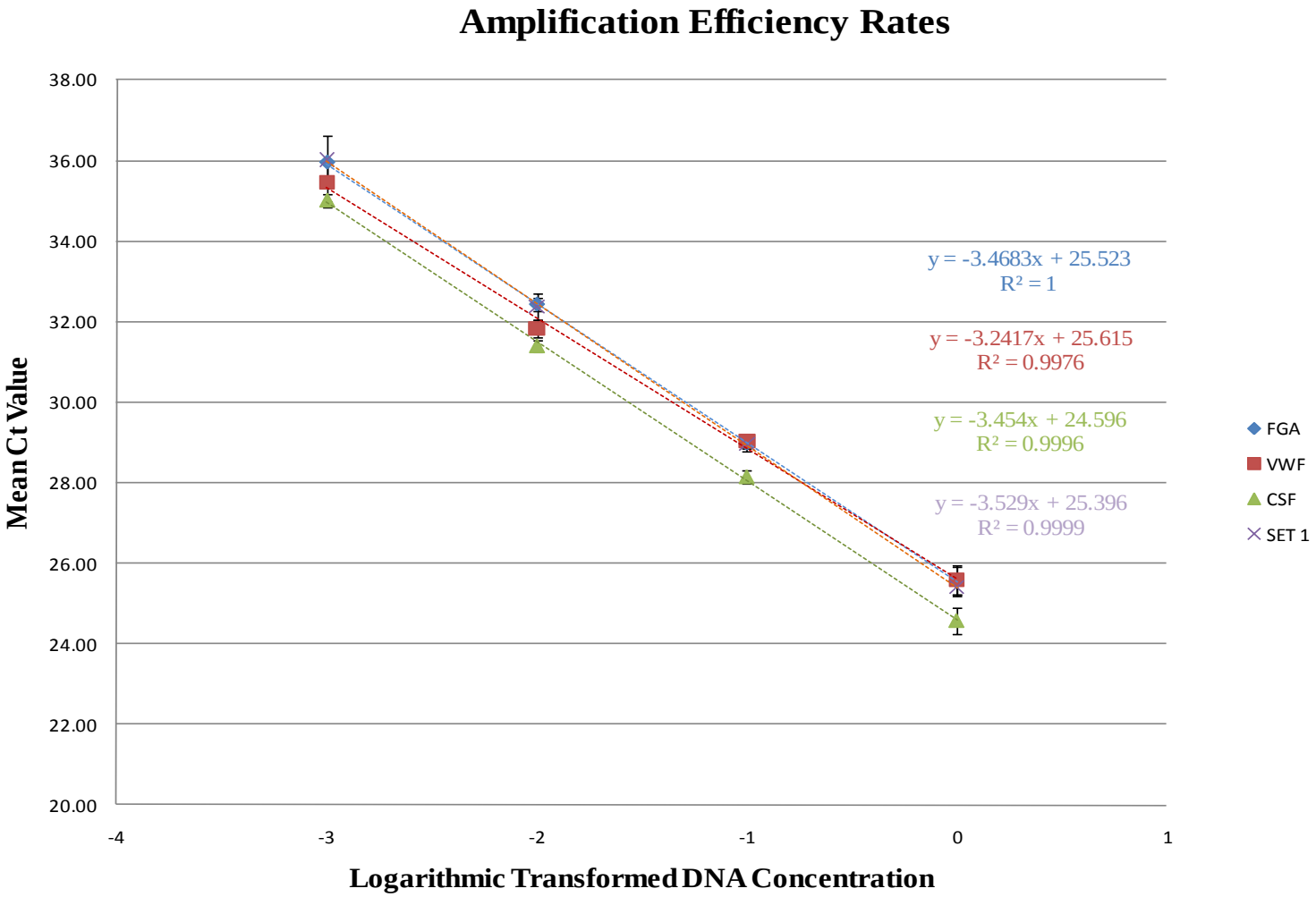


Figure 25: Serial Dilution Assay for VWF, FGA, CSF and Set 1: The slopes of the trend lines from the serial dilution assay were used to calculate the Amplification efficiency rate of each target. The correlations were strong ranging between 0.9976 (VWF) and 1(FGA) and the variation observed in the data was small. The error bars represent ± 1SD. From this data the amplification efficiency rates for each reaction was calculated.

4.3.2 Target Stability

Figures 26 to 29 show the change in the Ct values of each of the four targets, FGA, VWF, CSF and Set 1, over the course of the 50 day ageing period.

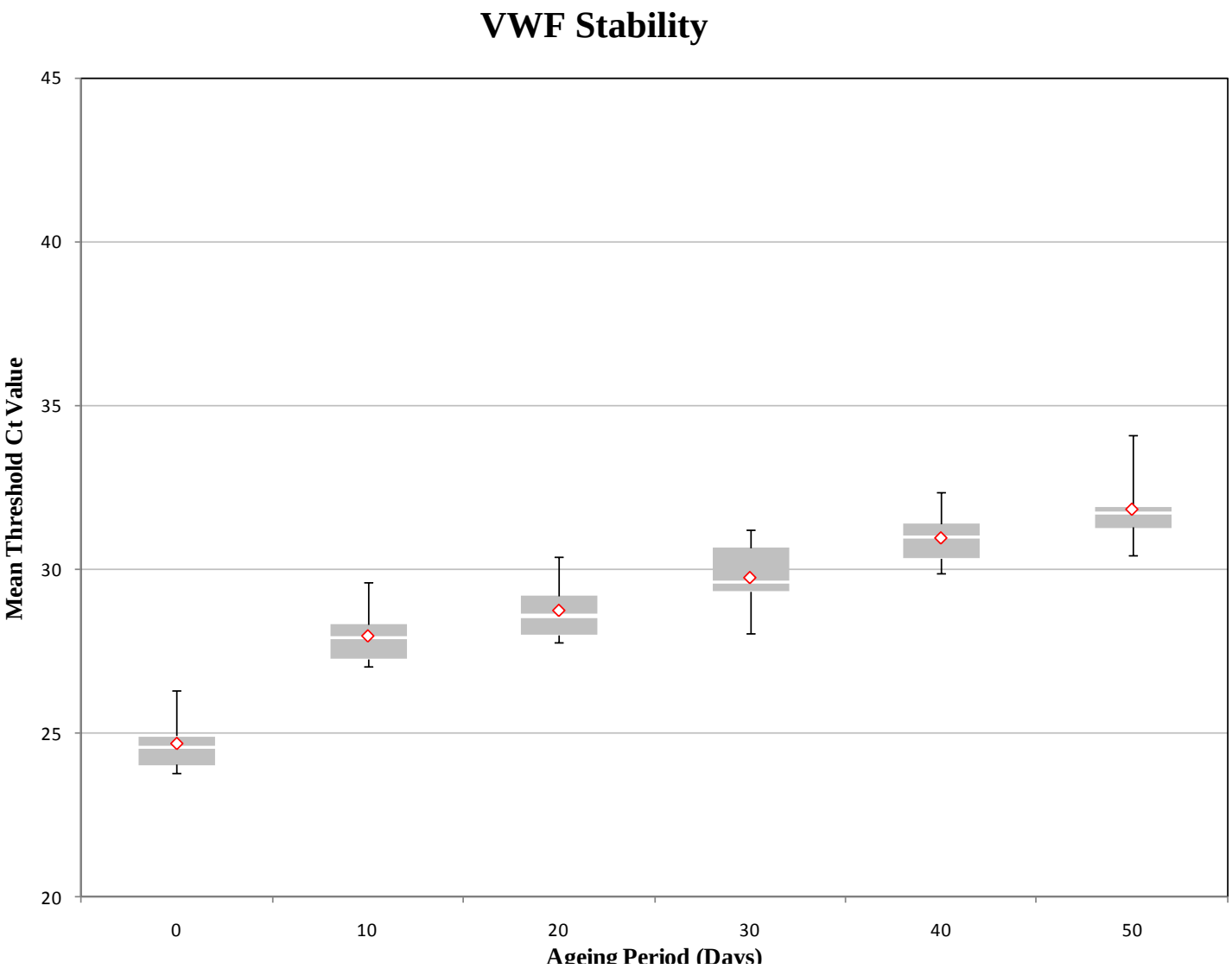


Figure 26: Box and Whisker plot describing the raw Ct values of VWF from all six donors. The mean value increased from 24.70 (day 0) to 31.84 (day 50) over the 50 days, which was expected. These results are indicative of the expression level of VWF and the degradation rate of the sample.

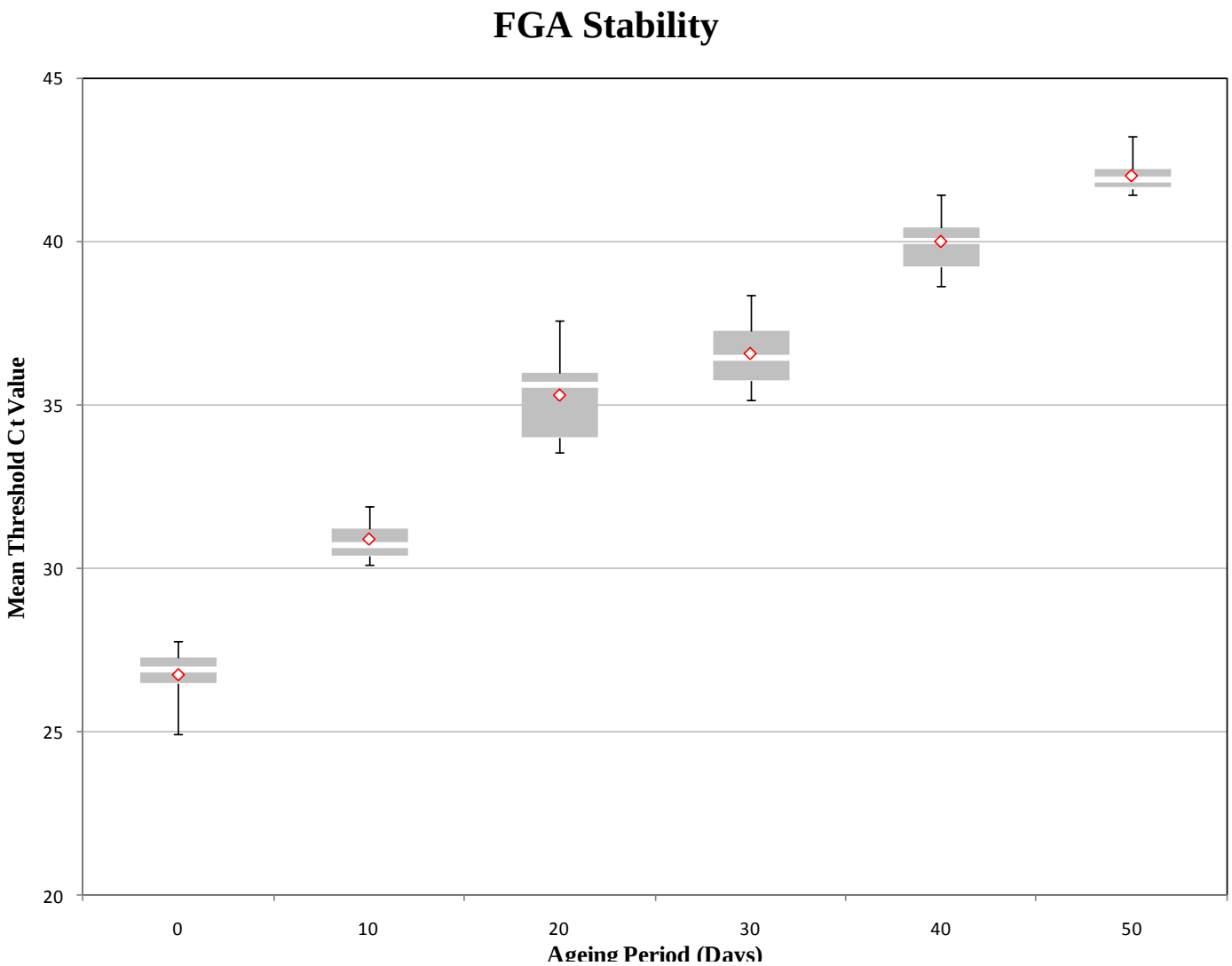


Figure 27: Box and Whisker plot of the raw Ct values for FGA. The change in the mean Ct value is greater than that observed with FGA. The mean value at time 0 is 26.72, which increases to 41.98 at day 50. The level of variation observed was moderate and did not increase with time.

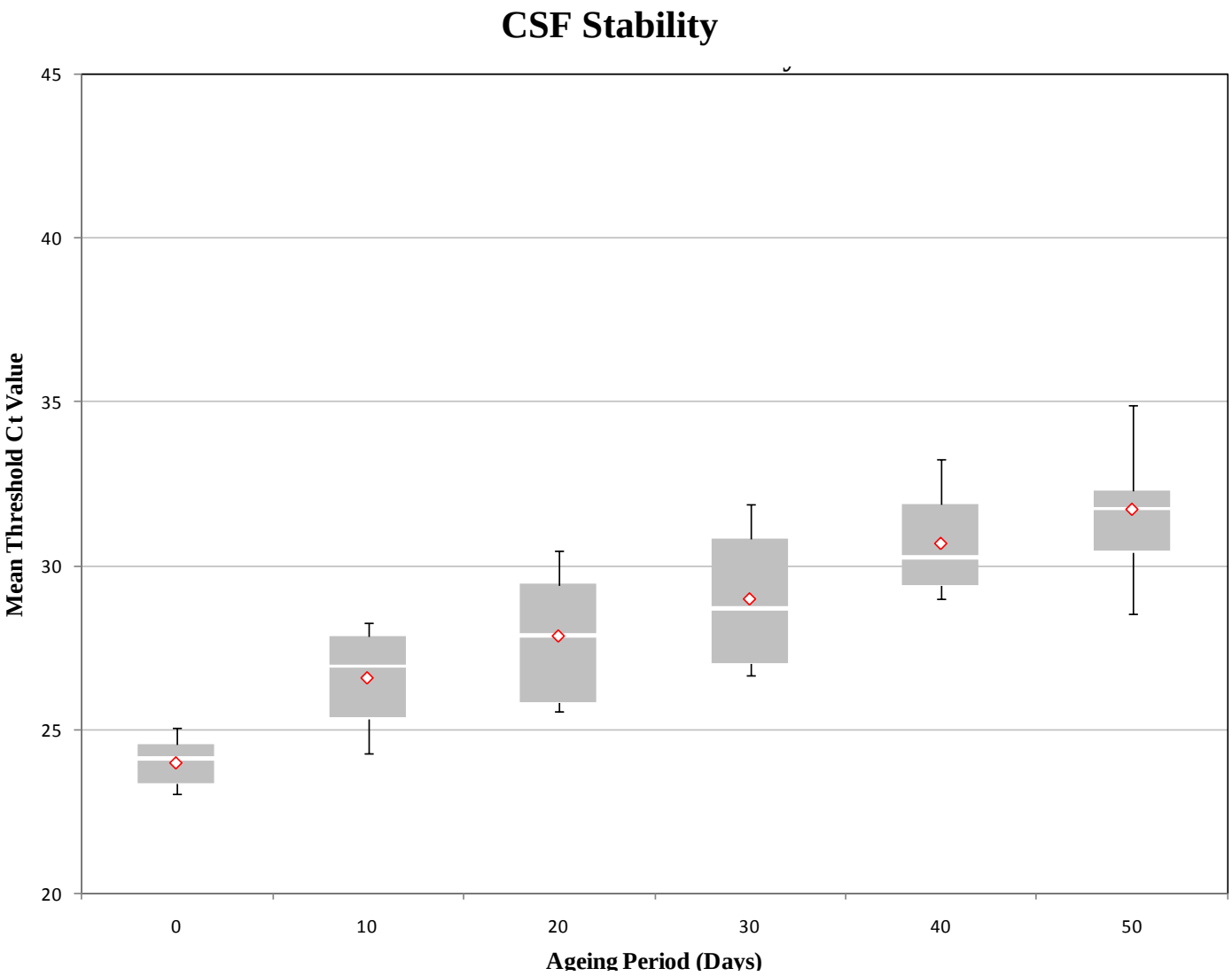


Figure 28: Box and Whisker plot of the raw Ct values for CSF. The change in the mean Ct value was comparable to VWF and Set 1. The mean value at day 0 was 24.12, which increases to 31.72 at day 50. The level of variation observed increased slightly with ageing time with the standard variation ranging from 0.7 (day 0) to 2.05 (day 30)

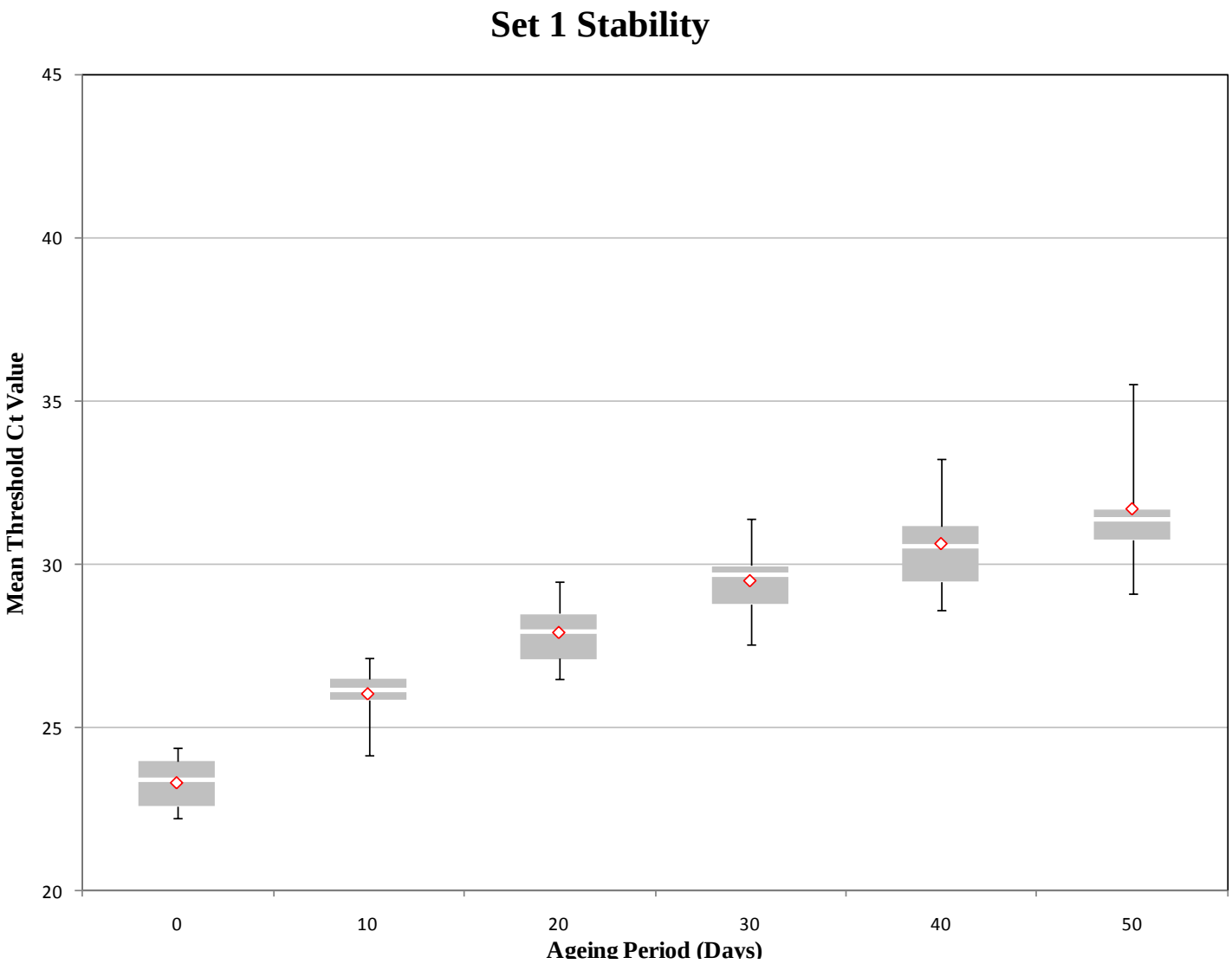


Figure 29: Box and Whisker plot of the raw Ct values for Set 1. The change in the mean Ct value was comparable to VWF and CSF. The mean value at day 0 was 23.29, which increases to 31.70 at day 50. The level of variation observed was again very small though there was a mild increasing trend with ageing time suggesting an environmental effect was present in the degradation process.

4.3.3 Relative Expression

Each blood sample was left to age for the specified period of time before being quantified using qPCR. The raw Ct values obtained were converted into RE values using Equation 4, described in section 2.8 on page 94. The time 0 samples were used as calibrator samples whilst 18S rRNA was used as the reference sample.

Table 19: Relative Expression Values. The Ct values obtained from each sample were converted to RE values and are presented in this table. Each sample was analysed using each of the four targets. The results show that the relative amounts of each target decreases as the sample ages. This was common to all donors.

	Ageing Period (days)	VWF	FGA	CSF	Set 1
Donor 1	0	1.0000	1.0000	1.0000	1.0000
	10	0.1895	0.0603	0.6361	0.3007
	20	0.1306	0.0011	0.2054	0.2108
	30	0.0520	0.0013	0.1226	0.0903
	40	0.0282	0.0001	0.0252	0.0659
	50	0.0226	0.0000	0.0303	0.0575
Donor 2	0	1.0000	1.0000	1.0000	1.0000
	10	0.1189	0.0468	0.1785	0.1380
	20	0.0725	0.0117	0.0826	0.0449
	30	0.0375	0.0042	0.0839	0.0246
	40	0.0196	0.0001	0.0272	0.0089
	50	0.0179	0.0001	0.0116	0.0080
Donor 3	0	1.0000	1.0000	1.0000	1.0000
	10	0.0837	0.0787	0.0542	0.1671
	20	0.0535	0.0019	0.0239	0.0574
	30	0.0469	0.0008	0.0107	0.0154
	40	0.0272	0.0002	0.0085	0.0125
	50	0.0199	0.0000	0.0077	0.0090
Donor 4	0	1.0000	1.0000	1.0000	1.0000
	10	0.1911	0.0714	0.3235	0.1620
	20	0.1098	0.0109	0.2111	0.0300
	30	0.0566	0.0039	0.1311	0.0170
	40	0.0294	0.0003	0.0486	0.0127
	50	0.0128	0.0000	0.0161	0.0064

	Ageing Period (days)	VWF	FGA	CSF	Set 1
Donor 5	0	1.0000	1.0000	1.0000	1.0000
	10	0.0937	0.0000	0.1125	0.1111
	20	0.0767	0.0000	0.0313	0.0346
	30	0.0618	0.0000	0.0165	0.0135
	40	0.0305	0.0000	0.0069	0.0042
	50	0.0126	0.0000	0.0033	0.0013
Donor 6	0	1.0000	1.0000	1.0000	1.0000
	10	0.1687	0.0298	0.3731	0.4807
	20	0.1009	0.0008	0.3159	0.1120
	30	0.1034	0.0005	0.1596	0.0650
	40	0.0522	0.0001	0.0586	0.0398
	50	0.0451	0.0000	0.0340	0.0187

4.3.4 18S rRNA Gene Reference Stability

18S rRNA is a common housekeeping gene used for normalisation in gene expression assays (Kuchipudi *et al*, 2012). In this experiment it was used to assess the reaction kinetics of the degradation process of these samples. The Ct values for 18S rRNA across the 50 day ageing period are presented in Appendix V, with the mean results illustrated in Figure 30 on page 153.

The relative expression of 18S rRNA was calculated according to Equation 3 described in section 2.8 on page 94. The stability of 18S rRNA in blood samples has been reported by various authors [Nussbaumer *et al*, (2006); Anderson *et al*, (2005); Gopee and Howard (2006)] so obtaining expression data from this target would provide a good indication of the degradation rates observed under the specified experimental conditions. The calculated RE values are illustrated in Figure 32.

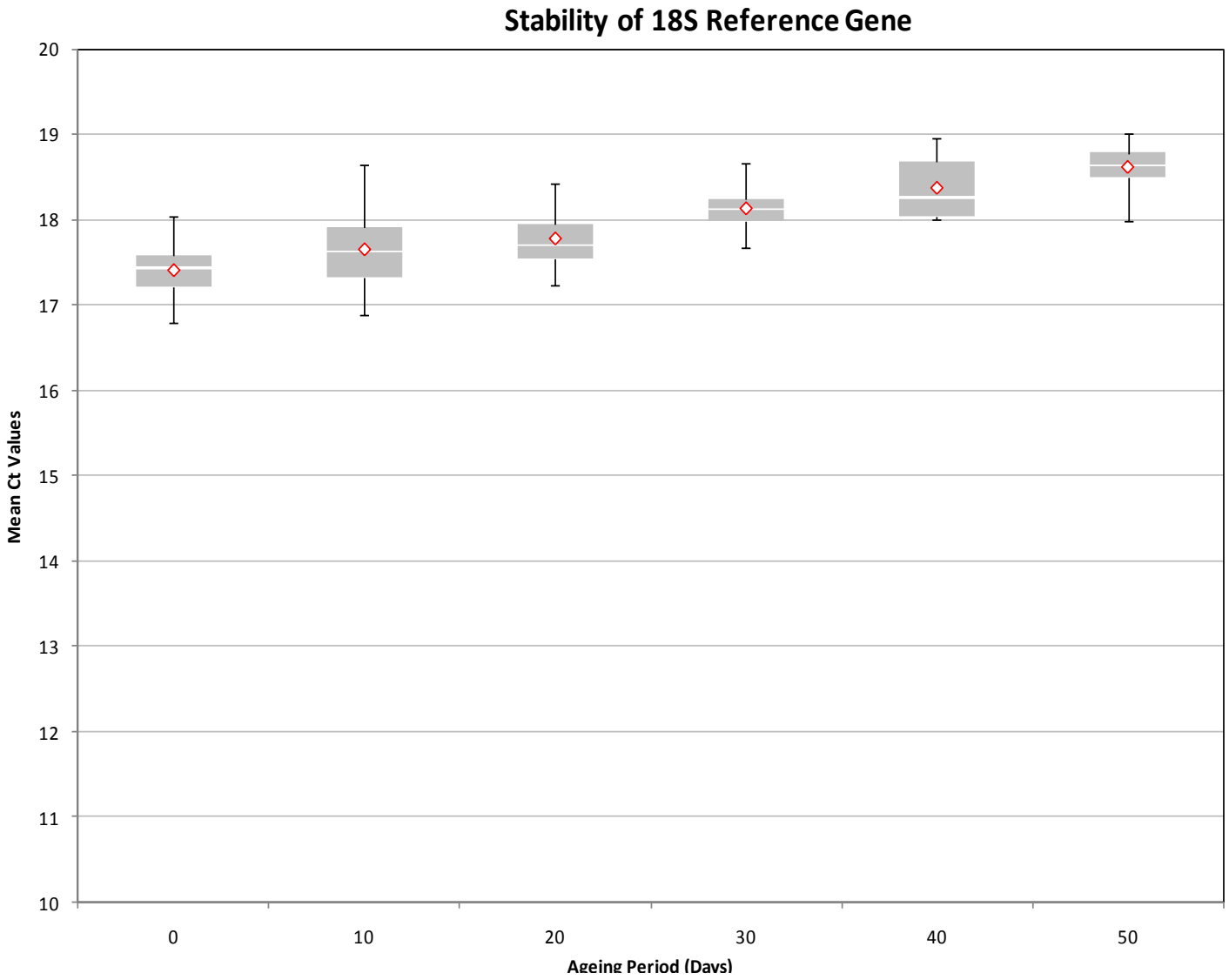


Figure 30: Box and Whiskers plot of the 18S Stability data. The mean Ct value increase from 17.53 to 18.61 over the 50 day period. This increase is small indicating the stability of 18S rRNA. The variation in the data is also relatively small (mean SD of 0.37). The variation at time 0 is also 0.37, which indicates that the extraction method was consistent and robust as similar quantities of DNA were initially recovered from each donor.

4.4 Discussion

The aim of this study was to determine whether there were any differences in the susceptibility to degradation of four particular loci commonly used in DNA profiling (Applied Biosystem) and degradation studies [Tvedebring *et al,* (2009); Hicks *et al,* (2009); Gil *et al,* (2008)] . The main criteria considered for degradation studies has been the target size but there has been minimal research into influential factors and limitations of this approach. The most suitable sized targets will depend on the size of DNA fragments in the sample (Bauer *et al*, 2003) therefore optimisation and a thorough understanding of the degradation kinetics for any environment is important. The work in chapter three identified a potential anomaly that suggested loci susceptibility may affect results from this type of investigation. This could limit the accuracy and precision of size based techniques used to measure degradation levels. Given different parts of the DNA strand have different degrees of susceptibly to degradation factors (Dillon *et al,* 2010) it is unlikely that experiments designed around size alone will not be loci transferable. As such size and loci optimisation should be conducted with any experiment of this nature.

Based on the theory proposed by Bauer *et al,* (2007) that the size of the amplicon is a primary factor in the rate of degradation, a similar change in expression was expected from the four similar sized targets used in this experiment. The results from the 18S rRNA stability study indicate similar amounts of DNA were used in each sample therefore Ct values can be examined directly to establish generalised expression trends, albeit in a logarithmic form (Bustin, 2000). However the amplification efficiency rates of the four targets used here were considerably different (differ by as much as 11.43%) therefore when converting data to a lineal form (relative quantities/expression) any generalised trends that were observed with the Ct values may be lost (as RE values compensate for the difference in amplification rates). For these reasons both Ct values and relative expression values will be discussed.

4.4.1 Interpretation of Target stability to identify Loci susceptibility

Figures 26 to 29 show the change in the Ct values of each of the four targets, FGA, VWF, CSF and Set 1 over the course of the 50 day ageing period. The mean Ct value obtained from VWF increased from 24.70 (day 0) to 31.84 (day 50). This increase was indicative of degradation. The degradation rate of VWF was comparable to that observed in earlier work (chapter three) using similar amplicon sizes and environmental conditions. It was also comparable to the degradation rates of CSF and Set 1 in this study. FGA however, degraded at a significantly greater rate than the other targets. The mean Ct values for FGA increased from 26.72 (Day 0) to 41.98 (Day 50). Given most of the influential variables were kept constant and all targets had nearly identical amplicon sizes it was expected that the change in expression of each target would be similar. Despite this, FGA displayed a significantly greater rate of degradation. These results support the notion that the loci itself can affect the degradation rate and that assay design should not be based on amplicon size alone. To confirm these results the relative expression levels were calculated and will be discussed shortly. The inter-donor variation observed was again very small. Expressed as percentage covariance the values ranged from 0.63% (Day 50) to 3.64% (Day 20).

The relative expression was calculated using calibrator samples (Time 0). Values were converted to a logarithmic form for illustration purposes. Figure 31 on page 156 illustrates the difference in expression levels and hence rate of degradation between FGA and the other three targets. A tight cluster of trend lines can be observed throughout the data for VWF, CSF and Set 1 ranging from 0 to -2.89 (negative is indicative of a decrease in expression) across the 50 day ageing period. This tight cluster is not present with the FGA data. The spread of time dependant trend lines indicates a greater rate of degradation with this particular target. The rate of increase is similar between donors 1,2,3,4 and 6. At Day 50 these samples all have values around -5 (log scale) having all started from 0 at Day 0.

Comparison of the Relative Expression for Similar Sized Targets

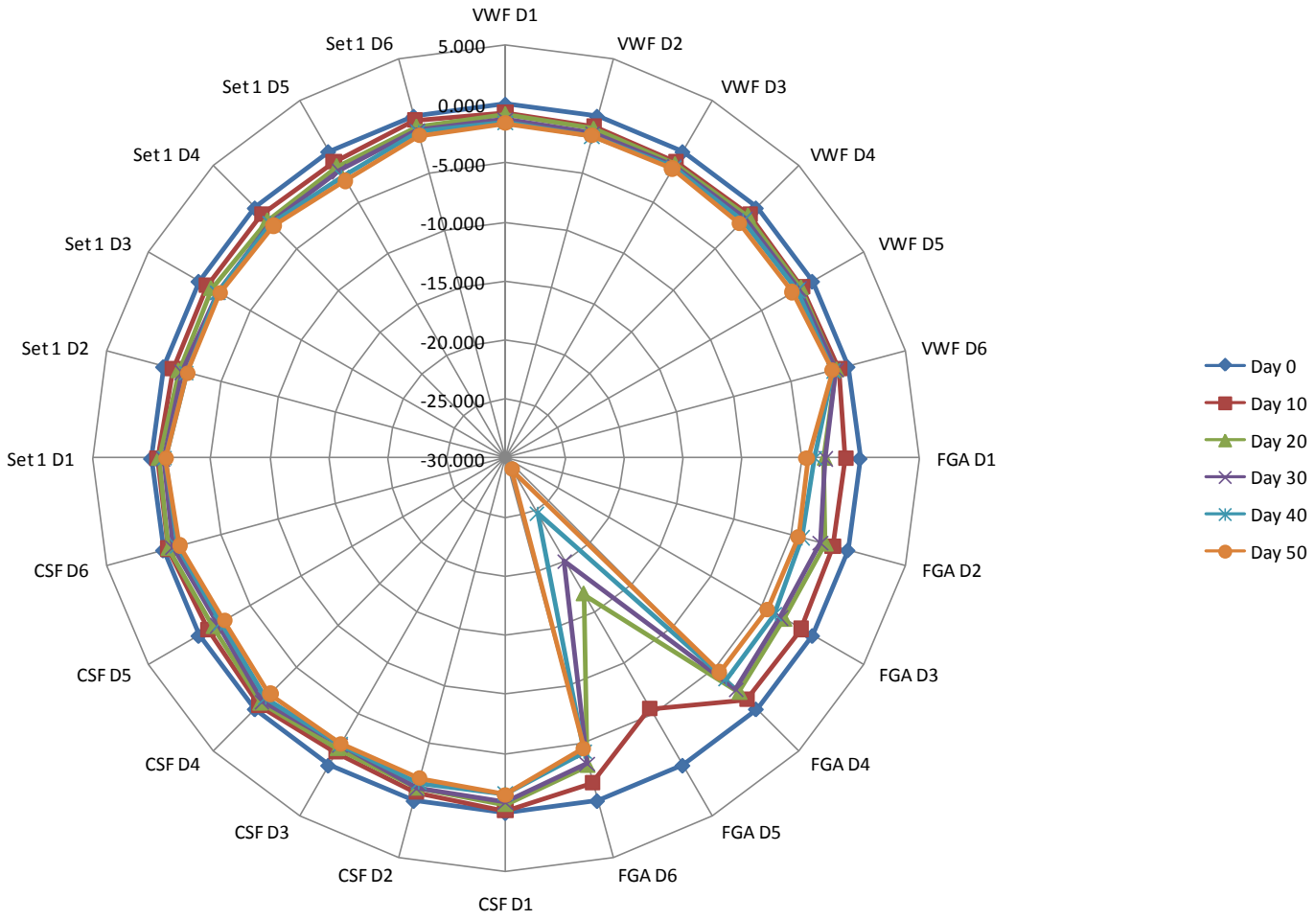


Figure 31: Comparison of the Relative Expression levels for Similar Sized Target Amplicons. The data presented above indicates the similar expression levels between FGA, CSF and Set1 targets. VWF although having a similar sized amplicon showed markedly lower expression levels, indicating a greater rate of degradation. These results support the hypothesis that loci susceptibility exists and careful consideration must be taken when selecting targets for degradation and age studies.

There was an anomaly in the results from donor five. The FGA samples from donor five showed a larger than expected level of degradation reaching relative log expression levels of almost -30. Given 18S rRNA values from these particular samples were similar to the other donor samples, the lower expression levels could not be attributed to a smaller sample volume (or initial quantity). FGA underwent a greater rate of degradation which may have been caused by some form of loci susceptibility to degradation although this does not explain the increased degradation rate in donor five as all samples were aged under the same conditions.

It is well recognised that the size of the target amplicon can affect the rate of degradation (Hicks *et al*, 2010). Hicks *et al* (2010) examined thousands of partial DNA profiles from the Swiss National DNA database to determine the number of times a locus was found to be absent. They found that the percentage of allele dropout was related to the size of the allele. FGA being the third biggest of the 10 STR targets used in the SGM+ profiling platform had the third highest dropout rate (27%), behind D18 (55%) and D2 (49%), which were the largest and second largest targets respectively illustrating the size affect. The influence of size was also noted by Balding and Buckleton (2009) during their attempts to interpret low template DNA profiles. During this study they analysed real crime scene samples where FGA, D18, D7 and CSF could not be detected. Although not stated the increased susceptibility of the D18S51 site, which is located at 18q21.33, may be due to a fragile site FRA18B which is located in the same area.

In this experiment we observed an increase rate of degradation that was not related to amplicon size. FGA was approximately the same size as the other three targets (VWF, Set 1 and CSF) tested under the same conditions. This high rate of degradation could be explained by the FGA amplicon having an increased susceptibility to degradation factors. Various authors [Tvedebrink *et al,* (2009); Gil *et al,* (2008)] have observed this potential vulnerability in FGA despite it being a common target used in many of the DNA profiling platforms around the world (Butler, 2006). Tvedebrink *et al,* (2009) estimated the probability of allelic drop out of STR alleles in real crime case samples. They noted that FGA had the highest occurrence (18) of dropout amongst all loci tested (see Table 20).

Table 20: Observed dropout in the data set stratified by locus. All dropouts were single contributor alleles (Tvedebrink et al,2009).

	D3	VWF	D16	D2	D8	D21	D18	D19	THO	FGA
Observed	306	356	322	398	362	375	315	220	258	313
Drop outs	10	11	11	14	11	7	10	10	17	18
Proportion	0.03	0.03	0.03	0.04	0.03	0.02	0.03	0.05	0.07	0.06

Tvedebrink *et al,* (2009) concluded there was a significant difference ($p = 0.01$) in the dropout rate between loci but there was no significant change in the drop out probability caused by the size of the allele. They suggested the larger alleles within the same locus have the same probability of dropping out as smaller alleles therefore the increased dropout rate must be caused by some other factor than amplicon size. This observation is supported by this study where the size alone was not an accurate gauge in determining degradation rate. Tvedebrink *et al,* (2009) were cautious in stating that the difference between the larger and smaller alleles used in this study was relatively small (400 bp) and may have masked any possible size effect. Gil *et al,* (2008) examined the robustness of complex DNA profiles and whether empirical methods could be used for interpretation. They examined the peak heights for each locus which related directly to the amount of amplified product. FGA presented with the lowest mean peak height at 170 rfu (relative fluorescent units) per allele. This fell well short of the highest mean observed with loci D16 (696 rfu). Given the samples used in this study were real case samples exposed to the various degradation factors we cannot exclude increased loci susceptibility to degradation as a possible cause of the lower quantities.

Mateiu and Rannala (2008) highlighted the degradational changes and effects at a base level that can cause problems for PCR analysis and as such may explain the low expression values obtained. If PCR is inhibited, degradation rates can be overestimated as low quantification may be a result of problems associated with the amplification procedure rather than an actual reduction in the amount of target species (Mateiu and Rannala, 2008). Biochemical processes subsequent to cell death cause the reduction of nucleotide sequence information in many ways including the breakage of the DNA into 100- to 500-bp fragments, fragmentation of bases and sugars and the loss of amino groups (O'Connel, 2002). Several of these post-mortem modifications can block amplification during PCR whereas others allow PCR products to be

obtained, but with incorrect bases incorporated and maintained in the amplification products (Brotherton *et al,* 2007). The latter would have a minimal effect on inaccurate expression levels unless sequence changes resulted in reduced probe binding efficiency (Brotherton *et al,* 2007). The continuous improvement of amplification techniques has reduced the number of such artefacts, but the precise rate or pattern, of occurrence of miscoding lesions remains difficult to estimate and can ultimately affect quantification and estimated degradation rates (Hofreiter *et al,* 2001). An approximate rate of post-mortem damage was calculated by Hofreiter *et al,* (2001) by comparing the PCR products of older samples with a database reference sequence. They concluded that miscoding lesions are unlikely to occur in more than 0.1% of the genome. The prevalence of these lesions would not account for the large difference between FGA and the three other similar sized targets but should be considered when carrying out degradation studies of this nature.

4.4.2 18S rRNA Reference Gene Stability

Reference genes, which are often called, housekeeping genes, are typically used in gene expression assays to ensure the data obtained is reliable (Bustin *et al* (2002). To be suitable, a housekeeping gene must remain constant over the course of a study. Many housekeeping genes have been identified as suitable reference genes for blood samples although there is often debate regarding performance (Dudek *et al,* 2004). 18S rRNA is one housekeeping gene that has been identified and validated by numerous authors [Cheung *et al,* (2009); Anderson *et al,* (2005); Nussbaumer *et al,* (2006)]. Ribosomal RNA is the central component of the ribosome, the protein manufacturing machinery of all living cells (Moss *et al,* 2006). More specifically, the 18S rRNA in eukaryotes, forms part of the small ribosomal subunit, and is 1900 nucleotides in length (Allan and Greenwood, 2007). The human genes that code for rRNAs are organized in approximately 300-400 tandem repeats, which are present in five clusters on chromosomes 13, 14, 15, 21 and 22 (Allan and Greenwood, 2007). Various authors [Zubakov *et al,* (2008); Anderson *et al,* (2005)] have suggested that 18S rRNA remains stable in blood for extended periods of time (up to 180 days) however, it was necessary to test the stability of 18S rRNA under the specific conditions of this study using aged and degraded blood samples.

The mean Ct values are presented in Figure 30 on page X. Although Ct values are depended on the amount of starting material, the spectrophotometric quantification results suggest that similar amounts of initial DNA was used for each sample therefore the Ct values could be

examined for trends. According to these results 18S rRNA is relatively stable under the conditions of this experiment as the mean Ct value only increased from 17.53 to 18.61 over the 50 day period. To accurately gauge this level of change, the relative expression values were calculated. Although the Ct values suggested the expression of 18S rRNA was relatively stable, the relative expression data suggests otherwise. The mean relative 18S rRNA expression for all donors is presented in Figure 32 on page 161. There was a trend, albeit subtle regarding a decrease in the rate of degradation during the first 10 days (although donor 3 and 5 actually showed an increase in 18S rRNA expression during this time, which could be explained by experimental error). This trend continued over the course of the study. Donors 1, 2, 3, 4 and 5 all experienced similar reduced rates of expression of 18S rRNA. After 50 days the RE values were 0.526, 0.523, 0.512, 0.469 and 0.448 for donors 1 to 5 respectively indicating the quantity of 18S rRNA had approximately halved in theses samples over the length of the study. Donor 6 was an exception having a RE value of 0.265 after 50 days. This higher rate of degradation cannot be explained given all samples were aged under similar conditions. Although the ability of 18S rRNA as a housekeeping gene is well documented in clinical samples (Cheung *et al*, 2009), these results suggest that 18S rRNA is not a suitable housekeeping gene under these conditions. Given the samples in this experiment were aged and degraded it is unlikely that any recognised housekeeping gene would maintain a constant level of expression over the course of experiment (50 days).

Although 18S rRNA was not suitable as a reference gene under these conditions, the mean expression levels of 18S rRNA did correlate with the age of the sample ($R^2 = 0.99$). Using a lineal regression analysis the age of a sample in this study can be calculated using the following formula:

$$\text{Age} = - (\text{RE}-0.9961) / 0.0112$$

Although establishing a method for ageing bloodstains was not a direct aim of this study, these findings provide some interesting data for future work. It should be noted that the variation observed is high as depicted by the error bars in Figure 32. The %CV for each time period varied between 19.4% (day 10) and 30.3% (day 0) so resolution in age estimations remains poor. Furthermore similar amounts of DNA were extracted from all samples. This is possible to achieve in a controlled laboratory environment however it would be difficult to achieve using case samples. Therefore this approach could not be used for real forensic work.

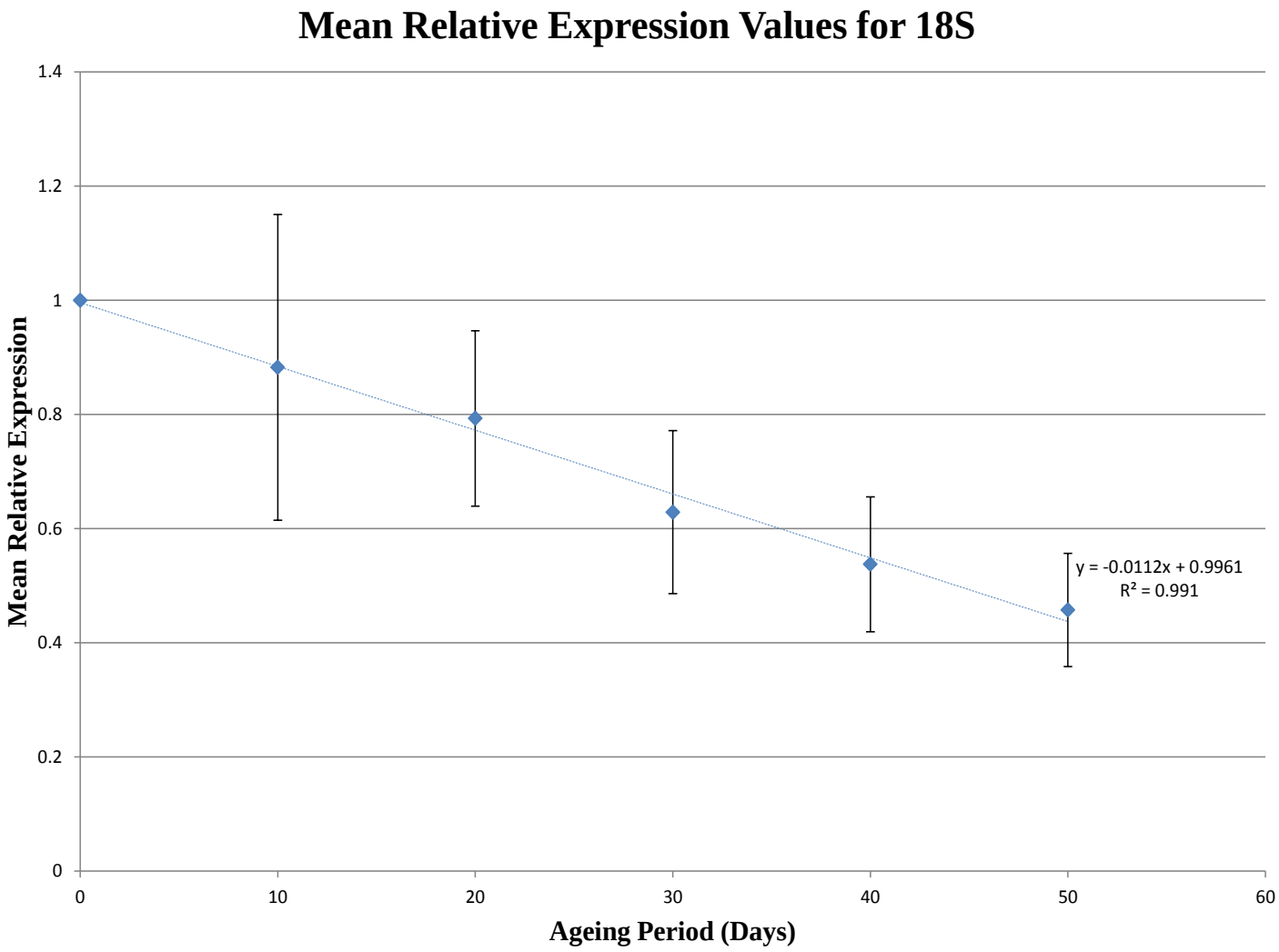


Figure 32: Mean Relative expression data for 18S rRNA. Values from all six donors were averaged and are presented here. There is a strong correlation between RE and sample age however there is significant variation between donors which results in poor resolutions when calculating age. This variation ranged from 19.4% to 30.3% (as expressed as %CV).

4.5 Conclusion

This experiment showed that whilst the amplicon or target size obviously has a significant effect on the degradation results, the behavioural characteristics of the loci are also important if accurate and methods for determining the degradation level and ultimately the age of a sample, are to be adopted. It is only after the loci stability has been assessed should degradation levels be estimated based on the selection of different sized loci.

There are a number of studies that have utilised different sized amplicons to measure the degradation levels of biological samples [Bauer *et al*, (2003), Swango *et al*, (2006), Hudlow *et al*, (2008)]. The results here have shown that even if identical targets sizes are used, there is likely to be a large variation in the level of degradation stated because of the different degrees of loci susceptibility to degradation. In order to achieve a universal approach, where sample and studies can be compared, further research is required to establish loci that exhibit similar characteristics. For forensic purposes this study should be carried out using sample conditions likely to be found at crime scenes.

Under the conditions stipulated in this study the relative expression of 18S rRNA was reduced by approximately 50% after 50 days. A lineal regression equation could be applied to this data to estimate the age of the blood. However due to the high inter-donor variation the resolution for such estimations remains poor. This approach is also not suited for forensic samples due to the lack of calibrator sample though it has provided some interesting information for further investigation.

CHAPTER 5: THE EFFECT OF A MOISTURE FREE ENVIRONMENT ON THE INTEGRITY OF AGED BLOOD SAMPLES

5.1 Introduction

Degraded samples are problematic for forensic laboratories and law enforcement agencies and all care is generally taken to ensure the preservation of biological crime scene samples (Lee *et al*, 2012). Evidence collected for DNA analysis is often recovered using standard cotton swabs, which are sometimes air dried, before packaging. The swabbing technique is susceptible to contamination and often results in incomplete drying, especially in high humidity environments (O'Brien, 2012). The swabs are generally stored and transported at room temperature in containers that are not necessarily sealed from the external environment. During sample storage, which can be days, weeks, or even years, DNA degradation can and does occur (Garvin *et al,* 2013). As such the time frame and conditions, at which samples are transported and stored, have a direct effect on the quality of the results that are achieved in the laboratory (Lee *et al,* 2012) and therefore every effort should be made to preserve the samples. Different preservation mechanisms can be used to halt the growth of bacteria present in a sample. For example, samples can be either dried (McCabe, 1991), cooled, frozen (Ahmad, 1995), freeze-dried (Takahashi *et al*, 1995) or chemically treated (Zhong *et al*, 2001). The preservation capabilities of these techniques vary. No preservation method can completely prevent cellular and DNA damage and are generally only useful in preserving samples for short periods of time (Leal-Klevezas *et al*, 2000).

The requirements for preserving and storing DNA vary depending on the interests of the scientist. For example, DNA required for pharmaceutical testing may only need to be stable for a few years, whereas samples used in evolutionary biology need to have been adequately preserved for thousands or even millions of years (Anchordoquy and Molina, 2007). Forensic scientists are concerned with the short term preservation and storage of biological material as it moves from the crime scene to the laboratory (Lee *et al,* 2012) as well as the long term storage of DNA after it has been extracted and profiled. Long term storage is often required for replicate or repeat testing, confirmation of results and to accommodate future testing with new technologies (Lee *et al,* 2012).

Repeated freezing of biological samples such as blood and saliva will degrade a sample (Ross *et al*, 1990) as will the long term storage of DNA in an aqueous solution (Bonner and Klibanov, 2000). Despite these issues forensic biological samples collected for DNA analysis should ideally be frozen to prevent significant degradation to the evidentiary material. Short term freezing (less than 8 years) of these samples is the most effective method of reducing degradation (Lee *et al*, 2012). However freezing samples is not a common practice in the UK according to the National Police Improvement Agency crime scene investigation training manual. The cost of fitting out crime scene vehicles with freezers is likely to be the main impediment to utilising this preservation technique.

The use of chemically treated cards is a cheaper and more practical way to preserve DNA in the short term. Commercial manufacturers of these cards claim that biological material can be stored at room temperature for days or months if stabilising procedures are performed (www.promega.com). The use of cards (Isocards and FTA cards) for the collection of different human biological samples has become a frequent practice in forensic laboratories (Prieto *et al*, 2006) and with law enforcement agencies. The chemical treatment of these cards acts to preserve the DNA against bacterial and fungal degradation activity (Barbaro and Cormaci 2004). It is also suggested that the chemical compounds lyse the cells, dissociate proteins from nucleic acids and destroy nucleolytic enzymes (Barbaro and Cormaci 2004). Whilst it has been suggested that this allows for the indefinite storage of these samples at ambient temperature (Barbaro and Cormaci 2004), independent findings suggest otherwise. The ability of these cards to preserve the biological material was discussed in Prieto *et al* (2006). In one experiment only 40% of the samples stored on IsoCode cards resulted in amplification of DNA. Zhong *et al*, (2001) obtained comparable results when analysing whole blood samples. They found that DNA was only recovered from 42% of samples stored on Isocode whilst DNA from 63% of samples stored on FTA cards was successfully extracted (Zhong *et al*, 2001). Mass *et al*, (2007) discovered that IsoCode and FTA cards were less effective with blood samples that were not dried prior to isolation.

Drying is a well recognised method of preserving DNA as it neutralises most of the factors that cause DNA degradation, including bacteria, fungi, enzymes and humidity (Lindahl, 1993). Several different methods may be used to dry a sample, including active drying, passive drying, and air drying (Garvin *et al*, 2013). Air drying is a form of passive drying in which the sample is not protected from outside contamination, therefore increasing the likelihood of

sample contamination (Garvin *et al,* 2013). All forms of passive drying are dependent on climatic conditions as higher relative humidity in the external environment leads to a lower drying rate (Garvin *et al,* 2013). Active drying is performed independent of the climatic conditions however it is still susceptible to contamination.

Whilst various authors have highlighted the role moisture plays in the degradation process of DNA [Garvin *et al,* (2013); Overballe-Peterson *et al,* (2012)], its effect has not been quantified. Unfortunately for the forensic scientist the DNA molecule is one of the least stable molecules within a cell. Specific cellular enzymes called endonucleases rapidly degrade DNA as does although these enzymes require energy therefore their degradational capabilities may be limited in dead or dying cells (Mouttham and Schwarz, 2010). Bacteria and fungi are also effective at degrading DNA although the degradation is often incomplete. Chemical degradation via hydrolysis and oxidation can occur rapidly and over sustained periods of time causing significant DNA degradation (Mouttham and Schwarz, 2010). Figure 33 shows the sites on the DNA molecule that are susceptible to these types of processes, which are explained on page 182. These factors limit the lifespan of DNA *in vivo* and within the environment (Mouttham and Schwarz, 2010).

The aim of this study was to quantify the effect that moisture plays in facilitating DNA degradation. A secondary aim concerned the development of a hermetically sealed dry swab that could preserve biological evidence to a standard that will alleviate the need for freezing or refrigerating blood samples recovered from crime scenes.

Figure 33: Susceptible sites on the DNA molecule. The schematic diagram shows the various bonds on the DNA molecule that are susceptible to hydrolytic attack, oxidative and alkylation damage. Image adopted from http://socserv.mcmaster.ca

5.2 Methods

5.2.1 Blood Collection and Storage

Blood samples were prepared as described in section 2.1.5 on page 84.

5.2.2 Swab Design

The standard swabs were set up using Sterilin cotton swabs (Copan, Italy), placed inside 20ml Universal tubes (Copan, Italy). The swabs were secured with Bostik blu tack so that the tips did not come into contact with the sides of the universal tube. A standard screw top lid was used but not screwed down. This created a dependant (on the outside environment) internal swab environment.

The dry swab was designed to ensure favourable conditions between collection and laboratory analysis. It was specifically constructed to ensure that the drying of samples was independent of the environmental conditions, whilst reducing the risk of contamination. Each dry swab unit was self-contained and hermetically sealed, obviating the need for specialised drying equipment and preventing contamination from the moment the swab was returned to the unit. The dry swabs were created in a similar manner to the standard swab with the following modifications to make it a hermetically sealed dry unit. A silica based drying agent (Merck, Germany) was added to the universal tube, which contained a colour indicator to monitor the internal environmental conditions and therefore ensure that a moisture free environment was maintained. The dry swabs were sealed with a rubber sealed lid to ensure it maintained an independent environment.

5.2.3 Aging Conditions

Once deposited with blood, the standard swabs were returned to their normal packaging (not sealed from the external environment) and left to age. The dry swabs, once deposited with blood were placed into the sealed environment which contained a silicon drying agent to remove any moisture from the internal environment. All samples were aged at either 30°C, room temperature, 4°C or frozen (-20°C) thus providing temperature dependant data.

5.2.4 TH01 SYBR Green Assay

In chapter three a singleplex SYBR green assay was developed that targeted five different amplicons on the TH01 gene (Set 1- Set 5). Stringent optimisation studies were carried out and the results indicated that this assay was robust and suitable for assessing the degradation levels of blood samples, although it fell short of providing a reliable method for ageing the samples. The two optimal targets from that assay, Set 1 (64bp) and Set 4 (239bp), were used to measure the relative quantities of DNA recovered in this experiment. The samples in this experiment were aged during the winter months, as opposed to earlier work where samples were aged over summer. This meant a comparison could be made between samples aged in an internal environment during different times of the year.

5.2.5 Sample Processing

DNA was isolated from the blood samples using an organic DNazol BD extraction method (Invitrogen, US) according to manufacturer's instructions with one exception. Longer incubation times (up to 50%) were used due to the difficult nature of the clotted samples. The detailed protocol can be found in section 2.4.3 on page 90. The concentration of extracted DNA in all test and control samples was determined using a NanoPhotometer and LabelGuard™ Microliter Cell (Implen, Germany) as previously described in section 2.5 on page 90. DNA amplification was achieved using Applied Biosystems 7500 thermocycler using a SYBR green detection approach. Samples were loaded into MicroAmp® Optical 96-well reaction plates (Applied Biosystems, California, USA) containing 2 x Power SYBR Green PCR Master Mix (Applied Biosystem, California, USA). Forward and reverse primers of the target amplicons, Set 1 (TH01) and Set 4 (TH01), previously described on page 104, were added at the optimal concentration of 400nM. Template DNA and nuclease-free water was added to the respective wells to create a final reaction volume of 20 µl. The reaction plates were then loaded onto the ABI 7500 and run according to the standard protocol that is described in section 2.7.2 on page 93. A dissociation stage was included in each PCR run to confirm the absence of non-specific amplification. The data was analysed using Applied Biosystems 7500 Real-time PCR SDS software (version 1.4.0) using the automatic baseline feature. An identical threshold was used for both targets. A passive reference dye (ROX), which was included in the Power SYBR Green master mix was used to correct any well to well variation in the background fluorescence. An exogenous standard control (human DNA) was

used to adjust for any instrumental variation between runs. Human genomic control DNA (Bioline, UK) was used for the positive controls whilst negative control assays were also run with every batch to detect any false positive results (or possible contamination) using nuclease free, PCR grade water. All samples were run in triplicate.

5.3 Results

5.3.1 Assay Optimisation

The amplification efficiency of each of the two targets (Set 1 and Set 4) had previously been determined (see page 105). Using the optimal primer concentrations the amplification efficiency rate of Set 1 was 96.8%, whilst Set 4 amplified at 106.0% efficiency. To ensure these expected values correlated to the actual amplification rates, five test samples were chosen at random and the efficiencies determined.

Table 21: Mean amplification efficiency rates of five random test samples. Although the amplification efficiency rates of Set 1 and Set 4 were previously determined as described on page 105. it was necessary to check that the rates, which were determined using high quality human DNA was the same with the blood samples used in this experiment. Five random blood samples and serial dilutions were performed. Each target was amplified at each dilution and the Ct values obtained were used to calculate the efficiency rate of the amplification.

Target Amplicon	Sample	Slope	Efficiency (%)
Set 1	Test Sample 1	3.390	**97.1**
	Test Sample 2	3.363	**98.3**
	Test Sample 3	3.432	**95.6**
	Test Sample 4	3.353	**98.7**
	Test Sample 5	3.437	**95.4**
Set 4	Test Sample 1	3.361	**98.3**
	Test Sample 2	3.388	**97.3**
	Test Sample 3	3.311	**100.4**
	Test Sample 4	3.446	**95.0**
	Test Sample 5	3.365	**98.2**

The test samples were run in triplicate. The mean efficiency rates were 97.0% and 97.9% for Sets 1 and 4 respectively. The actual rate for Set 1 was comparable to the expected value (96.8%) however, there was a significant difference between the expected (106.0%) and actual (97.9%) rate of Set 4. The variation between test samples was small (Standard deviation of 1.95%) therefore it was more appropriate to use the actual rate for quantification purposes.

5.3.2 DNA Quantification

The quantity of DNA extracted from each sample was determined. A summary showing the difference in the recovery rates between the standard and dry swabs are presented in the following tables.

Table 22: Average Percentage Increase of DNA recovered from Dry Swabs Compared to Standard Swabs at each given Ageing Period.

N° conditions Examined	Ageing Time Frame (Days)	Median recovery from standard swabs (ng/µl)	Median recovery from dry swabs (ng/µl)	Percentage Increase (%)
22	0	**4.11**	**4.60**	**11**
22	10	**2.84**	**4.02**	**41**
22	20	**2.51**	**4.06**	**61**
22	30	**2.23**	**3.20**	**43**
22	60	**2.24**	**3.27**	**45**

Table 23: Comparison of DNA recovered from dry and standard swabs. Each aging condition was examined to determine the amount of DNA recovered from the dry and standard swabs. With the exception of the two sets of frozen samples (negative values), a greater amount of DNA was consistently recovered from the dry swabs when compared to the standard swabs. The difference was calculated as a percentage and the results are presented in here.

Ageing Time Frame	Frozen	4°C	Room Temperature	30°C
0	-10.2	14.1	19.9	14.2
10	-10.9	58.1	49.3	42.2
20	10.2	57.6	10.8	45.6
30	8.7	83.4	36.0	9.6
60	-9.2	7.3	67.6	61.3

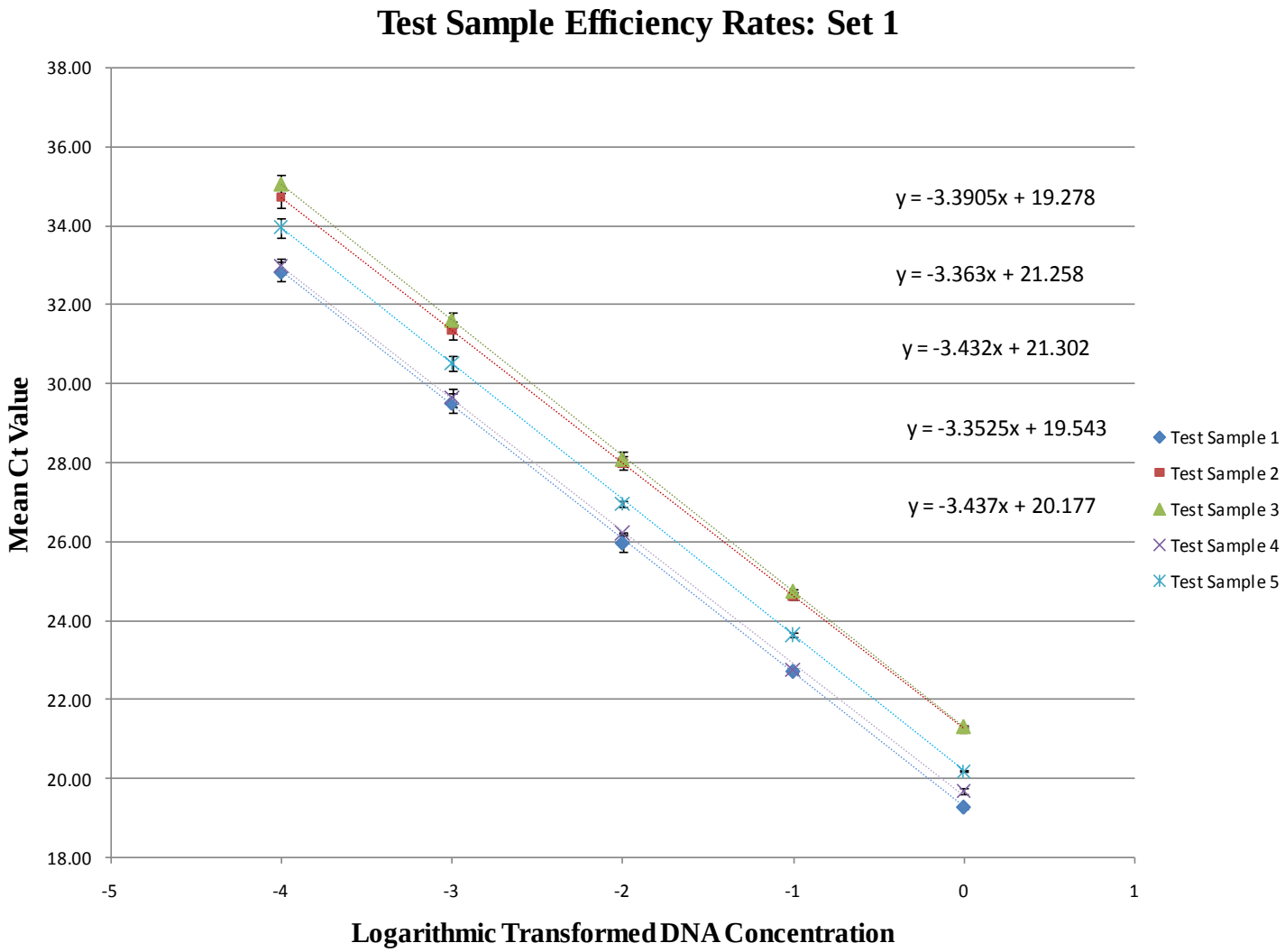


Figure 34: Test sample Amplification Efficiency Rate (Set 1). Five test samples were chosen at random and the amplification rates were calculated using a standard serial dilution method. The correlations (R^2) for each sample were very strong for all five samples ranging from 0.995 to 0.999. The calculated mean rate (97.0%) was very similar to the expected rate (96.8%) as previously determined during the optimisation of this TH01 singleplex assay (refer to chapter three for complete optimisation details)

Note: The trend equations presented on the graph are in the same descending order as presented in the legend.

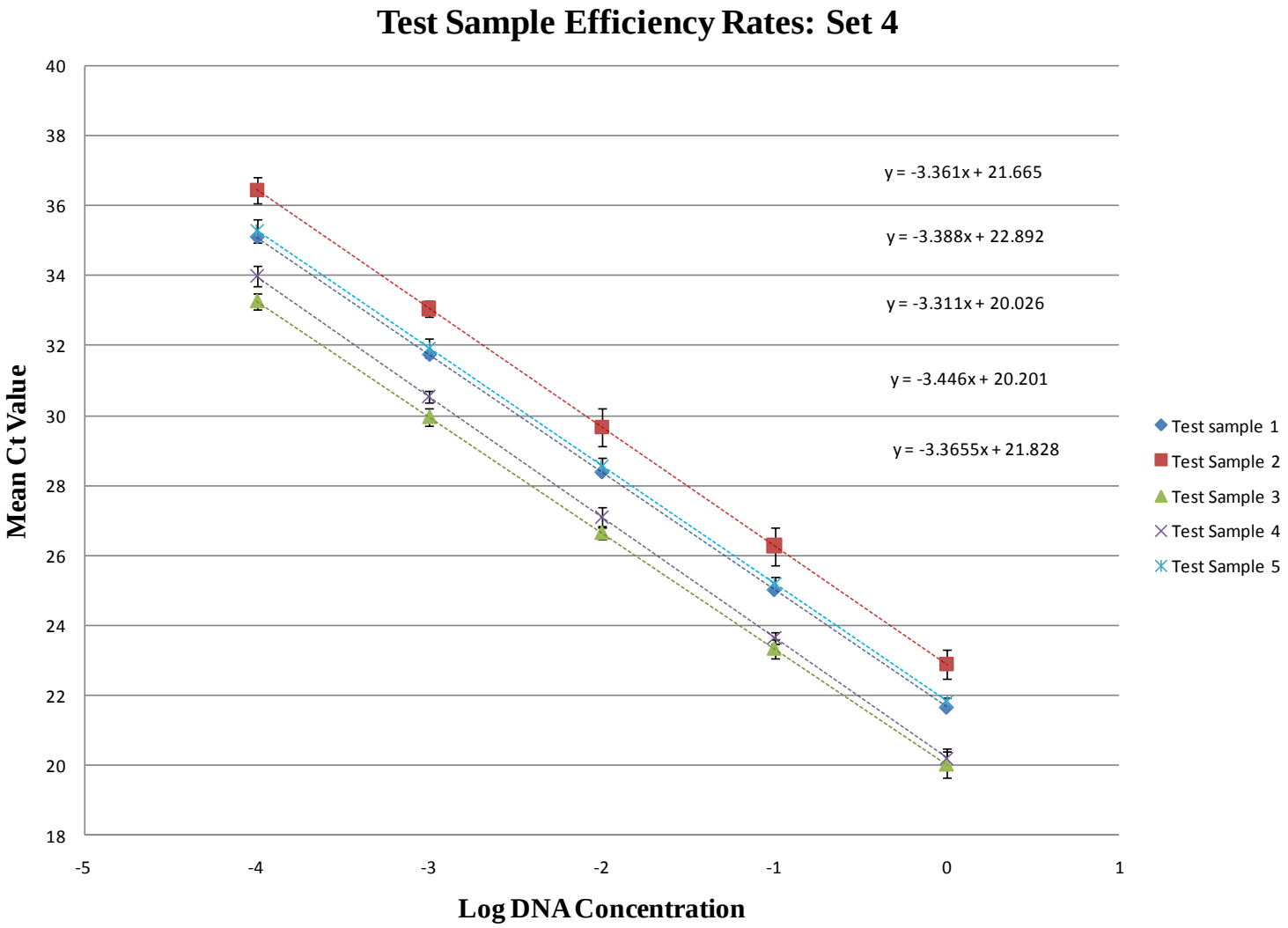


Figure 35: Test Sample Amplification Efficiency rate (Set 4). The same five test samples as previously examined were again used to measure the amplification rate of the 239 bp target. Again strong correlations were seen with all five samples across the dilutions series (R^2 values ranged from 0.997 to 0.999). The mean actual amplification rate was 97.9%, which was significantly less than the expected rate of 106.0%. The variation is this data set was small (1.95%) therefore it was more appropriate to use the actual rate for quantification purposes.

Note: The equations on the graph and are in the same descending order as presented in the legend.

5.3.3 Relative Quantity Ratios (RQR)

The relative quantities (RQ) of each amplicon (Set 1 and Set 4) for each sample were calculated from the Ct values using equation 2 described in section 2.8 on page 94. The relative quantity ratios (RQR) were then calculated using the following formula:

$$RQR = RQ\ (Set1)/\ RQ\ (Set\ 4)$$

The RQRs were used to assess the level of degradation of each sample. Set 1 and Set 4 are different sized amplicons (64 and 239 base pairs respectively) and therefore based on the theory that larger amplicons are more susceptible to degradation (Bauer *et al*, 2003), the RQR should increase as a sample becomes more degraded. The RQRs for each sample at each aging period are presented in Tables 24-28.

The abbreviations used in the tables are:

S: Swabs were soiled with garden soil prior to the deposition of blood
C: Swabs remained clean prior to the deposition of blood
30: Swabs were aged at 30°C
RT: Swabs were aged at room temperature (22-24°C)
4: Swabs were aged at 4°C
F: Swabs were aged at -20°C (frozen)

Table 24: The Relative Quantity Ratios for all Standard and Dry swab samples aged for 0 days. A RQR value of 1.0 indicates an equal amount of Set 1 amplicon and Set 4 amplicon was amplified. This indicates the sample is of high quality. As samples become more degraded the RQR will increase.

Standard Swab			Dry Swab		
Set 1 Relative Quantity	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**	**Set 1 Relative Quantity**	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**
7.05E-09	7.80E-09	**0.90**	1.07E-08	1.21E-08	**0.89**
2.89E-08	1.74E-08	**1.66**	5.00E-08	4.56E-08	**1.10**
1.09E-08	1.27E-08	**0.86**	2.97E-08	3.47E-08	**0.86**
1.51E-08	1.48E-08	**1.02**	1.74E-08	1.69E-08	**1.03**
3.68E-08	3.68E-08	**1.00**	3.80E-08	3.26E-08	**1.17**
4.06E-08	4.95E-08	**0.82**	1.74E-08	1.62E-08	**1.07**
5.41E-08	5.65E-08	**0.96**	5.56E-09	7.59E-09	**0.73**
8.78E-09	9.41E-09	**0.93**	8.78E-09	9.74E-09	**0.90**
3.46E-08	3.52E-08	**0.98**	1.78E-08	1.73E-08	**1.03**
1.64E-08	1.68E-08	**0.98**	2.03E-08	3.70E-08	**0.55**

Table 25: The Relative Quantity Ratios for all swab samples aged for ten days. With the exception of the frozen samples, the RQR of all samples increased during the first ten days indicating that samples were degraded during this period. The increase observed was greater with the standard samples than the dry swab samples.

Conditions		Standard Swab			Dry Swab		
		Set 1 Relative Quantity	Set 4 Relative Quantity	Relative Quantity Ratio (RQR)	Set 1 Relative Quantity	Set 4 Relative Quantity	Relative Quantity Ratio (RQR)
S	**30**	1.01E-08	6.02E-10	**16.85**	4.84E-09	1.00E-09	**4.82**
S	**30**	4.50E-09	3.57E-10	**12.59**	4.99E-09	8.47E-10	**5.89**
S	**30**	7.29E-09	5.56E-10	**13.10**	1.57E-09	4.67E-10	**3.35**
S	**RT**	5.34E-09	8.70E-10	**6.14**	2.55E-09	6.27E-10	**4.06**
S	**RT**	6.31E-09	5.83E-10	**10.82**	2.73E-10	1.23E-10	**2.22**
S	**RT**	5.27E-09	7.07E-10	**7.46**	8.56E-10	2.78E-10	**3.08**
S	**4**	2.52E-09	1.05E-09	**2.41**	3.91E-09	1.08E-09	**3.61**
S	**4**	9.69E-09	1.31E-09	**7.39**	4.62E-09	1.61E-09	**2.87**
S	**4**	5.64E-09	7.56E-10	**7.46**	6.22E-09	5.54E-09	**1.12**
S	**F**	2.32E-08	2.02E-08	**1.15**	5.54E-09	3.96E-09	**1.40**
S	**F**	9.11E-09	9.74E-09	**0.94**	3.78E-09	4.19E-09	**0.90**
C	**30**	5.24E-09	3.78E-10	**13.85**	4.79E-09	1.57E-09	**3.06**
C	**30**	3.54E-09	2.73E-10	**12.95**	9.06E-09	1.78E-09	**5.08**
C	**30**	6.68E-09	5.32E-10	**12.54**	1.14E-08	2.67E-09	**4.27**
C	**RT**	5.04E-09	9.74E-10	**5.17**	8.52E-09	4.13E-09	**2.06**
C	**RT**	8.04E-09	1.36E-09	**5.88**	4.48E-09	9.42E-10	**4.76**
C	**RT**	7.75E-09	1.69E-09	**4.59**	3.63E-09	1.69E-09	**2.14**
C	**4**	9.49E-09	4.26E-09	**2.23**	7.86E-09	7.16E-09	**1.10**
C	**4**	5.45E-09	2.59E-09	**2.11**	6.14E-09	4.26E-09	**1.44**
C	**4**	9.70E-09	4.42E-09	**2.19**	3.21E-09	1.75E-09	**1.83**
C	**F**	2.53E-08	1.60E-08	**1.58**	1.56E-08	1.93E-08	**0.81**
C	**F**	3.08E-08	1.52E-08	**2.02**	9.30E-09	1.18E-08	**0.79**

Table 26: The Relative Quantity Ratios for all samples aged for 20 days. Again the general trend was an increase in the RQR values for all samples (with the exception of the frozen swabs) indicating further degradation. Comparatively speaking the dry swabs presented with a lower mean RQR, and therefore degradation level, than the standard swabs.

Conditions		**Standard Swab**			**Dry Swab**		
		Set 1 Relative Quantity	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**	**Set 1 Relative Quantity**	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**
S	**30**	2.04E-09	5.34E-11	**38.21**	3.83E-09	3.16E-10	**12.10**
S	**30**	1.90E-10	1.03E-11	**18.47**	6.01E-10	1.05E-10	**5.72**
S	**30**	4.79E-10	2.55E-11	**18.76**	1.05E-08	9.67E-10	**10.93**
S	**RT**	5.11E-09	2.47E-10	**20.63**	2.83E-09	3.15E-10	**8.98**
S	**RT**	1.56E-10	9.08E-12	**17.28**	2.68E-09	4.95E-10	**5.42**
S	**RT**	2.14E-09	1.59E-10	**13.43**	3.46E-09	8.04E-10	**4.30**
S	**4**	3.01E-09	3.16E-10	**9.51**	9.06E-10	2.10E-10	**4.31**
S	**4**	3.07E-10	3.81E-11	**8.08**	1.78E-09	3.13E-10	**5.69**
S	**4**	1.69E-09	2.05E-10	**8.26**	8.82E-10	3.31E-10	**2.66**
S	**F**	5.46E-09	3.62E-09	**1.51**	1.79E-09	1.80E-09	**0.99**
S	**F**	1.12E-08	9.31E-09	**1.21**	3.45E-09	4.51E-09	**0.76**
C	**30**	2.60E-09	7.94E-11	**32.80**	2.11E-09	3.61E-10	**5.86**
C	**30**	3.48E-09	1.92E-10	**18.12**	4.00E-09	4.38E-10	**9.14**
C	**30**	1.36E-09	4.02E-11	**33.91**	3.15E-10	6.40E-11	**4.92**
C	**RT**	2.41E-09	2.95E-10	**8.17**	3.19E-09	6.81E-10	**4.66**
C	**RT**	5.89E-10	8.44E-11	**6.98**	2.26E-09	5.18E-10	**4.38**
C	**RT**	3.51E-09	4.87E-10	**7.20**	3.28E-10	8.27E-11	**3.97**
C	**4**	7.46E-09	1.66E-09	**4.50**	1.25E-09	4.35E-10	**2.88**
C	**4**	3.87E-09	8.32E-10	**4.65**	8.09E-09	2.93E-09	**2.76**
C	**4**	1.99E-09	7.59E-10	**2.63**	1.22E-10	7.99E-11	**1.53**
C	**F**	1.49E-09	1.01E-09	**1.47**	8.18E-09	1.25E-08	**0.65**
C	**F**	1.28E-10	1.10E-10	**1.17**	8.32E-09	7.26E-09	**1.15**

Table 27: The Relative Quantity Ratios for all samples aged for 30 days. The same general trends as previously described were observed with the 30 day samples.

Conditions		Standard Swab			Dry Swab		
		Set 1 Relative Quantity	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**	**Set 1 Relative Quantity**	**Set 4 Relative Quantity**	**Relative Quantity Ratio (RQR)**
S	30	3.32E-09	4.3997E-11	**75.57**	1.16E-09	7.57E-11	**15.32**
S	30	1.69E-09	5.3089E-11	**31.85**	1.06E-09	9.20E-11	**11.63**
S	30	1.85E-09	3.5600E-11	**52.00**	6.63E-10	6.47E-11	**10.26**
S	RT	6.41E-10	4.9414E-11	**12.99**	6.28E-10	5.94E-11	**10.58**
S	RT	9.43E-10	3.5600E-11	**26.51**	2.99E-10	3.79E-11	**7.89**
S	RT	1.08E-09	5.1658E-11	**20.99**	1.21E-09	1.34E-10	**9.05**
S	4	3.60E-10	4.9078E-11	**7.35**	3.27E-10	3.58E-11	**9.13**
S	4	9.96E-10	8.3046E-11	**12.00**	1.20E-10	1.45E-11	**8.31**
S	4	2.35E-10	2.8318E-11	**8.32**	1.15E-09	4.19E-10	**2.75**
S	F	3.91E-09	1.6832E-09	**2.32**	4.22E-09	3.10E-09	**1.36**
S	F	3.69E-10	2.9893E-10	**1.24**	5.58E-09	6.20E-09	**0.90**
C	30	4.03E-10	1.6173E-11	**24.95**	2.19E-10	2.64E-11	**8.30**
C	30	3.70E-10	1.3775E-11	**26.92**	8.91E-10	4.64E-11	**19.18**
C	30	6.43E-10	1.6793E-11	**38.34**	4.43E-10	5.53E-11	**8.02**
C	RT	1.37E-09	1.2684E-10	**10.87**	9.12E-10	1.20E-10	**7.55**
C	RT	3.60E-10	2.5300E-11	**14.26**	1.79E-09	1.93E-10	**9.30**
C	RT	2.34E-09	1.4690E-10	**15.97**	1.26E-09	1.27E-10	**9.89**
C	4	5.56E-10	9.9525E-11	**5.59**	6.56E-09	1.09E-09	**6.02**
C	4	3.74E-09	4.4123E-10	**8.48**	6.37E-10	9.71E-11	**6.56**
C	4	8.07E-10	1.5046E-10	**5.37**	8.11E-11	3.83E-11	**2.11**
C	F	4.15E-09	2.0103E-09	**2.07**	3.82E-09	3.92E-09	**0.97**
C	F	7.75E-09	4.8194E-09	**1.61**	1.60E-09	2.10E-09	**0.76**

Table 28: The Relative Quantity Ratios for all samples aged for 60 days. The same general trends as previously described were observed with the 60 day samples.

Conditions		Standard Swab			Dry Swab		
		Set 1 Relative Quantity	Set 4 Relative Quantity	Relative Quantity Ratio (RQR)	Set 1 Relative Quantity	Set 4 Relative Quantity	Relative Quantity Ratio (RQR)
S	**30**	3.10E-10	2.42E-12	**127.98**	3.00E-11	1.01E-12	**29.68**
S	**30**	8.76E-10	3.90E-12	**224.29**	3.82E-11	1.07E-12	**35.49**
S	**30**	2.93E-10	1.64E-12	**178.95**	2.48E-10	9.82E-12	**25.32**
S	**RT**	2.60E-10	1.54E-12	**169.03**	2.60E-10	6.76E-12	**38.52**
S	**RT**	2.00E-09	1.16E-11	**171.74**	5.38E-10	2.87E-11	**18.74**
S	**RT**	1.40E-09	1.41E-11	**99.07**	6.26E-10	3.60E-11	**17.36**
S	**4**	2.22E-10	9.42E-12	**23.59**	3.24E-10	2.24E-11	**14.47**
S	**4**	7.67E-10	2.37E-11	**32.38**	3.30E-10	4.25E-11	**7.77**
S	**4**	1.70E-09	1.49E-10	**11.38**	6.52E-10	9.81E-11	**6.65**
S	**F**	4.12E-09	9.98E-10	**4.14**	1.21E-09	9.45E-10	**1.28**
S	**F**	3.95E-09	2.25E-09	**1.76**	1.43E-09	1.21E-09	**1.18**
C	**30**	3.12E-10	2.08E-12	**149.75**	8.19E-11	3.85E-12	**21.26**
C	**30**	2.58E-10	2.88E-12	**89.56**	2.04E-10	6.95E-12	**29.38**
C	**30**	7.00E-10	2.12E-12	**329.47**	3.32E-11	1.46E-12	**22.80**
C	**RT**	3.06E-10	5.80E-12	**52.85**	1.30E-09	6.16E-11	**21.17**
C	**RT**	2.07E-10	2.36E-12	**87.62**	1.36E-09	6.29E-11	**21.60**
C	**RT**	2.31E-10	4.66E-12	**49.65**	8.94E-10	3.25E-11	**27.45**
C	**4**	4.66E-10	1.99E-11	**23.35**	8.73E-11	8.16E-12	**10.70**
C	**4**	8.18E-10	3.63E-11	**22.53**	2.84E-09	1.89E-10	**15.04**
C	**4**	3.21E-10	1.75E-11	**18.32**	1.01E-09	9.78E-11	**10.36**
C	**F**	5.73E-10	2.83E-10	**2.03**	9.15E-10	1.20E-09	**0.76**
C	**F**	9.43E-10	5.73E-10	**1.64**	4.69E-10	4.38E-10	**1.07**

5.3.4 Statistical Analysis

A statistical analysis was carried out on the differences between the RQR values for the dry and standard samples for each of the ageing conditions. These results are presented in Tables 29-37 and are discussed in section 5.4 on page 182.

Table 29: Statistical Analysis Comparing RQRs of all dry and standard swabs

Statistical Parameter	Dry Swabs	Standard Swabs
Mean (RQR)	28.32	7.43
Number	32	32
Standard Error of Mean (SEM)	8.69	1.40
Lower 95% CI	10.59	4.56
Upper 95% CI	46.05	10.30
P value (Wilcoxon test)	<0.0001	

Table 30: Statistical Analysis Comparing RQRs from the clean/standard swabs with the clean/dry swabs samples.

Statistical Parameter	Clean Standard swabs	Clean Dry Swabs
Mean (RQR)	24.47	6.94
Number	16	16
P value (Wilcoxon test) (95% confident level)	0.00044	

Table 31: Statistical Analysis Comparing RQRs of all soiled and clean dry swab samples

Statistical Parameter	Soiled Dry swabs	Clean Dry Swabs
Mean (RQR)	7.93	6.93
Number	16	16
Standard Error of Mean (SEM)	2.13	1.90
Lower 95% CI	3.39	2.89
Upper 95% CI	12.47	10.98
P value (Wilcoxon test)	0.0155	

Table 32: Statistical Analysis Comparing RQRs of soiled and clean dry swab samples and soiled standard swabs

Statistical Parameter		Soiled Dry Swabs (A)	Clean Dry Swabs (B)	Soiled Standard Swabs (C)
Mean (RQR)		7.93	6.93	32.17
Number		16	16	16
Standard Error of Mean (SEM)		2.13	1.90	13.14
Lower 95% CI		3.39	2.89	4.17
Upper 95% CI		12.47	10.98	60.17
Rank sum difference (Dunn's Multiple Comparison)	Column A v B	-13.00	P > 0.05	
	Column A v C	-29.00	P > 0.001	
	Column B v C	-16.00	P > 0.05	

Table 33: Statistical Analysis Comparing RQRs from all Frozen Samples

Statistical Parameter	Dry Swab/ Clean	Dry Swab/ Soiled	Standard Swab/ Clean	Standard Swab/ Soiled
Mean (RQR)	0.8725	1.0975	1.7000	1.7825
Number	4	4	4	4
Standard Error of Mean (SEM)	0.02626	0.07565	0.1270	0.4176
P value (Non Para ANOVA)	0.0329			

Table 34: Statistical Analysis Comparing RQR values from all 4°C Samples

Statistical Parameter	Dry Swab/ Clean	Dry Swab/ Soiled	Standard Swab/ Clean	Standard Swab/ Soiled
Mean	5.195	5.777	8.495	11.510
Number	4	4	4	4
Standard Error of Mean (SEM)	2.391	1.547	4.391	3.724
P value (Non Para ANOVA)	0.0115			

Table 35: Statistical Analysis Comparing RQR values from all Room Temperature Samples

Statistical Parameter	Dry Swab/ Clean	Dry Swab/ Soiled	Standard Swab/ Clean	Standard Swab/ Soiled
Mean	9.910	10.852	22.435	48.005
Number	4	4	4	4
Standard Error of Mean (SEM)	4.675	4.836	13.763	32.967
P value (Non Para ANOVA)	<0.0001			

Table 36: Statistical Analysis Comparing RQR values of all 30º C Samples

Statistical Parameter	Dry Swab/ Clean	Dry Swab/ Soiled	Standard Swab/ Clean	Standard Swab/ Soiled
Mean	11.772	14.210	65.265	67.385
Number	4	4	4	4
Standard Error of Mean (SEM)	4.528	5.550	41.616	37.470
P value (Non Para ANOVA)	0.1415			

Table 37: Statistical data comparing all "like samples". All dry samples that had a corresponding standard sample (in terms of age and aging condition) were paired and the difference in the RQR values were statistically analysed.

Statistical Parameter	Dry Swab (Room Temperature)	Standard Swab (4°C)
Mean	10.381	10.003
Number	8	8
Standard Error of Mean (SEM)	3.119	2.726
P Value (Wilcoxon Paired Test)	0.6406	

5.4 Discussion

5.4.1 The Effects of Moisture on the Integrity of a Sample

The moisture level in a sample or in the surrounding environment can have an influential and negative effect on the integrity and preservation of biological evidence (Setzer *et al*, 2008). This is because moisture will sustain the existence of DNA degrading microorganisms (Garvin *et al*, 2013) and water itself can cause hydrolytic and oxidative damage to the DNA strand (Lindahl, 1993). The DNA molecule is particularly prone to hydrolytic damage (Mouttham and Schwarz, 2010) because the glycosides bond joining the bases to the sugars is susceptible to water (Lindahl, 1993) and when broken results in the loss of the purine (adenine and guanine) base (Höss *et al*, 1996). The pyrimidine bases (cytosine and thymine) are also open to hydrolytic deamination although the double helical structure offers greater protection from this form of degradation (Lindahl, 1993). Additionally water being an aggressive polar solvent can affect the hydrogen bonds between the base pairs in the DNA causing a weakening in the structure and reducing its structural resilience (Eglington and Logan, 1991). The phosphodiester bonds in the sugar-phosphate backbone of the DNA molecule are also labile and subject to quick hydrolytic cleavage (Demple and Harrison, 1994). It has been suggested that cleavage of the phosphate backbone is the most frequent type of hydrolytic damage (Mouttham and Schwarz, 2010). *In vivo*, in a fully hydrated system this event takes place about once every 2.5 hours (Mouttham and Schwarz, 2010). Most research concerning the effect of moisture has focussed on ancient DNA [Höss *et al*, (1996); Poinar and Stankiewicz, (1999)], where it is suggested that the long term survival of macromolecules such as DNA requires cool and dry conditions (Höss *et al*, 1996). In this experiment the focus was on the effect moisture has on biological samples, namely blood, stored for short periods of time (60 days). There is little data to quantify the effects of moisture on the integrity of blood samples over such short periods of time.

The TH01 singleplex assay developed in chapter three was used to measure the degradation levels in this experiment. Samples were set up so that the effects of moisture and bacteria on the degradation rate of DNA could be assessed. It was thought that the clean dry swab samples would reduce hydrolytic degradation in samples that were free of bacteria. The soiled dry swab samples would prevent hydrolytic damage whilst inhibiting DNA degradation by bacterial means. The clean standard swabs were thought to have no inhibition on the hydrolytic activity,

whilst the soiled standard swabs would allow both bacterial and hydrolytic degradation. Samples were paired (standard compared with dry) and comparisons made. The dry swab samples consistently produced lower RQRs than their respective standard swab samples. The mean RQR from all dry swab samples (all condition and all ages) was 7.43 compared to 28.32 from the standard swabs (see Figure 36 on page 184). The difference was statistically significant (Wilcoxon; $p = <0.0001$), suggesting a moisture free environment increased the preservation of samples across all temperature ranges (excluding frozen samples where no difference was observed and expected). Although it is uncertain which degradation process was inhibited this assessment it was clear that the removal of moisture had an effect, either via the reduction of hydrolytic, bacterial or enzymatic activity or more likely a combination of all three factors. This effect was immediate given the difference between the standard and dry swabs in the 10 day old samples was statistically significant (Wilcoxon; $p = 0.001$). Mouttham and Schwarz, (2010) suggested the rate of hydrolytic cleavage of the phosphodiester bonds decreased 20 fold in dry conditions. The effect of drying in this experiment was not as great. The results suggest that on average the degradation rate was decreased 4-fold in dry conditions. The difference in the rates observed may be due to the age of the samples. Samples in this experiment were only aged for 60 days whilst ancient samples were examined by Mouttham and Schwarz, (2010).

The clean/standard samples were compared with the clean/dry samples. The absence of bacteria in these samples meant that any differences in the degradation rate were likely to be due to hydrolytic effects. The mean RQR for the clean/standard samples was 24.47, whereas the mean value for the clean/dry samples was 6.94. These results suggest that samples stored in an environment containing moisture degrade at a rate four times greater than those samples stored in a moisture free environment. The effect appears to be greater over the course of time. After ten days the difference (RQR) between the two sets of samples was just over double (clean/standard swabs had an average RQR value 2.38 time greater than the clean/dry swabs), but this increased to 4.54 times by 60 days. A comparison of the differences between the two groups is shown in Figure 38. These results suggest that moisture plays a key role in the degradation process of DNA although its exact effect in this instance is unknown (Richter *et al,* 1988).

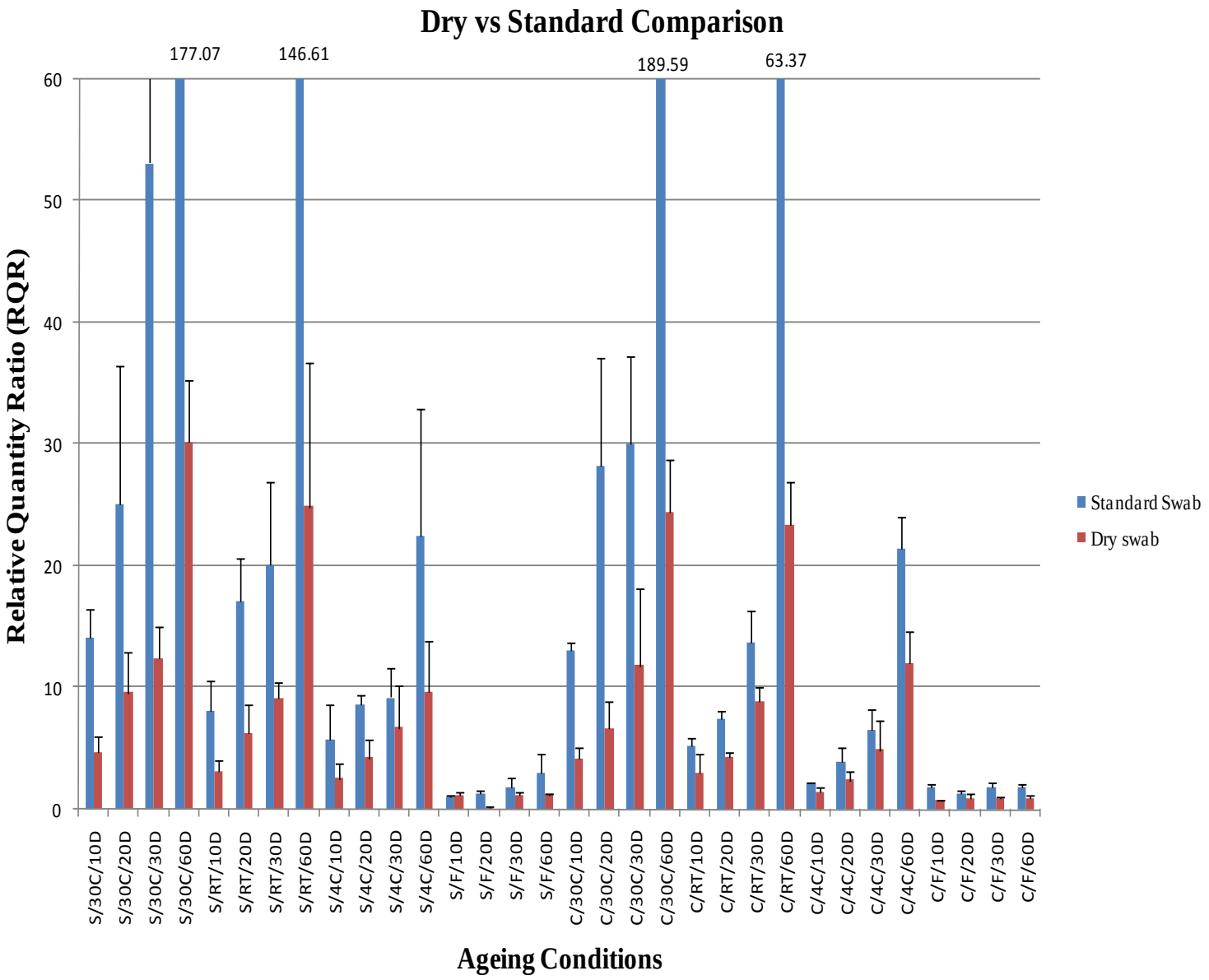


Figure 36: Comparison of the relative quantity ratios between dry and standard swab samples. RQRs were directly compared between paired samples. The RQRs from the Standard Swab samples were considerably higher in nearly all conditions suggesting a moisture free environment significantly contributes to the preservation of a blood sample. Note: Error bars represent 1 SD. Numerical values are included for samples that continued outside the range of the Y axis scale.

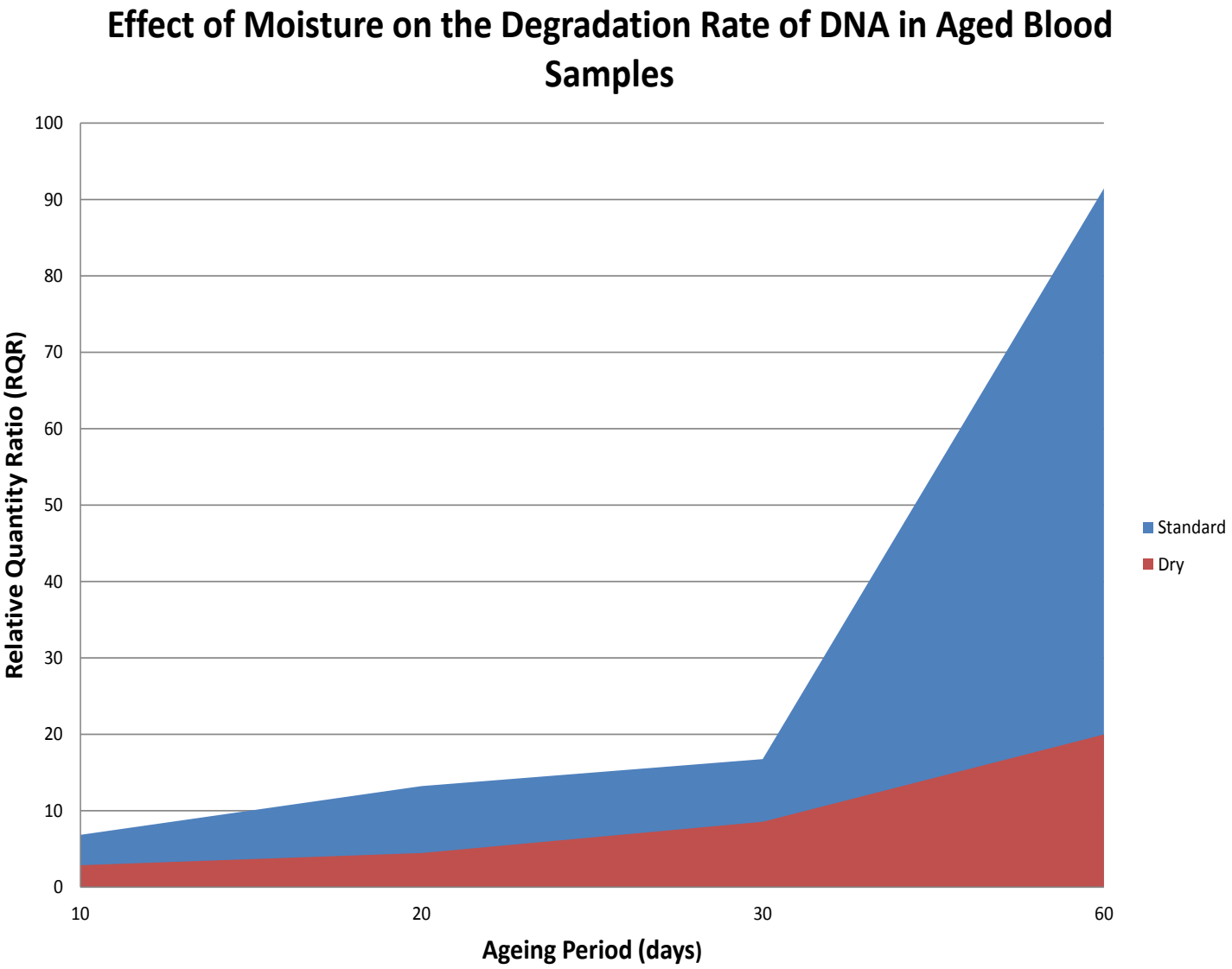


Figure 37: Effect of moisture on the relative quantity ratios (RQRs) of aged blood samples. The clean/standard swabs were compared to the clean/dry swabs to determine the effect moisture has on the DNA degradation levels in aged blood samples. The results clearly indicate that moisture has as significant effect on the degradation levels and this affect increases over time.

Although not as significant as the standard swab samples, samples stored in a dry environment still exhibited a degree of degradation over the course of 60 days. There are a number of reasons for this. First it is impossible to create a completely dry environment (Mouttham and Schwarz, 2010) therefore the DNA aged in the dry swabs were still susceptible to small amounts of hydrolytic degradation. Secondly, even when the environment is dry, DNA is still susceptible to oxidative attack. Free radicals (reactive oxygen species) such as hydrogen

peroxide, superoxide radical and hydroxyl radicals are all endogenously occurring products that cause DNA damage (Mouttham and Schwarz, 2010). These excited oxygen species are produced by UV radiation or are the by-products of aerobic metabolism (Richter *et al,* 1988). McCord *et al,* (2011) demonstrated the effect of these oxidizing compounds on DNA recovered from blood samples aged for three weeks. The blood samples were treated with hydrogen peroxide and bleach (NAClO) before being amplified using Powerplex 16 STR DNA profiling kit. The characteristic degradation curve seen with the loss of larger amplicons was due to the fragmentation of the genomic DNA (see Figure 36). The most vulnerable site for oxidative damage on DNA is on guanosine base, although the other three bases, adenosine, cytosine and thymine, can also undergo oxidative damage (Brock, 2013). The major site of oxidative attack on the DNA bases is the C=C double bond, leading to ring fragmentation and base modifications (McCord *et al,* 2011). Oxidative damage is a normal process and on any given day there is a low but detectable rate of damage present. In healthy cells the damage is proportional to the rate of metabolism as oxygen radicals are generated as side products during the conversion of glucose to energy (David *et al,* 2007). This rate of damage is balanced with DNA base excision repair, which involves removal and replacement of the oxidized base from the sugar-phosphate backbone (David *et al,* 2007). On first glance this oxidative damage is unlikely to explain the magnitude of the moisture effect observed in this experiment. However aged blood cells such as those used in this experiment may not have a functioning DNA base repair mechanism. Furthermore high levels of oxygen radicals are associated with cell death (David *et al,* 2007). This combination may explain the high DNA degradation rates observed. To determine whether oxidative stress was a major contributor to the levels of degradation observed, the levels of 8-oxo-2-deoxy guanosine can be measured (Evans *et al,* 2009). This should be considered in future studies. It is possible this well-accepted marker of oxidative stress may be a suitable target for ageing studies although increased levels are found in response to exposure to diverse external factors such as asbestos, ethanol, polycyclic aromatic hydrocarbons, metals, and ionizing radiation. Elevated levels are also associated with aging, diabetes, atherosclerosis, a host of cancers, as well as inflammatory and neurological diseases (Brock, 2013). Given levels of 8-oxo-2-deoxy guanosine can be so variable it may be difficult to standardise any forensic technique based on this variable.

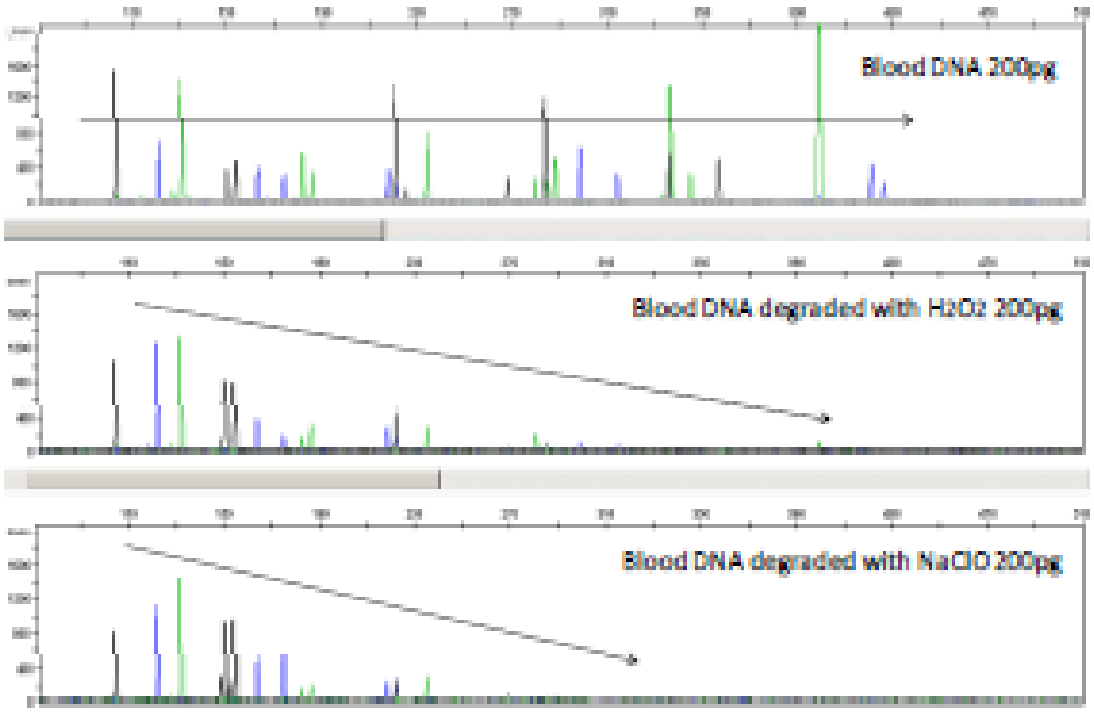


Figure 38: Effect of bleach and hydrogen peroxide on the integrity of DNA. A comparison of the amplification of a DNA sample extracted from blood with Powerplex 16 STR multiplex kit with the same sample treated with bleach and hydrogen peroxide. The data shows that the treatment of DNA with bleach and hydrogen peroxide results in allelic dropout, due to the chemical degradation of the DNA strand. Image adopted from McCord et al, (2011).

5.4.2 The effects of Bacteria on the Integrity of a Sample

Bacteria contain surface located endonucleases that quantitatively degrade extracellular DNA (Seto *et al*, 1975). It has been suggested that DNA is degraded by a cyclic mechanism that involves the attachment of DNA to the cell surface followed by nucleolytic attack (Seto *et al*, 1975). The breakdown of DNA results in fragments which are a recognised nutrient source for microbes (Overballe-Peterson *et al*, 2013). In order to determine the effect of bacteria on the integrity of DNA recovered from aged blood samples in this experiment, further comparisons were made. This was more difficult to determine as any environment that inhibit hydrolytic action, was also likely to inhibit bacterial growth and activity. The clean/standard samples were thought to be affected by hydrolytic activity only whilst the soiled/standard samples were

affected by both hydrolytic and bacterial activity. A comparison of the two groups can provide insight into the bacterial effect of degradation on blood samples.

The mean RQR for the soiled/standard samples was 32.17, whereas it was 24.47 for the clean/standard samples. On average the soiled/standard samples, which were exposed to both bacterial and hydrolytic degradation, had a 31.5% higher RQR value than samples that were exposed to hydrolytic activity only. Expressed as a percentage the RQR values of samples stored in a moisture free environment were 252% higher than those stored in an environment exposed to moisture. These results suggest that the degradational effect of bacteria is far less than that of hydrolytic (water) activity. After ten days the difference in the RQR values between these two groups of samples was 30% (7.28 compared with 5.58) whilst at 60 days the difference was 27% (87.27 compared with 69.05). This suggests that the effect of bacterial degradation is relatively constant. The difference between these two sets of samples is illustrated in Figure 39. The effect of temperature was also examined. Surprisingly at 30°C there was no significant difference in the mean RQR values. At 20 and 60 days the 30°C clean samples actually had mean RQRs greater than the respective soiled samples. This was not expected but could be explained if these samples had become contaminated thus allowing microbial growth. For samples aged at room temperature, there was a significant difference between soiled (mean RQR of 48.0) and clean samples (22.4). This 2-fold difference in the RQR values between these two sample sets suggests bacteria can double the degradation rate of samples aged at room temperature. The difference observed for samples aged at 4°C was less, with mean values of 11.5 and 8.5 for soiled and clean samples respectively. This suggests that the rate of DNA degradation caused by bacteria can be halved when the temperature is lowered from room temperature to 4°C. The effect of temperature is further discussed on page 192.

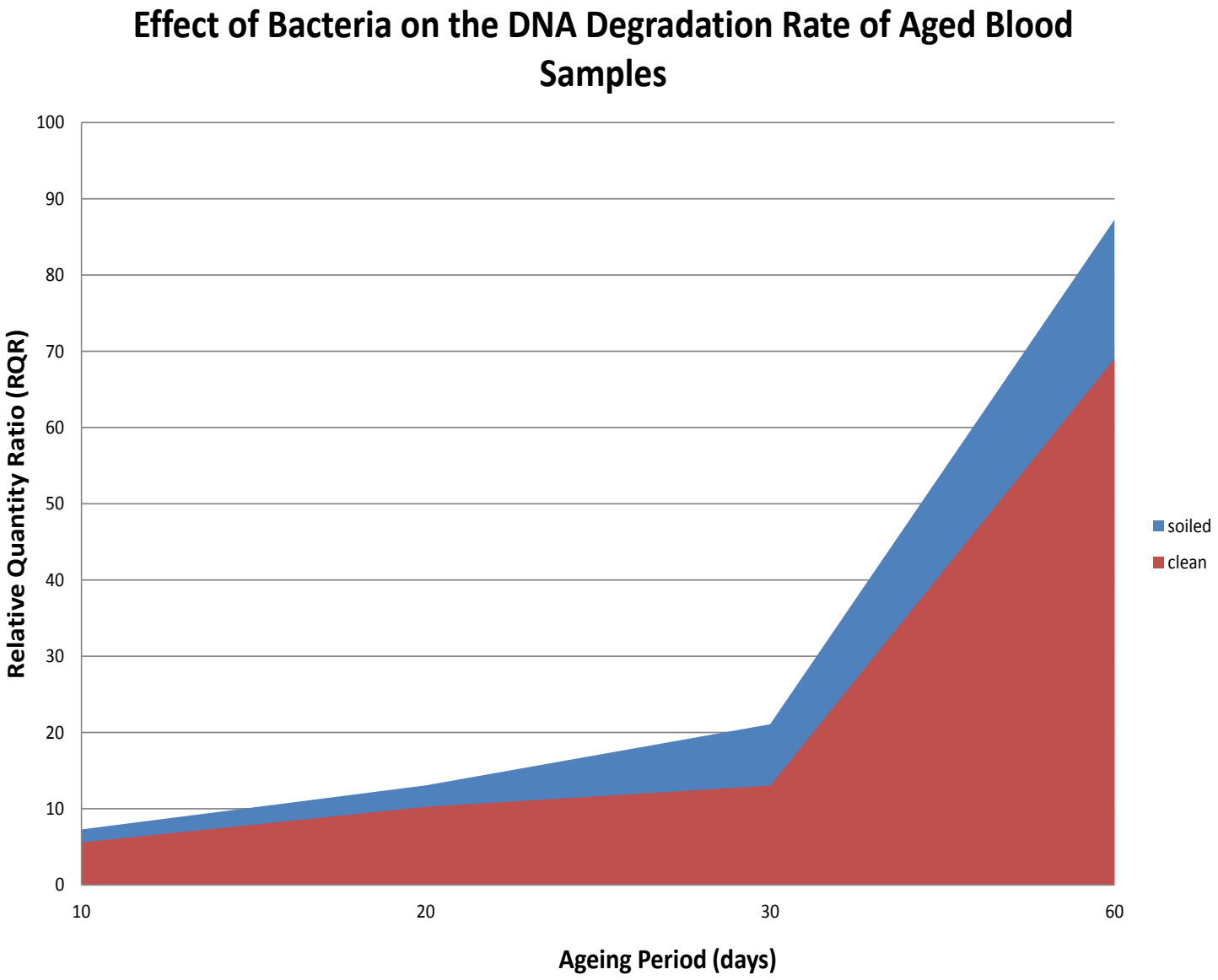


Figure 39: Quantifying the effect of bacteria on the integrity of blood samples. The RQRs varied marginally between the soiled and clean standard samples suggesting the degradational effect of bacteria on the integrity of a sample is less than that imposed by hydrolytic activity. The rapid increase in the RQR observed here, was similar to that observed when examining the effect of moisture. This suggests that the rate of degradation caused by bacteria is uniform or consistent over the 60 days.

The soiled and clean dry swab samples, which were thought to inhibit or reduce both hydrolytic and bacterial degradation, were compared with the soiled standard samples, which did not inhibit either. There was very little difference in the mean RQRs between the matched soiled (7.93) and clean (6.93) dry swab samples. This similarity suggests that the dry environment inhibited bacterial growth in the soiled samples. The similarities are illustrated in Figure 40 on page 191. The minor differences between these groups were still considered statistically significant (Wilcoxon; $p = 0.0155$) which could be explained if the removal of moisture was not immediate allowing some degree of bacterial and hydrolytic degradation during the initial days of the experiment. Even when bacteria and water were removed, the samples still underwent some level of degradation. This could be due to other environmental factors such as heat and UV light. It could also be due to the activities of endo and exonuclease enzymes that catalyse the cleavage of the phosphodiester linkages in the DNA backbone (Nishino and Morikawa, 2002) although these enzymes require energy and a cell without oxygen will deplete its energy source relatively quickly. As such nuclease degradation may cease relatively soon post mortem. Oxidative damage, which is independent of moisture is also a possibility as previously explained on page 182.

The RQR values from the clean dry swab samples were compared to the soiled standard swab samples which did not inhibit either degradation process. The mean RQR value for this set of samples was markedly higher (32.17). Using the RQR values as a guide the rate of degradation was approximately four times greater in samples that were not protected from bacterial and hydrolytic degradation processes.

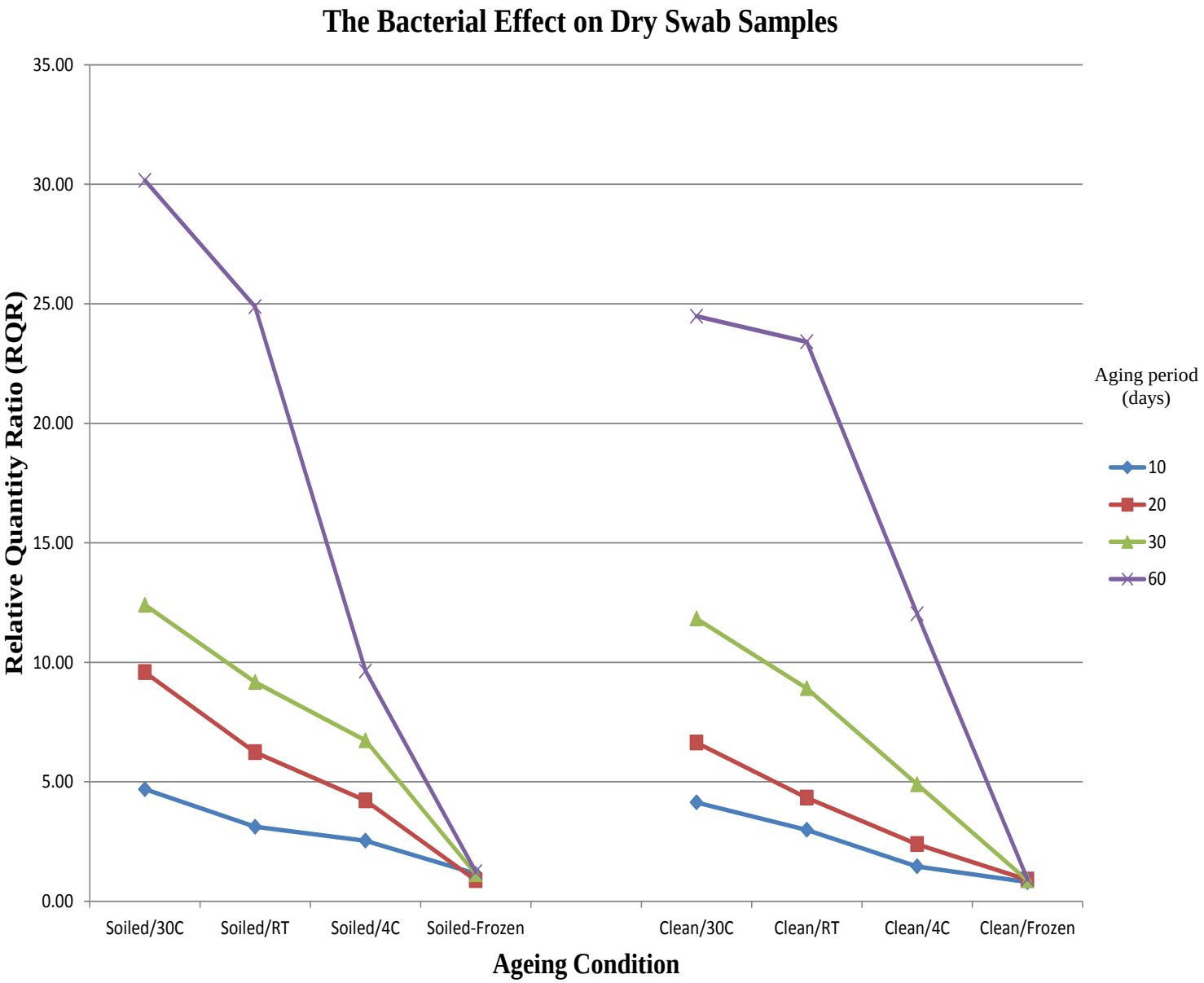


Figure 40: Effect of a dry environment of bacterial degradation. The RQRs varied marginally between the dry soiled samples and the dry clean samples, suggesting that the removal of moisture prevented any significant bacterial growth and hence bacterial degradation.

5.4.3 The Effects of Temperature on the Integrity of a Blood Sample

The samples used in this experiment were aged under various different temperatures therefore an assessment could be made as to what effect temperature has on the DNA degradation rate of blood samples (see Figures 42-45). In this experiment the frozen samples were primarily used for comparison purposes given freezing is considered the best approach for ensuring the DNA integrity of forensic biological samples (Garvin *et al,* 2013). The low RQR's obtained from the frozen samples, which ranged between 0.80 and 2.95, confirm this. These findings are supported by Cray *et al,* (2009) who found that blood samples stored over a period of 360 days were better preserved when stored at -70°C, as opposed to 4°C and -20°C. Furthermore Visvikis *et al,* (1998) found that long term storage at -70°C to -80°C had little impact on DNA yield or quality. Cushwa and Medrano (1993) conducted a study into the effect of temperature on the integrity of DNA. They reported that the optimal temperature for the short storage of blood (less than four weeks) was 4°C. Anything longer required freezing at temperatures below -70°C. They also concluded that blood stored at room temperature needed to be dealt with immediately and that blood stored at 37°C always yielded lower DNA amounts compared with blood stored at lower temperatures (Cushwa and Medrano, 1993). According to Roche, a manufacturer of biological kits and consumables, the ideal temperature varies depending on the source of the DNA. They suggest tissue is best stored in liquid nitrogen and stored at -80°C whilst buccal tissue and blood should be stored frozen at -20°C.

The mean RQR of the standard swab samples aged at 4°C in this experiment was 10.63. This was significantly greater than the results obtained from the frozen samples indicating samples stored at 4°C are affected by degradation processes. Halsall *et al,* (2008) examined the quality of DNA recovered from liquid and dried blood samples that were stored at 4°C for 24 hours. They observed no difference in the quality of either sample type when compared to fresh samples. Similar findings were observed by Lahiri and Schnabel (1993) who found there was little difference in the yield or quality of DNA extracted from blood stored at temperatures of 45°C, 37°C, 25°C, -20°C, or -70°C for 24 hours. Anderson *et al,* (1997) stored blood samples for up to 8 days at room temperature, 4°C and -20°C. They found no biologically meaningful changes in DNA damage after four days. The first measurement made in this experiment was after 10 days. The level of degradation of the standard swab samples stored at 4°C had increased 5-fold. By the conclusion of the 60 days the mean RQR of the standard swab samples was 22.45. These findings are consistent with those observation made by Richardson *et al,*

(2006). They found that long term storage of blood at 4°C had a detrimental effect on the amount and quality of DNA recovered. Richardson *et al*, (2006) used samples that were aged between 11 and 922 days. Although Cushwa and Medrano (1993) suggest blood can be stored by to four weeks without any significant effect on the quality of the DNA, the results in this experiment suggest otherwise.

This study was primarily concerned with determining whether a dry environment could reduce the rate of degradation of samples stored at room temperature. The mean RQR for standard swabs stored at room temperature, which were exposed to both the bacterial and hydrolytic, was 35.22. These results suggest the DNA in these samples was extensively degraded (35 times more degraded than at time 0). The mean RQR for the dry swabs stored at room temperature was 10.38, which suggests a 3-fold decrease in the rate of degradation with the removal of moisture at room temperature. Cushwa and Medrano (1993) examined blood samples aged at room temperature (23°C). They found that DNA could not be recovered from all of the samples tested. Those room temperature samples that DNA was recovered from produced lower yields than from samples stored at 4°C and -20°C. They demonstrated that refrigeration can slow the DNA degradation rate of blood samples. The results from this experiment support their findings. The mean RQR of the standard swab samples aged at 4°C was 10.64 (compared to 35.22 for the similar room temperature samples). This was comparable to the dry swab samples aged at room temperature (10.38). The difference observed was not statistically significant (ANOVA; $p = 0.64$), which suggests the removal of moisture from samples stored at room temperature has the same preservation effect as cooling samples to 4°C (see Figure 41 on page 194).

The results from this experiment suggest the preservation effect of refrigeration is more pronounced the longer the samples are stored for. After ten days the mean RQRs for the room temperature and 4°C samples were 8.14 and 5.75 respectively. After 60 days the RQR values were 146.61 and 22.24. Despite its obvious benefits, long terms storage of DNA at 4°C still resulted in degradation of the sample. These results support Abe *et al*, (2006) findings that samples stored at 4°C will still undergo some level of degradation but at a slower rate to samples stored at room temperature (Abe *et al*, 2006). Richardson *et al*, (2006) found that storing blood samples at 4°C for up to 922 days lead to a drop in DNA yield of approximately 20-25%.

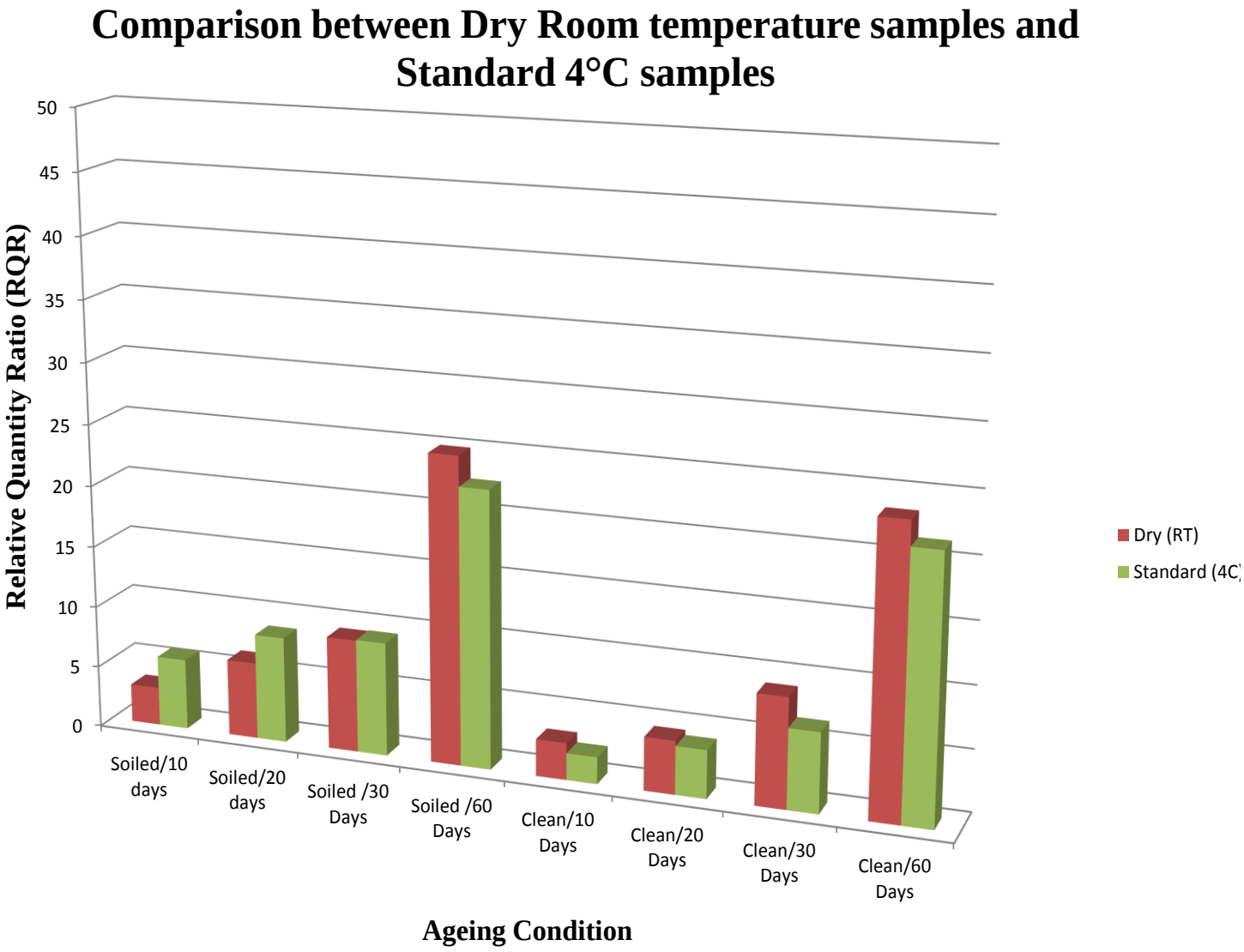


Figure 41: Quantifying the effect of moisture free environment. Like comparisons were made for the entire data set in an attempt to quantify the effect that the drying environment had on the integrity of the samples. If the variation that was observed is taken into consideration, the room temperature and 30°C dry swab samples had similar integrity levels as standard swabs that had been stored at 4°C.

There was a profound difference in the RQRs of samples stored at 30°C. Heat causes the hydrolytic cleavage of the N-glycosidic bond on the DNA strand, which results in the loss of the attached nucleic base (Zhang and Wu, 2005), as shown in Figure 41. This depurination process results in the formation of apurinic or "baseless" sites, which in turn create a change in the conformation shape of the DNA molecule (Lindhal, 1993). The change in the shape causes phosphodiester bonds to break which results in the cleavage of the DNA strand (Zhang and Wu, 2005). Although Karni *et al*, (2013) found that complete DNA degradation occurred at temperatures above 190°C, Sugiyama *et al*, (1994) suggested that thermal DNA degradation via the production of apurinic sites occurs at temperatures as low as 30°C. It is estimated that the rate of depurination is approximately one event every ten hours *in vivo*. Healthy living cells have efficient mechanisms to repair apurinic sites, such as base and nucleotide excision repair (Wu and Wang, 1999). The functionality of this repair mechanism in aged *ex vivo* blood cells is likely to be absent or minimal, making depurination one of the principle forms of DNA degradation in ageing samples (Vijg and Suh, 2013). It has been suggested that the rate of depurination increases and is dependent on temperature (Mouttham and Schwarz, 2010) and therefore is likely to have contributed significantly to the degradation of the samples aged at 30°C in this experiment.

The mean RQR for all standard swabs aged at 30°C was 66.32 which is a 200% increase from all samples aged at room temperature samples. McGrath (2010) suggested that the rate of DNA degradation increases significantly when the temperature is raised from 50°C to 70°C with a rapid initial reduction in DNA copy number followed by a more gradual period of degradation. The results from this experiment suggest the increase in degradation rate can begin occurring at temperatures as low as 30°C. The difference between these two temperature groups (30°C and room temperature) was 25.1% when the comparison was conducted on the dry swab samples. These results suggest that the effect of heat degradation is reduced in moisture free environments. Alternatively removing the moisture may reduce the number of apurinic sites that are created from the hydrolytic cleavage of the N-glycosidic. This is a more likely explanation. Cushwa and Medrano, (1993) suggested that the yield and quality of DNA recovered from samples stored at 37°C will always be compromised, even after storage for 24 hours. This contradicts the findings of Lahiri and Schnabel (1993), who reported no difference in the quality or quantity of DNA from blood stored in identical conditions after 24 hours. Samples in this experiment were not measured after 24 hours so no comparison could be made

however there was a significant decrease in the quality of the DNA after ten days (mean RQR of all 30°C after ten days was 9.06)

These findings highlight the need to deal with forensic biological samples so that they are not stored at 30°C. Even a reduction in storage to room temperature can have a significant effect on the DNA degradation rate of the sample.

Figure 42: Depurination and subsequent cleavage of the phosphodiester bond. In step 1 heat causes the hydrolytic cleavage of the N glycosidic bond, releasing the nucleic base. This causes a conformation change in the shape of the DNA molecule which results in the breakage of the phosphodiester bonds.

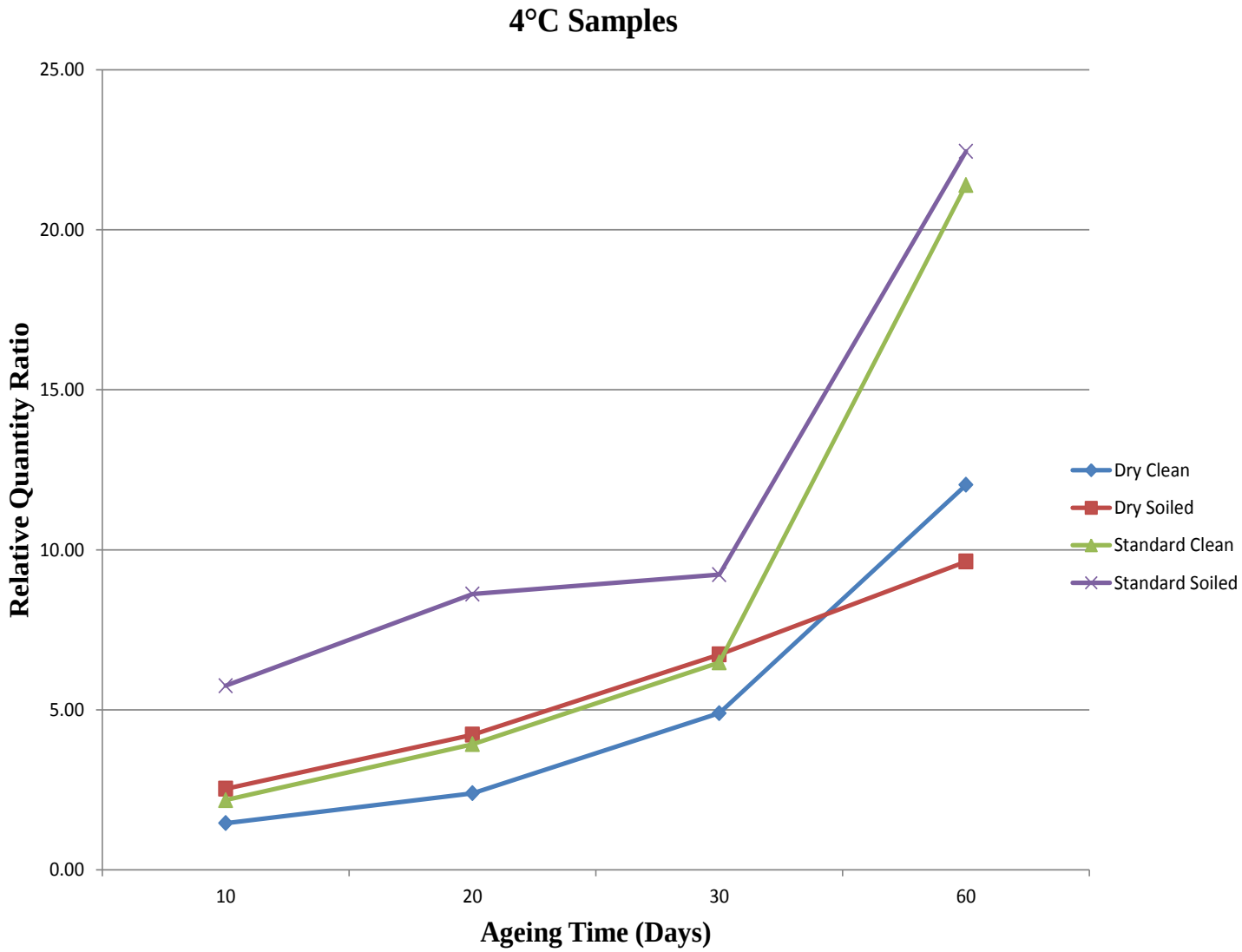


Figure 43: Comparison of all samples aged at 4°C. Lowering the ageing temperature to 4°C reduced the rate of degradation, although it fell short of preventing it completely. The dry samples (soiled and clean) and the standard clean samples presented with very similar mean RQRs. The one exception was the standard clean samples aged for 60 days.

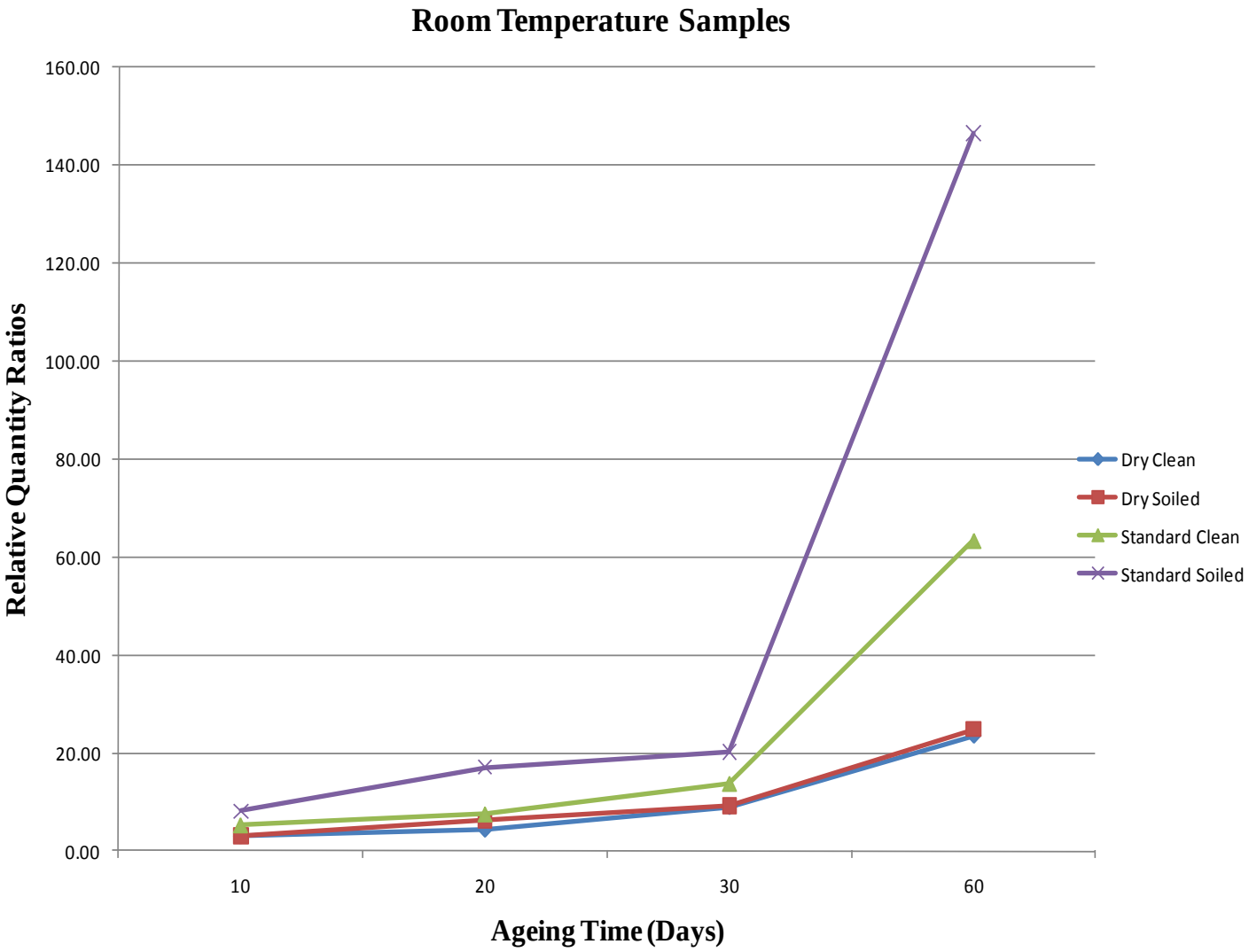


Figure 44: Comparison of all samples aged at Room Temperature. Trends were similar to those observed with the 4°C samples. The two categories of dry samples (soiled and clean) had very similar trends suggesting the inhibition of bacterial degradation. These samples had a greater integrity level than the Standard clean samples which may be explained by the dry environment reducing the effect of intra-cellular degradation processes. An important observation that has practical implications was that the dry swab samples did not preserve the samples to the same extent as the process of freezing.

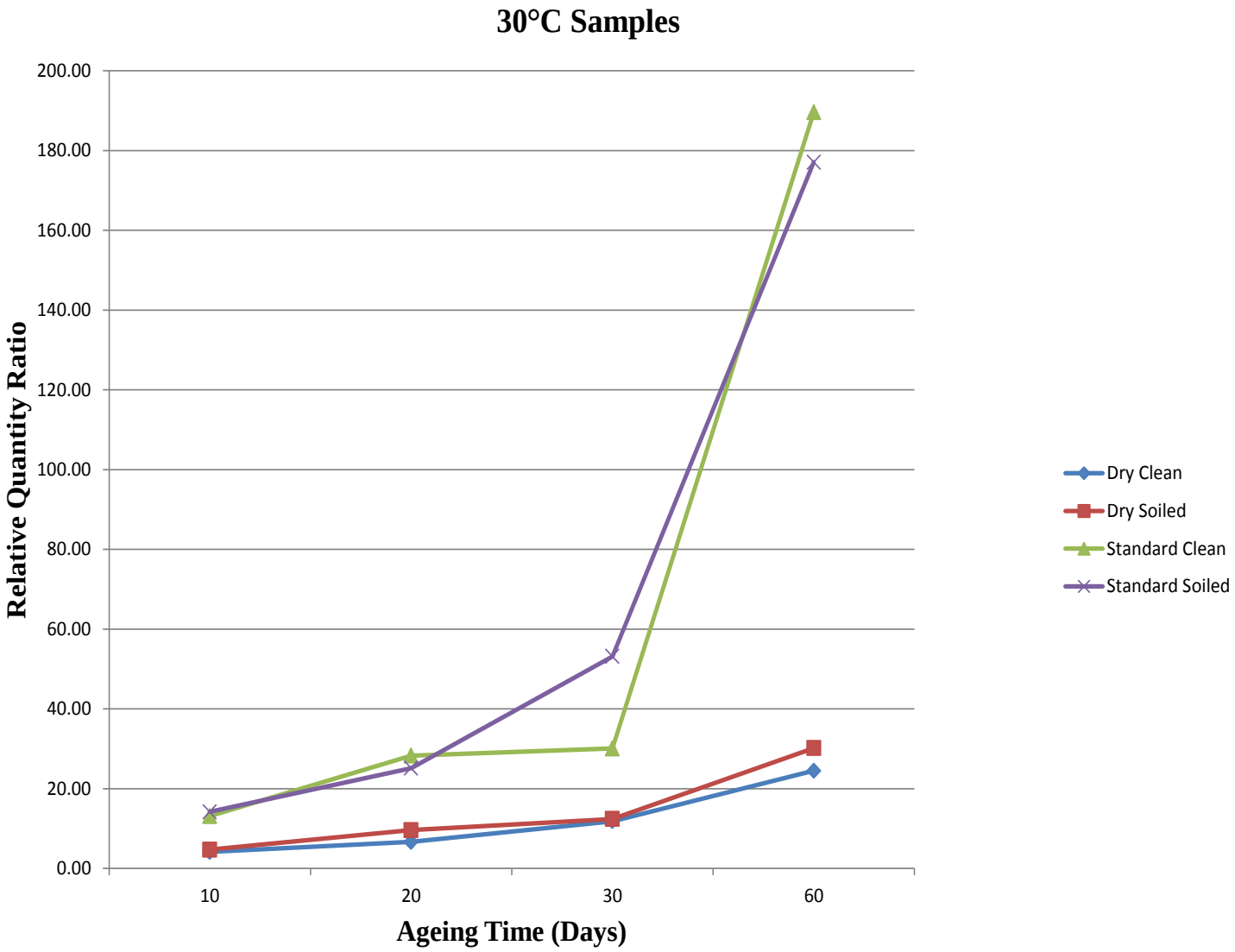


Figure 45: Comparison of all samples aged at 30°C. The dry swab samples again produced lower RQRs than their corresponding standards swab samples. The two categories of standard swab samples (clean and soiled) presented with similar RQRs.

5.4.4 Relationship between Degradation rate and Sample Age

The mean RQR values from the dry swab samples aged under each different condition were examined to determine if there was a correlation with the age of the sample. The correlations are shown in Figure 46. Samples aged at room temperature were important from a forensic perspective as most biological forensic samples are stored and transported at room temperature (Lee *et al*, 2012). There was a strong correlation with both the soiled and clean dry samples over the course of the 60 days [$R^2 = 0.985$ (soiled); $R^2 = 0.978$ (clean)]. This suggests there is a strong correlation between the two variables, mean RQR and sample age. This was unexpected given results from earlier experiments in chapter three suggested that DNA degradation rates were not a suitable measure of sample age. The samples in this experiment were stored differently, in that they were aged in a moisture free environment thus minimising degradation by hydrolytic and bacterial activity. These two factors may occur inconsistently or at different rates which would explain the large inter-donor variation observed in chapter three. Hoss *et al*, (1996) observed that environmental conditions have a greater influence on DNA degradation rates than time and these conditions are inconsistent and result in weak correlations between sample age and the preservation of DNA. The environmental conditions referred to included temperature, humidity (moisture) and pH (Hoss *et al*, 1996).

The intra-donor variation in the RQR values was examined to determine the reliability of this method as an age indicator. The variation when expressed as a percentage of the mean was very similar between the dry (26.3%) and standard samples (28.9%). Statistically the difference in the mean values were not significant (ANOVA; $p = 0.487$). The variation for the dry samples ranged from 1.8% to 53.7% however there was no observable trend with sample age or condition. The variation for the standard samples ranged from 2.8% and 65.8%, which was slightly greater than the dry samples.

The size of the variation observed was large and based on experience one would expect the inter-donor variation for this type of analysis to be even larger. Despite mean RQRs being somewhat useful for predicting the age of sample, the variation observed makes this approach unreliable for single sample use. The results provide further support regarding the inability of DNA fragment (based on size) analysis to reliably estimate the age of a sample.

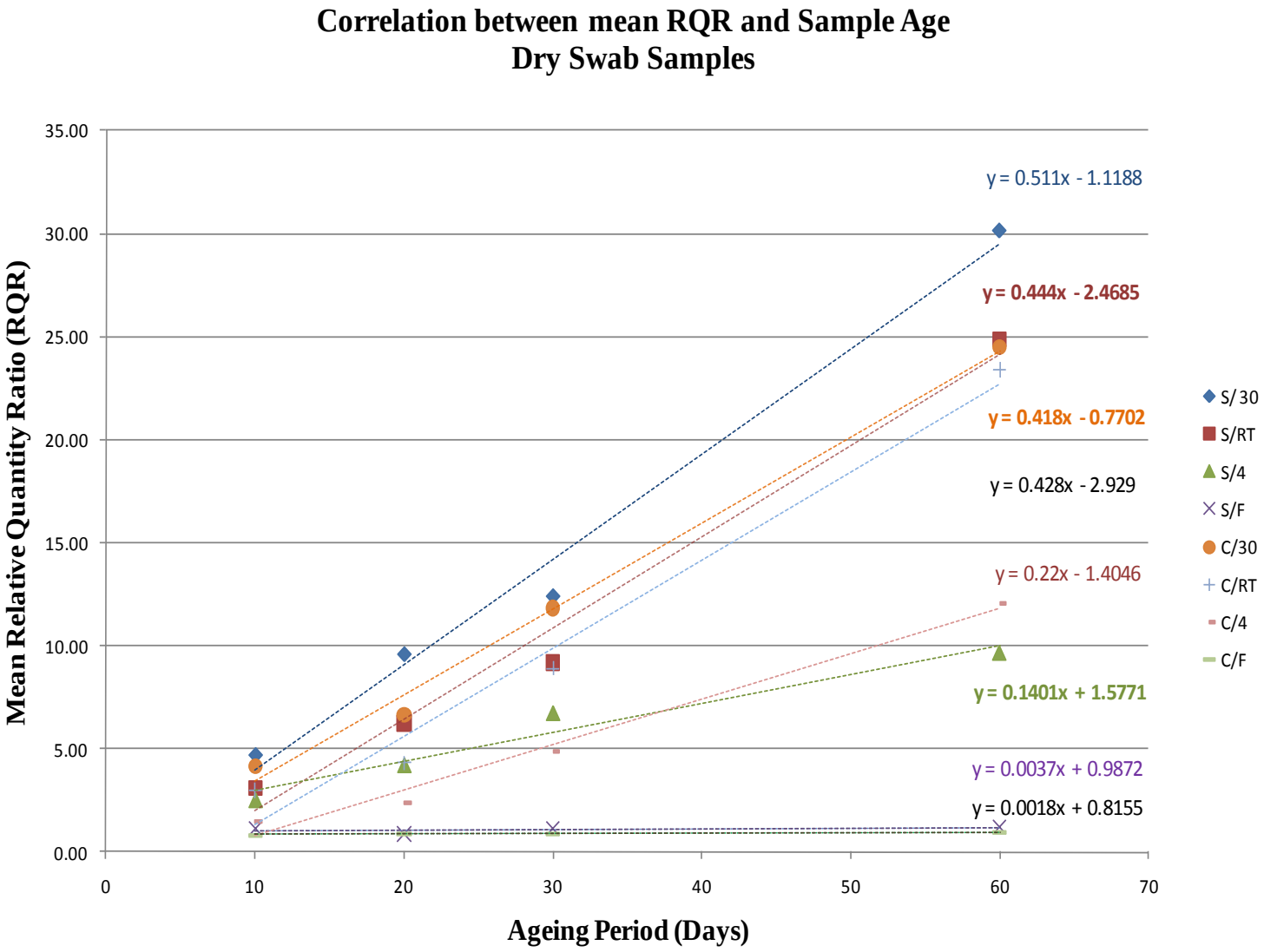


Figure 46: Correlation between mean RQR values and sample age (Dry swab Samples). The correlation between the RQR and sample age was strong ranging from 0.994 (Clean, 30°C) to 0.956 (soiled, 4°C). These correlations were greater than those obtained from the standard swab samples suggesting that the rate of degradation by bacterial means is variable between samples.

5.4.5 Stability of the "Internal Urban" environment

In chapter three it was hypothesised that an internal urban environment was a relatively stable environment and that any optimal sized targets determined for that environment could be applied to other similar environments. Samples in the previous chapter were aged over the summer months of June, July and August, whilst samples in this experiment were aged for a shorter period of time (only 60 days), but over the winter months of January and February. This meant a comparison could be made between like samples to determine whether seasonal variation had an effect on the "relatively stable" internal environment. The samples from this experiment that could be directly compared to samples from earlier work were those categorised as clean, standard swabs aged at room temperature.

The mean RQRs from chapter three were compared to the mean values obtained in this study, the results of which are presented in Figure 47 on page 203. The same target sizes were used (64 and 239bp) to maximise the reliability of the data. The results suggest the time of year has a significant effect on degradation levels despite being aged in an identical internal environment (Wilcoxon; $p = 0.125$). The seasonal effect observed was larger than expected suggesting prolonged differences in temperature may have an additive effect on the integrity of a sample. Based on the RQRs, samples aged over the summer months degraded at a rate of more than three times faster than those aged during the winter months. The results suggest this assay (based on two different sized amplicons) lacks the precision required of a forensic application. For example, a RQR of 60 could indicate the sample was between 30 and 60 days old, depending on the time of year. This range is large and more often than not would be unable to make a significant contribution to the investigation process. Samples aged over both seasons complicate the issue further.

The large variations between donors and now seasons, highlights the limitations of this type of DNA analysis to accurately predict the age of a sample, which confirms findings from earlier work. These results also show that an internal urban environment is not as controlled or stable as suggested and that prolonged temperature differences (even if only minor differences) can have a significant effect on the integrity of a biological sample.

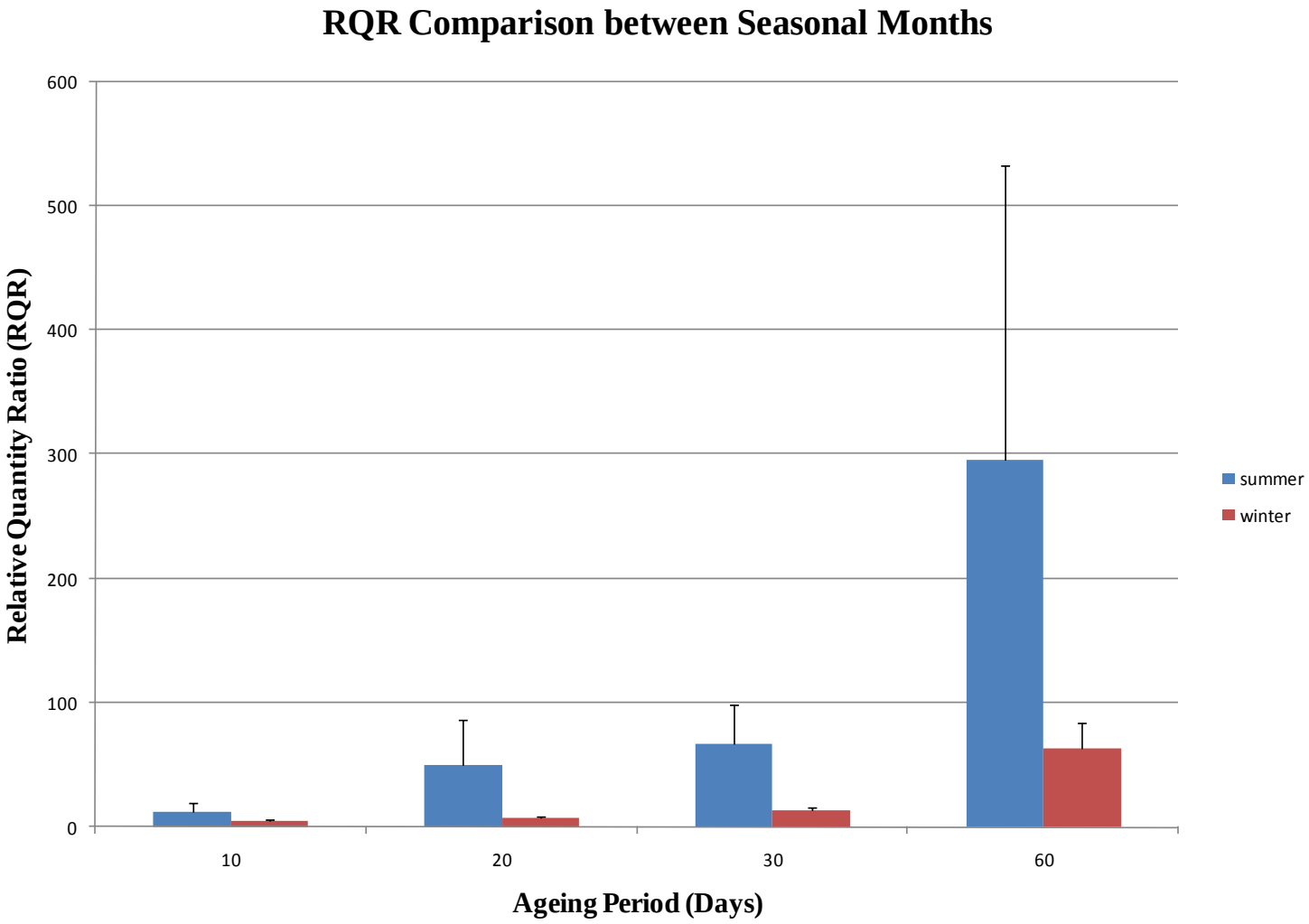


Figure 47: Seasonal comparison of aged blood samples in the same internal urban environment. The RQRs differed substantially between samples aged over different times of the year. The mean summer RQRs were higher at all ageing periods indicating a greater level of degradation level within these samples. These results suggest that an internal urban environment is not as stable as predicted. Error bars represent 1SD. For the summer samples the inter-donor variation is displayed but with the winter samples, only a single donor was used therefore the intra-donor variation is presented.

5.5 Limitations

One issue raised during this study was the possible contamination of the clean samples. No clean swabs were tested for bacterial growth and it was assumed that they were sterile and remained free from bacterial growth. There was evidence to suggest that the standard clean samples aged at 30°C may have been contaminated however because clean samples were not tested, this was an area of uncertainty that could not be resolved.

5.6 Conclusion

Moisture plays a significant role in the degradation of DNA (Garvin *et al*, 2013). It not only promotes bacterial growth, which digests DNA, but it also causes hydrolytic damage to the DNA strand (Bruskov, 2002). Whilst there are numerous methods for reducing the degradational effects of moisture, such as freezing, police forces require simpler and cheaper methods of preserving biological samples. The experiments conducted here were aimed at quantifying the degradational effect of moisture and to develop a hermetically sealed dry swab that could preserve biological evidence to a standard that will alleviate the need for freezing or refrigerating blood samples recovered from crime scenes. The results suggest freezing blood samples is still the most effective method for preserving the integrity of DNA. This study showed that storing forensic blood samples at 30°C can have a detrimental impact on the DNA integrity of blood samples stored for longer than ten days. Other authors have had similar findings where degradation levels are compromised with temperatures above 30°C [Cushwa and Medrano, (1993); Ade, *et al* (2006)]. Reducing temperature from 30°C to room temperature can have a significant effect on DNA degradation levels as shown in this experiment. The results from this experiment also suggest that removal of moisture from samples stored at room temperature has the same preservation effect as cooling samples to 4°C.

There have been numerous attempts to develop new methodologies to preserve the integrity of DNA in biological samples. Lee *et al*, (2012) tested the capability of SampleMatrix (SM; Biomatrica, Inc., San Diego, CA), a novel room temperature DNA storage medium, to preserve DNA in biological stains that had been aged for up to one year under varying conditions. The conditions included a typical ambient laboratory environment as well as exposing samples to three successive freeze-thaw cycles. They concluded there was no substantial differences between the quality of samples stored frozen in liquid and those samples maintained dry at

ambient temperatures protected in SM. For long-term storage and the storage of low concentration samples, SM provided a significant advantage over freezer storage through higher DNA recovery. Frippiat *et al*, (2011) examined two DNA storage technologies have been developed based on anhydrobiosis, Qiasafe (Qiagen) and Gentegra (Genvault). They found that both these technologies helped preserve the quality of DNA in biological samples, although results were dependent on the extraction method applied (Frippiat *et al*, 2011).

The results from the experiment conducted here suggest the hermetically sealed dry swab is also capable of preserving the integrity of DNA in aged blood samples. The ability to stabilize and protect DNA from degradation at ambient temperatures for extended time periods could have a tremendous impact in simplifying and improving sample storage conditions and requirements. These findings could potentially impact the way police constabularies handle biological evidence in the UK as most samples are stored at room temperature. Since completing this research dry swabs like those used in this experiment have been marketed (see Bode Technology) therefore police and forensic laboratories now have access to an alternative cheap, simple and effective method for preserving blood samples. The results from this experiment suggest that using a swab that removes moisture from the sample environment can significantly reduce both the bacterial and hydrolytic degradation processes up to 4-fold.

The singleplex assay used in this experiment was also assessed to determine whether it was suitable for ageing blood samples. Although strong correlations between RQRs and sample age were identified with samples that had been aged in moisture free environments, the variation observed significantly reduces the reliability of age estimations. This assay was used to compare samples used in chapter three, which were aged at different times of the year. The results suggest that that an internal "urban" environment is not as stable as predicted and that seasonal temperature variation can have a large effect on degradation rates therefore any optimisation must be done in accordance to the defined setting and time of year. This is a drawback for any potential forensic application.

CHAPTER 6: AN INVESTIGATION INTO WHETHER COMPONENTS OF THE INFLAMMATORY RESPONSE CAN BE USED TO AGE BLOODSTAINS

6.1 Introduction

Since the introduction of DNA profiling in 1984, DNA has been the molecule of choice when it comes to forensic biological examinations (Saferstein, 2006). Whilst it has primarily been applied for identification purposes, it has been used for various other applications such as determining the age of biological samples (Anderson *et al,* 2005) and assessing the "quality" of evidence (Niederstatter *et al,* 2007). However, most forensic laboratories do not routinely carry out a quality assessment of the DNA sample before performing STR profiling because the trialled DNA methodologies have not proven sufficiently reliable (Swango *et al,* 2007). There are various reasons for this, including the random and unpredictable nature at which DNA is degraded *ex vivo* (Craig *et al,* 2012), the differences in loci susceptibility to degradation (Hudlow *et al,* 2008), large inter-donor variation and the lack of optimisation of the relevant assays. These factors have meant that DNA degradation levels cannot be used to accurately and reliably estimate the age of bloodstains. Anderson *et al,* (2005), Bauer *et al,* (2003) and Zubakov *et al,* (2008) have explored the use of RNA to assess the age of biological samples. Although each author employed different methodologies, they all relied on the degradation rates of different RNA molecules to age samples. Whilst their results were encouraging they still lacked the necessary precision to be applied as a routine forensic tool. A successful RNA based ageing technique must be capable of reliably measuring the change of expression level of the target RNA species. A number of issues must be considered when developing such a technique including whether such methods are susceptible to the same limitations as the DNA methodologies described above. Selecting an appropriate RNA target will also largely determine the success of any technique (Zubakov *et al,* 2008). A suitable target is one that degrades at a consistent rate over time (Bauer *et al,* 2003) and exhibits little variation between individuals. This experiment aims to address these issues using components of the inflammatory response which is largely influenced by RNA levels.

The experiments carried out in chapters three, four and five involved blood samples that had aged for extended periods of time (up to 90 days). In this study the focus shifted to bloodstains aged for less than ten days. It was thought that the environmental and inter-donor variation observed in earlier work may be reduced to manageable quantities by using shorter time

frames. Although generalised patterns have been determined the variations observed in earlier work have limited these techniques and ultimately rendered them inadequate for forensic purposes [Bauer *et al*, (2003); Anderson *et al,* (2005)]. The shorter time frames were still applicable to forensic samples as crimes, particularly serious crimes involving the deposition of blood, are usually reported within ten days. Using shorter ageing periods presents its own set of unique problems. The main issue concerns the targets species themselves, which would need to exhibit a rapid, quantifiable change in expression during the specified time frame (Rogler *et al,* 2004). Rapid changes occur often and quite naturally *in-vivo* to ensure the processes involved in haemostasis are tightly maintained and regulated [Tang *et al,* (2006); Fuchs *et al,* (2013)]. However the complication lies in that forensic samples are essentially *ex-vivo* samples, therefore the change in expression needs to occur in blood samples that have been extracted from the body.

Pahl and Brune (2002) performed a time-wise analysis of three proinflammatory genes known to be sensitive to extracellular stimuli following phlebotomy. They observed changes in Tissue Necrosis Factor (TNF-α) and Interleukin 6 (IL-6) expression levels between sample acquisition and the beginning of analysis and even though these changes were unexplained, they were measurable over ten days. TNF-α is a pleiotropic inflammatory cytokine produced by a number of different cells, but primarily by macrophages (Olszewski *et al*, 2007). The cytokine serves a variety of functions including the regulation of immune cells, apoptotic cell death and inflammation (Swardfager *et al*, 2010). It possesses both growth stimulating properties and growth inhibitory processes, and it also appears to have self-regulatory properties (Murray *et al,* 1997). TNF-α is primarily produced as a 212-amino acid-long type II transmembrane protein arranged in stable homotrimer. From this membrane-integrated form the soluble homotrimeric cytokine (sTNF) is released via proteolytic cleavage by the metalloprotease TNF converting enzyme (Tang *et al,* 1996). The human TNF-α gene (*TNF*) maps to chromosome 6p21.3, spans about 3 kb and contains 4 exons. The 3' UTR of TNF-α contains an AU-rich element (ARE). Interleukin-6 (IL-6) is a monomer of 184 amino acids that acts as both a pro-inflammatory and anti-inflammatory cytokine (Janeway *et al*, (2001). It is secreted by T cells and macrophages to stimulate an immune reaction in response to trauma (Ray *et al*, (1989) such as infection, burns and neoplasia (Fernandez-Botran, (1995). Its functions range from key roles in acute-phase protein induction to B- and T- cell growth and differentiation. (Delves *et al*, 2011). At sites of chronic and acute inflammation the protein is secreted into the serum and induces a transcriptional inflammatory response through interleukin-6 receptor. The single

gene that codes for IL-6 is mapped to chromosome 7p21 and is 6119 bases in length (Ray *et al*, 1989). The expression changes in IL-6 and TNF-α observed by Pahl and Brune (2002) were thought to be caused by the collection and preparation techniques that occurred *ex vivo*. Blood deposited at a crime scene will not be exposed to anticoagulants or other stabilising reagents therefore these extraneous factors will not be relevant to this experiment. Of interest in this experiment was how the expression of IL-6 and TNF-α levels change in ageing bloodstains. Pahl and Brune (2002) demonstrated that the expression change of IL-6 and TNF-α increased around 20 fold after three days before showing a continual decline. These large changes over a short period of time made them a potentially suitable for aging studies using RNA degradation rates. Pahl and Brune (2002) also observed that the inter-donor variation with IL-6 and TNF-α was small which provided further support for the use of these particular target species in this study.

The aim of this experiment was to determine whether IL-6 and TNF-α expression levels changed in aged blood samples aged for up to ten days and if so whether these changes correlated with the age of the sample.

6.2 Methodology

6.2.1 Sample Preparation

After receiving ethical approval and informed consent blood samples were collected and prepared according to section 2.1.6 on page 84. Details of the eight Caucasian donors can be found in the table below. All donors confirmed that were healthy (to the best of their knowledge) at the time samples were collected.

Table 38: Age and Sex of Donors

Sample	D1	D2	D3	D4	D5	D6	D7	D8
Sex	Male	Female	Female	Male	Female	Female	Male	Male
Age (years)	25	21	21	22	21	21	21	30

6.2.2 Sample Processing

After samples had aged for the desired length of time, the RNA was extracted using a modified organic method (Invitrogen, US). The modifications were necessary because the blood samples had clotted. The modifications involved longer incubation times and more vigorous lysis steps. A detailed description can be found in section 2.2.1 on page 85. The extracted RNA was treated with DNase I in accordance with manufacturer's instructions (Invitrogen, US). This ensured the samples were free of any contaminating DNA. Details can be found in section 2.3.1 on page 88. All RNA samples were measured using a NanoPhotometer (Implen, Germany). This device has a number of key advantages over standard spectrophotometry, such as its ability to analyse trace samples. Samples as small as 1 µl can be measured, which is significantly smaller than the 15 µl required by standard spectrophotometry using a fluorescent RiboGreen dye (Anderson *et al*, 2005). Furthermore the nanophotometer has a fast analysis time and is more accurate than a standard single wavelength spectrophotometer reading as each sample is measured at four different wavelengths from which the concentration and purity is calculated (www.implen.de). Given that it cannot distinguish between DNA and RNA, all RNA samples were treated with DNase I (to remove residual DNA) prior to any analysis. Settings and protocols can be found in section 2.5 on page 90.

All RNA samples were subsequently converted to cDNA. The reverse transcription was carried out using Superscript II enzyme and random hexamers (Invitogen, UK) and according to the manufacturer's instructions. Details can be found in section 2.6.1 on page 90. The resulting cDNA was then amplified using a standard Real Time PCR run protocol and a SYBR green detection system. A reaction volume of 25 µl was used which contained 2x Applied Biosystem's TaqMan Universal SYBR Green PCR Master Mix (Applied Biosystems, US), cDNA, RNAse free water (Eppendorf, US) and the appropriate volume of primers such that final concentrations (both forward and reverse) of TNF-α and IL-6 were 400nM and 150nM for 18S rRNA. The standard run protocol described in section 2.7.2 on page 93 was used. Data from the real-time PCR was analysed by the Applied Biosystem 7500 SDS software (version 1.4.0).

6.2.3 Primer Sequences

Primer sequences were obtained directly from the authors, Pahl and Brune (2002). NCBI Blast searches were carried out on each sequence to verify the correct target. The TNF-α and IL-6 primers were obtained from MWG (Germany), whilst the 18S rRNA primers were obtained from Applied Biosystems (US). The sequences, where available are shown in Table 39.

Table 39: Primer Sequences for Il-6 and TNF-α

Primer Name	Forward Primer	Reverse Primer
TNF-α	ATTCTCCTTCCTGATCGTGGC	GGGTTTGCTACAACATGGGC
IL-6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
18S rRNA	Primers supplied by Applied Biosystems therefore sequences not available	

6.3 Results

6.3.1 Assay Optimisation

The amplification efficiency rates for each target species were calculated using methods previously described in 3.3.1.2 on page 105. The results from the serial dilution can be found in Figure 48, whilst the calculated efficiency rates are presented in Table 40.

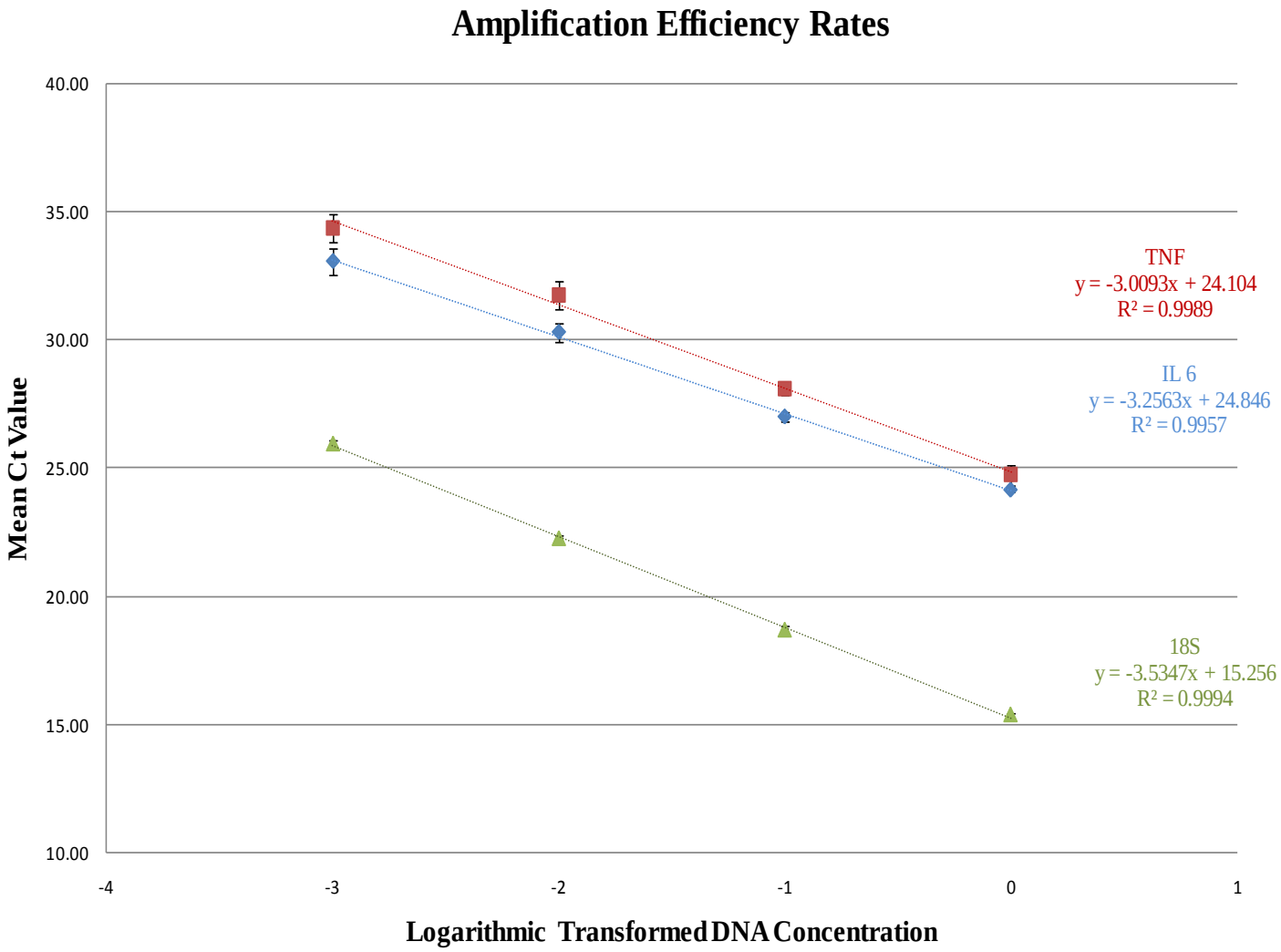


Figure 48: Serial Dilution Assay. Amplification efficiency rates of TNF-α, IL-6 and 18S r RNA were calculated for each of the target species using a serial dilution method. The trend lines all presented with strong correlations as indicated by the R^2 values. The error bars (represent ±1SD) were small indicating the reproducibility was good

Table 40: Mean Ct values and Amplification Efficiency Rates for IL-6 and TNF-α. To calculate the amplification efficiency rate of each target (TNF-α. and IL-6) and the 18S rRNA reference gene, a 10-fold serial dilution was made using high quality human genomic DNA (promega). This dilution series was then measured by each target using qPCR. The mean Ct values of each target for each dilution were plotted and the efficiency rate was determined from the slope of the lineal regression line of best fit.

Target	Concentration (relative)	Mean (Ct value)	Std Dev	Difference	Slope	Efficiency Rate (%)
TNF-α	Neat	24.13	0.04			
	1/10	26.99	0.17	2.86	**3.01**	**114.9**
	1/100	30.29	0.39	3.30		
	1/1000	33.06	0.50	2.76		
IL6	Neat	24.73	0.41		**3.26**	**102.8**
	1/10	28.08	0.22	3.34		
	1/100	31.75	0.53	3.67		
	1/1000	34.36	0.55	2.61		
18S rRNA						
	Neat	15.36	0.09		**3.53**	**91.8**
	1/10	18.67	0.18	3.31		
	1/100	22.25	0.12	3.58		
	1/1000	25.95	0.15	3.70		

6.3.2 18S rRNA Stability: Suitability as a Reference Gene

Although 18S rRNA is widely used and accepted amongst the scientific community as a housekeeping gene for whole blood (Bas *et al*, 2004), the results from chapter four highlight the need to test its stability under the given conditions of the particular experiment in question. In order to achieve this, three donors were randomly chosen and blood samples extracted and processed. Samples were measured in triplicate with the mean values presented in Figure 49. There was no significant increase in the Ct value over the ten day period indicating the stability of 18S rRNA target under these conditions for this period of time.

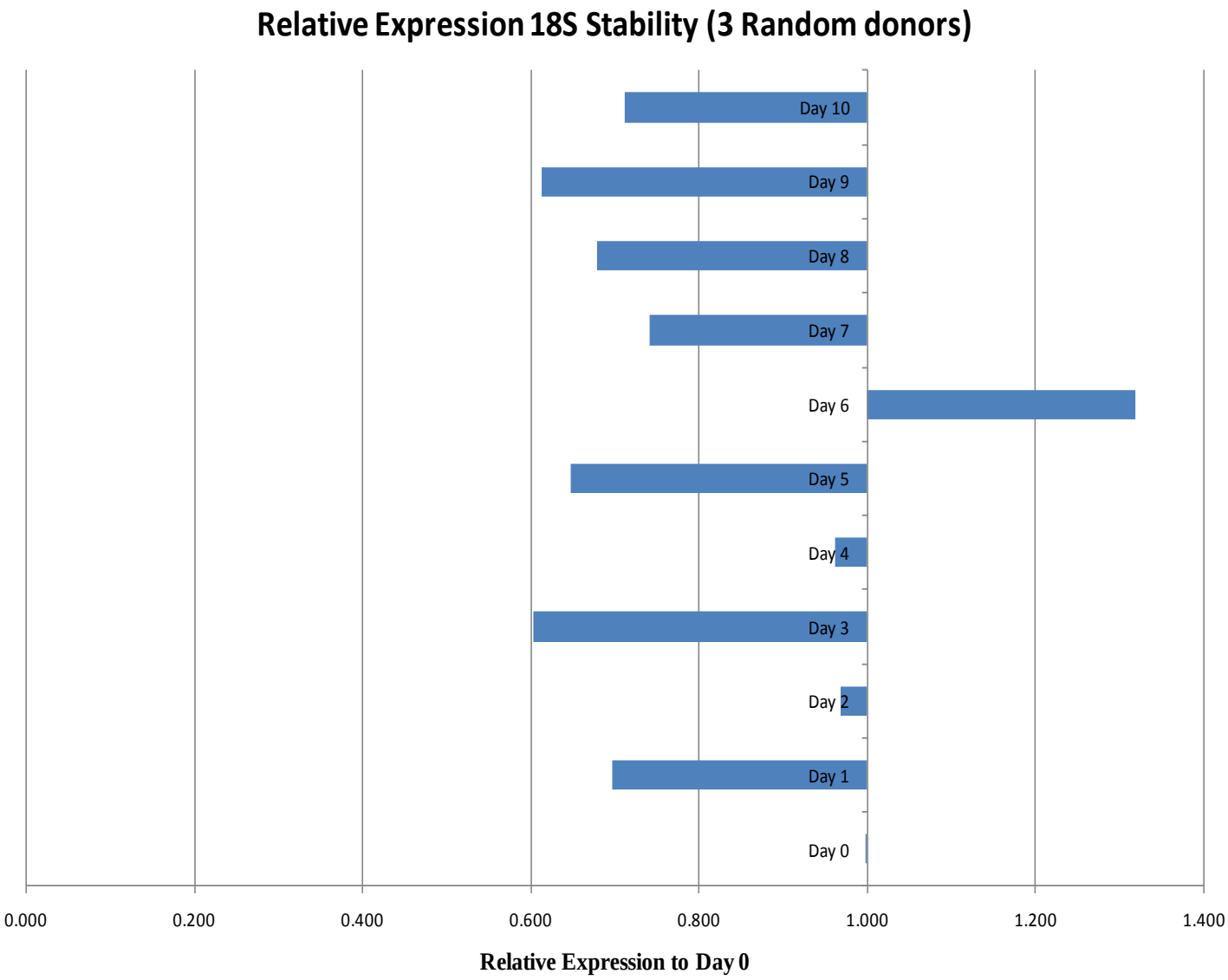


Figure 49: 18S rRNA Stability Assay: The stability of 18S rRNA was assessed by measuring the expression levels from three randomly chosen donors over the ten days. Each donor contributed a sample for each time period and each sample was measured in triplicate. Results from all donors and all triplicates were averaged and the relative expression values (according to day 0 samples) were calculated and are presented here.

6.3.3 Relative Expression Values

The Ct values for each donor at each ageing period (duplicate samples) are presented in Appendix VI. The relative expression values (RE) were calculated from the Ct values as previously described in Equation 3 in section 2.8 on page 94. The RE values for TNF-α are presented in Table 41 whilst the RE values for IL-6 are contained in Table 42.

Table 41: Relative Expression of TNF-α. The quantities of TNF-α were calculated for each donor at each time period (daily). The quantities were converted to a relative expression (compared to time 0 samples) and the expression values are presented in this table.

Relative Expression of TNF-α								
Aging Period (days)	**Donor 1**	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**	**Donor 6**	**Donor 7**	**Donor 8**
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
1	1.392	0.304	1.067	0.645	1.894	0.858	0.585	0.576
2	0.609	0.096	0.040	0.681	0.683	0.410	0.205	0.443
3	0.726	0.028	0.041	0.268	0.433	0.207	0.012	0.114
4	0.025	0.023	0.012	0.696	0.088	0.039	0.006	0.029
5	0.026	0.012	0.006	0.100	0.103	0.051	0.006	0.024
6	0.007	0.013	0.005	0.070	0.093	0.004	0.002	0.010
7	0.005	0.008	0.001	0.002	0.037	0.002	0.005	0.002
8	0.000	0.002	0.000	0.001	0.011	0.000	0.003	0.005
9	0.001	0.000	0.000	0.041	0.004	0.002	0.007	0.002
10	0.000	0.001	0.008	0.002	0.002	0.002	0.002	0.002

Table 42: Relative Expression of IL-6. The quantities of IL-6 were calculated for each donor at each time period (daily). The quantities were converted to a relative expression (compared to time 0 samples) and the expression values are presented in this table.

Relative Expression of IL-6								
Aging Period (days)	**Donor 1**	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**	**Donor 6**	**Donor 7**	**Donor 8**
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
1	2.584	1.467	4.848	0.601	1.000	0.909	0.881	1.363
2	2.953	1.688	2.129	1.498	0.527	0.325	0.729	1.677
3	3.423	0.976	1.235	1.018	0.145	0.362	0.334	1.275
4	0.247	0.318	0.774	0.755	0.169	0.185	0.325	0.638
5	0.276	0.542	0.325	0.455	0.090	0.055	0.334	0.301
6	0.592	0.578	1.103	0.163	0.063	0.083	0.013	0.016
7	0.057	0.011	0.249	0.311	0.060	0.099	0.010	0.002
8	0.003	0.010	0.037	0.195	0.005	0.002	0.009	0.002
9	0.003	0.005	0.045	0.158	0.002	0.004	0.012	0.004
10	0.001	0.004	0.049	0.017	0.009	0.003	0.004	0.003

6.3.4 Inter-Donor Variation

The variation in the relative expression values between donors (inter-donor variation) was calculated and expressed as percentage co-variance (%CV). Results are presented in Table 43.

Table 43: Inter-donor variation of IL-6 and TNF-α. The relative expression values were compared between donors to see if levels of IL-6 and TNF-α were similar at each age period. The high variation (%CV) shows the quantities of IL-6 and TNF-α at each age period varied significantly between donors.

%CV	**Ageing Period (Days)**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
IL-6	53.01	60.84	100.87	192.05	90.97	128.56	141.10	121.48	179.74	94.17
TNF	77.10	57.38	88.42	55.78	51.75	112.99	109.46	188.98	173.77	133.01

6.4 Discussion

The ability to calculate the age of a bloodstain left at a crime scene is something that continues to elude forensic scientists around the world (Bremmer *et al*, 2012). Many different methods have been used to try and solve this problem, such as electron paramagnetic resonance spectroscopy (Fujita *et al*, 2005), enzymatic methods (Ballantyne, 2008) and DNA degradation methods (Anderson *et al*, 2005). Some of the best results to date have involved the analysis of RNA (Zubakov *et al*, 2008). Most of these studies have involved longer ageing periods and have been limited by the large inter-donor variations observed. This particular study aimed to address this issue by using bloodstains that had been aged for shorter periods of time (ten days) and by analysing RNA targets that had shown rapid changes in expression levels *ex vivo*. Pahl and Brune (2002) identified two species, IL-6 and TNF-α, that displayed measurable changes in expression for up to seven days following extraction from the body making them a suitable target for this particular forensic investigation.

The relative quantities or expression levels of each target species were determined using a normalisation process involving 18S rRNA as a reference gene. It is generally accepted that 18S rRNA is a relatively stable component in blood tissue over extended periods of time (Bas *et al*, 2004), although the exact period of time differs between authors [see for example Anderson *et al* (2005); Bas *et al*, (2004)]. The findings in chapter four suggested that the 18S rRNA stability was maintained for at least 20 days in blood samples that are stored at room temperature. This finding is consistent with Anderson *et al*, (2005) and Thellin *et al*, (1999). In this experiment 18S rRNA expression remained relatively stable over the ten days (see Figure 49) and therefore was a suitable normalisation gene for this study.

6.4.1 IL-6

Based on the work carried out by Pahl and Brune (2002), IL-6 was expected to show elevated expression levels over the first few days. This expected change was only observed in donor 1 and donor 3 and was limited to the first three days only (see Figure 50 on page 218). Although an increase was observed it did not reach the magnitude (20 fold increase) reported by Pahl and Brune (2002). The greatest increase was slightly less than 5-fold (donor 3, day 1). Three of the eight donors (donor 5, 6 and 7) showed no increase in IL-6 expression at all. IL-6 expression is normally low and levels are usually nondetectable in the absence of inflammation (Ershler *et*

al, 1993) suggesting the extraction of blood from these donors did not trigger an inflammatory response. There are a number of possible reasons for this. The inflammation process is a natural immune response to tissue damage and individual variation in the response is common (Franco *et al*, 2013). A low immune function (underactive immune system) can result from lifestyle habits such as stress, lack of exercise, a low nutritional diet as well as age (Castaneda *et al*, 1995). Immunodeficiency disorders, which predominately target the thymus, lymph nodes and bone marrow, prevent the body from acting against pathogens and tissue damage (Delves *et al*, 2011) and can reduce IL-6 levels (Ishiyama *et al*, 1994). The inflammatory response involves a cascade of cytokines such as IL-1, chemokines and TNF-α (Labow *et al*, 1997). Any reduction in these factors can lead to a reduced response and therefore lower IL-6 expression levels. Furthermore TNF-α is one of many factors that induce IL-6 production (Huang *et al*, 2009). In this experiment low TNF-α levels were observed therefore the results suggest there was a reduced amount of IL-6 stimulating factors present. There are a variety of other IL-6 stimulating factors such as lipopolysaccharide, which is bacteria derived, and platelet derived growth factor (Tosato and Jones, 1990) which were not examined, but may have contributed to the low levels observed. There are also pathological conditions, such as haemodialysis, that are hypo responsive to IL-1 and TNF-α stimulates (Le Meur *et al*, 1999). Any pathological condition that results in a hypo responsive effect will effectively reduce IL-6 expression levels. Any of these factors could have resulted in the absence of the expected increase in IL-6 expression.

There is an alternative disposition in that an increase in IL-6 expression was present, but masked by internal and external degradation factors. The environment in which the samples were aged was not protected from microbes, which are known to degrade nucleic acids (Siuda and Chrost, 2000). Certain types of bacteria have the ability to rapidly cleave and inactivate IL-6 through the release of arginine- and lysine-specific proteinases (Banbula *et al*, 2002). The bacteria use multiple cleavage sites within the N and C-terminal region of the IL-6 polypeptide chain (Banbula *et al*, 2002). After the initial proteolytic cleavage, IL-6 is further degraded by other enzymes released by the bacteria.

Whilst the results from the first three days were inconsistent as collectively donors presented with elevated, reduced or unchanged expression levels of IL-6, there was a consistent decrease in expression after day four, although the size of the decrease varied between donors. This pattern generally continued for the remaining six days.

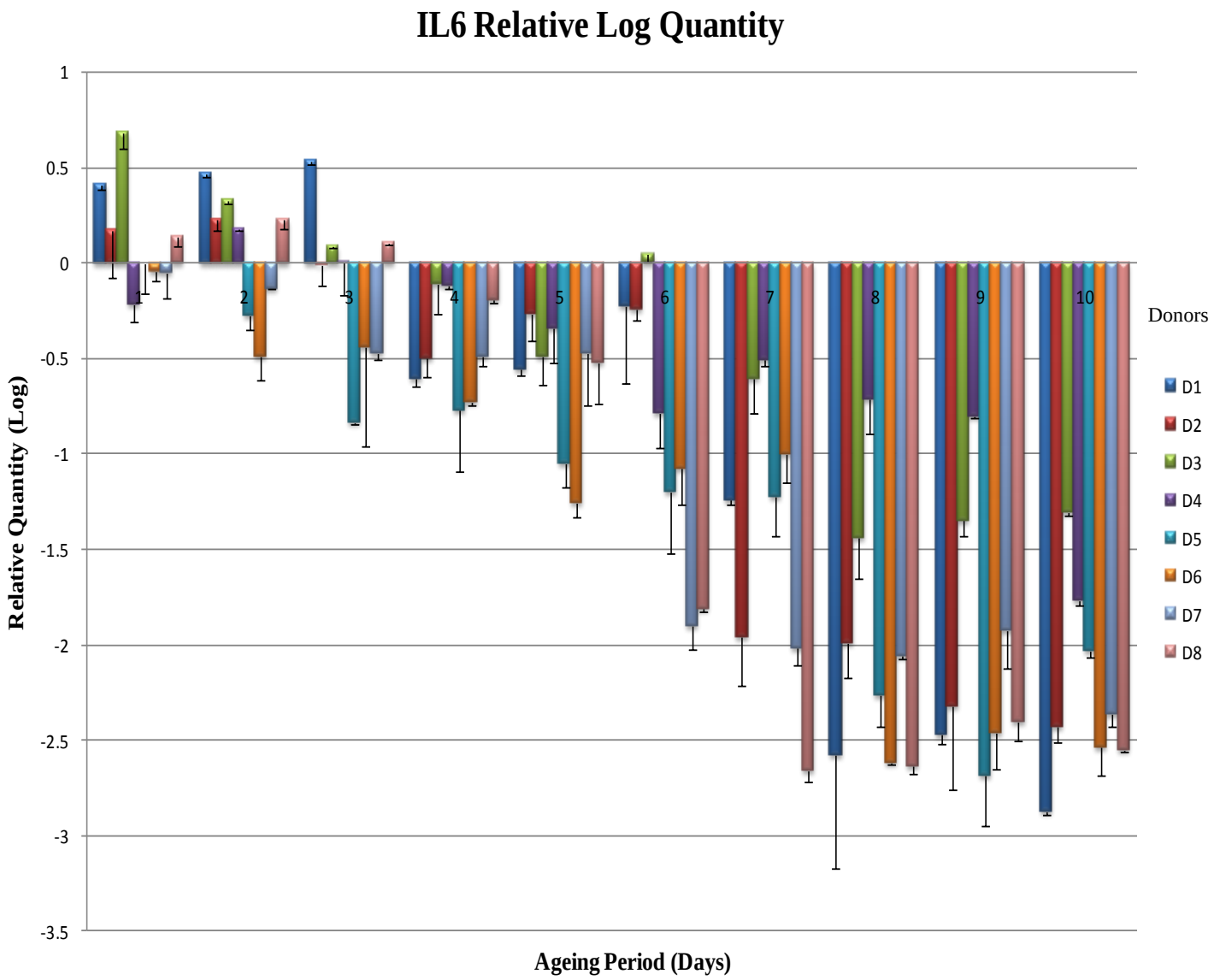


Figure 50: Relative Log quantities of IL-6 for each donor across each of the ten days. A number of donors showed an increase over the first three days as expected however this observation was not consistent amongst the entire population. After day four there was a downward trend in expression levels from all donors. Results from day nine and ten did not follow the general trend with a plateau effect being observed as the decline in expression levels tapered off. Error bars represent 1SD.

Mean values for this data were determined and are presented in Figure 51 on page 221. Whilst the mean data suggests that expression levels of IL-6 were elevated for the first 24 hours only, this increase was less than 2-fold. The mean expression levels of IL-6 continued to decrease after day 1 and this trend was consistent for the entire ten days (though there was little different between the mean values at day eight, nine and ten). The observed degradation was not unexpected. Both the IL-6 and TNF-α targeted in this assay are both species of messenger RNA. *In vivo* RNA expression is tightly regulated by various transcriptional and translational processes, according to the requirements of the particular microenvironment (Cooper and Sunderland, 2000). These processes include the regulation of transcription by specific factors that alter the specificity of RNA polymerase for a given promoter, the binding of repressors that impede RNA polymerase and the activities of activators, enhancers and silencers (Austin and Dixon, 1992). Post-transcriptional events such as the capping, splicing and addition of a poly-A tail (Cooper and Sunderland, 2000), as well as translational processes that are regulated by RNA binding proteins (Babitzke *et al,* 2009) all play a role in RNA expression. A cell transcribes more RNA than accumulates which indicates the cell has an active RNA degradation system. In general, RNA is degraded at the end of its useful life, which is long for a ribosomal RNA but very short for excised introns or spacer fragments, and is closely regulated for most mRNA species (Houseley and Tollervey, 2009). RNA molecules with defects in processing, folding, or assembly with proteins are identified and rapidly degraded by the surveillance machinery (Houseley and Tollervey, 2009). Environmental factors, such as UV light and heat (Pichon *et al,* 2012) and ribonuclease activity largely account for RNA degradation *ex vivo* (Auer *et al,* 2003). These processes cause a shortening of the poly-A tail and the removal of the G cap, which then allows nuclease enzymes to degrade the mRNA molecule (Rainen *et al*, 2002).

There was an unusual anomaly observed in the data obtained from day nine and ten samples. The mean IL-6 expression at these time periods were similar to the values obtained from day eight, suggesting the degradation rate was either significantly reduced or there was an increase in IL-6 expression that was equal to the rate of degradation. Pahl and Brune (2002) noted significant increases in IL-6 after three to four days however if these results were due to an increase in IL-6 it occurred much later in this study. Mandl *et al,* (1995) noticed that one of the degradation products of fibrinogen, FDP-D stimulated IL-6 production in *in vitro* monocytes, macrophages and leucocytes. Like blood deposited at a crime scene, the samples extracted and stored in this experiment were exposed to the normal clotting processes. It is therefore possible

that the results observed were caused by an increase in IL-6 production which resulted from the formation of FDP-D. It has been suggested that the stimulatory effect of FDP-D on IL-6 production might be the consequence of elevated IL-1 levels (Csala *et al*, 1999). Whether FDP-D and IL-1 were responsible remains unknown as these components were not measured. There were a number of other anomalies observed in the relative expression data (RE) which included an increase in IL-6 expression (1.10) by donor three at day six. Donor four presented with an immediate decrease in expression at day one (0.66), which was then followed by an increase the following day (1.50). These anomalies remain unexplained.

The mean relative expression levels show a definite decreasing trend in the levels of IL-6 over the ten day period however the inter-donor variation observed in this experiment meant that samples could not be accurately aged. Whilst it was possible to distinguish the older samples (aged for eight, nine or ten days) from the younger samples (aged less than five days), it was not possible to distinguish samples between each day. The inter-donor variation observed could be due to any number of factors. Some of these have already been discussed and include deficiencies in the immune and inflammatory response to the trauma experienced (extraction of blood) by each donor (Franco *et al*, 2013). There are other situations and conditions that can cause elevated levels of IL-6 (Birx *et al*, 1990). IL-6 is significantly elevated with exercise which is significant in terms of the activities that often lead to bloodshed of a criminal nature. Volunteers used in this study were not subjected to any exercise before extracting blood therefore samples tested here may not be representative of certain forensic samples. Elevated levels of IL-6 are often found in a wide range of inflammation-associated disease states, including susceptibility to diabetes mellitus and systemic juvenile rheumatoid arthritis (Nawata *et al*, 1989). Increased IL-6 levels are associated with hypertetomenia in patients with diabetes (Jain *et al*, 2003) and human immunodeficiency virus (Birx *et al*, 1990). IL-6 is also highly expressed in tumour microenvironments (Roca *et al*, 2009). Furthermore IL-6 has major effects on haematopoiesis and thrombopoiesis and there are often elevated levels associated with malignant cells [Fernandez-Botran, (1995); Delves *et al*, (2011)]. Low levels of IL-6 have been found in patients with autoimmune liver disease, including primary biliary cirrhosis (Tovey *et al*, 1989). IL-6 has both pro and anti-inflammatory functions. Smooth muscle cells in blood vessels produce IL-6 as a pro-inflammatory cytokine and additionally osteoblasts secrete IL-6 to stimulate osteoblast formation (Marriott *et al*, 2004). IL-6 role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-α and IL-1, and activation of IL-1 and IL-10.

IL-6 has numerous roles across many different biological and pathological pathways and expression levels can change in response to many different stimuli. Given the nature of IL-6 the variable levels of expression between donors observed in this study is more likely to be a normal rather than an abnormal phenomenon. This variability is detrimental to any forensic ageing methodology and as such it is unlikely IL-6 is suited to this type of analysis.

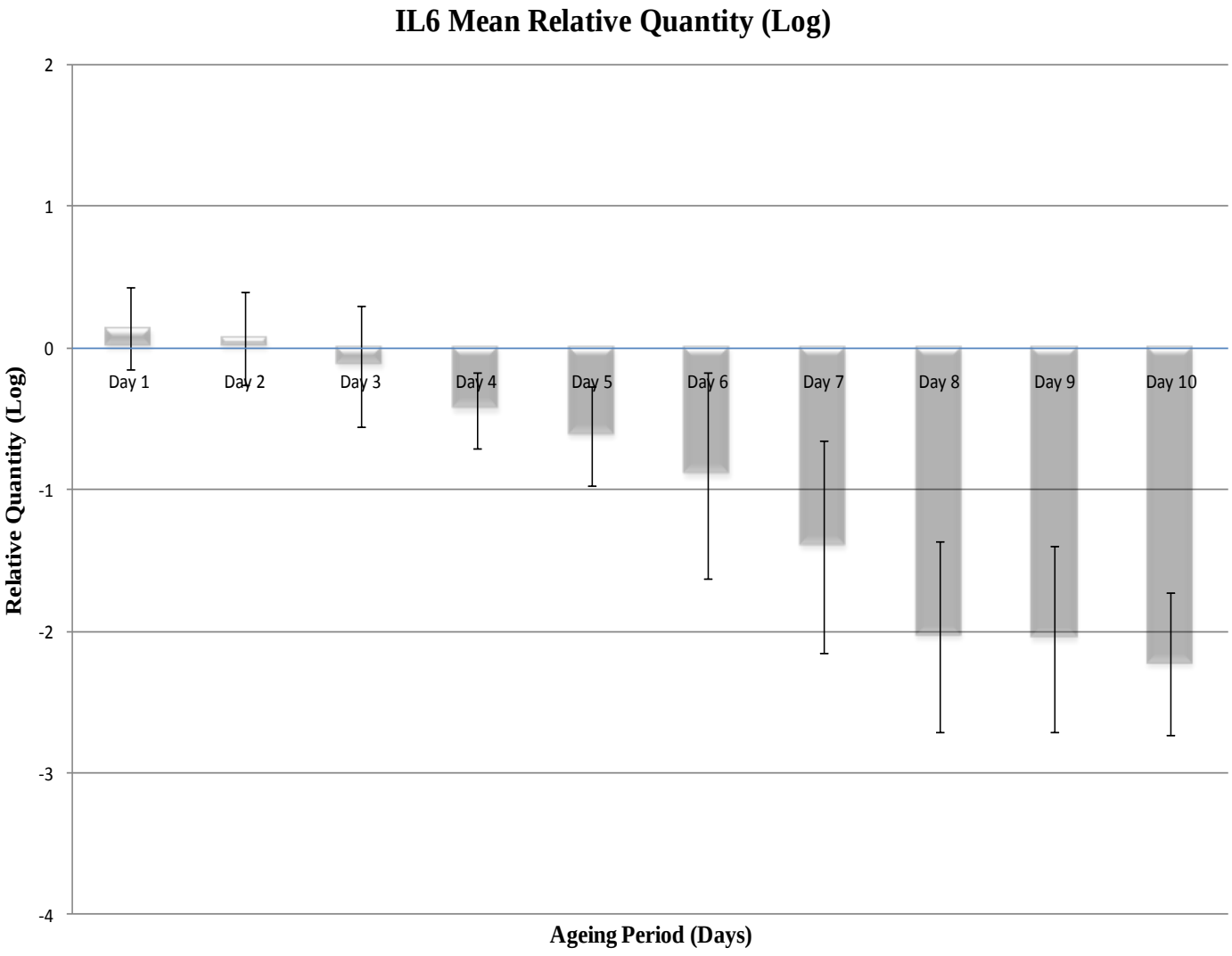


Figure 51: Mean Relative Log quantities for all eight donors are presented above. Mean values show there is a definitive trend over the ten days. Values of IL-6 are mildly elevated over the first 24 hours before decreasing steadily to day eight. The rate of decrease was significantly less at day nine and ten. The error bars represent 1 standard deviation.

6.4.2 TNF-α

With the exceptions of donor 1, 5 and 3 (marginally), there was no increase in TNF-α expression levels observed at any point during the study. In those samples where an increase was observed, it occurred during the first day of the aging period and like IL-6, the increase in expression was significantly less than that observed by Pahl and Brune (2002). The largest expression change was less than 2-fold (1.89). This observation was unexpected given TNF-α, a proinflammatory cytokine, plays a primary role in the inflammation process (Frasca *et al*, 2011). It is likely the cause of the significant increase in TNF-α expression observed by Pahl and Brune (2002) was either absent or had only a minimal effect in this particular study. A number of possible causes exist. Peripheral blood RNA isolation methods can critically impact differential expression, particularly where fold-change differences are small and there is inherent variability within biological cohorts (Asare *et al*, 2008). Since most cellular components of peripheral blood are specialized to respond quickly to exogenous stimuli it has been suggested that there is a need to use sample procurement methods that reduce the overall impact of *ex vivo* changes in gene expression studies (Debey-Pascher *et al*, 2009). Pahl and Brune (2002) supported this position stating there was a need to standardise and stabilise clinical blood samples after phlebotomy as blood cells are exposed to a variety of extracellular agents, which may influence their activation status. Technical issues such as prolonged sample handling or temperature changes during blood storage, handling and processing can all affect gene expression levels (Debey-Pascher *et al*, 2009). Changes in the environment and exposure to different surfaces are also likely to affect gene expression (Pahl and Brune, 2002). In this experiment blood was extracted in a controlled manner using a small tipped syringe, which caused minimal trauma to the donor. The size of the needle used by Pahl and Brune (2002) was not referenced, but if significantly larger than the one used in this study it could account for the 30-fold increase they observed in IL-6 and TNF-α expression levels. Unfortunately with forensic samples, many of the technical issues that can cause changes in expression levels will be impossible to control and will be largely unknown. Blood recovered from a crime scene will have originated from some form of trauma however these observations suggest the type of trauma will have an effect on the expression levels of IL-6 and TNF-α. The immune response to a small needle prick is likely to be significantly different to that experienced by someone who has bled as a result of a large stab wound, blunt force trauma or a gun shot. Pahl and Brune (2002) also suggested there was a potential surface effect on the expression levels of IL-6 and TNF-α specifically. There are an endless number of possible surfaces and environmental

conditions that forensic samples may be deposited on. Wood, concrete, tiles, laminate, carpet, and metallic surfaces as well as wet, dry, hot and cold surfaces are all external environmental factors which could affect the expression levels of IL-6 and TNF-α. Lahat *et al*, (2008) demonstrated that cell hypoxia causes a 2-fold reduction in the intracellular levels of TNF-α by enhancing its degradation in the lysosomes and inhibiting its secretion via secretory lysosomes (Lahat *et al*, 2008). It is likely that the processes involved in transporting oxygen across the blood cell walls will breakdown quickly following cell and tissue death resulting in intracellular hypoxia. Hypoxia could explain the lack of increased TNF-α expression observed during the initial phase of this study. If an increased expression did occur, it may have been masked by an increase in the degradation rate of TNF-α. There are a number of proteolytic enzymes that can breakdown TNF-α, such as pancreatic proteases (Alsfasser *et al*, 2005), although these proteases are not expected to play a significant role in the degradation of TNF-α from blood samples. Ribonucleases that specifically target the AU-rich sequences in the 3' untranslated region of the TNF-α mRNA can cause rapid degradation of the TNF-α mRNA molecule (Caput *et al*, 1986). It has also been suggested that specific proteins with binding activity for this sequence act as a precise target for ribonculease attack to the AU-rich motif in TNF-α mRNA (Suk and Erickson, 1996). Certain peptidases, such as serine peptidase also participate in TNF-α degradation (Bauvois *et al*, 1992), whilst there are also environmental degradation factors such as UV light and heat that will contribute to the *ex vivo* degradation of this species of mRNA.

Over the course of the ten days there was a discernable trend in the mean amount of TNF-α. This trend can be seen in Figure 53 on page 227. This trend was consistent up until day eight, at which point the mean amount of TNF-α remained relatively constant (for days nine and ten). This observation was unexpected and unexplained, but mirrored the IL-6 findings. It was thought that this anomaly could have been caused by contamination therefore the experiment was repeated. The results (not presented in this thesis) were markedly different, which illustrates the variability in the expression of TNF-α. The repeated experiment was conducted during the winter months which meant ageing conditions varied from the initial trial, which was conducted in summer. The variation could be due to the changing environmental conditions and if so highlights this techniques vulnerability to varying environmental conditions. Alternatively the variation in TNF-α could be a natural occurrence and this could explain the significant inter-donor variation observed despite using relatively controlled conditions. The surface type, temperature and humidity were all controlled in this experiment

and yet the inter-donor variation observed was large, ranging from 51.7% (day five) to 189.0% (day eight). This was comparable to the variation observed with IL-6, where the range was 53.0% (day one) to 192.1% (day four). Although there was no distinct pattern in terms of the size of the inter-donor variation over the ten day period, the size of the variation was comparable to the longer studies carried out in chapters three and four, which was disappointing.

Marucha *et al*, (2005) suggested that levels of TNF-α were variable between individuals and this variability was a result of different TNF-α responses to social activities. A variable response to social activities is just one mechanism that can result in inter-donor variation of TNF-α expression. Like IL-6, there are a number conditions and stimuli that can cause a change in TNF-α expression. The expression of TNF-α is regulated by both transcriptional and post-transcriptional mechanisms [Crawford *et al*, (1997); Han *et al*, (1990); Prichett *et al*, (1995); Raabe *et al*, (1998)]. TNF-α is a key mediator in the local inflammatory immune response, which initiates a cascade of cytokines and increases vascular permeability (Royall *et al*, 1989). Increased TNF-α levels are therefore associated with inflammatory conditions such as rheumatoid arthritis, alcoholic liver disease, pancreatitis and Chohn's disease (Marriott *et al*, 1998). Septic shock, transplantation rejection, multiple sclerosis (Titelbaum *et al*, 2005), cachexia, diabetes (Swaroop *et al*, 2012), ischemia-reperfusion injury (Zhang *et al*, 2006), adult respiratory distress syndrome and cancer can all cause increased TNF-α levels (Janeway *et al*, 1999). B cells also contribute to the inflammatory process by secreting TNF-α in a wide range of autoimmune diseases (Lund and Randall, 2010). Inflammation also plays an important role in the pathogenesis of many diseases typical of old age (Ferrucci *et al*, 2005). Elevated levels of both IL-6 and TNF-α have been associated with functional disability and mortality of the elderly (Fulop *et al*, 1999). Aging is also characterized by a dysregulation of inflammatory and anti-inflammatory networks, which results in a low-grade chronic proinflammatory status (Franceschi *et al*, 2000) and the age-related increase in circulating inflammatory mediators, such as cytokines and acute-phase proteins. Age related alterations in responses to immune stimulation (e.g., chronic T cell stimulation with viruses, such as CMV) also contribute to low-grade inflammation by increasing the level of proinflammatory mediators, such as TNF-α (Sansoni *et al*, 2008). The factors suggest the baseline expression of TNF-α may vary between the young and the elderly, which would be problematic for analysing forensic samples as often the age of the donor will be unknown. TNF-α has the ability to induce apoptotic cell death and to inhibit tumorgenesis and viral replication and is commonly found in high levels where

bacterial, fungal and parasitic invasions occur (Biermer *et al*, 2003). The lipopolysaccharide from bacteria cell walls is an especially potent stimulus for TNF-α synthesis (Tracey and Cerami, 1993). B lymphocytes secrete TNF-α in response to infectious agents, such as *Toxoplasma gondii, Heligomosomoides polygyrus, and Pneumocystis carinii* (Frasca *et al*, 2011). Elevated levels are also associated with viral infections, such as dengue virus, HCV and HIV (Wati *et al*, 2011). Both interferon-γ (IFN- γ) and interleukin-4 (IL-4), which are secreted by T lymphocytes, enhance the secretion of TNF-α in macrophages (Suk and Erickson, 1996). The secretion of TNF-α by macrophages assists in the blood clotting process (Janeway *et al*, 1999). The clotting process occurs in *ex vivo* samples and is visually evident in crime scene samples (James *et al*, 2005). These issues give rise to the possibility that the size of the bloodstain or volume of blood can potentially affect the levels of TNF-α as the volume will determine the amount of activation components, the number of macrophages present and the degree of clotting.

Tissue trauma, which will be relevant to most forensic samples, also results in elevated TNF-α levels (Locksley *et al*, 2001), It has also been shown that there can be unintentional gene expression induced by exposure to the contents of lysed cells, such as haemoglobin (Rainen *et al*, 2012). This has the potential to change expression levels as a bloodstain ages and deteriorates. Furthermore there is potential for different expression levels between stains that are deposited at the beginning of the traumatic incident compared to those deposited towards the end, especially if the trauma is prolonged and severe. This would be due to the development of the immune reaction to any trauma and the extra time available for the migration of stimulating factors. At the other end of the scale there are conditions that result in low levels of TNF-α. Reduced TNF-α expression is common in maintaining homeostasis by regulating the body's circadian rhythm (Pan *et al*, 2002), AIDS (Hober *et al*, 1989) and in conditions that require re-modelling or replacement of injured and senescent tissue.

What is clear is that there are a multitude of factors that have the potential to cause changes in TNF-α expression. The variation observed in this experiment was large, preventing this technique from accurately determining the age of blood samples. At best this method could be used to determine an age range only. The collaboration of these findings suggests it may be necessary to find a molecule which is less susceptible (in terms of expression changes) or plays a less dynamic role in the immune response.

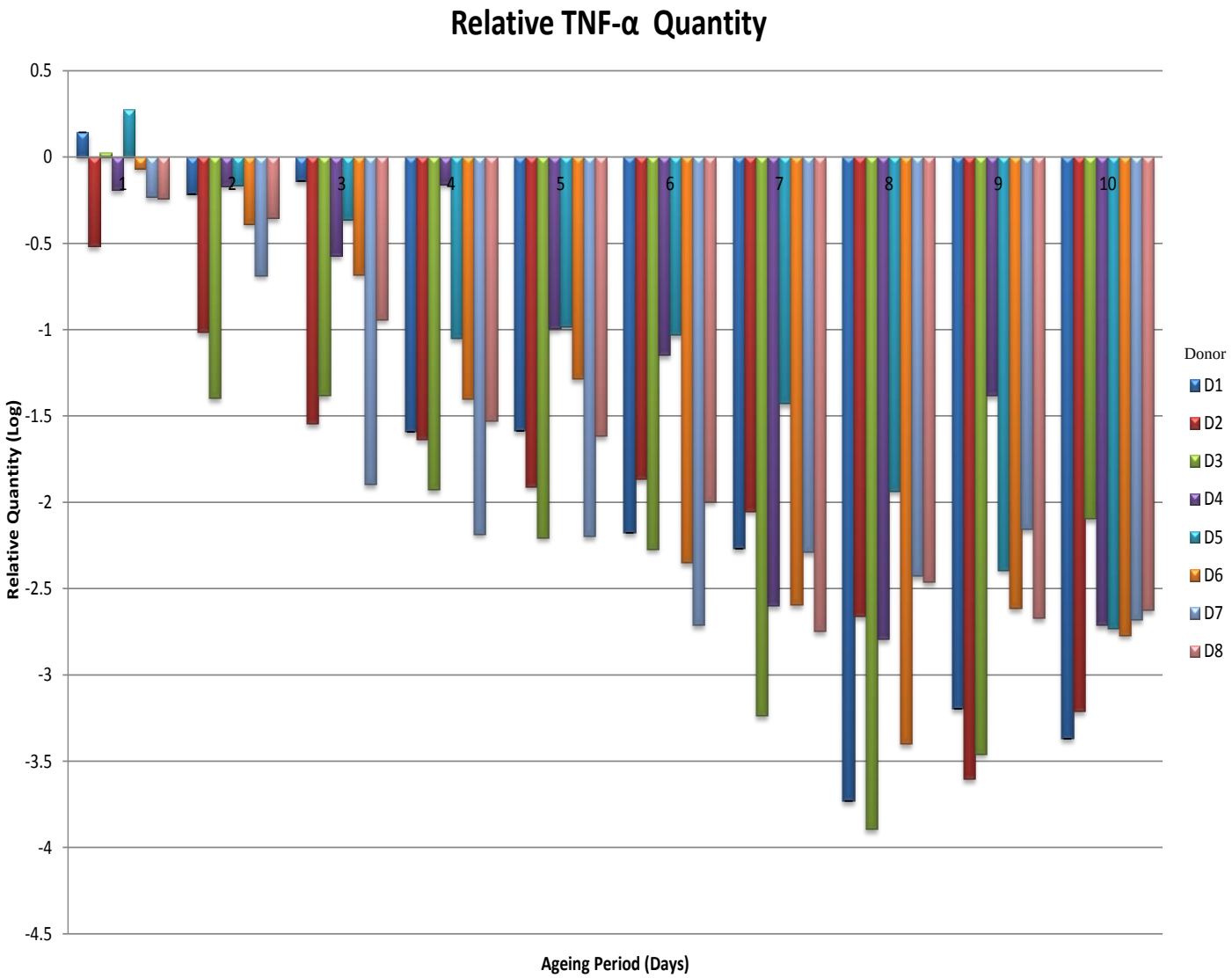


Figure 52: The quantities of TNF-α for each donor was measured at daily intervals. The relative (log) values are presented above. The data shows the levels of TNF-α decreased over the first eight days followed by a slight increase during day nine and ten. The inter-donor variation in the TNF-α levels was significant.

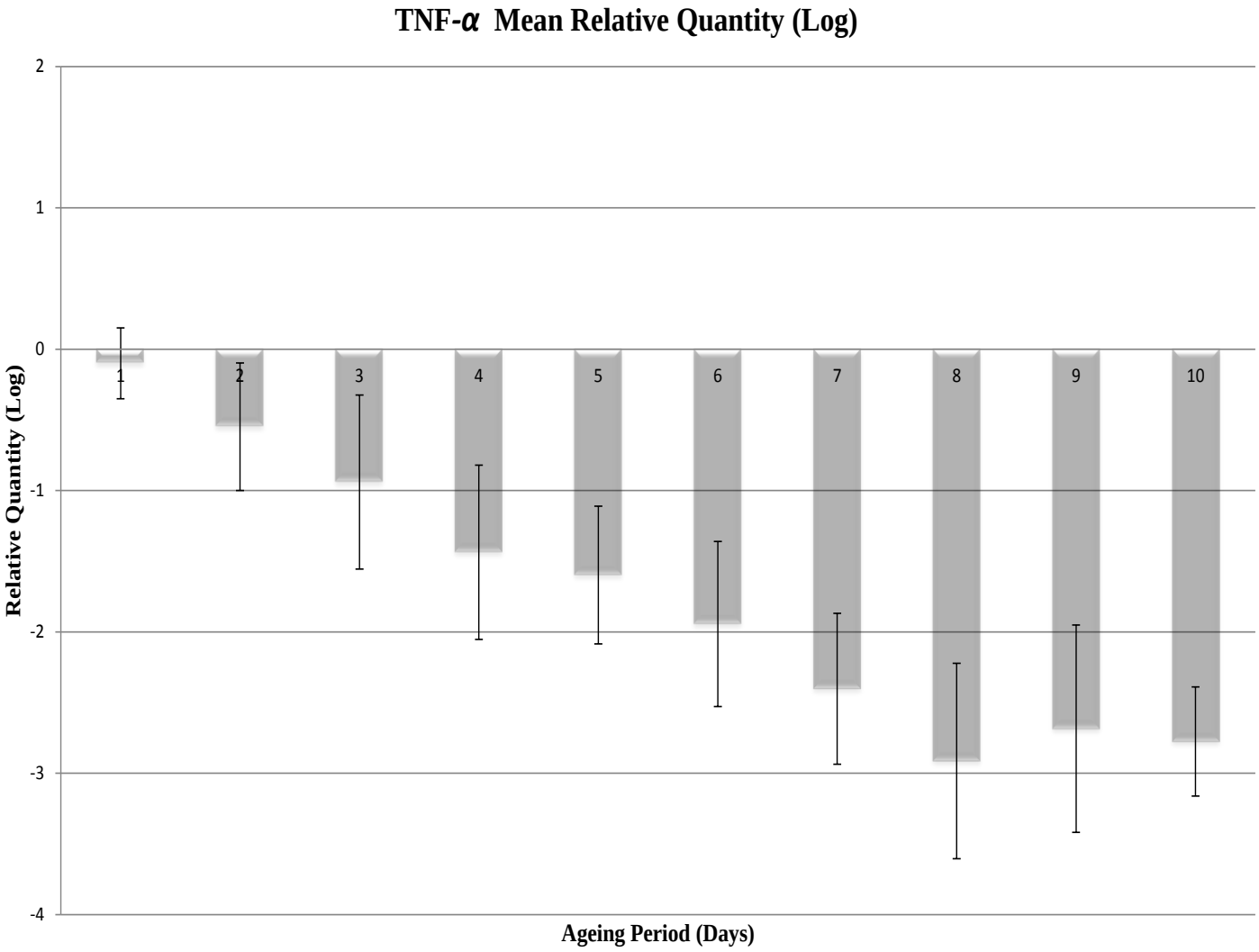


Figure 53: The mean TNF-α values across the ten days, expressed on a logarithmic scale. The increase in TNF-α that was expected, was not observed. Instead there was a continual decline in expression levels until day nine, where an unexpected increase were observed. The error bars represent 1 standard deviation.

6.5 Conclusion

The aim of this study was to identify a suitable RNA target and develop an appropriate forensic method for determining the age of fresh bloodstains that were less than ten days old. Whilst a general decrease in the relative amounts of IL-6 and TNF-α was observed, the inter-donor variation was large. Accurate quantification of mRNA in whole blood is made difficult by the simultaneous degradation of gene transcripts and unintended gene induction caused by sample handling or uncontrolled activation of coagulation (Rainen *et al,* 2002). Furthermore the targets chosen in this experiment are heavily involved in the immune response and therefore changes in expression are a natural and common phenomenon. As such this technique lacks the robustness, accuracy and reproducibility expected from a forensic application. It remains a possibility that nucleic acids, in particular RNA, may still provide the solution to the challenging task of accurately ageing bloodstains however it may be more appropriate to target a molecule that does not play a major role in the immune response. Given biological variability will always exist an extensive investigation is required into identifying appropriate targets that can minimise this variability down to manageable levels. IL-6 and TNF-α satisfied certain criteria in that their expression levels changed rapidly over short periods of time but because of their dynamic roles in a number of biological processes, expression levels between donors were highly variable, unpredictable and therefore not suitable for this type of forensic analysis.

CHAPTER 7: A NOVEL 3'/5' RNA METHOD FOR AGEING BLOODSTAINS

7.1 Introduction

RNA has become the focus in age determination studies largely due to the development of new RNA methodologies [Anderson *et al*, (2005); Vennemann and Koppelkamm, (2010)], and the recent data suggesting its stability is greater than first thought (Zubakov *et al*, 2008). The inability to establish a reproducible and accurate ageing method using DNA has also encouraged the shift towards RNA (Bremmer *et al*, 2012). Traditionally, RNA degradation has been determined using spectrophotometric methods (Fleige and Pfaffl, 2006) or by analysing the ribosomal RNA bands on agarose gel (Lehrach *et al*, 1977), both of which lack qualitative and quantitative accuracy (Fleige and Pfaffl, 2006). More recent methods have involved the use of qRT-PCR to measure the relative quantities of two different sized target amplicons in much the same way as DNA degradation rates have been measured (Bauer *et al*, 2003). It can be assumed that many of the problems and limitations associated with the DNA methodology would be relevant to RNA, making this approach potentially unreliable.

The introduction of the Agilent Bioanalyser (BioRad) has provided an alternative method of analysis. This microfluidic capillary electrophoresis system claims to provide an objective measure of RNA quality by measuring several parameters on the electropherogram generated (Schroeder, 2006) and assigning a RNA Integrity number (RIN) value to a sample. Fleige and Pfaffl, (2006) performed an exhaustive study to compare the two benchmark techniques, the Agilent Bioanalyser and qRT-PCR. The authors extracted RNA from various tissue types and subjected them to controlled degradation. The samples were then measured using the Bioanalyser. All samples had RIN values between four (almost no evidence or rRNA bands) and ten (apparently intact material). The quantity of each individual transcript was then measured using a qRT-PCR assay. The relationship between the quantity and RIN was relatively weak and varied between tissue types (Fleige and Pfaffl, 2006). They concluded that moderately degraded RNA samples can be reliably analysed and quantified using qRT-PCR as long as the amplicons are kept short and expression is normalised against a reference gene (Fleige and Pfaffl, 2006). Their conclusions highlighted the deficiency of assigning a RIN and the limitations of this approach but remained ambiguous when it came to the effectiveness of the qRT-PCR assay.

In the absence of a reliable measure of RNA integrity, Nolan *et al,* (2006) proposed a novel 3'/5' qRT-PCR assay, which was independent of ribosomal RNA integrity. It was suggested that this method, which was modelled on the standard approach adopted by microarray users and long accepted conventional techniques applied to end point PCR, provided a reasonable measure of the transcripts of interest (Nolan *et al,* 2006). One potential limitation of this assay is that it measures the integrity of ubiquitously expressed mRNA (specified by the target gene) based on the assumption that the target species is representative of the entire RNA population in the sample. Blood is a highly dynamic tissue as there are many physiological and pathological processes that will cause changes in the levels of blood components. This includes mRNA expression levels therefore using a single species to predict the overall integrity of a sample could be problematic. The tissue type, storage conditions, processing techniques and even the species of RNA will all affect the degradation rate of an RNA transcript (Nolan and Bustin, 2008). These factors warrant the need to design, optimise and purposely select target genes specific for the samples and conditions being tested.

For the purpose of ageing bloodstains in a forensic setting, the integrity of the entire RNA population is not required. Rather a single RNA species can be examined therefore this method is potentially suitable for the forensic application of ageing bloodstains. Given its proposed performance abilities, the assay designed by Nolan *et al,* (2006) was tested on bloodstains to establish whether sample age could be more accurately determined than previous DNA and RNA methodologies.

7.1.1 Target Species Selection

Due to the dynamic nature of blood, individual cell characteristics were considered during the selection process as each individual cell type has its own characteristic mRNA profile, which is dependent on the roles and activities of a particular cell. RNA recovered from whole blood originates from the nuclear material found within the white blood cells (leukocytes) (Lodish *et al,* 2012). There are five major groups of white cells that exist in human blood. They include neutrophils, lymphocytes, monocytes, basophils and eosinophils and each play their own distinct biological role in the haematopoietic process (Lodish *et al,* 2012). The mRNA expression profile of each individual cell line changes depending on the developmental stage of the cell and the haemostatic state of the individual (Frenck *et al,* 1998). The differentiation and cell proliferation status of a cell has a direct impact on the absolute copy number of mRNA

transcripts and therefore would have a direct implication on our proposed methodology as it is essentially a quantification method. Cells originating from the lymphoid lineage (lymphocytes) often have key roles in the immune response and as such their replication cycle can be erratic and unpredictable (Lodish *et al*, 2012). On the other hand the myeloid progenitor cells, which includes neutrophils, eosinophils, basophils and monocytes differentiate in the bone marrow and then migrate into the peripheral blood where they remain for a relatively short period of time (lifespan of hours to days) (Lodish *et al*, 2012). Mature granulocytes are terminally differentiated cells and do not undergo cellular division (Weng, 2001). It was thought that because myeloid cells, and in particular granulocytes, do not undergo the mass replication and differentiation that lymphocytes experience when the immune response is triggered, the expression levels of specific mRNA species from cells within this lineage might be more consistent. It was thought that by selecting a target from this cell lineage, the inter-donor variation which has been a common problem throughout the work presented in this thesis, may be reduced.

To increase the chance of obtaining successful results using this methodology, it was thought that the target RNA species needed to satisfy further criteria. It was thought that targets should be stable in blood tissue, be sufficiently expressed to allow detection from minute samples, should maintain a relatively uniform expression independent of the physiological state of the body and must have a consistent time-wise degradation rate. From a forensic perspective, it would also be desirable if the RNA species was only expressed in blood, therefore simultaneously identifying the biological stain. To select suitable targets, data from the whole genome gene expression analysis of time-wise degraded blood samples conducted by Zubakov *et al*, (2008) were examined. They used a Affymetrix U133 plus2 Genechip containing >54,000 mRNA probe sets, which encompasses most known and predicted human genes, to identify stable mRNA markers specific in certain tissue types, such as blood, saliva and semen. Further refinement of the gene sets was achieved by comparing the selected candidates against the GNF SymAtlas tissue database (Zubakov *et al*, 2008). This database is a tool for the visualization of tissue-specific gene expression based on comprehensive microarray data. Nine stable mRNA markers specific for blood were identified showing expression in stains aged up to 180 days (Zubakov *et al*, 2008) making them potential candidates for this research. Three suitable blood targets identified by Zubakov *et al*, (2008) were chosen for this experiment. Two species (MNDA and AMICA1) are known to be highly or even specifically expressed in the myelocyte cell lineage (Zubakov *et al*, 2008) and therefore given their respective haemopoeitic

status, were considered the most likely to produce successful results. Adhesion molecule interacts with CXADR antigen-1 (AMICA1) is a blood specific gene located on human chromosome 11 (11q 23.3). The AMICA1 mRNA transcript is expressed in hematopoietic tissues and is prominently expressed in granulocytes (Moog-Lutz *et al,* 2003). The human myeloid cell nuclear differentiation antigen (MNDA) is expressed specifically in cells of the granulocyte/monocyte lineage as well as the early myeloid lineage (Zubakov *et al,* 2008). The MNDA gene is a single-copy gene that is localized to human chromosome 1q 21-22 within the large linkage group conserved between mouse and human (Briggs *et al*, 2006). To test the theory on cell lineage, a third target was chosen. Caspase-1 (CASP1), also known as interleukin-1α-converting enzyme, is expressed in all peripheral leukocytes (Lin *et al,* 2000). The CASP1 gene encodes a protein which is a member of the cysteine-aspartic acid protease family. The caspases are a family of aspartic acid-specific proteases that fulfil various roles in apoptosis or in the proteolytic activation of cytokines (Jee *et al*, 2005). CASP1 is responsible for the activation of cytokines such as IL-1 and IL-18, which are involved in inflammation, septic shock and wound healing processes (Schumann *et al,* 1998). The levels of these interleukins are likely to be highly variable between individuals making standardisation for age determination studies difficult. A fourth RNA species, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is a common housekeeping gene (Barber *et al,* 2005) was included for normalisation purposes.

The 3'/5' assay is based on the traditional qRT-PCR technique in that it involves a reverse transcription step, followed by the amplification of target species using real time PCR (Nolan *et al,* 2006). This traditional approach has been used throughout this thesis however there are a few noticeable differences with the 3'/5' assay. The RT reaction is primed using oligo-dTs, rather than random hexamers. This causes the cDNA synthesis to begin at the end of the transcript (poly-A tail) and will continue until there is a break in the transcript (Nolan *et al*, 2006). The greater the level of degradation, the sooner the polymerising enzyme will encounter this discontinuation and synthesis of the cDNA will cease (Nolan *et al,* 2006). Two amplicons are carefully designed so that they are spatially separated, one towards the 5' end and the other towards the 3' end of the mRNA sequence. The ratio of amplicons will be representative of the success of the reverse transcription reaction to proceed along the entire length of the transcript. The ability of the RT enzyme to reach the 5' end target will depend on the integrity of the mRNA. Nolan *et al,* (2006) suggests that a 3'/5' ratio of around one indicates high quality, whereas anything greater than five suggests degradation. With forensic samples, degradation is

likely to be more advanced than the samples tested by Nolan *et al* (2006). The primary aim of this experiment was to determine whether this ratio can be directly correlated with the age of the sample.

7.2 Methodology

7.2.1 Primers and Assay Design

All primers were specifically designed using Beacon designer Version 7 (Sigma, UK) and where possible addressed key elements concerning runs of bases, G/C content, and the avoidance of complementary runs in both the sense and anti-sense primers. Doing so reduces the formation of secondary structures such as hairpins and primer dimers, which decrease the efficiency of the PCR reaction. A BLAST (NCBI) search was conducted on all primers to identify any cross homology to unwanted gene targets. It was also important to consider amplicon size as the work in chapter three demonstrated that different sizes have different resolving capabilities depending on the level of sample degradation. In this assay, the distance between the 3' and 5' targets is important in the overall success of the assay (Nolan and Bustin, 2006). If the distance is small, the assay will be incapable of distinguishing between degraded samples. If the distance is large, the 5' target is unlikely to amplify in degraded samples as there will be a higher probability that the DNA strand will be cleaved between the two targets (Nolan and Bustin, 2006). Details of the targets are given in Table 44.

7.2.2 Sample Preparation

Samples were prepared as described in section 2.1.7 on page 84.

Table 44: Details regarding primer and assay design. Primer sequences, amplicon size and spatial distance between 3' and 5' targets are listed for all genes tested.

Target	Primer	Primer Sequence	Amplicon Size	Distance between 3' and 5'
AMICA1 3'	Forward	5' – TGTGTCCTGGGCCACTCTAC – 3'	134bp	
	Reverse	5' – TCCAAATTCTCCATCTTCAGTGTATTG – 3'		
AMICA1 5'	Forward	5' – TGGTAATCAGTTGGTGATCATTGTG – 3'	121bp	351 bases
	Reverse	5' – CTCTTATTTCCACAGGTCTTCTTCAC – 3'		
MNDA 3'	Forward	5' – CAAGGAAGGACCAATGAATGTTAATTG – 3'	195bp	
	Reverse	3' – TCCAGAAAGCAAACTAATCTATAACTCAG – 5'		301 bases
MNDA 5'	Forward	5' – AGGTGGATGCAAGAAGAAATGTTC – 3'	153 bp	
	Reverse	3' – TCACATGGAAATATTGAGTCTTACTGG – 3'		
CASP1 3'	Forward	5' – TACTACAACTCAATGCAATCTTTAACATG – 3'	146 bp	
	Reverse	3' – TCCAGAAACTCCTACTGAATCTTTAAAC – 5'		421 bases
CASP1 5'	Forward	5' – AGTTATGGATAAGACCCGAGCTTTG – 3'	106 bp	
	Reverse	3' – TGCCAGGTAACTGTCTTCTTCAC – 5'		
GAPDH 3'	Forward	5' – AGTCCCTGCCACACTCAG – 3'	174 bp	
	Reverse	3' – TACTTTATTGATGGTACATGACAAGG – 3'		121 bases
GAPDH 5'	Forward	5' – GTGAACCATGAGAAGTATGACAAC – 3'	140 bp	
	Reverse	3' – CATGAGTCCTTCCACGATACC – 5'		

7.2.3 Sample Processing

Once samples had been aged for the specified period of time RNA was extracted using the QIAamp RNA Blood Mini kit (Qiagen, UK) according to manufacturer's instructions. There were a few minor variations which incorporated extended incubation times due to the difficult nature of the samples (aged). A detailed description of the protocol can be found in section 2.2.2 on page 86. Samples were stored at -20°C until subsequent treatment steps.

Each RNA sample was treated with DNase (Invitrogen, US) to remove any residual DNA that may be present following the extraction procedure. A detailed description of the digestion protocol can be found in section 2.3.1 on page 88. Following the enzyme treatment, each RNA sample was quantified to ensure known amounts of RNA could be added to the Reverse Transcription step. Quantification was achieved using Implen's spectrophotometer and according to manufacturer's instructions. The detailed protocol can be found in section 2.5 on page 90. The purified total RNA was then reverse transcribed into cDNA using oligo-dT priming (Invitrogen, US) using the manufacturer's standard protocol, which is described in section 2.6.2 on page 91. Each sample was run in triplicate and all appropriate positive and negative controls were run with each assay. Finally, the cDNA was amplified using a standard Real time PCR methodology using a SYBR green detection system and according to the standard protocol outlined in section 2.7.2 on page 93. Final primer concentrations of 400nm were used. The threshold was set at 0.2 for all runs.

7.3 Results

7.3.1 Efficiency Assays

There were two amplified products (3' amplicon and 5' amplicon) for each target species (AMICA1, MNDA, CASP1 and GAPDH). It was necessary to determine the rate of amplification for each amplicon (eight in total) to ensure accurate qPCR quantification. Efficiency assays were carried out as previously described using a serial dilution technique. Details can be found in Tables 45 to 48. The relevant Figures can be found in Appendix VII.

Table 45: Ct values and Amplification efficiency rates for AMICA1 3' and 5' targets

AMICA1 3'	R1	R2	R3	Mean	Std Dev	Difference	Slope	Efficiency Rate
Neat	28.48	28.81	28.63	28.64	0.17		**3.51**	**92.6**
1/10	32.08	31.84	31.87	31.93	0.13	3.29		
1/100	35.29	35.65	35.67	35.54	0.21	3.61		
1/1000	40.02	38.91	38.51	39.15	0.78	3.61		
AMICA1 5'	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	25.87	26.08	26.01	25.99	0.11		**3.55**	**91.3**
1/10	29.29	29.55	29.43	29.42	0.13	3.43		
1/100	33.24	32.95	33.42	33.20	0.24	3.78		
1/1000	36.47	36.23	36.99	36.56	0.39	3.36		

Table 46: Ct values and Amplification efficiency rates for MNDA 3' and 5' targets

MNDA 3'	R1	R2	R3	Mean	Std Dev	Difference	Slope	Efficiency Rate
Neat	24.27	24.60	24.64	24.50	0.20		**3.32**	**100.1**
1/10	27.59	28.25	27.58	27.81	0.38	3.31		
1/100	31.42	31.18	31.36	31.32	0.12	3.51		
1/1000	34.38	34.44	34.43	34.42	0.03	3.10		
MNDA 5'	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	21.90	21.76	21.74	21.80	0.09		**3.58**	**90.3**
1/10	25.36	25.05	24.34	24.92	0.52	3.12		
1/100	28.29	28.68	28.57	28.51	0.20	3.59		
1/1000	32.31	33.33	32.01	32.55	0.69	4.04		

Table 47: Ct values and Amplification efficiency rates for CASP1 3' and 5' targets

CASP1 3'	R1	R2	R3	Mean	Std Dev	Difference	Slope	Efficiency Rate
Neat	21.90	21.76	21.74	21.80	0.09		**3.44**	**95.3**
1/10	25.36	25.04	25.35	25.25	0.18	3.45		
1/100	28.29	28.67	28.57	28.51	0.20	3.26		
1/1000	32.30	32.10	32.18	32.19	0.10	3.68		
CASP1 5'	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	20.77	20.42	20.54	20.58	0.18		**3.77**	**84.2**
1/10	23.47	23.55	23.72	23.58	0.13	3.00		
1/100	27.23	27.31	28.38	27.64	0.64	4.06		
1/1000	31.36	32.10	30.96	31.47	0.58	3.83		

Table 48: Ct values and Amplification efficiency rates for GAPDH 3' and 5' targets

GAPDH 3'	R1	R2	R3	Mean	Std Dev	Difference	Slope	Efficiency Rate
Neat	23.56	23.51	23.89	23.65	0.21		**3.67**	**87.3**
1/10	27.07	27.06	27.58	27.24	0.30	3.59		
1/100	31.05	31.16	30.89	31.03	0.14	3.79		
1/1000	34.60	34.59	34.71	34.63	0.07	3.60		
GAPDH 5'	**R1**	**R2**	**R3**	**Mean**	**Std Dev**	**Difference**	**Slope**	**Efficiency Rate**
Neat	22.63	22.48	22.57	22.56	0.08		**3.57**	**92.0**
1/10	26.12	26.37	26.29	26.26	0.13	3.70		
1/100	29.80	30.03	30.08	29.97	0.15	3.71		
1/1000	32.99	33.01	33.28	33.09	0.16	3.12		

7.3.2: Relative Quantity Ratios from the 3'/5' Assays

The mean Ct values, relative quantities (RQ) and 3'/5' expression ratios for AMICA1, MNDA, CASP1 and GAPDH can be found in Appendices VIII, IX, X and XI respectively. Figures 54 to 57 illustrate the observed trends in the 3'/5' expression ratios. The ratios obtained for AMICA1 were poor due to the inability to obtain results from the 5' amplicon in samples aged for more than 40 days. There was no general pattern observed between the ratio and the age of the sample. A weak trend was observed between the MNDA 3'/5' expression ratio and sample age, although it should be noted that the ratios observed were excessively high. Some of the samples aged for 60 days had ratios in excess of 4000. Given Nolan *et al*, (2006) suggested that degraded samples would produce ratios of approximately five; values in the thousands indicate these samples were highly degraded. Although a trend was observed it was weak and prone to large inter-donor variation. The 3'/5' expression ratios for CASP1 showed no obvious trend and results could not be obtained from samples aged for more than 40 days. The ratios obtained from the housekeeping gene, GAPDH produced the best results. There was a definite increase in ratio over time however the variation in the ratio between donors at each age point was still large.

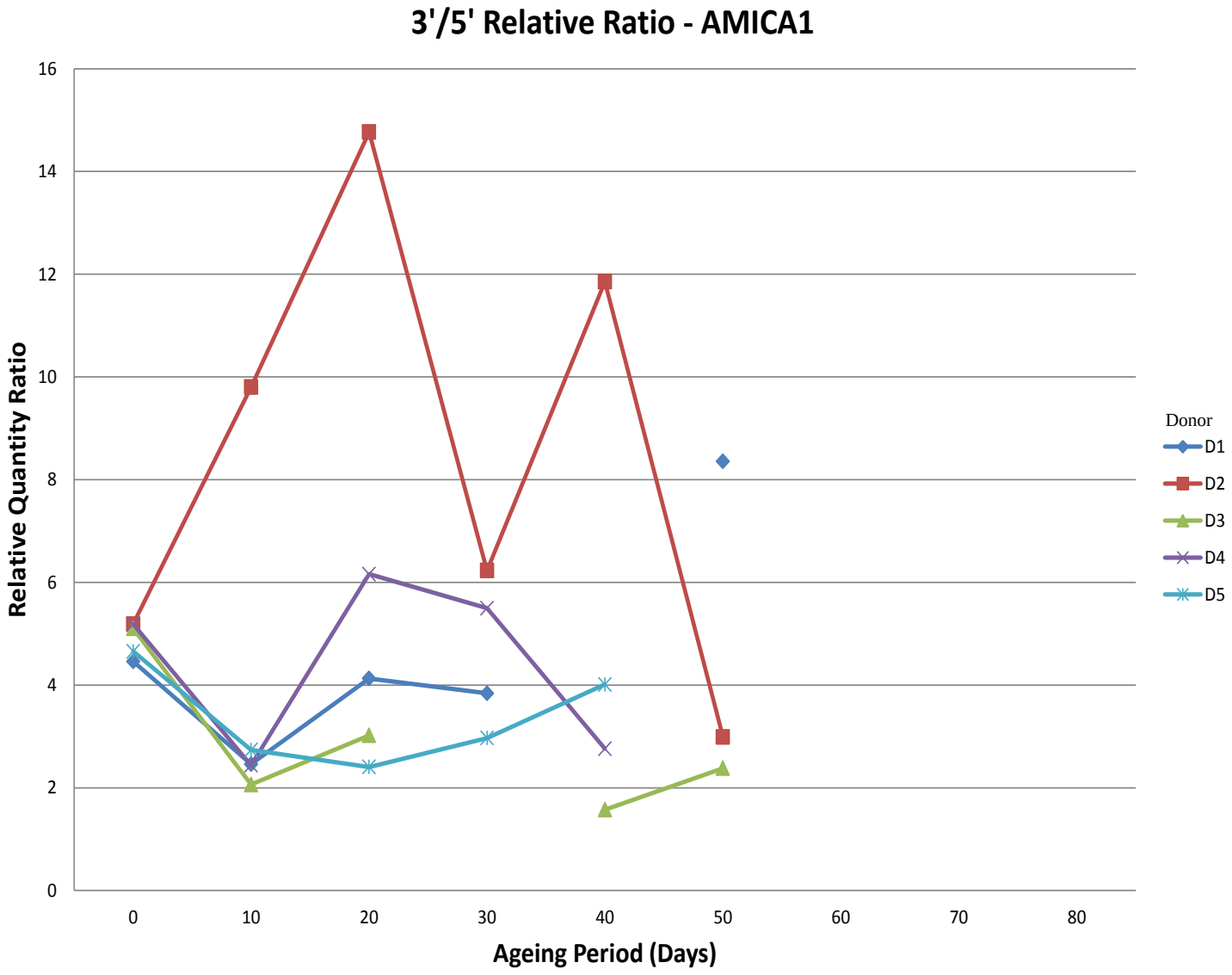


Figure 54: Time dependant analysis of the 3'/5' AMICA1 relative quantity ratio. There was no obvious trend identified between the 3'/5' expression ratio of the AMICA1 target and the age of the sample. The amplification of the 3' and 5' targets was somewhat erratic over the course of the 80 days. No results were obtained from samples aged for 60 days or more due to the inability to amplify the 5' target, whereas only three of the five donors produced results for samples aged for 50 days. Donor 1 failed to produce a result at 40 days whilst donor 3 failed at 30 days.

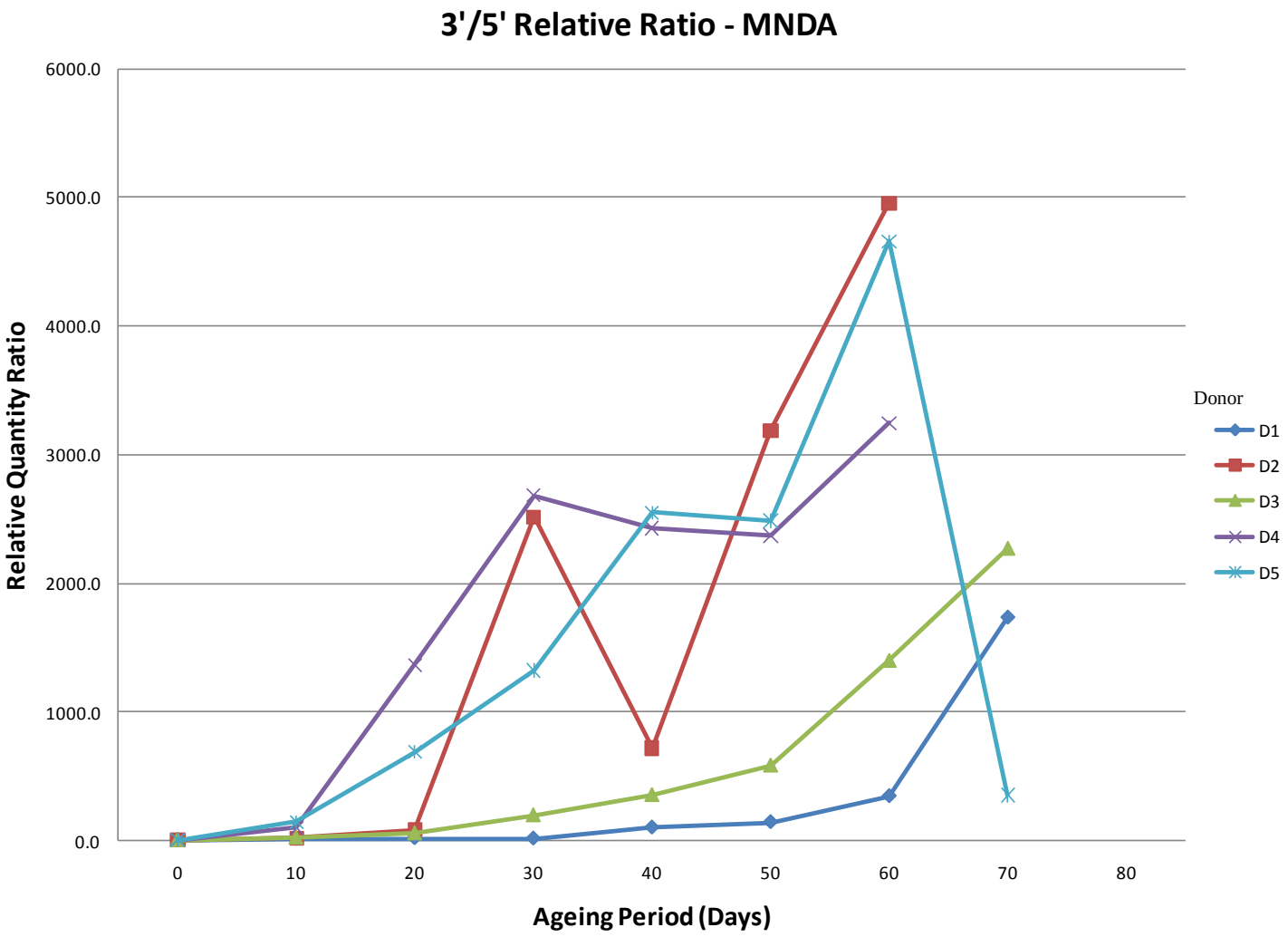


Figure 55: Time dependant analysis of the 3'/5' MNDA relative quantity ratio for each donor. The 3'/5' expression ratio values obtained from MNDA were considerable larger than the ratios obtained from AMICA1 suggesting these samples were considerably more degraded. The data shows a generalised pattern with increasing ratio over time however the variation between each donor is large. Limited or no data at all was obtained from samples aged for 70 and 80 days respectively due to the inability to amplify the 5'target.

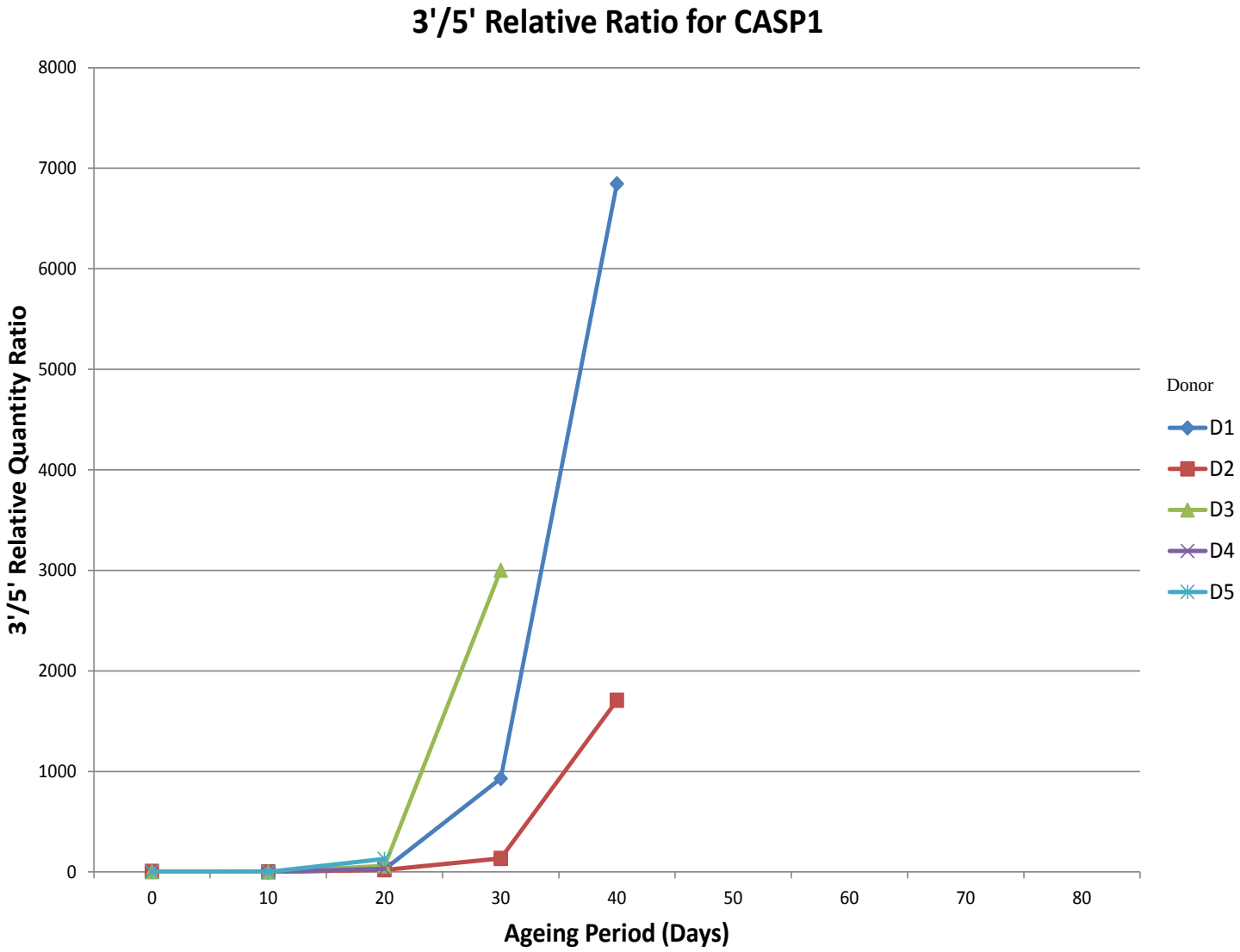


Figure 56: Time dependant analysis of the 3'/5' CASP1 relative quantity data. The results for CASP1 were poor and as such no correlation between age and ratio was observed. Obtaining values for the 5' target proved difficult and as such a ratio could only be obtained from limited samples. There was not enough data to make any further conclusions.

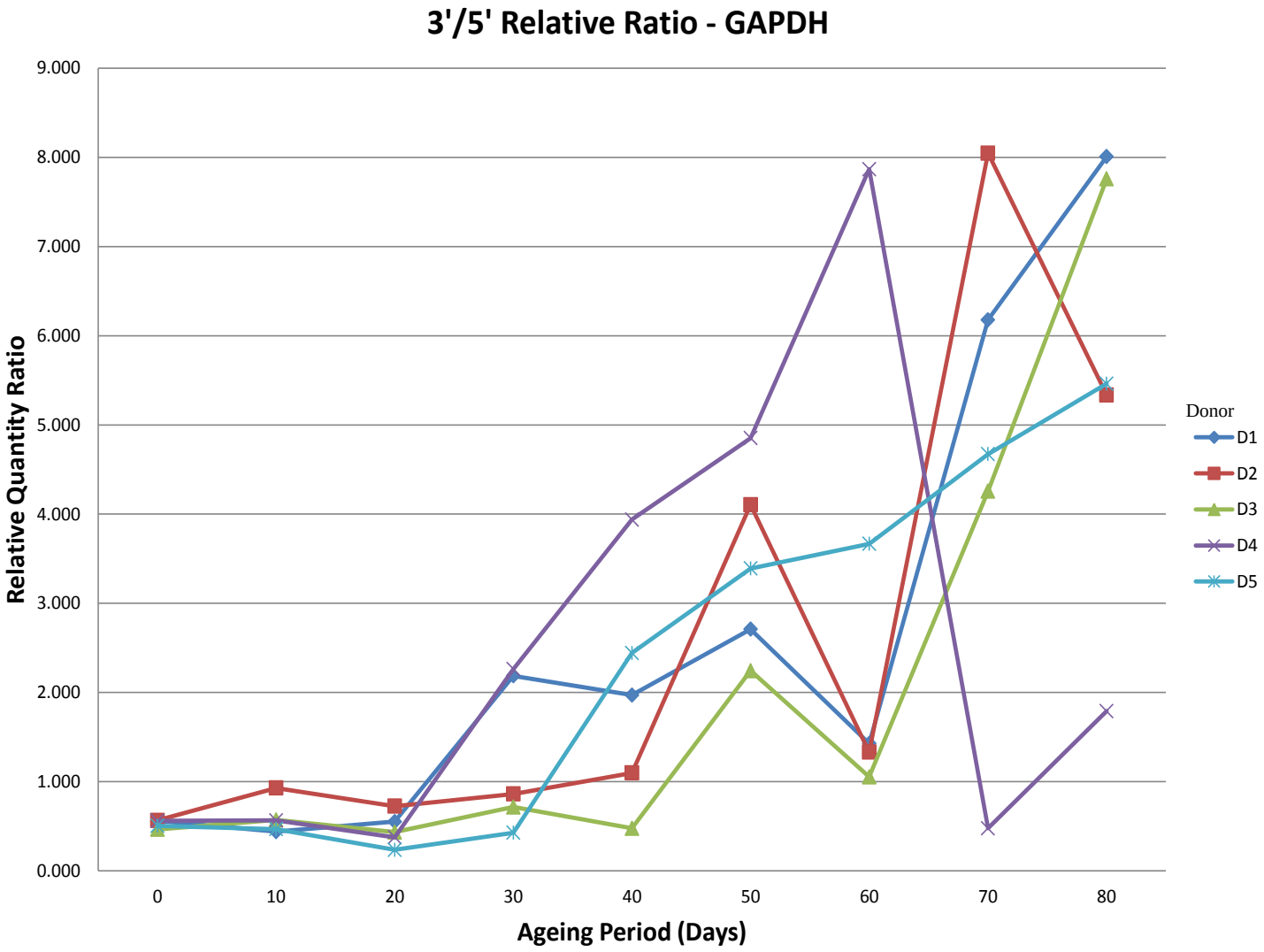


Figure 57: Time dependant analysis of the 3'/5' GAPDH relative quantity data. The 3'/5' GAPDH ratio showed a good correlation with sample age. In terms of discriminating power, GAPDH outperformed all other targets tested. The ratio remained relatively constant up to 20 days suggesting no significant change in the degradation levels of the GAPDH target. This is consistent with its housekeeping ability. After day 20 a weak trend can be observed, with the ratio increasing as the samples aged. This trend was weak with large differences being observed between donors for each age point.

The mean ratios were calculated and presented in Figure 58 on page 244, although because of the large differences the data did not graph well. The AMICA1 target did not present with a consistent or definable relationship between sample age and the 3'/5'ratio. The CASP1 assay provided very little information as a ratio could only be obtained for samples aged for less than 40 days. For the samples that did provide results there was an obvious increase in the ratio over time but the correlation was relatively weak (R^2 = 0.75) thus making age estimations unreliable. MNDA values were more encouraging. The results obtained from the day 70 samples did not follow the general trend of the rest of the MNDA data and when included in the statistical analysis the correlation between the ratio and sample age is similar to that seen with CASP1 (0.72). However, removal of the 70 day samples (which appeared to be outliers) significantly strengthen the relationship between the two variables (R^2 = 0.91). In terms of a correlation between the Relative 3'/5' Ratio and sample age, the GAPDH target performed the best, despite its reason for inclusion in this study (for normalisation purposes). Values were obtained across the entire length of the study and presented with a moderately strong linear relationship (R^2 = 0.9166).

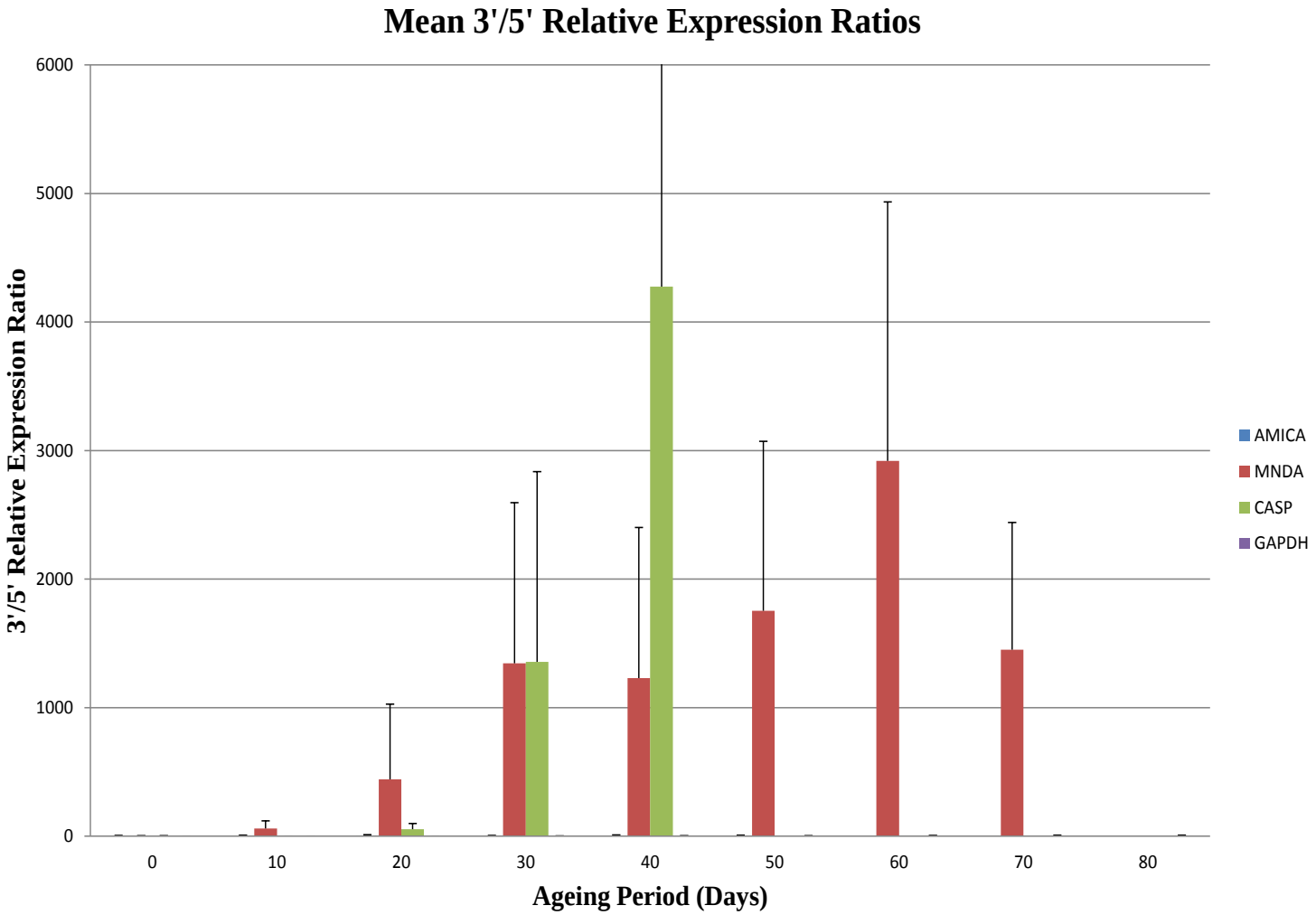


Figure 58: Mean Relative Ratios for each target. Due to the large variation in ratio size, the data does not graph well. Data was only obtained up to day 40 for CASP1, day 70 for MNDA and day 50 for AMICA1. GAPDH provided results across the entirety of the study. Each individual target species is presented in a more detailed layout shortly. Error bars indicate 1 SD.

7.4 Discussion

There have been a number of difficulties with establishing a robust RNA technique for estimating the age of bloodstains. Identifying a suitable RNA species that degrades in a consistent and predictable manner has been difficult (Bauer, 2007). There is literally hundreds of possible RNA analytes that can be examined from blood cells however the difficulty lies in finding one that changes expression in a manner that correlates with the age of the sample (Bauer, 2007). A second difficulty concerns whether the technique itself is capable of accurately measuring the degradation change of the RNA analyte (Nolan and Bustin, 2008). Whilst qRT-PCR is considered a robust and reliable method for quantifying RNA (Pfaffl, 2001), it has been used in various different ways to measure the degradation levels of a sample [compare for example the different approaches adopted by Anderson *et al,* (2005) and Bauer *et al*, (2003)]. The purpose of this study was to explore an approach (3'/5'assay) described by Nolan *et al* (2006) in the hope it would provide a more accurate method for determining the degradation levels of RNA transcripts in forensic type blood samples. This approach has not as yet, been used to age bloodstains. The 3'/5'assay establishes a degradation ratio based on the quantity of two amplified products that are positioned at different distances from the poly-A tail of the RNA molecule. Nolan *et al,* (2006) suggests that a ratio of greater than five was indicative of a degraded clinical sample. Ratios of greater than five were expected in this experiment given samples were aged over a period of 80 days.

Three target species that had proven to be robust and reliable markers for aged blood stains (Zubakova *et al,* 2008) were used to measure the degradation level. The 3'/5'relative expression ratio were calculated and compared to the age of the sample to determine whether a correlation existed.

7.4.1 AMICA1

The mean 3'/5'relative expression ratio for AMICA1 remained relatively consistent for samples aged up to 50 days (4.86). The reliability of these results is questionable given the ratio at day 0 was 4.92 suggesting these samples were more degraded than those that had been aged for 50 days. Despite this apparent stability, the 3'/5' ratio could not be calculated for samples older than 60 days because the 5' amplicon could not be detected from any donor. The 3' amplicon was only marginally better being detected in only 33% (14 of 45) of the older

samples. These results suggest there was a rapid and pronounced rate of degradation between 50 and 60 days. A rapid rate of degradation between 30 and 60 days was also observed in chapter five, although the degradation concerned DNA. It is uncertain whether this was coincidental or whether nucleic acids become particular susceptible to degradation after this period of time. The inability to detect the 5' amplicon after 50 days was a concern. Zubakov *et al,* (2009) was able to detect AMICA1 in blood stains that had aged for 16 years whilst Lindenbergh *et al,* (2011) was able to detect AMICA1 in stains aged up to 28 years. Given the similarity in the ageing conditions these findings suggest the 3'/5'assay employed in this experiment is not as suitable as the methods applied by these authors for this type of work.

The size of the inter-donor variation observed was smaller than previously observed in chapter six, but was still too large for any forensic application. The variation (%CV) was smallest at Day 0 (6.8%) but was significantly larger at all other ageing periods recorded. The inter-donor variation ranged from 6.8% to 91.9% (Day 40) with a mean value of 61.8%. The variation observed in chapter six peaked at 189%. This improvement provides support for the hypothesis that cell lineage may have an effect on the size of the inter-donor variation as AMICA1 is derived from monocytic cell line which tends to have a more consistent and stable life cycle (Lodish *et al*, 2012).

Overall these results indicate the AMICA1 target was not suited to this type of analysis as there was no correlation between the 3'/5'ratio and the sample age and therefore this approach lacked the ability to distinguish between aged samples. Alternatively the problem may exist with the amplicon spacing and design which is discussed on page 257, rather than the AMICA1 species itself.

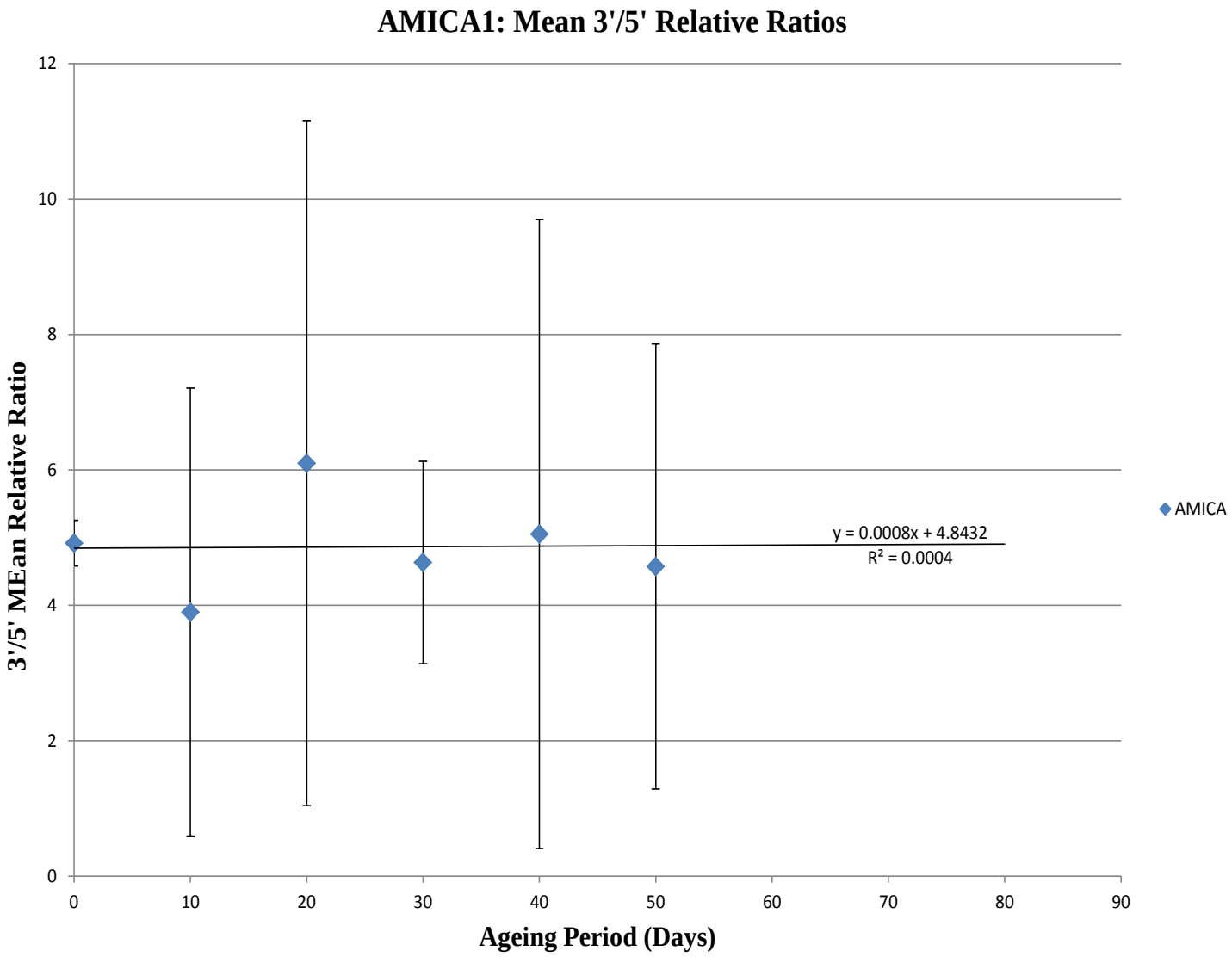


Figure 59: Mean AMICA1 3'/5' ratio data for all five donors across the 80 day ageing period. Data was not obtained for any samples aged for longer than 50 days. This is possibly due to the large distance (351 bp) between the 3' and 5' targets. There is no correlation between the age of the sample and the ratio (R^2 = 0.0004) and the inter-donor variation observed was large. Error bars represent ± 1SD.

7.4.2 MNDA

The MNDA target was chosen because it had previously shown good stability (Zubakov *et al*, 2008) and is exclusively expressed in cells from the myeloid lineage (Briggs *et al*, 2005). It was thought that by selecting a target from this cell lineage there may be a reduction in the inter-donor variation as the cell cycle, proliferation rates and general haemopoietic functions were more predictable and consistent (Lodish *et al*, 2012).

Eukaryotic mRNA is degraded in a number of different ways. For example some specific sequences trigger decay independently of deadenylation (Beelman and Parker, 1995). This pathway results in specific transcripts being degraded via deadenylation-independent decapping followed by 5' to 3' degradation (Beelman and Parker, 1995). An alternative pathway is dependent on deadenylation and involves the shortening of the poly-A tail followed by decapping and 5' to 3' exonucleolytic degradation of the transcript (Beelman and Parker, 1995). mRNA degradation can also occur by both specific and nonspecific endonucleolytic cleavage in the transcript body (Esnault and Malter, 1999), whilst bacteria are known to consume and degrade mRNA transcripts (Mathy *et al*, 2007). Additionally MNDA mRNA is specifically cleaved by caspases during neutrophil apoptosis (Milot *et al*, 2012) Given the numerous decay mechanisms in existence, a decrease in the MNDA mRNA expression was expected over the course of this experiment. The MNDA data from the first 60 days presented with a moderately strong linear relationship (R^2 = 0.913) between the mean relative 3'/5' expression ratio and sample age. This correlation weakened when the entire data was included (R^2 = 0.716). Nolan *et al*, (2006) suggested that a ratio of five was indicative of a degraded sample. This assessment was made in reference to fresh clinical samples. Aged degraded samples such as those used in this experiment were expected to have higher ratio values. The MNDA expression ratios were far greater than those observed with AMICA 1 and Nolan *et al*, (2006). Whilst the mean ratio for AMICA1 fluctuated between 3.90 and 6.09, the MNDA ratio varied from 3.20 (day 0) to 2919.9 (day 60). This MNDA ratio peaked at 4915, suggesting these samples were severely degraded. Whilst elevated ratios were expected, the size of the increase was not. Such high ratios could be explained if there was an increase in the normal transcript numbers (increasing both 3' and 5' products) as well as a subsequent increase in the degradation of 5' MNDA amplicon. An increase in MDNA mRNA expression has been observed at sites of inflammation (Briggs *et al*, 2005) and in some cases of acute myeloid leukaemia (Briggs *et al*, 1994). Monocytes have been shown to increase MNDA mRNA

secretion upon stimuli from TNF (Trinchieri *et al,* 1986). Furthermore Briggs *et al,* (2005) suggested that MNDA mRNA is involved in the regulation of programmed cell death in human myeloid hematopoietic cells. Any of these conditions could result in an increase in transcript numbers. Unfortunately absolute quantification was not performed on these samples to determine if this was in fact the case. The length of the ageing process and therefore exposure to the various degradation factors could account for the high rate of degradation of the MNDA mRNA species. No results were obtained from the 80 day samples due to the fact that the 5' target could not be detected from any of the donors. The results from the older samples (>60 days) were generally poor due to the inability to reliably amplify and detect the 5' target. Whilst there are a number of factors that play a role in the degradation of MNDA mRNA, Mathy *et al,* (2007) suggests the most significant of these is the action of exoribonucleases. These enzymes predominately degrade mRNA in the 5′-to-3′ orientation (Mathy *et al,* 2007), which means that nucleotides from the 5' end of the molecule are cleaved first. The activity of these exonucleases could explain why the 5' amplicon was not detected in the older samples and why the MNDA ratio is so high. Moving the 5' amplicon closer to the 3' amplicon may prevent this dropout and provide more conclusive results. This should be considered for future work. There was one stand out anomaly in the data. The 70 day samples did not follow the general trend established in the first 60 days of the study. A mean value of 1450.9 was observed, which was equivalent to almost half the value obtained from the earlier 60 day samples. This anomaly remains unexplained but could be due to contamination.

Like AMICA1, the variation (%CV) observed with MNDA were less than the values reported using various other techniques on similar type samples [see for example; Anderson *et al,* (2005); Bauer *et al,* (2003)]. This was encouraging and provided further support to our initial hypothesis (regarding criteria for target selection). Despite the improvement the %CV still ranged from 19.7% (Day 0) to 132.4% (Day 20) with a mean variation of 81.6%, which is still too great for a forensic application. The mean variation for MNDA was greater than AMICA1 (61.8%) but less than CASP1 (87.5%). CASP1 is expressed in both the myeloid and lymphoid cell lines (all leukocytes) therefore these results suggest that cell lineage may potentially affect the size of the inter-donor variation. It should be noted that the CASP1 results were rather poor and limited given that no results were obtained from samples older than 40 days. Given the population size used in this study, a definitive conclusion regarding whether cell lineage affected the variation observed cannot be reached however there is enough evidence to warrant further investigation.

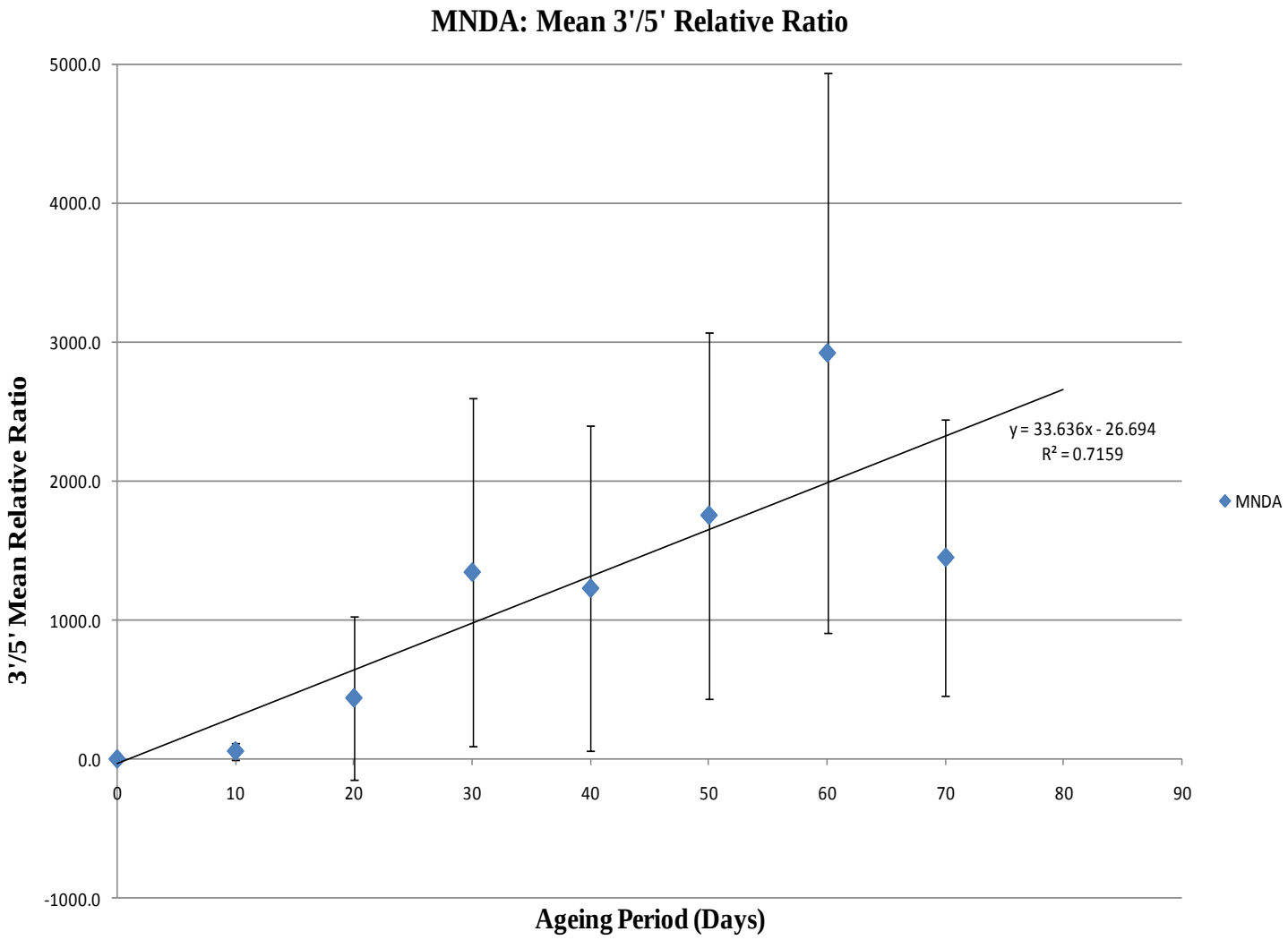


Figure 60: Mean MNDA 3'/5' ratio for all five donors across the 80 day ageing period. Data could not be obtained for the 80 day old samples. There was a moderately strong correlation between sample age and ratio (R^2 = 0.7159). The mean value at 70 days appears to be an outlier as it does not follow the trend of the data. The inter-donor variation of this data set is large and limits the reliability of an estimation made. Error bars represent ±1SD

7.4.3 CASP1

The CASP1 data was very poor. The mean 3'/5' ratio ranged from 3.4 (day 0) to 4274.8 (day 40), which in terms of size was similar to that seen with MNDA. No results were obtained from any sample aged for longer than 40 days. Although the 3' amplicon was still detected in 97.5% (39/40) of these samples, the 5'amplicon was not detected at all. This again could be due to the exonuclease 5'-3' degradation activity as described earlier. It is also possible there was a problem with the amplification setup. During the efficiency assays, it was determined that the 5' amplicon had an amplification efficiency rate of 84.2%, which is low given the ideal samples and conditions used. Though this is likely to have affected the quantification process, it does not explain the inability to produce any results at all. An alternative and more probable explanation is that there was a problem with the assay design and in particular the distance between the 3' and 5' products which is discussed on page 257.

The degradation of CASP1 mRNA occurred in an exponential manner over the course of 40 days. Although this generalised trend was observed, the correlation between the CASP1 ratio and age was relatively weak ($R^2 = 0.75$). The exponential pattern of degradation suggests that CASP1 mRNA was relatively stable for up to 20 days. This was followed by a rapid rate of degradation between 20 and 40 days. This pattern (rate of degradation) can be distinguished from the MNDA degradation which appeared relatively constant throughout the ageing period. The apparent stability over the first 20 days could have been due to minimal degradation however there is no evidence to suggest CASP1 mRNA species is afforded any specific protection from the various mRNA pathways previously explained. Alternatively, an increase in CASP1 expression during the first 20 days may have balanced any degradation giving the appearance that CASP1 was relatively stable during this period. CASP1 is an aspartate-specific cysteine proteases known for regulating programmed cell death and inflammation (Shao *et al*, 2007). CASP1 is found in cells as an inactive precursor and is activated in response to inflammatory triggers, including pathogen-derived molecules, as well as danger signals released from infected or dying cells (Scott and Saleh, 2007). It is therefore possible that CASP1 levels could have been initially elevated in blood samples used in this experiment.

The inter-donor variation observed from the CASP1 data was slightly larger than AMICA1 and MNDA. The mean variation (%CV) was 87.5% however values were only obtained from samples aged up to 40 days. This variation may have increased if ratios were obtained from older samples. There are a number of possible causes of the variation observed. All samples

were aged under similar conditions therefore environmental degradation rates should have been relatively consistent. It is possible that initial CASP1 expression levels varied between donors. Although all donors were assumed to be healthy, CASP1 levels are increased with certain types of bacterial infection, including *Salmonella typhimurium, Pseudomonas aeruginosa, Listeria monocytogenes, and Legionella pneumophila* [Amer *et al,* (2006); Franchi *et al,* (2006); Ren *et al,* (2006)], septic shock (Shao *et al,* 2007) and autoimmune inflammatory disorders (Martinon *et al*, 2009). There are a number of different haplotypes of the CASP1 gene with some alleles being more prominent in certain disease states (Blankenberg *et al,* 2006). A study carried out by Blankenberg *et al,* (2006) investigated the genetic variability of the CASP1 gene and plasma levels of CASP1 in relation to cardiovascular risk. They demonstrated that haplotypes carrying particular alleles such as the A^{in6} were associated with lower mRNA expression, which could pose problems if this technique was to be used on the general population. Jee *et al,* (2005) also observed similar variability in certain disease states such as gastric cancer where significant expression loss was observed. Their findings also suggested that epigenetic events such as DNA methylation and histone deacetylation play important roles in the regulation of CASP1 (Jee *et al,* 2005). Epigenetics, which is the inherited changes in the phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence (Spector, 2012), could pose problems for accurate analysis using this approach. These changes may remain through cell division and for the remainder of the cells life being passed on to multiple generations (Bird, 2007). Although there may be no change in the underlying DNA sequence, non-genetic factors may cause the genes to behave, or express themselves differently therefore standardisation may be very difficult. It has been demonstrated that CASP1 can activate nucleases which would increase the rate of RNA degradation by nuclease attack (Nagata *et al,* 2003). Fahy *et al* (2008) observed lower relative copy numbers of inflammatory mRNAs, including CASP1, in monocytes from patients with septic shock compared to those with critically ill control subjects. These findings are relevant to this experiment as any proposed method involving the measurement of mRNA transcripts would ideally require a target that had a similar expression rate between donors (inter-donor variation was minimal). Blankenberg *et al,* (2006) suggests this may not be the case for CASP1. It has been suggested that exposure to UV-B light also inhibits mRNA degradation and deadenylation (Gowrishankar *et al,* 2005), therefore samples aged in the direct sunlight may actually have a lower rate of CASP1 degradation than samples not exposed to sunlight. Given the size of the variation associated with the CASP1 and the absence of data from any sample aged for longer than 40 days, the CASP1 ratio was not suitable for determining the age of a blood sample.

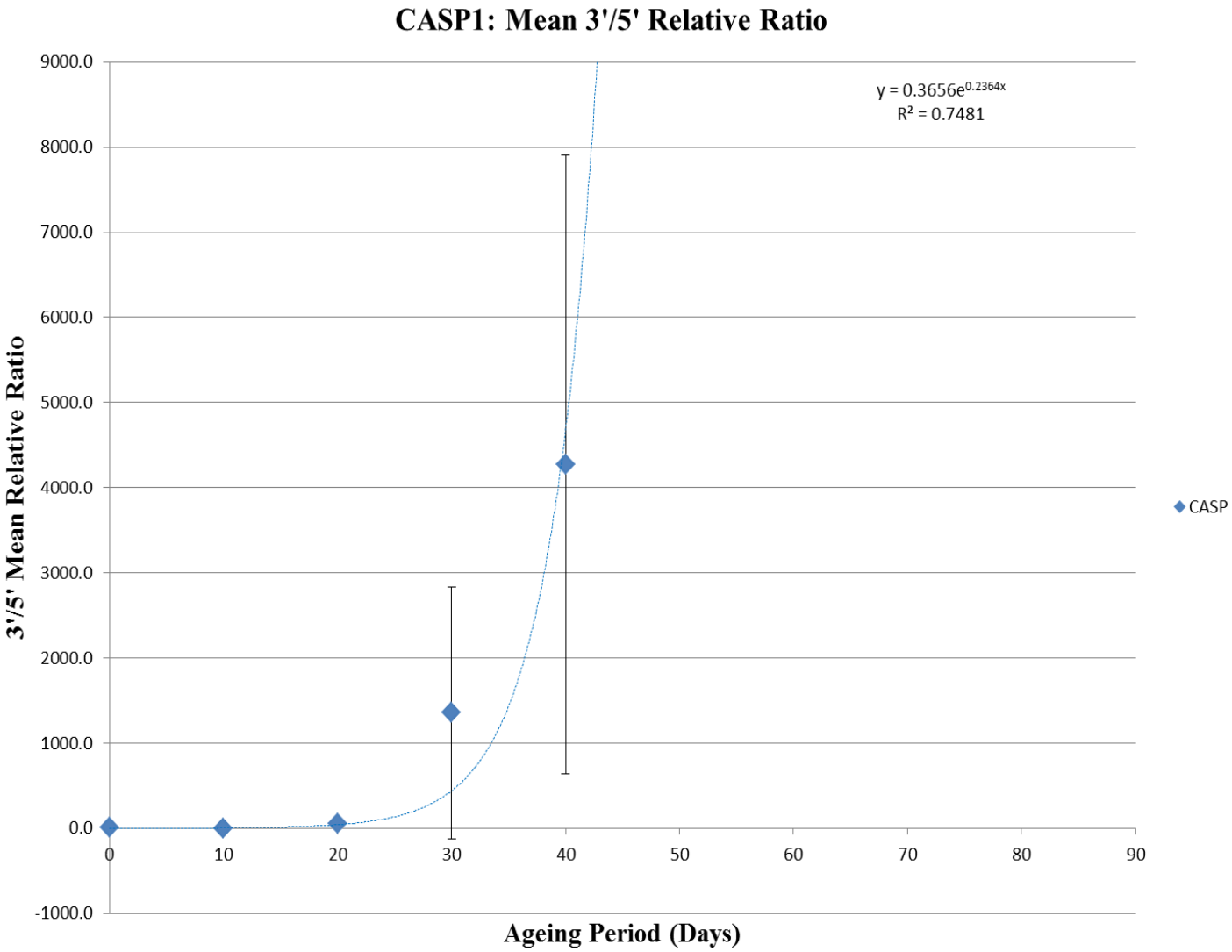


Figure 61: Mean CASP 3'/5' ratio from all five donors over the 80 day ageing period. No data could be obtained from samples older than 40 days. This is likely due to the spatial distance of 421 base pairs between the 3' and 5' targets. The correlation determined from the data that was obtained suggested a relatively weak relationship did exist between sample age and ratio however combined with the large inter-donor variation observed, any estimation based on this data would be unreliable. Error bars represent ±1SD.

7.4.4 GAPDH mRNA

GAPDH mRNA was initially selected for use as a reference or normalisation gene. GAPDH is not specific to blood tissue, nor had it been rigorously tested by Zubakov *et al* (2008) whose ageing conditions were similar to those used in this study. The mean 3'/5' ratio data for GAPDH is shown in Figure 62. As a normalisation marker, GAPDH was only stable for the initial 20 days as indicated by the 3'/5' expression ratio, which barely changed. Values of 0.53, 0.59 and 0.46 were obtained which suggests there was twice as much 5'target as there was 3' target. These results were not expected. Using this technique it is not possible to amplify the 5' target without amplifying the 3' target given the RNA is transcribed from the 3'end (Nolan and Bustin, 2008), however the reverse is not true therefore the ratio should always be greater than 1. These results could be explained if the 3' target became degraded between the reverse transcription and the real time PCR step. Alternatively these results could be explained if the 3' target amplifying at a lower rate than the 5' target. According the amplification efficiency assays the 3' target was expected to amplify at 87% whilst the 5' target was calculated at 92%. This 5% difference could account for the 2-fold difference observed. Whilst this would be detrimental for an absolute quantification method, the relative method used here is unaffected and trends observed can still be relied upon. GAPDH mRNA expression levels changed significantly after 20 days. The general increase in 3'/5' expression ratio confirms that GAPDH mRNA degraded consistently over the remainder of the ageing period. Despite GAPDH not being specifically selected for correlation purposes, the relationship between its 3'/5' expression ratio and sample age was stronger than that observed with any of the other selected targets (R^2 = 0.92). This correlation is strengthened to 0.96 if the data from the first 20 days, when GAPDH expression was stable, is ignored. The degradation of GAPDH in ageing blood samples was expected given the various degradation pathways of mRNA previous explained (Mathy *et al*, 2007). Furthermore it has been shown that lysosomes exhibit specific GAPDH mRNA degradational properties (Hopsu-Havu *et al*, 1997). Environmental conditions such as heat and UV light can also degrade GAPDH mRNA (Yoon *et al*, 2013) however what effect this had on GAPDH mRNA expression in this experiment is unknown.

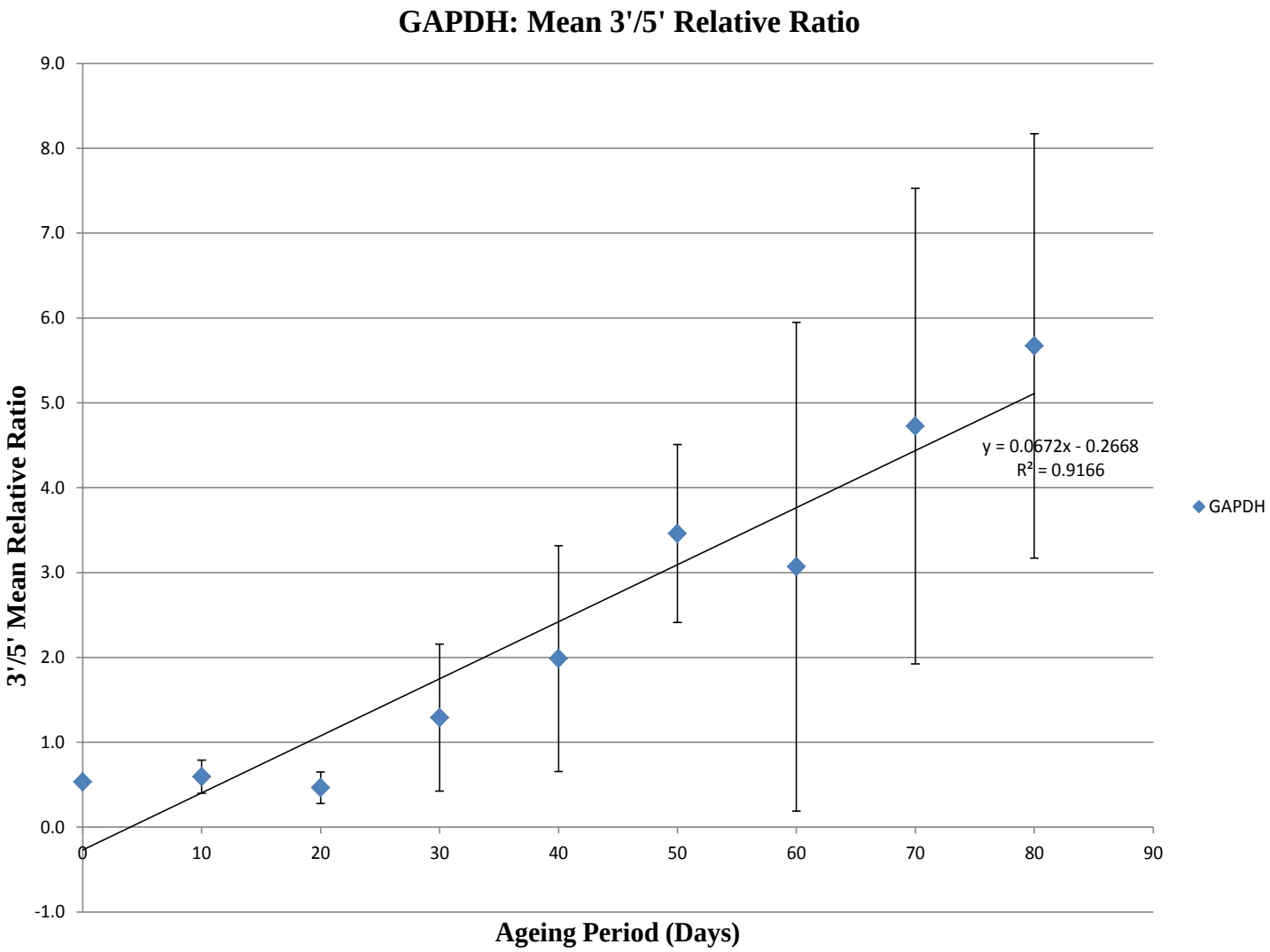


Figure 62: The mean GAPDH 3'/5' ratio data for all five donors across the 80 day ageing period. Data was obtained across the entire study. GAPDH produced the strongest correlation between sample age and ratio out of all targets tested. This corresponded to the smallest distance between the 3' and 5' targets which is likely to be an influential factor in these types of studies. The inter-donor variation was small during the early stages (less than 50 days) of this study however increased significantly in the latter stages. Error bars represents ±1SD.

The correlation that was observed between the GAPDH mRNA ratio and sample age in this experiment supports findings from various other authors [Wang *et al,* (2011); Wu, *et al,* (2010)]. Both Wang *et al,* (2011) and Wu, *et al,* (2010) identified negative correlations between the degradation rate of GAPDH mRNA and post mortem interval. Although GAPDH mRNA expression decreased over the course of the ageing period in this experiment, the decrease was small compared to that observed with CASP1 and MNDA. The mean 3'/5' expression ratio across the entire study remained below the defined degradation limit (5) described by Nolan *et al,* (2006), which was for clinical samples. This suggests GAPDH was relatively stable throughout the ageing period. These findings are consistent with comments made by Anderson *et al,* (2005) who stated GAPDH mRNA decayed at a slower than other types of mRNA, including IL-3. Of equal importance is that the results suggest the 3'/5' method was capable of detecting the small degradational changes, highlighting the sensitivity of this technique. Jorges and Bishop (2008) failed to detect mRNA GAPDH in blood spots that had aged up to 4 weeks (28 days) whereas levels were still detected using the 3'/5' assay after 80 days. Samples were aged under similar conditions, which suggest the differences observed in the ability to detect targets may be attributed to the sensitivity of the assay. It should be noted that although the ageing conditions were similar it has already shown in chapter five that even small changes can have dramatic effects on the degradation levels of nucleic acid targets. The small changes that were observed also correlated with age suggesting the target RNA species and methodology may be suitable for determining the age of blood samples aged for less than 80 days. These findings suggest the 3'/5' assay may be more sensitive at measuring degradation rates than traditional RT-qPCR methodologies if the spacing between the amplicons is optimised.

The inter-donor variation observed was again a limiting feature of this methodology. Although still excessive for an accurate forensic technique, the variation was considerably less than all previous targets tested. The smallest variation (%CV) was 8.4% (day 0), indicating that the natural variation between donors is small. The largest variation was 93.7% and this occurred towards the end of the study (day 60). The overall mean variation for GAPDH was 49.2%. There are many factors that can cause change in GAPDH mRNA expression, which would in effect potentially cause variation between donors. GAPDH mRNA expression will change depending on the requirement of the GAPDH protein. The GAPDH protein has a variety of biological properties (Sirover, 1997). As a membrane protein, GAPDH functions in endocytosis whilst in the cytoplasm it is involved in the translational control of gene

expression. In the nucleus GAPDH mRNA has roles in nuclear tRNA export, DNA replication and DNA repair (Sirover, 1997). The requirements of each of these processes can result in a change in GAPDH mRNA expression. GAPDH mRNA expression is also enhanced in certain pathological conditions such as cardiac syndrome X (Dabek *et al*, 2010) and neurodegenerative disorders, especially those characterized at the molecular level by the expansion of CAG repeats (Sirover, 1997). Activation of T lymphocytes during an immune response can also result in fluctuating GAPDH mRNA expression levels by as much as 70-fold (Bas *et al*, 2004). Expression levels of GAPDH mRNA can be increased up to 75% in hypoxic conditions (Zhong and Simons, 1999) which could be of significance with aged blood stains that containing dead or dying cells. Barber *et al*, (2005) observed significant differences in the expression levels of GAPDH mRNA between tissue types and between donors of the same tissue. The inter-donor variation observed by Barber *et al*, (2005) is a concern for the suitability of GAPDH in ageing studies.

Given the various roles and factors that affect GAPDH mRNA levels it is not unexpected that Dheda *et al*, (2004) reported that GAPDH mRNA expression in blood was more variable (> 30-fold maximal variability) than 12 other housekeeping genes measured. Bustin (2002) suggested the same. Although no specific effect of either age or gender was observed on GAPDH mRNA expression, the inter-donor variation observed was consistent to the findings in this experiment and are ultimately detrimental to applying this technique as a forensic tool.

7.4.5 Amplicon Size and Spacing

There was a large variation in each of the targets ability to resolve a samples age based on the 3'/5' ratio. There are two likely explanations for this. Firstly, the target itself may not behave or degrade in a manner that is consistent with the age of the sample. Secondly, the assay conditions in terms of amplicon length and the spatial distances between the 3' and 5' targets and the distance from the poly-A tail may not have been ideal. Optimisation of these issues should be considered for future studies, although a study of this nature was outside the scope and cost/time constraints of this research. The results in this experiment suggest that the amplicon size does not have a significant influence on the quality of the data. In terms of the correlation between age and ratio, GAPDH performed the best ($R^2 = 0.92$) with amplicon sizes of 174 and 140. AMICA1 performed the worst ($R^2 = 0.0004$) with target sizes of 134 and 121. MNDA and CASP which had very similar correlation strengths (0.72 and 0.72 respectively)

had amplification products of 195, 153, 146 and 106 base pairs. All targets were not excessively long therefore amplification difficulties that can sometimes be seen with longer targets were not an issue. When the data was considered holistically, there was no obvious pattern between the correlation strength and amplicon sizes.

The spacing or distance between the 5' and 3' targets is likely to be an influential variable given the mechanics of the 3'/5' technique. Because the reverse transcription step relies on the integrity of the RNA sample between the two targets, it is expected that the larger the distance between the 5' and 3' targets, the greater the chance of encountering structural damage. This structural damage will prevent the conversion of RNA into cDNA. This has an exponential effect on older, more degraded samples therefore any optimal distance determined in one study using one particular set of samples will likely to be different from another study using a different set of samples. This poses a problem with forensic samples.

For the samples in this experiment (aged over 80 days in an internal urban environment), a relatively short spatial distance between the two targets provided the best results in terms of the ratio to age correlation. The spatial distance between the 5'and 3' GAPDH amplicons was only 121 bp, whilst the distance between the AMICA1, MNDA and CASP1 amplicons were 351, 301 and 421 base pairs respectively. A spatial distance of 421 appears too large for samples aged for 50 days or more whilst a spatial distance of approximately 300 appears too large for samples aged for 80 days or more. The data obtained from this study suggests a distance of less than 300 base pairs is required to obtain results across the entire ageing period using blood samples aged naturally in an internal urban environment. GAPDH amplicons which were separated by 121 base pairs produced the best results for this data set and future optimisation studies should concentrate on distances between 100 and 250 base pairs. Nolan *et al*, (2006) looked at two different tissue types using this methodology. Their assay was designed so that the 3' target was 429 base pairs from the poly-A tail whilst the 5'target was 1072 base pairs away. The distance between the 3' and 5' targets was 547 base pairs, which was rather large compared the distances used in this study however the sample types differed significantly. Nolan *et al,* (2006) used fresh and formalin fixed colonic biopsies. The fresh samples produced ratios of between 1 and 5 whereas the formalin fixed samples presented with ratios as high as 500. The ratios obtained from the GAPDH data were comparable to those fresh samples of Nolan *et al,* (2006). Ratios as high as 5000 were obtained from the MNDA data which significantly exceeded the formalin fixed samples. Despite the numbers, these studies cannot

be compared because of the different primer and assay conditions used. Similar to the problems outlined in chapter four, unless there is complete optimisation and the characteristics of targets are known, it is very difficult to compare data between studies.

7.5 Limitations of the 3'/5' RNA assay

This method is limited by the ability and efficiency of the reverse transcription reaction step. To ensure reliable results complete optimisation of this step as well as the PCR step is required, therefore making this technique labour intensive. Using target specific primers are the most specific and sensitive method for converting mRNA to cDNA (Bustin, 2004) but are only recommended when RNA quantity is not a limiting factor. Unfortunately, with forensic samples, the amount of RNA will often be limited. The final limitation may explain the poor results obtained. Oligo-dT priming, which is essential to the 3'/5'assay, should only be used with intact RNA. Degraded RNA will often lose their poly-A tail which is the binding site for the reverse transcription primer. This could mean that the amount of RNA in degraded forensic samples will be underestimated. There was a relatively consistent expression in CASP1, MNDA and GAPDH over the first 20 days. There was a sharp decrease in expression after this point which may indicate that the poly-A tail is stable for only 20 days under the conditions of this study. If this is true, the Oligo-dT priming method may not be suitable for forensic type samples.

7.6 Future work

There is clear evidence to suggest that the spatial distance between the 3' and 5' targets will influence the quality of the results. A complete optimisation study should be carried out to determine the optimal distances between the two targets as well as the optimal distances from the poly-A tail. These studies should be carried out on GAPDH as well as other housekeeping genes. An investigation into the degradation regularity on the different sites of a particular gene is also warranted. Ren *et al,* (2009) recently examined six different sites between the 5'-cap and the 3'-end of the GAPDH mRNA sequence to determine whether the degradation rate correlated with the post mortem. They found that all Ct values from each of the different sites correlated with PMI. They observed a stronger correlation between the two variables (PMI and degradation rate) in sites closer to the 3' end. The authors went on to conclude that the different

sites showed different rates of degradation, even within the same tissue type that had been aged under the same conditions (Ren *et al,* 2009). This variability should be considered in future work.

7.7 Conclusion

The results obtained from this experiment were encouraging and have provided direction for future studies. It was established that the spatial distance between the 3'and 5' targets is a key factor in the assays ability to distinguish between degraded samples. In this experiment the best results came from GAPDH, which had a spatial distance of 121 base pairs between the two amplicons. This distance will require optimisation if more accurate and reliable results are to be obtained. Unfortunately the results obtained from this experiment were again limited by the recurrent issue of inter-donor variation. The variation was high across all target species and even though these values were generally lower at Day 0, there was no obvious pattern with regards to ageing time. It was hoped that by selecting particular targets of specific cell lineage that the inter-donor variation could be reduced however it appears to have had very little effect. These types of studies which use molecular targets to estimate the age of a sample are likely to be more successful by using target species that have limited or very regulated roles in the haemostasis process. Targeting species that have multiple functions and are affected by multiple components and processes are likely to result in large variations between individuals. Given the nature of blood, identifying suitable candidate targets though possible, may be an exhaustive process. Housekeeping genes and in particular, GAPDH mRNA may provide the answer as it outperformed all other targets tested here and given its relative abundance will generally provide a better signal to noise ratio. Unfortunately the diversity of mRNA decay pathways, in addition to different rates of decay for individual mRNAs within one pathway, allows for a wide spectrum of mRNA half-lives and for their differential regulation (Beelman and Parker, 1995). This highlights the difficulties in identifying a suitable target for this type of analysis.

CHAPTER 8: AGE DETERMINATION OF HAIR (Anagen Phase) USING RNA ANALYSIS

8.1 Introduction

Bauer *et al,* (2003) and Anderson *et al,* (2005) have shown that RNA degradation rates can be used to estimate the age of bloodstains albeit with limited resolution. Anderson *et al,* (2005) suggests the technique they adopted may be applied to other tissue types, but following a thorough literature review there was no evidence this had been done. In chapter seven it was suggested that blood components, and in particular RNA species, are prone to a high degree of inter-donor variability, which limits the accuracy and precision of RNA based ageing techniques. This chapter investigates the use of hair, which is a commonly found and forensically important type of biological evidence found at crime scenes (Saferstein, 2006). It was thought that hair, being a less dynamic tissue type than blood may produce more consistent and predictable results when it comes to ageing samples. The theory underpinning the work done by Anderson *et al,* (2005), namely that different RNA molecules from different parts of the cell will degrade at different rates, was sound and therefore adapted for use in this research with the ultimate aim of determining a method for ageing hair samples.

To declare the method adopted in this experiment relates to ageing hair samples is slightly misleading as the RNA recovered was obtained from the follicular tag attached to the hair, rather than the actual hair shaft itself. The follicular tag is often referred to as the root or sheath cells but is effectively the collection of cellular material attached to the bulbous part of the shaft (Jolles *et al,* 1997). Not all hairs contain a follicular tag therefore the method proposed will not be applicable to all hair samples (Saferstein, 2006). The hair shaft itself would be the ideal sample location but the peculiarities of hair (intricate structure and biochemistry) make it a difficult tissue to process (Bender and Schneider, 2006). The shaft consists of three main sections, each containing differentiated cells originating from stem cells in the hair follicle (Jolles *et al,* 1997). Melanin granules, which are predominately ovoid in shape, are responsible for determining hair colour (Goldman and Steinert, 1990). These granules are generally found in high concentrations throughout the length of a strand of hair (predominately in the cortex and medulla) (Petraco, 1999). They play an important role when dealing with forensic samples as the granules can lead to PCR inhibition (Eckhart *et al,* 2000), a process most forensic DNA and RNA procedures rely on. The position of the cell nuclei is another reason why hair samples

are notoriously difficult to process (Cooper and Sunderland, 2000). The nuclei are thought to be positioned between the keratin filaments therefore limiting the effectiveness of standard DNA and RNA extraction procedures (McNevin *et al*, 2005). A number of recent studies have attempted to address this problem, with varying success. Most have relied on modifications to the PCR process, such as extended cycles (Barbaro and Falcone, 2005), or primer design to isolate very short amplicons (Butler *et al*, 2003), which is a common technique used for highly degraded biological samples. Recent research has focussed on the extraction step. McNevin *et al*, (2005) describes an optimal extraction procedure for genomic DNA in keratinised hair samples, which involves soaking hairs in simple digestion buffers containing Tris-HCl, a salt and a chelating agent without prior cleaning of the hair shafts. Their results were encouraging as full DNA profiles using Profiler Plus (Applied Biosystems, US) were consistently obtained from hair shafts. They did however conclude that the amount of genomic DNA recovered was very small. They found that anagen hair roots yield approximately 1% of the quantity of DNA found in buccal swabs whilst telogen phase hairs yielded as little as 0.1%. The problems described here have concerned the extraction of DNA, though it can be assumed RNA is affected in much the same way.

The human hair cycle is an important feature of this type of tissue and directly affects the types and evidentiary value of hair samples recovered from crime scenes. There are three phases of the human hair growth cycle (Saferstein, 2006). These include the anagen (active growth) phase, the catagen (transitional) phase and the telogen (resting) phase (Saferstein, 2006). Some authors suggest a fourth, "exogen" or shedding phase (Stenn and Paus, 2001) but this is not consistently documented in the literature. The anagen phase is the longest of the phases, lasting 2 to 4 years (Robbins, 2002). At any one time, approximately 75% to 95% of all follicles in humans are in this phase (Ackerman and Viragh, 1993). The catagen phase begins when mitosis in the hair follicle germ cells, ceases (Robbins, 2002). This phase lasts between two and three weeks and is followed by the third and final telogen phase. The telogen phase, which lasts about three months is characterised by apoptosis or cell programmed death. This results in keratinisation of the cell proteins and necrosis (Robbins, 2002). As hair is extruded out of the hair follicle, cells become dehydrated and protein is keratinised and cross linked via cysteine rich disulphide bridges (Ackerman and Viragh, 1993). The keratinisation process results in hairs containing less than 10% water and causes cytolysis and hence loss of genomic DNA and RNA in the cortex (Ackerman and Viragh, 1993). Apoptosis of the hair cells is characterised by the disappearance of cell organelles and by the cleavage of genomic DNA by specific

deoxyribonucleases (Fischer *et al*, 2011). This may represent an upper limit to the size of amplicons that could be possibly recovered from keratinised hair. Other authors have agreed this nuclease attack is probably the most important factor in the degradation of DNA and RNA in forensic samples [Fischer *et al*, (2011); McNevin *et al*, (2005)]. The inability to routinely recover usable quantities of nucleic acids from hair shafts has been a real problem given that telogen phase (which do not contain follicular tags) hairs are shed naturally (50-150 hairs per day) and are the most likely type to be found at a crime scene (Saferstein, 2006).

Given the complications and difficulties associated with keratinised hair, anagen phase hairs were chosen for this study. The aim was to measure the degradation rates of 18S rRNA and B-actin mRNA found within the cellular material adhered to the anagen phase hairs and to determine whether this degradation was consistent with the sample age. If the method proved successful then other, more difficult hair types could be trialled. The methodology used by Anderson *et al*, (2005) was adopted in this work which allowed a direct comparison between the different tissue types (hair v blood). It was thought this may provide some evidence as to whether blood is in fact a notoriously difficult sample type for this type of investigation. Furthermore there was no currently known research on the use of RNA in this manner to age hair samples, which supported the novelty aspect of this study.

8.1.1 Assay Considerations

King *et al* (2001) suggested hair samples are the preferred source of RNA (compared to blood) given that they are easy to transport and pose little to no health and safety risk. However, cases involving hair evidence can be limited to a single hair strand or a single root cell from which RNA can be extracted and therefore extracting measurable quantities of RNA can be problematic. King *et al* (2001), Kim *et al* (2006) and Bradley *et al* (2005) have demonstrated the successful isolation of RNA from trace amount of starting material but the published figures regarding the exact quantities vary considerably. This is primarily due to the variation in the number of cells that are attached to, or present within the follicular tag, which includes the root of the hair itself, as well as any attached follicular tissue. King *et al* (2001) isolated RNA from between 10 and 20 hair roots that had been aged for up to 10 days. These samples were obtained by plucking the hairs directly from the head, eyebrows and chest of the donor. They suggested that RNA is better protected from extra-cellular degrading agents in the hair root than RNA located within blood lymphocytes, as they failed to isolate RNA from the

lymphocytes after ten days. The inability to obtain RNA from 10-day-old blood samples is unusual given Anderson *et al,* (2005) recovered RNA from samples aged for 150 days, whilst Bauer *et al,* (2003) and Zubakov *et al,* (2008) isolated stable mRNA markers from blood and saliva stains aged up to 16 and 6 years respectively. Bradley *et al,* (2005) added to the findings of King *et al,* (2001) by effectively isolating RNA from between three and ten hair follicles up to six weeks after storage at room temperature. These findings were further supported when Kim *et al,* (2006) not only isolated RNA from as a little as one hair follicle (up to three hair follicles) but quantified the amount recovered. From 36 volunteers they determined that the average RNA yield was 112.5ng per hair follicle. Kim *et al,* (2006) also distinguished between RNA species and found that the ratio of ribosomal to messenger RNA was smaller than expected, which may indicate hair follicle cells do not produce as many proteins as other cell types and therefore may not be actively proliferating as much as other tissue types.

Extraction of RNA is commonly carried out using manufactured extraction kits or organic methods. Many researchers have successfully used RNA isolation kits in order to extract a high quantity of RNA from various tissue types [Gopee and Howard, (2006); Heinrich *et al*, (2007)]. A disadvantage with most of these kits is that they do not allow for the simultaneous extraction of DNA. This could pose a problem with forensic samples where DNA will almost always be required. Where both DNA and RNA is required an organic isolation technique capable of co-isolating both DNA and RNA is favoured by many researchers [Juusola and Ballantyne, (2003); Bauer and Patzelt (2003)]. The sensitivity of this technique is sufficient for forensic trace samples as Bauer and Patzelt (2003) demonstrated that genetic profiles could be obtained from small volumes of blood and semen (1 µl). Given the quantity of recoverable RNA was a concern and the forensic requirement to preserve the DNA from the sample, an organic Trizol (Invitrogen, UK) method was chosen to isolate and extract total RNA from the hair samples. Ten hair roots per sample were used to ensure enough RNA could be recovered.

8.1.2 Target Selection: 18S rRNA and B-actin mRNA

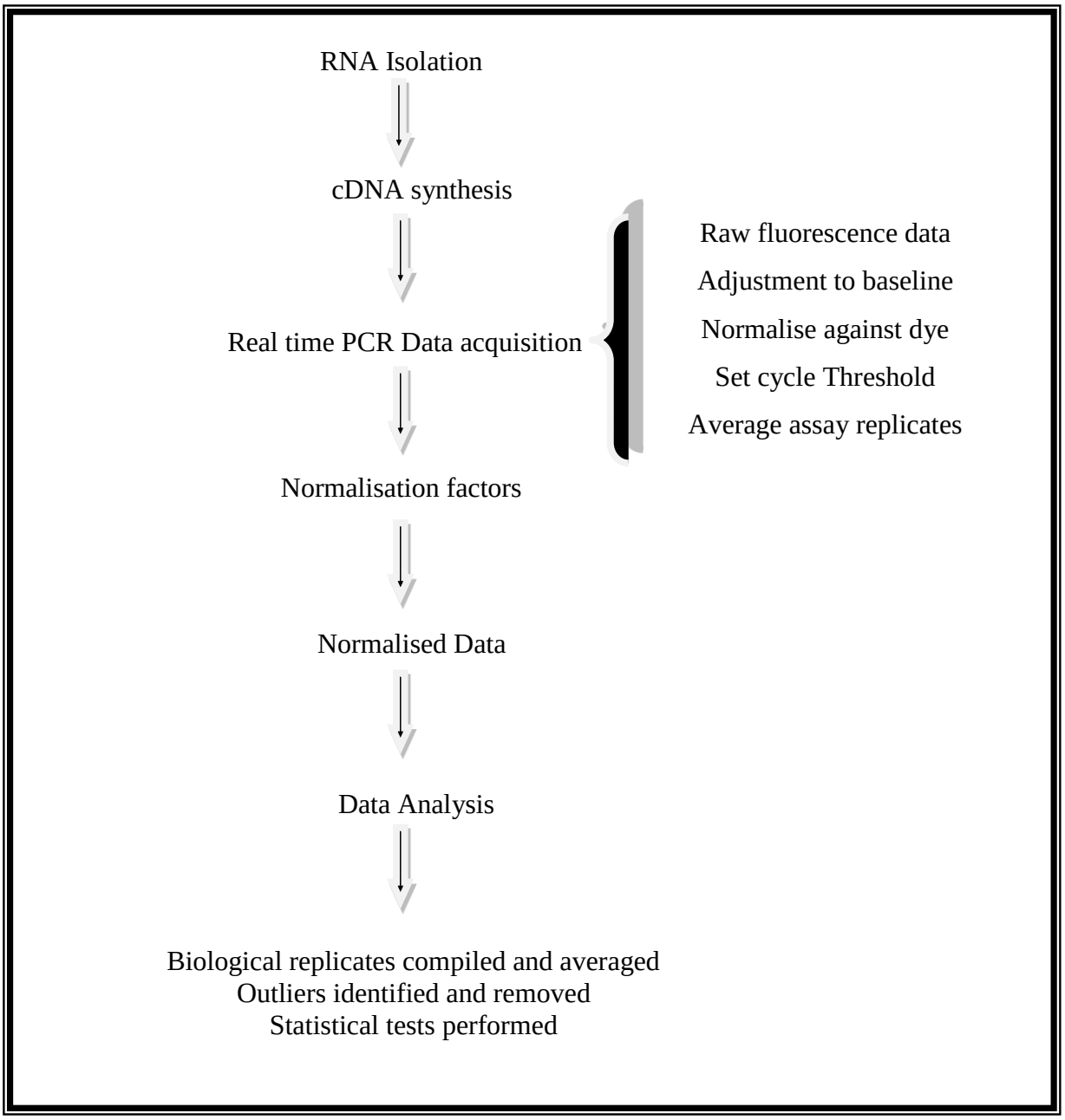
The results from chapter six and seven suggest that using stable, housekeeping targets for degradation studies are likely to provide the best results. For this reason, the two species of RNA chosen for this study were 18S rRNA and B-actin mRNA. There were also a number of other reasons for choosing these targets. Previous studies have been successful at expressing both targets from biological samples such as blood, saliva and semen [Anderson *et al,* (2005);

Juusola and Ballantyne, (2003)]. Being housekeeping genes, they are both universally expressed in all cell types and they exist in relatively high quantities, which was an important factor when dealing with minute forensic samples. 18S rRNA is believed to be more stable than mRNAs as it forms a part of a ribosomal sub-unit complex within the cytoplasm, which protects it from nuclease attack in a similar manner to which DNA is protected by histones (Anderson *et al,* 2005). The stability of B-actin mRNA has been well documented as it has been consistently recovered in bloodstains that have been aged for 9 months (Juusola and Ballantyne, 2003) and more recently, for as long as 2 years (Setzer *et al,* 2008).

The approach of using two different targets to estimate the degradation levels, and hence age of the sample, relies on selecting two targets that have different yet consistent degradation rates. It has been hypothesised that B-actin mRNA will degrade at a faster rate than 18S rRNA because it lacks the protective environment of the ribosome thus satisfying part of this pre-selection criterion (Anderson *et al,* 2005). The work in chapter four demonstrated that 18S rRNA is a suitable reference gene for blood samples up to a given period of time. Wong and Medrano (2005) suggested that validating the stability of the control gene with one's own samples in the defined reaction conditions is essential. As such the stability of 18S rRNA was examined in this experiment.

8.2 Methodology

The entire process beginning with the extraction of RNA to the interpretation and data analysis is described simply in the flow chart below.



8.2.1. Sample collection (Hair)

Hair samples were collected from a total of ten individuals, all of who were white Caucasian of either English or Irish ethnic background; five individuals were aged between 20-25 years old (two females and three males) and five individuals were aged between 40-45 years old (three females and two males) (details are provided in Table 49). The removal and preparation of the hair samples is described in section 2.1.8 on page 85.

Table 49: Age and Sex details of all ten hair donors

Donor	Sex	Age (yrs)
1	F	40-45
2	F	40-45
3	M	40-45
4	M	40-45
5	F	40-45
6	F	20-25
7	F	20-25
8	M	20-25
9	M	20-25
10	M	20-25

8.2.2 Sample Ageing Conditions

The major factors involved in post-mortem degradation of 18S rRNA and B-actin mRNA are endogenous nucleases and environmental factors such as light and bacteria (Gopee and Howard, 2006). To test the robustness of the proposed method, these external factors were not controlled to any great extent within this research. Samples were subsequently aged at room temperature, room humidity and in the presence of daylight.

8.2.3 Sample Processing

These processes are described in chapter two. In brief, an organic extraction method was used to isolate and extract total RNA from the hair samples. Trizol reagent (Sigma, UK), which is a monophasic solution of phenol and guanidine isothiocyanate was the commercial preparation of choice as it is suitable for isolating total RNA, DNA and proteins from a single sample (refer to section 2.2.3 on page 86)

Note: A sensitivity study was performed to determine the number of hair strands that were able to produce reliable quantification results. The organic method was replaced by a more sensitive commercial kit, detailed of which can be found in section 2.2.4 on page 87)

Following the extraction process each sample was treated with DNase I (Invitrogen, UK) to remove any residual DNA that may have carried over during the extraction process. This process was carried out according to manufacturer's instructions (section 2.3.2 on page 88). The concentration of total RNA in all test and control samples were then determined using a NanoPhotometer (Implen, Germany) and LabelGuard™ Microliter Cell and according to manufacturer's instructions. The analysis parameters can be found in section 2.5 on page 90.

The extracted and purified RNA samples were converted to cDNA using a Reverse transcription method involving random priming and Superscript II Reverse transcriptase (Invitrogen, UK). These were carried out according to manufacturer's instructions. Details can be found in section 2.6.3 on page 92.

The Real-time analysis was performed using an ABI 7500 Real time PCR instrument (Applied Biosystems, US). The run conditions are presented in section 2.7.1 on page 92. Primer concentrations were 200nM B-actin probe (FAM dye) and 300nM for both the forward and the reverse B-actin primers; 200nM 18s rRNA probe (VIC dye) and 100nM for both the forward and the reverse 18s rRNA primers. The threshold bar was set at 0.20 to minimise any background noise. Forty cycles were chosen to maximise the chance of successful amplification.

8.2.4 18S and B-actin Primer Optimisation

A complete primer optimisation was performed for both the 18S rRNA and B-actin mRNA targets using the same method employed in earlier chapters. Samples were loaded into MicroAmp® Optical 96-well reaction plates (Applied Biosystems, US). Each well contained Power SYBR Green PCR Master Mix (Applied Biosystems, US), the appropriate volumes of 18S and B-Actin primers, control cDNA (which had been reverse-transcribed from control Human RNA (Applied Biosystems, US)) and nuclease free water. The reaction plate was centrifuged for 15 seconds prior to real-time analysis. Samples were then run on an ABI 7500 Real time Thermocycler according to the protocol outlined in section 2.7.1 on page 92.

The data were analysed using Applied Biosystems 7500 Real-time SDS software (v 1.4.0). The threshold bar was set at 0.100 rfu for B-actin and 0.15 for 18S, which was significant to block out any background noise and yet fell within the exponential amplification phase of all the samples. A passive reference dye (ROX) was included in the Power SYBR green master mix (Applied Biosystems, US) to account for any subtle differences in PCR master mix volumes (pipetting errors). A negative control using nuclease free water was run with every batch as well as a positive PCR control using genomic DNA (Bioline, UK). All samples were run in triplicate. Normalisation of the data was required to correct sample to sample variation.

8.2.5 Efficiency of the 18S rRNA/ B-Actin mRNA Multiplex Reaction

The amplification efficiency of each target was again calculated using a serial dilution assay. A total reaction volume of 25 µl was used and the amplification was performed as previously described (refer to section 2.7.1 on page 92). Negative and positive controls were included to ensure reliable operating conditions. The data was used to calculate the amplification efficiencies of both 18S rRNA and B-Actin as previous described in 3.3.1.2 on page 105.

8.3 Statistical analysis

Ct values were exported into GenEx software (version 4.3.7) (GQ, Germany) which is a specific statistical software for qPCR data. Values were pre-processed which involved the normalisation of the raw Ct values against interplate calibrators (which accounts for any minor

run to run variation), individual efficiency rates, qPCR repeats and reference controls. The normalisation process addressed key issues raised by Pfaffl (2002) and the resulting Ct values were corrected according to his compensating formula. Results were tested for normal distribution (Gaussian distribution) and upon conformation were subjected to regression analysis (geometric mean, standard deviation, SEM, %CV and independent t-tests) using InStat 3 statistical software (version 3.06). The relative expression ratios were calculated from the normalised data in Excel.

8.4 Results

8.4.1 RNA Quantification: Levels of Detectability

The quantity of RNA recovered was measured for each sample across the entire ageing period. These values are presented in Appendix XII. The quantities were used to gauge the amount of RNA required for the reverse transcription step. The mean amount of RNA recovered at each age period is provided in the table 50. Despite initial concerns, a sufficient amount of RNA was recovered from all samples.

Table 50: Mean Quantity (ng) of total RNA recovered from Hair Samples. The mean amount of RNA was calculated from all ten donors (including their replicate samples) at each age period.

	Age Period								
	0	**1**	**5**	**15**	**30**	**45**	**60**	**75**	**90**
Mean Quantity (ng)	123.36	125.13	126.84	117.62	99.59	75.51	52.03	49.24	24.94

8.4.2 Primer Optimization (Matrix) studies

Using a singleplex SYBR green qPCR approach the optimal primer concentrations were determined for both species. The concentration range tested for the B-actin mRNA assay was 100nM to 600nM (in 100nM increments), whilst for 18S rRNA the range was between 25nM and 250nM. Each combination was run in triplicate with the results presented in Figures 63 and 64. The real time amplification plots are shown in Appendix XIII. The optimal combination as determined by the lowest Ct values were 300/300nM (forward/reverse) for B-actin mRNA and 100/100nM (forward/reverse) for 18S rRNA. The results from the primer optimisation studies were used to determine suitable primer combinations for the multiplex RT-PCR reactions.

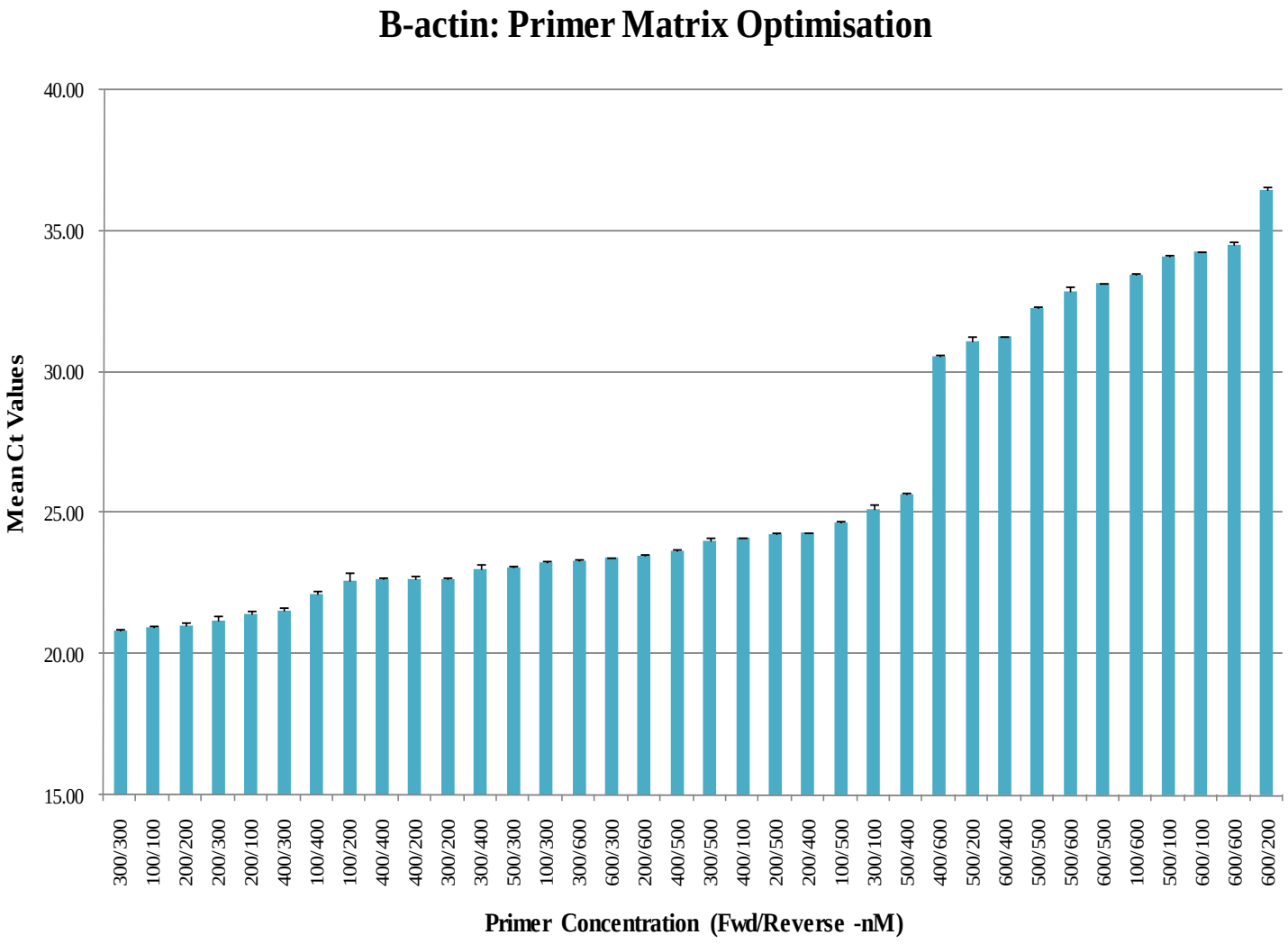


Figure 63: A complete Primer optimisation was performed to determine the optimal concentrations. The B-actin primer combination of 300nM for both forward and reverse primers was the most efficient as indicated by the lowest mean Ct value. A singleplex SYBR green detection system was used for the analysis. Error bars represent +1 Standard deviation.

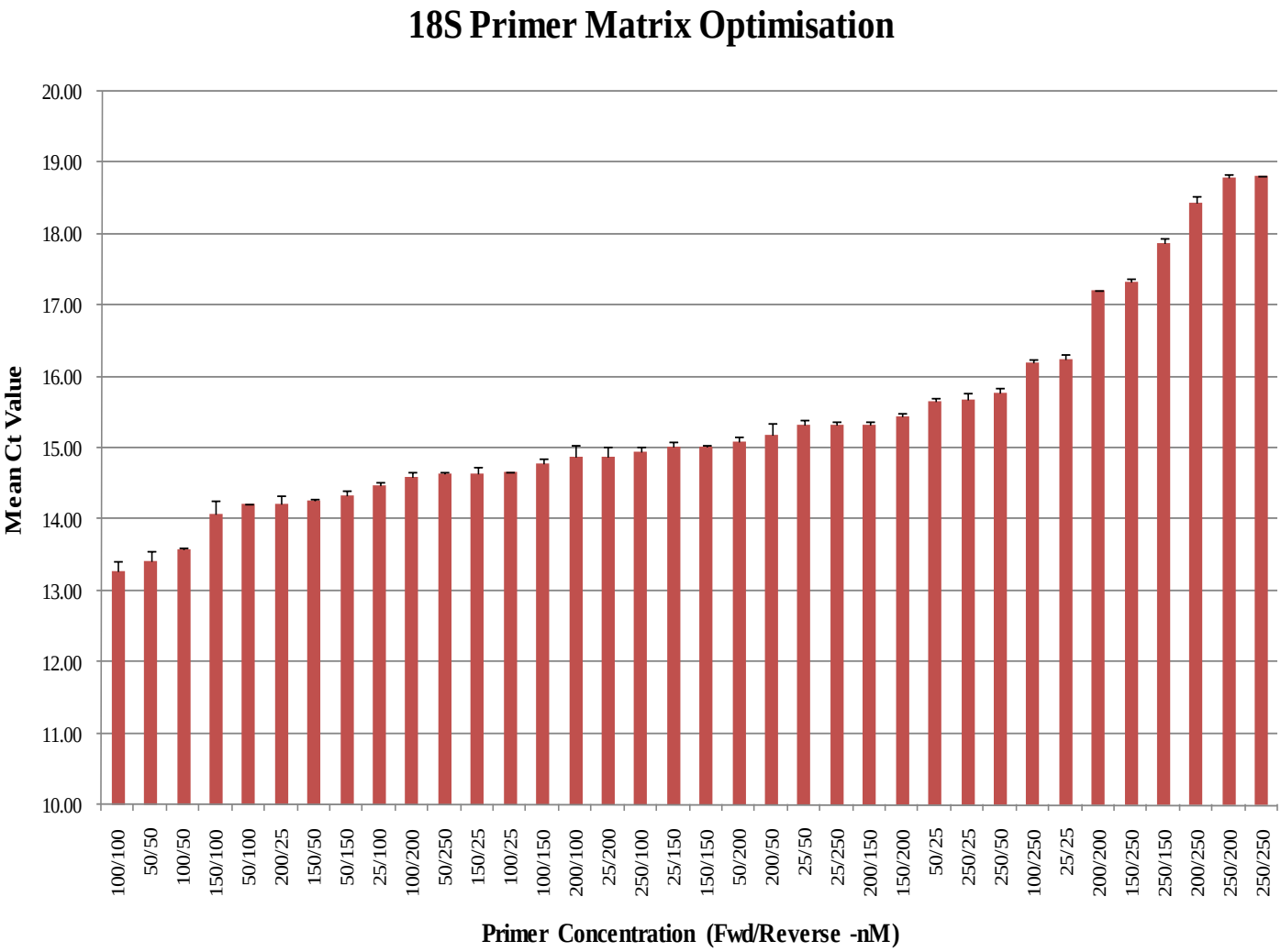


Figure 64: A complete Primer optimisation study was performed to determine the optimal concentrations for the 18S rRNA amplicon. Primer concentrations of 100nM for both forward and reverse primers produced the most efficient reaction rate. Error bars represent +1 Standard deviation.

8.4.3 Efficiency of the 18S rRNA and B-Actin mRNA Multiplex Reaction

The primer combinations that had the greatest efficiency in the singleplex optimisation study were tested as a multiplex assay. Multiple combinations were trialled although the results from the two most efficient assays are presented here. The real time amplification curves for the multiplex reaction can be found in Appendix XIV.

Assay 1:

B-Actin mRNA forward and reverse primers concentrations: 300nM
18S rRNA forward and reverse primers concentrations: 50nM

The primer concentrations is assay 1 were incidentally the same as those used by Anderson *et al*, (2005).

Table 51: 18S/B-Actin efficiency Assay 1: A 10-fold serial dilution of high quality DNA was created and used to measure the amplification efficiency rates of 18S rRNA and B-Actin mRNA. The Ct values were determined for each dilution and the slope of the lineal regression line was used to calculate the efficiency rates of each target.

Concentration	18S Ct Value	ΔCt value	Slope of regression line	B-Actin Ct Value	ΔCt value	Slope of regression line	Difference in Ct Values
Neat	16.25			24.80			**8.55**
1/10	19.71	**3.46**	-3.649	28.45	**3.65**	-3.794	**8.74**
1/100	23.38	**3.67**		32.31	**3.86**		**8.93**
1/1000	27.19	**3.81**		36.16	**3.85**		**8.97**

From the above data the slope of the regression line and amplification efficiency rates for 18S rRNA and B-actin mRNA were determined using Equation 1 described in section 2.8 on page 94. The amplification efficiency rate for B-actin mRNA was 83% whilst the amplification rate for 18S rRNA was 88%.

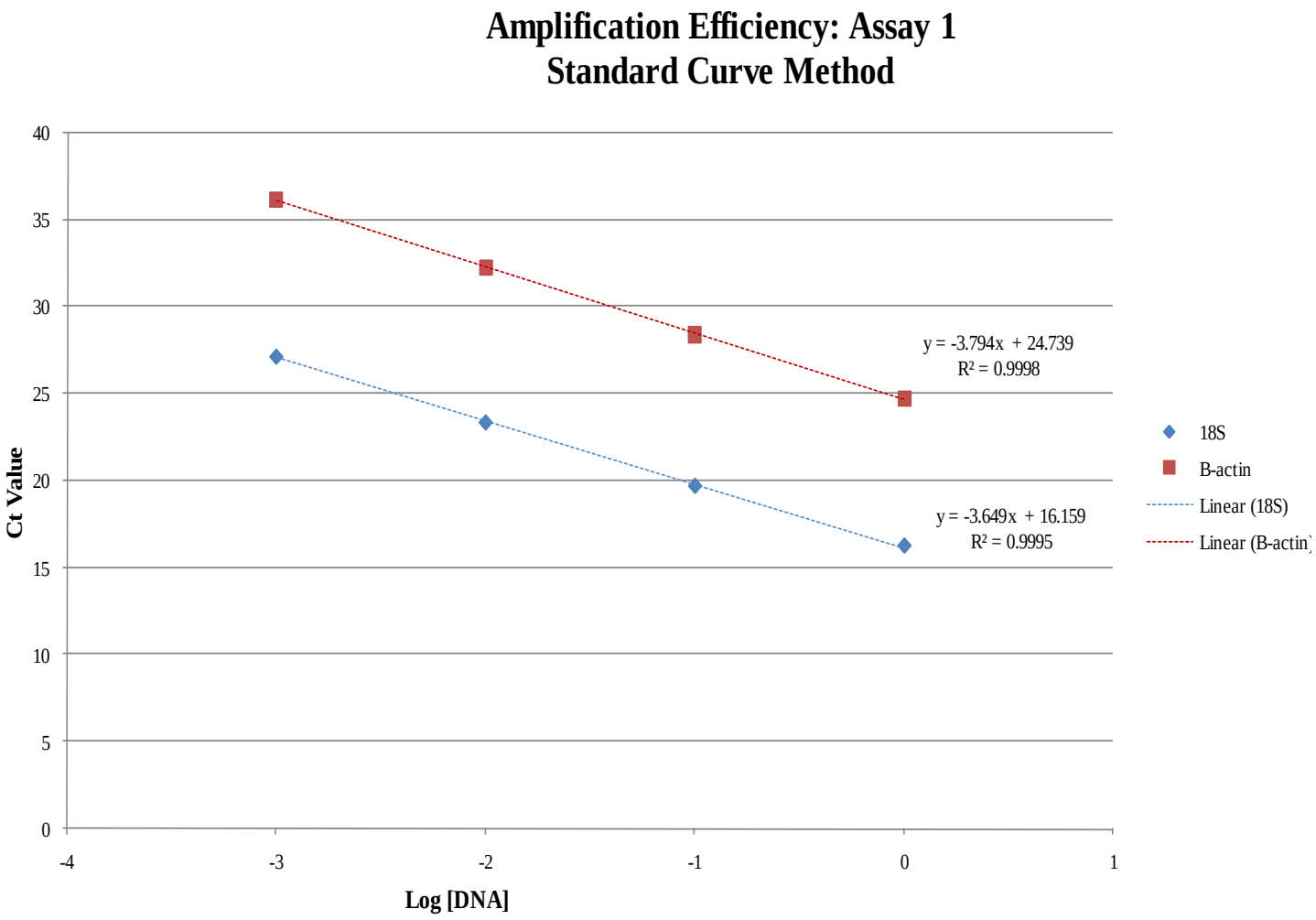


Figure 65: Amplification Efficiency Assay 1: The standard curve of the logarithmic transformed RNA concentration was used to calculate the amplification efficiency rates of the two target species. Mean Ct values (samples were run in triplicate) were used to generate the above trend lines which showed a strong correlation. Error bars represent ±1 Standard deviation.

As described in section 1.6 on page 68, there are a number of quantification strategies that can be used on this data. To test whether the Comparative Ct method ($2^{-\Delta\Delta Ct}$) was appropriate the difference in efficiency rates between 18S rRNA and B-actin mRNA were examined. According to Livak and Schmittgen (2001) the log concentration versus Ct difference for each dilution can be plotted and the slope of the trend line can be used to determine the suitability of this method for the experiment conditions. A value of less than 0.1 indicates that the target amplification rates are similar enough to be analysed using the Comparative method. The results obtained from assay 1 were tested and are shown in Figure 66.

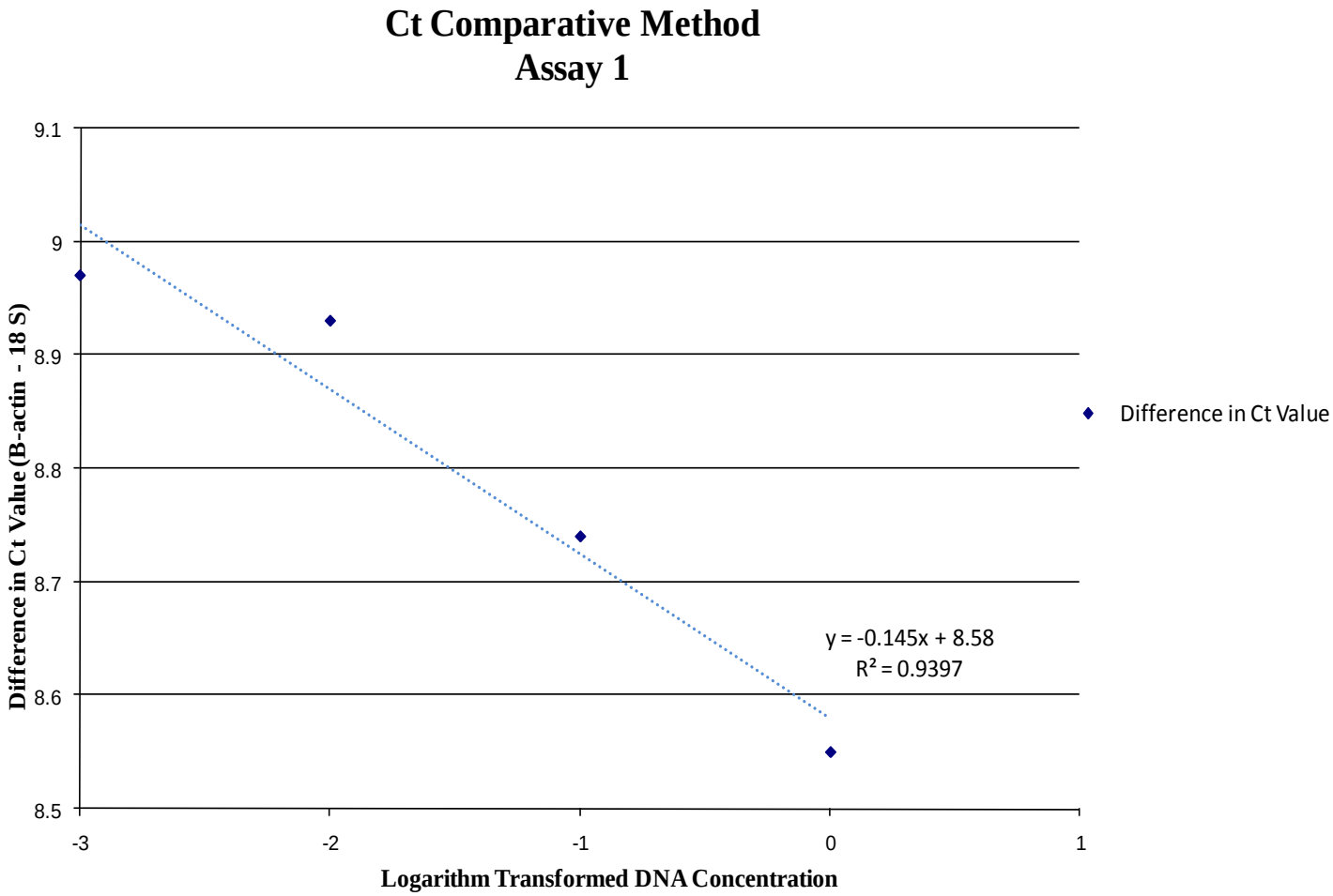


Figure 66: Ct Comparative Method – Assay 1: The difference in the Ct values from B-actin mRNA and 18S rRNA at each dilution was calculated and plotted against the logarithmic transformed DNA concentration. To determine the similarity of the efficiency rates the slope of the trend line is examined. For rates to be considered similar, the slope of the trend line should not exceed 0.1. The slope of the trend line from assay 1 was 0.145 which was greater than the defined limit therefore these two rates were not similar enough to be used by the comparative method and any attempt to do so would result in unreliable data.

Assay 2:

B-Actin forward and reverse primer concentrations- 300nm
18S forward and reverse primer concentrations - 100nm.

Table 52: 18S/B-Actin efficiency Assay 2: A 10-fold serial dilution of high quality DNA was created and used to measure the amplification efficiency rates of 18S rRNA and B-Actin mRNA. The Ct values were determined for each dilution and the slope of the lineal regression line was used to calculate the efficiency rates of each target.

Concentration	18S Ct Value	ΔCt value	Slope of regression line	B-Actin Ct Value	ΔCt value	Slope of regression line	Difference in Ct Values
Neat	15.98			25.60			**9.62**
1/10	19.24	**3.26**	-3.442	29.03	**3.43**	-3.515	**9.79**
1/100	22.76	**3.52**		32.59	**3.56**		**9.83**
1/1000	26.28	**3.52**		36.13	**3.54**		**9.85**

Efficiency rates were again determined using Equation 1 described in section 2.8 on page 94. The amplification efficiency rate for B-actin mRNA was 92% whilst the amplification rate for 18S rRNA was 95%.

The values obtained from assay 2 demonstrate that the reaction efficiency again failed to reach 100% however this particular combination of primers still outperformed all other combinations tested. The data from assay 2 was analysed to determine if the reaction efficiencies were similar enough to be used in the Comparative Ct method, the results of which can be seen in Figure 68 on page 280.

The absolute value of the slope for assay 2 was 0.073. These results indicate that the efficiency rates were similar enough to be analysed by the Comparative Ct method but the method described by Pfaffl (2002) was preferred for subsequent data analysis.

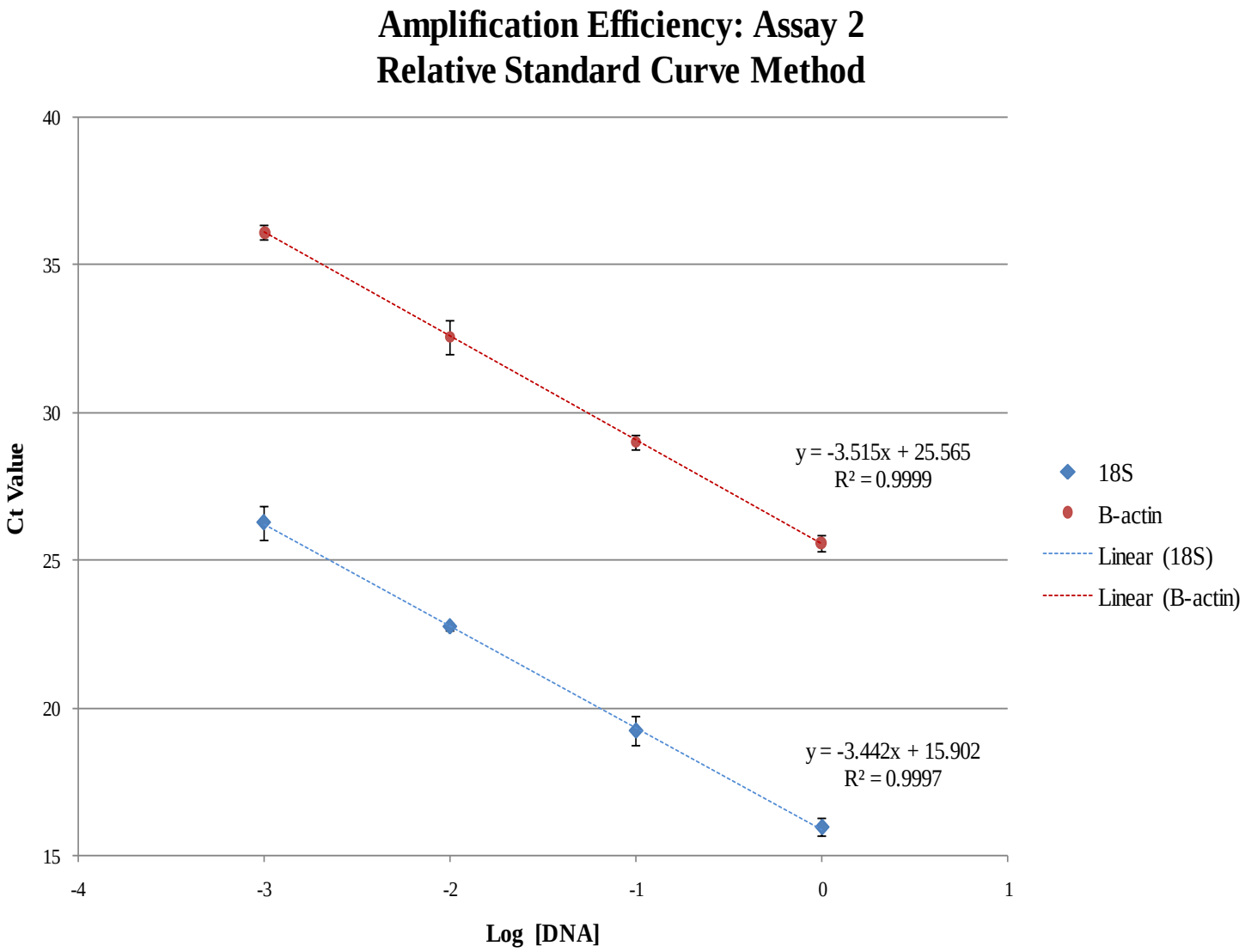


Figure 67: Amplification Efficiency – Assay 2. The slope of the above trend lines can be used to calculate the amplification efficiency of each of the target species in a reaction. Both these trend lines show a strong correlation between the two variables with high correlation values. The calculated efficiency for B-actin mRNA was 92.5% and 95.2% for 18S.The efficiency rates were greater in these conditions compared with those stipulated in assay 1.Error bars represent ± 1 standard deviation.

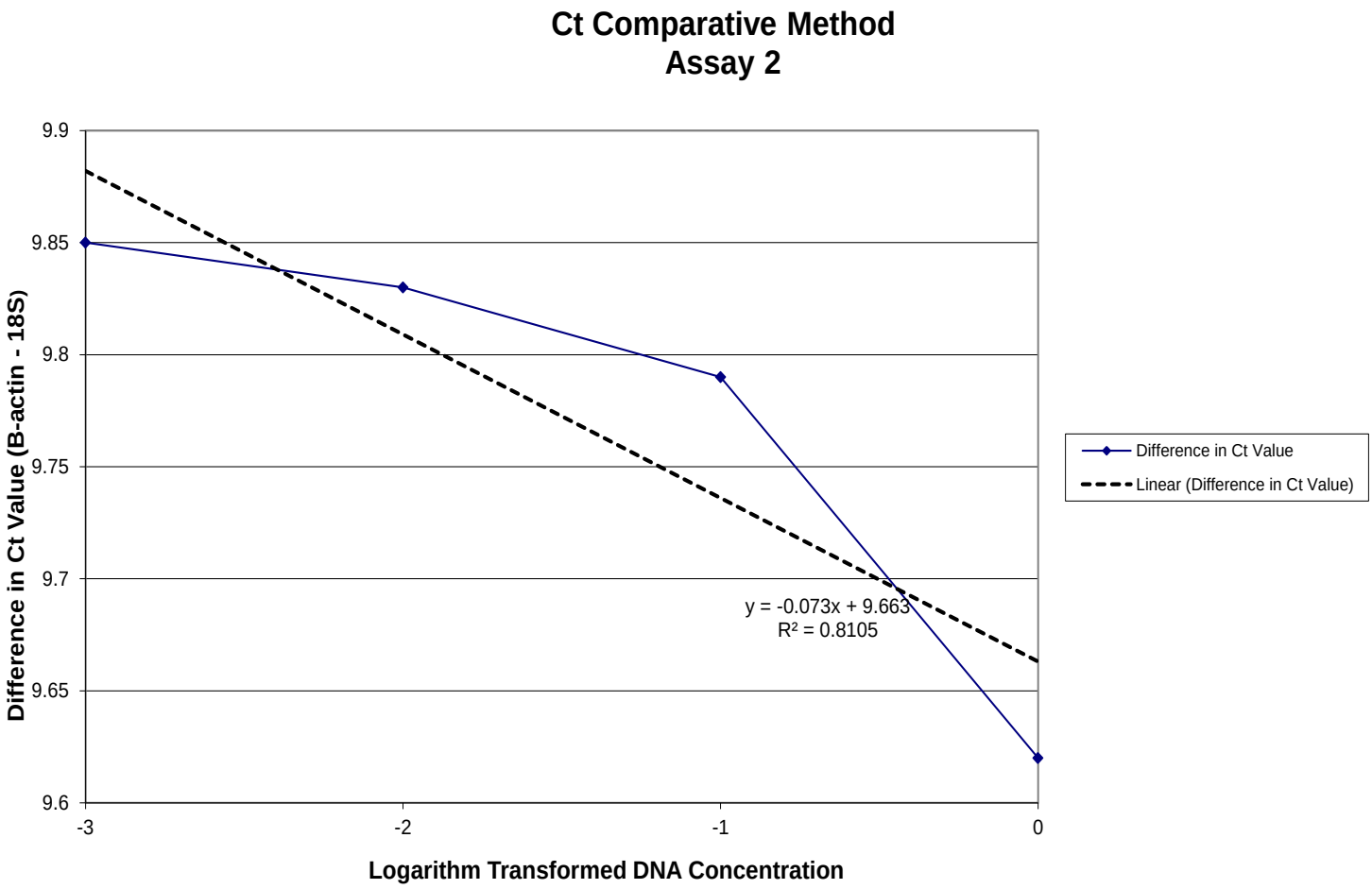


Figure 68: Ct Comparative – Assay 2: The slope of the trend line was again used to measure the similarity between the two efficiency rates. The data from assay 2 resulted in a trend line with a slope of 0.073 which was below the defined acceptable limit therefore this data could be analysed using the comparative method with confidence in any interpretation.

8.4.4 Sample Efficiency Rates

The efficiency rates of five samples (actual), chosen at random, were tested and compared to the rates obtained from the multiplex efficiency assay (expected). Due to the low RNA concentrations in the initial (neat) samples, a two-fold serial dilution (rather than the preferred 3-fold) was performed. Samples were run in triplicate with the mean values presented in the table below.

Table 53: Mean Ct values of five randomly selected test samples. A 2-fold serial dilution was performed to test the amplification efficiency of the targets in the test environment.

Sample	Mean Ct Value (18S)			Mean Ct Value (B-actin)		
	Neat	1/10	1/100	Neat	1/10	1/100
Sample 1	20.76	23.99	27.35	25.15	28.56	32.34
Sample 2	21.28	24.50	27.84	27.42	30.45	34.29
Sample 3	19.84	23.34	26.57	27.01	30.23	33.85
Sample 4	24.52	27.91	31.49	32.15	35.39	38.84
Sample 5	25.60	29.08	32.68	33.08	35.53	40.24

Efficiency rates were determined using Equation 1 described in section 2.8 on page 94, the results of which are presented in Table 54.

Table 54: Comparison of the efficiency rates between samples and the optimised multiplex assay (using control reagents)

	Efficiency Rate (%)					
	Optimised Multiplex Assay	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
18S	95.2	100.9	101.7	98.2	93.6	91.6
B-actin	92.5	89.7	95.5	96.0	99.0	90.2

The efficiency rate of 18S rRNA in the optimised assay was 95.2%, which was comparable to the rate obtained from the random samples (mean efficiency rate of 97.2%). The efficiency rate of B-Actin mRNA in the optimised assay was 92.5% which was also comparable to the rate obtained from the random samples (mean efficiency rate of 94.1%).

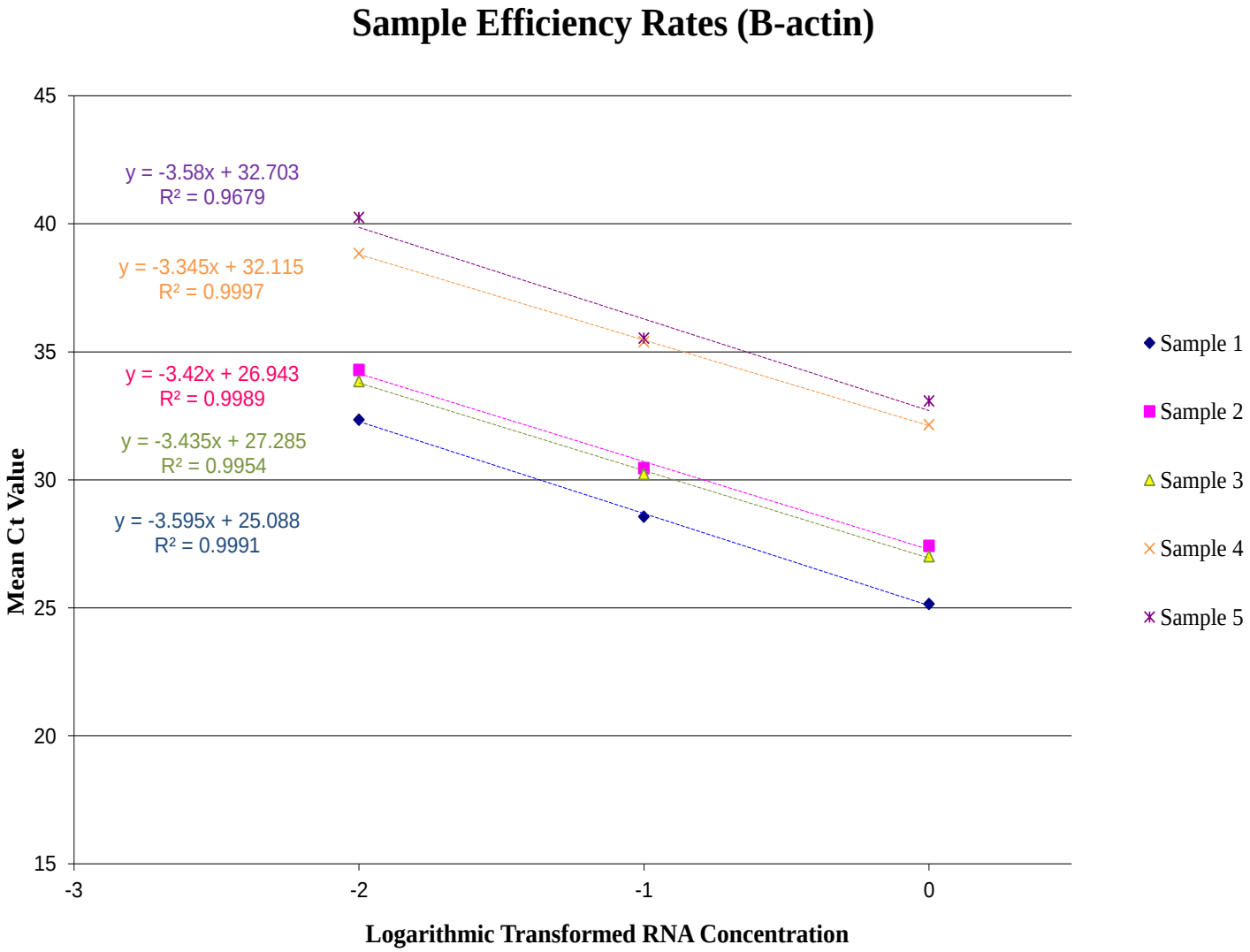


Figure 69: Efficiency rates of Test Samples - B-actin mRNA: The efficiency rate of B-actin mRNA in the actual samples was tested to ensure there were no major variations to those determined using controlled conditions in the optimisation assay.

Details of the random samples:

Sample 1:	*4A*	*0 days*
Sample 2:	*9B*	*5 days*
Sample 3:	*3A*	*45 days*
Sample 4:	*1B*	*75 days*
Sample 5:	*7A*	*90 days*

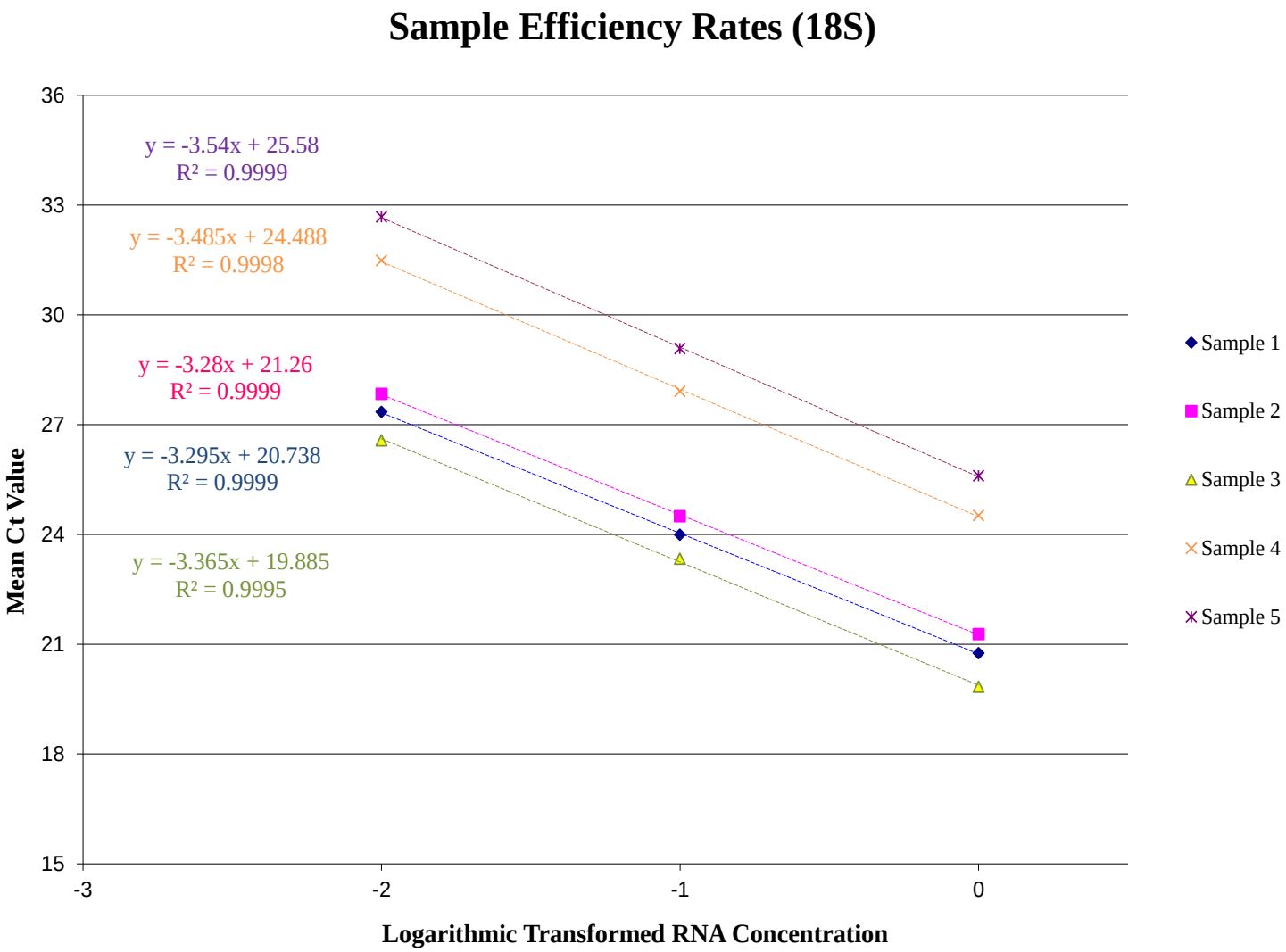


Figure 70: Efficiency rates of Test Samples -18S rRNA. The efficiency rate of the 18S rRNA in the actual samples was tested to ensure there were no major variations to those determined using the controlled conditions in the optimisation assay. There was no significant difference in the rates.

Details of the random samples:

Sample 1:	*4A*	*0 days*
Sample 2:	*9B*	*5 days*
Sample 3:	*3A*	*45 days*
Sample 4:	*1B*	*75 days*
Sample 5:	*7A*	*90 days*

8.4.5 Dynamic Range: Multiplex (18S/B-actin) Assay

The dynamic range of the multiplex assay was defined using a standard curve method. Control RNA was reverse transcribed and then serial diluted before being amplified using a Real Time PCR process. The variability of the Ct values were determined and are presented in Table 55.

Table 55: Variation in the Replicate Samples from the 10-Fold Serial Dilution Assay. The table below shows the coefficient of variance (%CV) for the serial dilution of control RNA. The variance was used to define the dynamic range of the multiplex assay

RNA Concentration	Ct average	Ct Standard Deviation	%CV
Standard 1	8.46	0.327	3.86
Standard 2	11.69	0.111	0.95
Standard 3	14.90	0.079	0.53
Standard 4	18.35	0.122	0.67
Standard 5	21.35	0.135	0.63
Standard 6	24.37	0.104	0.43
Standard 7	27.74	0.105	0.38
Standard 8	31.06	0.140	0.45
Standard 9	34.33	0.279	0.81
Standard 10	37.37	0.350	0.94
Standard 11	41.87	1.034	2.47

A variation (%CV) of less than 1% was the pre-defined acceptable limit for this assay. Therefore any test samples producing Ct values of less than 11.69 or greater than 37.37 were considered to be outside the reliable dynamic range of the 18S/B-actin multiplex assay.

8.4.6 Mean Ct Values and Relative Expression Ratios

The Ct values for all donors across the entire ageing period are presented in Appendix XV. The relative expression ratios (RER) were calculated from the Ct values according to Equation 5 described in section 2.8 on page 94. The results are presented in Table 56.

Table 56: Mean Relative Expression Ratios for all donors across the 90 day ageing period.

	Mean Relative Expression Ratio at each Ageing Point							
	1	**5**	**15**	**30**	**45**	**60**	**75**	**90**
Donor 1	1.602	1.879	2.567	3.922	4.177	5.021	5.198	6.002
Donor 2	1.496	1.925	2.378	3.390	3.978	4.685	5.749	5.280
Donor 3	1.600	1.801	2.408	3.467	4.531	4.797	5.064	5.595
Donor 4	1.348	1.949	2.162	3.150	3.849	5.104	5.678	5.968
Donor 5	1.498	1.831	2.534	3.178	4.230	5.344	5.362	5.978
Donor 6	1.523	1.805	2.249	3.499	4.598	4.946	5.229	4.866
Donor 7	1.459	1.949	2.346	3.767	3.926	4.533	5.646	5.267
Donor 8	1.310	1.933	2.643	3.267	4.189	4.899	5.387	5.397
Donor 9	1.406	1.964	2.397	3.401	4.246	5.534	5.125	4.767
Donor 10	1.502	1.901	2.299	3.097	4.063	4.764	4.823	5.223
				Statistical Details				
Mean	1.474	1.894	2.398	3.414	4.179	4.963	5.326	5.434
Std Dev	0.096	0.061	0.148	0.266	0.243	0.303	0.297	0.446
CI	0.059	0.038	0.091	0.165	0.150	0.188	0.145	0.276
SEM	0.030	0.019	0.046	0.084	0.076	0.095	0.094	0.141
CI lower (95%)	1.405	1.849	2.292	3.223	4.004	4.745	5.113	5.115
CI upper (95%)	1.543	1.937	2.504	3.604	4.352	5.179	5.538	5.753

8.4.7 Sensitivity of 18S/B-actin Assay

A sensitivity study was performed to determine the number of hair strands that were able to produce reliable quantification results. The results for both the 18S rRNA and B-actin mRNA species are presented in the following tables.

Table 57: 18S rRNA Sensitivity Assay. To determine the sensitivity of the assay the number of hair strands used for each sample was varied. The mean Ct values are shown here. RNA was recovered from all samples containing three or more hairs, suggesting the number of hairs required for the reliable extraction of RNA using this methodology is three. RNA was recovered from all but one donor (7) when samples consisted of only two hairs. Samples containing a single hair produced mixed results with RNA being recovered from only four out of the ten donors.

Number of Hairs	Donor (mean Ct Value)												
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	mean	Std Dev	%CV
10	20.30	20.67	21.21	19.74	20.36	21.26	20.35	20.70	19.78	20.66	20.50	0.51	2.50
9	21.65	21.10	21.78	21.34	21.51	22.67	21.24	20.98	21.22	21.94	21.54	0.50	2.32
8	22.63	22.43	22.52	22.58	23.09	23.00	23.07	21.73	22.98	23.59	22.76	0.50	2.21
7	23.94	23.94	24.43	23.27	23.79	22.70	24.55	24.58	24.40	24.04	23.96	0.60	2.51
6	25.99	27.42	25.92	26.79	26.33	26.45	27.42	28.54	25.30	27.36	26.75	0.95	3.55
5	29.78	31.11	32.67	29.23	31.82	30.66	31.74	31.59	30.76	33.09	31.25	1.20	3.83
4	34.94	32.59	33.55	35.19	35.63	35.00	36.75	35.42	37.14	37.19	35.34	1.48	4.20
3	37.73	37.19	34.13	36.98	37.28	37.14	38.06	37.56	37.02	38.64	37.17	1.19	3.20
2	39.30	41.99	44.09	42.91	43.69	42.14	UD	42.59	42.24	43.12	42.45	1.38	3.24
1	44.67	UD	UD	40.02	UD	UD	43.98	UD	44.23	UD	43.23	2.16	4.99

**UD = Undetected*

Table 58: B-actin Sensitivity Assay. To determine the sensitivity of the assay the number of hair strands used for each sample was varied. The mean Ct results are shown here. The results indicate that the sensitivity of the B-Actin assay was less than the 18S assay. Recovery of RNA was only reliable when five or more hairs used per sample. RNA was recovered from 80% of the samples when four hairs were used whilst RNA was recovered from only five of the ten donors where the samples were composed of three hairs. RNA could only be recovered from 30% of the samples that contained two hairs whilst no RNA was recovered from any samples containing a single hair.

Number of Hairs	Donor (mean Ct Value)												
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	mean	Std Dev	%CV
10	25.59	24.84	25.50	24.96	25.39	25.83	24.98	25.48	25.60	25.59	25.38	0.33	1.31
9	26.13	26.55	26.23	26.93	26.57	26.15	25.12	26.79	26.79	26.23	26.35	0.52	1.97
8	28.24	27.99	28.50	27.34	26.37	26.91	28.52	29.26	28.51	29.69	28.13	1.02	3.63
7	28.83	29.71	27.41	28.62	29.11	27.76	28.60	28.72	28.44	29.48	28.67	0.70	2.45
6	32.77	29.57	33.82	33.42	33.75	34.74	34.08	33.43	33.83	34.75	33.42	1.48	4.42
5	34.57	34.83	35.30	35.81	36.35	35.49	35.08	36.04	36.30	36.17	35.59	0.64	1.79
4	38.13	38.43	37.66	37.18	39.11	UD	39.90	39.69	38.87	UD	38.62	0.96	2.47
3	42.94	39.70	UD	42.17	UD	44.56	40.49	UD	42.50	UD	42.06	1.75	4.16
2	44.89	UD	UD	UD	43.41	UD	UD	41.29	UD	UD	43.20	1.81	4.19
1	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	n/a	n/a	n/a

**UD = Undetected*

8.4.8 Gender effect on the Relative Expression Ratio

The possibility that a gender effect may occur in hair samples was examined. The mean RERs were calculated for each donor at each time point (see Figure 71) and grouped according to their gender. This information was then statistically compared. The slope of the trend lines between sexes are almost identical (slope 0.047 vs 0.046 respectively) and despite the fact the correlations were not perfect, they were still relatively strong [R^2 = 0.950 (males); R^2 = 0.959 (females)]. An independent unpaired t-test was performed using Instat statistical software (Graph pad, US) on the mean relative expression ratios for both genders over the entire time frame in order to determine if the slight differences observed were significant. According to the t-test the differences observed were not significant (ANOVA; p = >0.1).

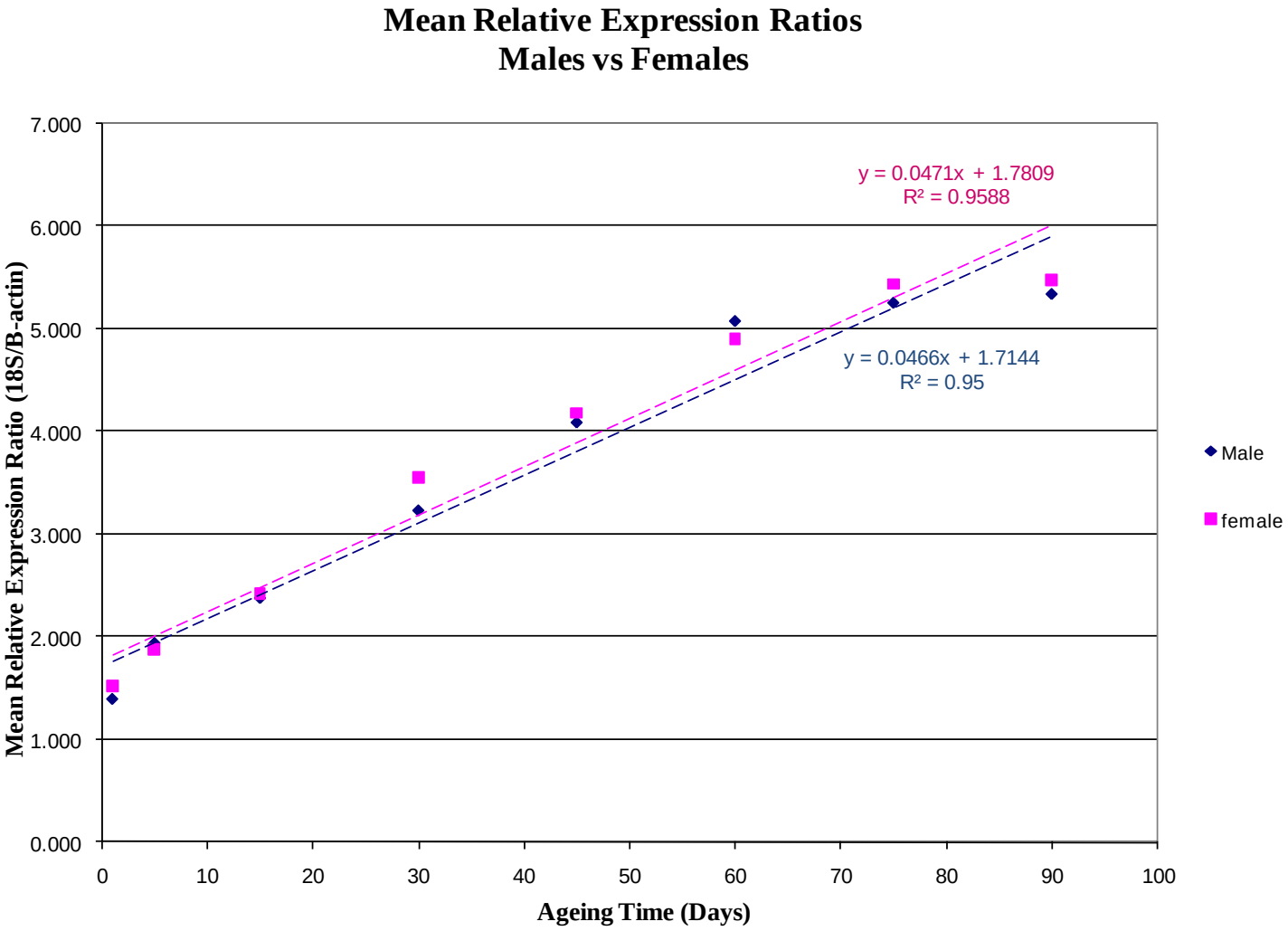


Figure 71: Mean Relative Expression Ratios Comparing Males and Females. The mean Ct values for males and females were calculated and compared to determine if there were any significant differences. Both trend lines display similar strength correlations across the 90 days whilst statistically the difference was not significant.

8.4.9 Age effect on the Relative Expression Ratios

A comparison of the RERs was made between individuals from different age categories (20-25yrs and 40 -45yrs). The strength of the correlation was greater in the older group (R^2 = 0.98) than in the 20-25 age group (R^2 = 0.93). The difference observed increased with time and a statistical analysis was carried out to determine whether this difference was significant and suggestive of an age bias. The data presented with a normal (Gaussian) distribution. The t-test indicated that the difference in the RER's between the age groups was not significant (ANOVA; p = >0.1).

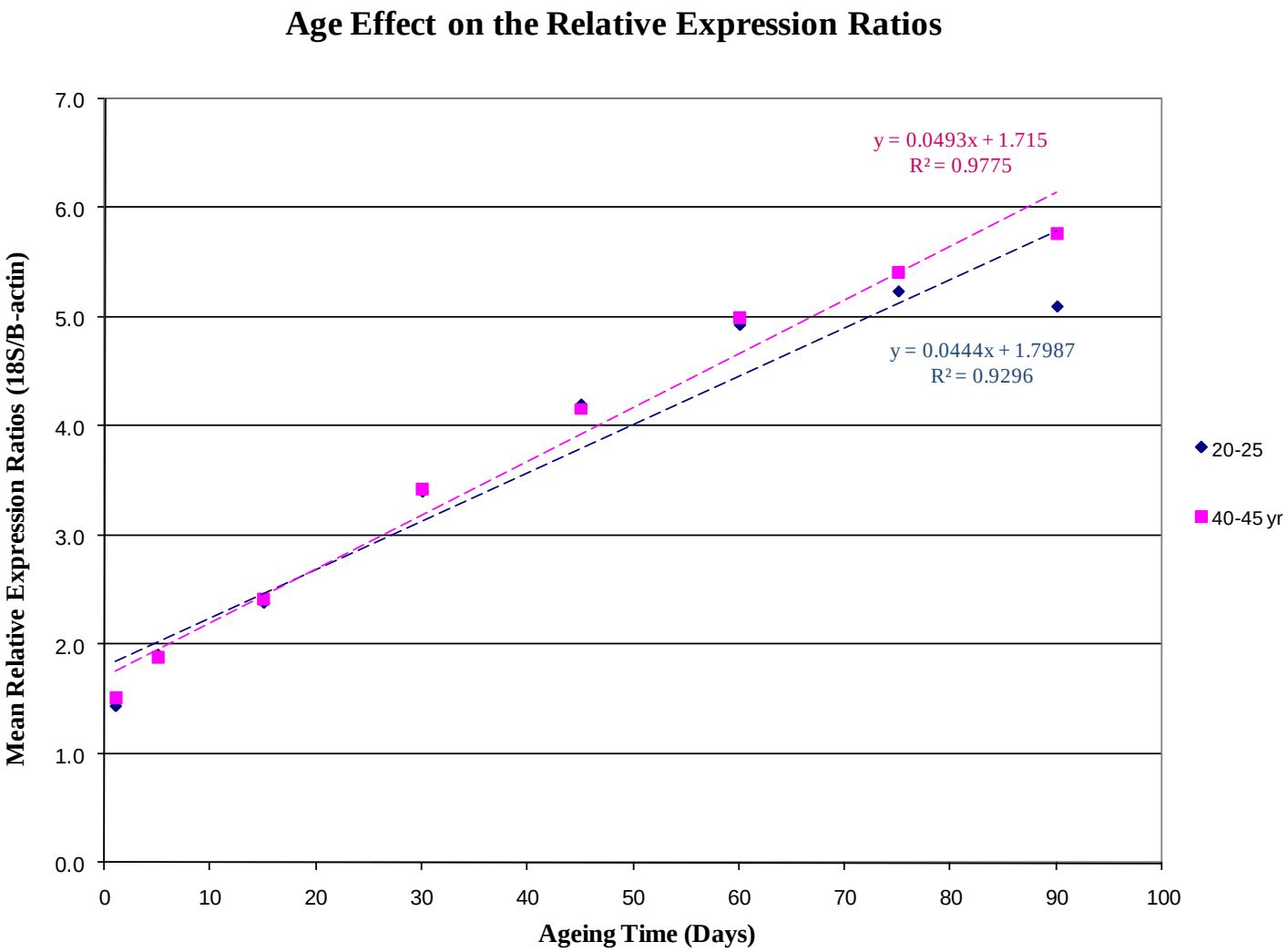


Figure 72: Age Effect on the Relative Expression ratios for Hair samples. The mean relative ratios for volunteers in each age bracket were compared. The female population displayed a stronger correlation than the male data set. The plateau effect observed in the mean RER's after 60 days appears to be predominately caused by the male data.

8.5 Discussion

The purpose of this study was to determine whether the relative expression ratios (RER) of 18S rRNA and B-actin mRNA extracted from the follicular tag of hair samples changed in a lineal manner, dependent of time, allowing an estimation of the age of the sample. Hair was chosen due to its common occurrence at crime scenes (Sachs, 1997) and the fact limited success has been obtained in this thesis by using blood samples.

8.5.1 Method Optimisation

Real-time reverse transcription PCR was chosen as the most suitable method for the analysis due to its sensitivity, reliability and success other researchers had at using it to analyse small concentrations of RNA [Bauer *et al*, (2003); Anderson *et al*, (2005); Gopee and Howard, (2006)]. However, a major disadvantage of the qRT-PCR (which is predominately caused by its high sensitivity) is that it requires good experimental design and an in depth understanding of the efficiency and normalisation techniques. Even today, data normalisation remains problematic and subject to frequent criticism (Bustin and Nolan, 2004). Normalisation is essential to control experimental error and several different strategies that can be used on RNA expression data (Guenin *et al*, 2009). It is essential to address these areas if accurate interpretation of results is to be achieved. As such a number of stringent optimisation tests were carried out.

The optimal primer concentrations were determined to ensure that one species was not preferentially amplified in the multiplex assay. The results from the singleplex assays indicated the suitability of a number of different primer concentrations however when multiplexed the 18S rRNA amplified rapidly which consumed the master mix ingredients prior to the complete amplification of the B-Actin mRNA. Concentrations had to be adjusted to ensure that the 18S rRNA was the rate limiting step. The optimal primer concentrations were also determined to ensure the maximum amplification efficiency was achieved, and the rate obtained was accurate. Accurate amplification efficiency rates are essential as they are used to calculate the quantity of target species (Pfaffl, 2006). Determining these rates can often be difficult because there are a number of experimental factors, including primer concentrations that can effect on the calculated rate (Pfaffl, 2006). Pipetting errors, primer dimer amplification and variable amplification rate throughout the reaction (declining to 0 during the plateau phase) can all

contribute to non-ideal and error in the rates calculated (Pfaffl, 2006). Even small variations between actual rate and calculated rate can have a significant influence on the resulting quantities, as demonstrated in Table 59.

The first of the multiplex assays ("assay 1") consisted of primer concentrations that were used by Anderson *et al* (2005), namely 50nm (18S) and 300nm (B-actin). Under these conditions the amplification rate of 18S rRNA was 88%, whilst B-actin mRNA amplified at 83%. Both of these rates were less than ideal and any values obtained from an absolute quantification perspective would be an underestimation of the actual amount of PCR product present. Because absolute values were not required for this study, comparative Ct methods were examined to determine their suitability. Comparative methods do not depend on having ideal amplification efficiencies but they are based on the assumption that the efficiency rates of both species are similar. The results from assay 1 suggest the amplification efficiency rates between the two target species were too different and therefore the comparative method could not be used. It is unclear whether efficiency tests were performed by Anderson *et al*, (2005) however if these issues were not addressed it would significantly affect the accuracy or validity of their results. A second multiplex assay ("assay 2") was examined. Assay 2 consisted of primer concentrations that were deemed to be the most efficient from earlier optimisation studies. Under these conditions 18S rRNA amplified at 95% efficiency whilst the B-actin mRNA displayed an amplification rate of 92%. Both these values were an improvement on the conditions stipulated by Anderson *et al*, (2005) although they were still not ideal. The efficiency rates were examined to determine their suitability with the Comparative Ct method. The rates were similar enough to be used with this method but there was still a small difference in the amplification rates which would incorporate a degree of error into final quantity calculations. As such the compensated formula proposed by Pfaffl (2002), which corrects for any slight differences in amplification rates, was used thus eliminating this error.

Table 59: The effect of amplification rate variation in the amount of PCR product produced over 30 cycles. This figure shows how a small change in the amplification efficiency rate can result in large change in the amount of product produced. This demonstrates the importance of accurately calculating the efficiency rate of each target species.

Cycle No	Amount DNA 100% Efficiency	Fold Decrease at 90%	Fold Decrease at 80%	Fold decrease at 70%
0	1	1.0	1.0	1.0
1	2	1.0	1.0	1.0
2	4	1.0	1.3	1.3
3	8	1.1	1.3	1.6
4	16	1.2	1.6	2.0
5	32	1.3	1.7	2.3
6	64	1.4	1.9	2.7
7	128	1.4	2.1	3.1
8	256	1.5	2.3	3.7
9	512	1.6	2.6	4.3
10	1024	1.7	2.8	5.1
11	2048	1.8	3.2	6.0
12	4096	1.9	3.5	7.0
13	8192	1.9	3.9	8.3
14	16384	2.1	4.4	9.7
15	32768	2.2	4.9	11.4
16	65536	2.3	5.4	13.5
17	131072	2.4	6.0	15.8
18	262144	2.5	6.7	18.6
19	524288	2.7	7.4	21.9
20	1048576	2.8	8.2	25.8
21	2097152	2.9	9.1	30.4
22	4194304	3.1	10.2	35.7
23	8388608	3.1	11.3	42.0
24	16777216	3.4	12.5	49.4
25	33554432	3.6	13.9	58.1
26	67108864	3.8	15.5	68.4
27	134217728	4.0	17.2	80.5
28	268435456	4.2	19.1	94.7
29	536870912	4.4	21.2	111.4
30	1073741824	4.7	23.6	131.0

Despite using this modified formula, a problem still exists. Efficiency rates are calculated from the slope (absolute value) of the lineal regression curve that is generated from the serial dilution assay Pfaffl (2002). The lineal regression curve is based on the mean value of the replicate samples used at each dilution. Because the mean value is used, the variation between replicates (standard error) is not accounted for and is generally not carried forward to the rate calculations. If it was the efficiency rate should be given as a range rather than a specific value. Using the data obtained from assay 2 and allowing for the standard error the following range of efficiency rates were obtained:

18S:
SE (slope): 0.09765
T Critical: 2.353
Slope (95% confidence): -3.646 < -3.442 < -3.237
Efficiency Range (95% confidence): 87.4% < 95.2% < 102.9%

B-actin:
SE (slope): 0.08452
T Critical: 2.353
Slope (95% confidence): -3.692 < -3.515 < -3.338
Efficiency Range (95% confidence): 89.4% < 92.5% < 95.6%

In relation to 18S rRNA the efficiency range due to the variation in the replicate samples is between 87.4% and 102.9%. When using the efficiency rate to calculate the amount of target species the mean rate is used (95.2%) however as demonstrated this mean rate could be out by as much as 7%, greatly reducing the accuracy of any target quantification. The range is slightly less for B-Actin mRNA varying from 89.4% to 95.6%. To reduce the range and increase the confidence in the amplification rate the number of serial dilutions and repeats can be increased. This should be considered an essential part of the protocol with this type of analysis however this limitation will still exist.

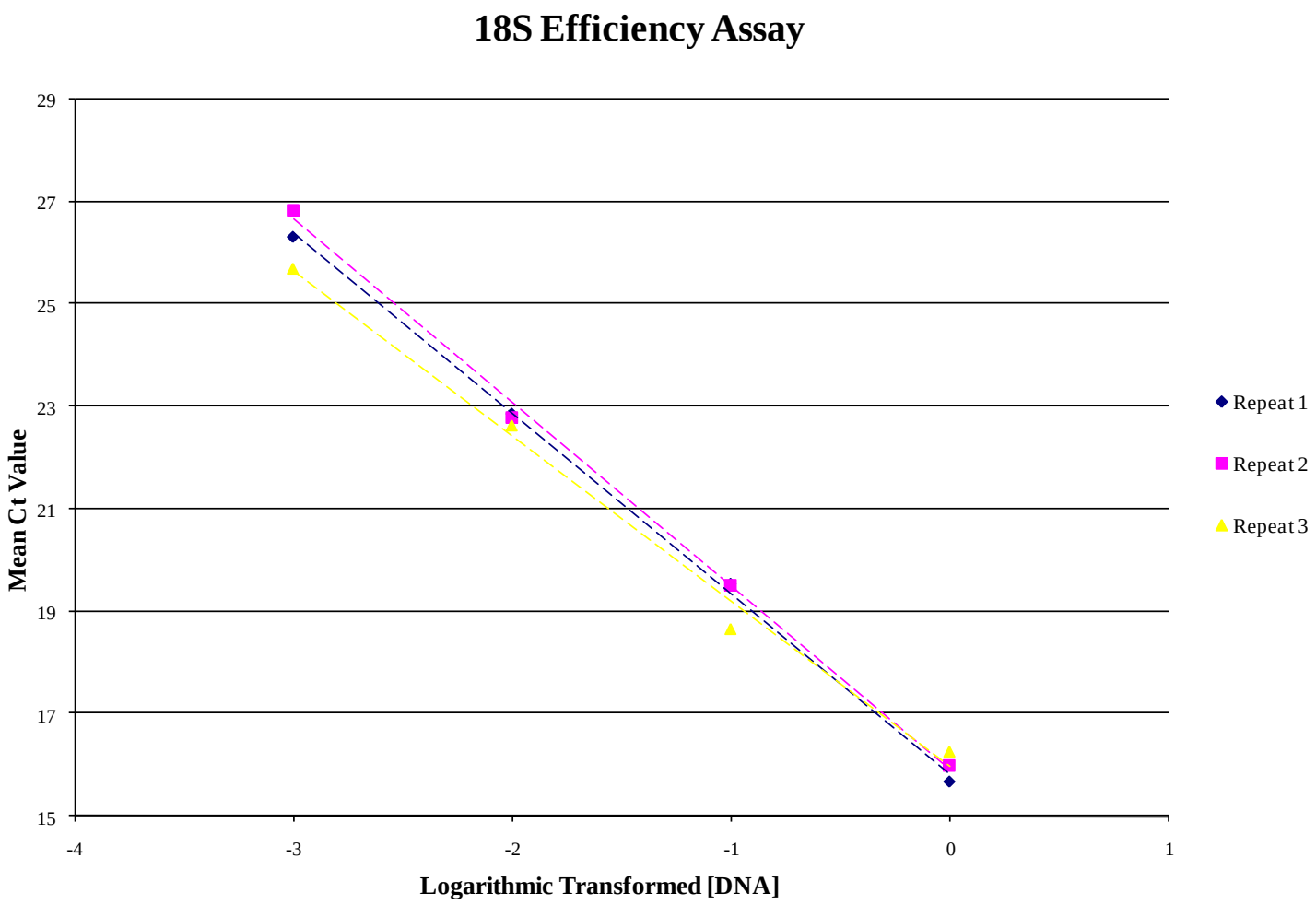


Figure 73: 18S rRNA Efficiency Assay: When determining the efficiency rate a lineal regression curve is constructed from the mean values of the replicate samples. However the data shown here indicates there is variation between replicates which is often ignored when calculating efficiency rates (as the mean value is used only). The number of replicates and serial dilutions used when creating standard curves can have a significant effect on standard error of the slope, which then in turn, has an effect on the calculated efficiency rate.

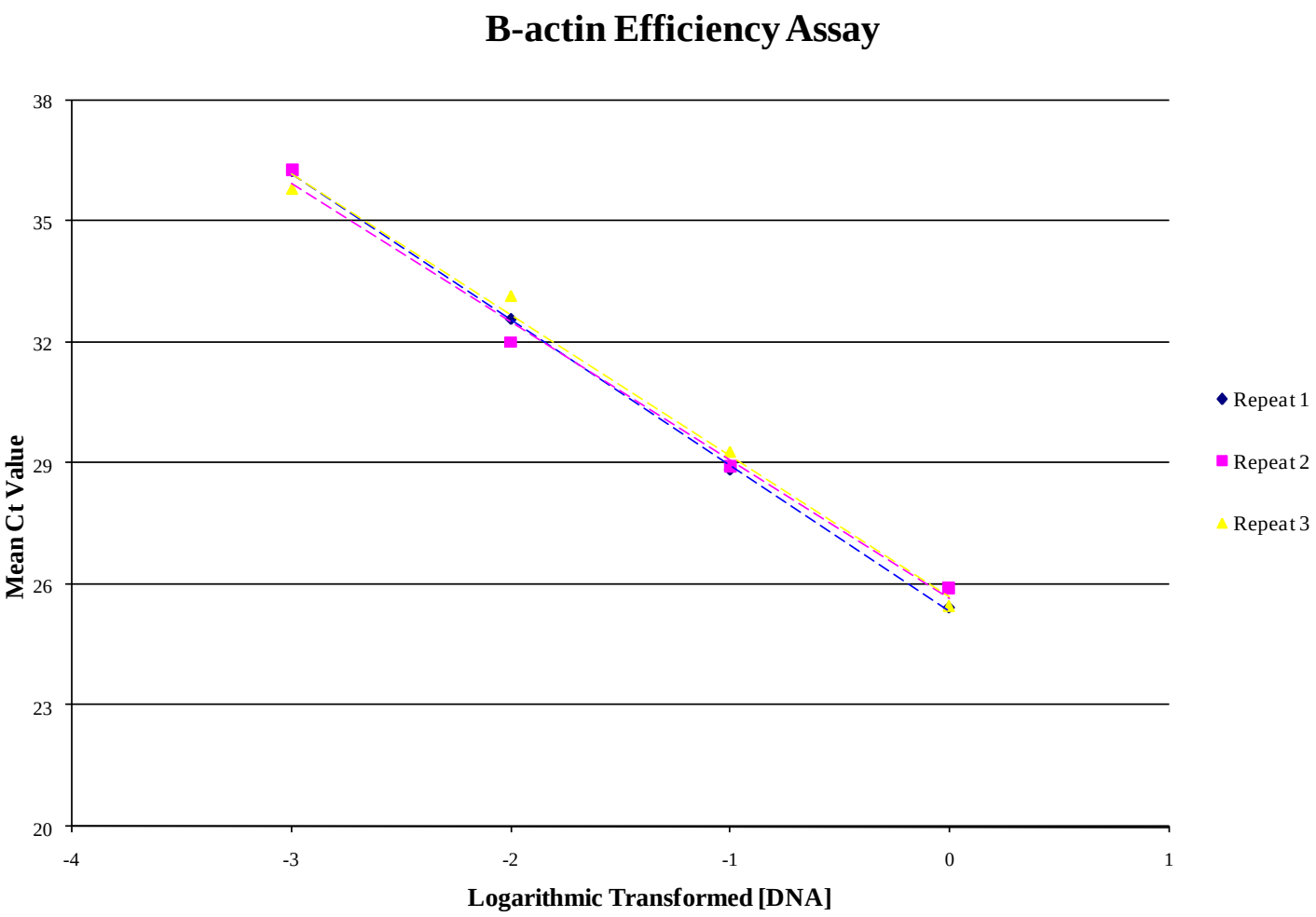


Figure 74: B-actin mRNA Efficiency Assay. As stated on the previous page, the number of replicates and serial dilutions used when creating standard curves can have a significant effect on standard error of the slope, which then in turn, has an effect on the calculated efficiency rate. Minor variations as seen in the above figure with the B-actin data should be more accurately described as an efficiency range of 89.4% to 95.69% at a 95% confident level, rather than a single value.

It was necessary to establish whether the efficiency rates of 18S rRNA and B-actin mRNA in the actual samples were the same as those calculated from the standard curve (serial dilution). Any difference would result in inaccurate quantification of the target species (Pfaffl, 2006). It could also indicate the presence of any inhibitor within the aged hair samples (Suslov and Steindler, 2005). Five randomly selected test samples were selected and the efficiency rates determined in a manner identical to those used to produce the standard curve. Given the low initial RNA (cDNA) concentrations in these samples, the size of the serial dilution was restricted. This affected the SEM and confidence interval of subsequent rate estimations. To compensate for this deficiency the number of replicates that were tested were increased. The mean efficiency rates of the five random samples were 97.2% (18S) and 94.1% (B-actin) which was comparable to the values from the Optimised Multiplex Assay, 95.2% (18S) and 92.5% (B-actin) respectively. The reduction in the rate observed was not enough to indicate the presence of any inhibitors. The differences between the expected and actual rates were small [18S (2%); B-actin (1.6%)] therefore the expected rates [95.2% (18S) and 92.5% (B-actin)] were used to calculate the quantity of target species. Whilst this introduces a small degree of error in the final quantification this approach would be acceptable for a high throughput technique as it would become too laborious to measure the efficiency rates of every sample. In forensic situations where this technique may only be applied to a single sample, it may be more appropriate to measure and use the efficiency rate of the individual sample, provided there is sufficient sample quantity.

The dynamic range of the assay was tested to ensure that Ct values obtained from the actual samples fell within the acceptable limits of the multiplex assay. Most of the test samples throughout the entire study fell within these limits adding confidence to result interpretation.

8.5.2 RNA Quantification

The Trizol (Sigma) extraction method was sensitive enough to extract measurable quantities of RNA from hair samples that had been aged for up to 90 days. There was a continual decline in the amount of RNA extracted over the 90 days (refer to Figure 75). The correlation between "mean RNA quantity" and "time" during this period was strong ($R^2 = 0.99$). This may indicate a possible consistency in the degradation processes involved (as all samples were aged under the same environmental conditions). However, this data does not provide any information regarding the quantity of individual RNA species as it was only total RNA that was quantified.

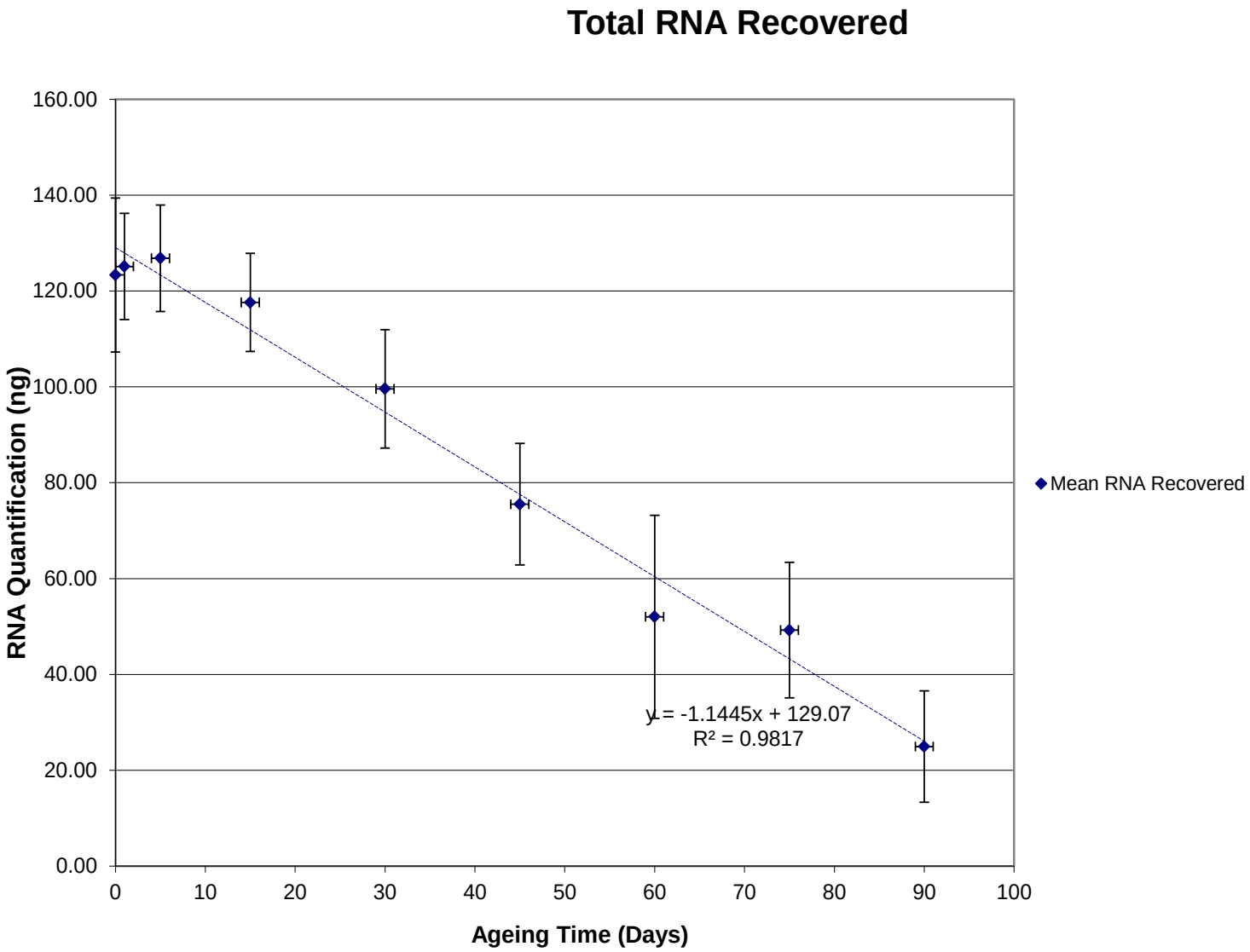


Figure 75: Total Quantity of RNA Recovered. The Figure above displays the general decline in the mean amount of total RNA extracted from the samples over the 90 days. Despite the strong correlation between quantity recovered and age there was a variation observed between donors as indicated by the spread of values at each ageing point.

The lower than expected yield of RNA from these samples was an area of concern. The mean total RNA (from ten hair roots) was determined to be 88.25ng across all donors and age points. From fresh samples (0 days) the mean was only slightly greater at 123.36ng. This is considerably less than amounts observed by Bradley *et al*, (2005). They extracted 112.5ng per singular hair follicle. Various other authors [King *et al*, (2001); Kim *et al* (2006)] have also suggested larger volumes are obtainable from similar number of cells, whether it be blood or another tissue type, though direct comparisons with other tissue types may be misleading as Kim *et al* (2006) suggested that hair follicles have lower quantities of RNA than other tissue types such as blood. Kim *et al* (2006) also suggests the ratio of ribosomal to messenger RNAs in hair follicular cells is smaller than expected, though they stopped short of assigning any value to their assumption. The data from this experiment (Ct values from Day 0) suggest there is approximately 35 times more copies of 18S rRNA than B-actin mRNA in follicular hair cells. Given that Kim *et al*, (2006) suggests rRNAs are smaller than expected in hair cells, levels of B-actin mRNA must also be low given the relative difference in quantities.

The number of cells that can be attached to or within the hair follicles (follicular tag) when hairs are forcibly removed can vary, and this to some extent may explain the lower than expected yields. King, *et al* (2002) and Kim *et al*, (2006) provided no details as to the exact number of cells that were attached to each follicle. This particular variable is difficult to control unless the follicular tag is dissected and microscopically examined. Although a visual examination was conducted during this study to ensure a follicular tag was attached, cell counting was not performed. The variation in the number of cells may also explain the large inter-donor variations that were observed. The inter-donor variation ranged from 8.7% (5 days) to 46.6% (90 days) with an initial variation of 13.0% (Day 0). Similar inter and intra-donor variations were reported by Andreasson *et al*, (2006) who extracted nuclear and mitochondrial DNA from various hair samples. Even though this study involved DNA, many comparisons can be made with RNA as they both derive from the same cellular source. Both plucked and naturally shed hairs were examined using a multiplex quantification assay based on probe based detection system and a large variation in DNA content was found between the two types of hairs (Andreasson, Gyllensten and Allen, 2002). The difference is likely to be an indirect result of the growth cycle of hair.

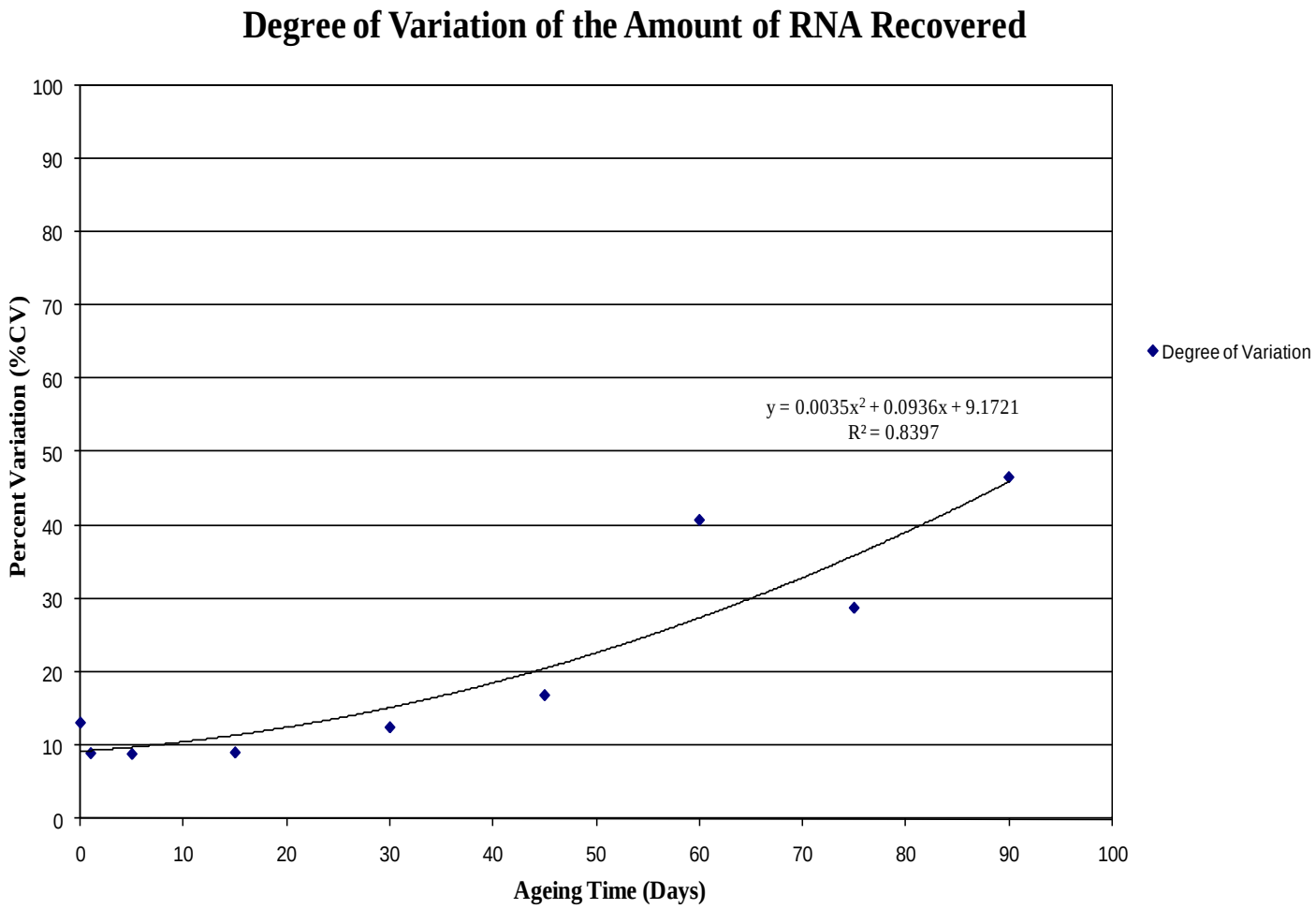


Figure 76: Variation in the quantity of RNA recovered over time. Despite there being a general increase in the variation (expressed as percent coefficient) as the ageing time increase (2nd order polynomial with correlation value of 0.839), it was not consistent. It is suggested that the initial variation at time 0 can be largely attributed to the natural growth cycle of hair samples.

The different hair types will have different amounts of cellular material adhering to them (Bender and Schneider, 2005). One could assume that plucked hairs (sample type used in this particular study) are predominately in either the catagen or anagen phase of the cell cycle and thus rich in cellular material, whereas naturally shed hairs are in the telogen phase, which contain significantly less cellular material. Despite every sample in this study consisted of ten strands of hair, the growth phase of each individual hair was not determined and this could explain the variations observed.

The organic extraction methodology used may have also contributed to the low quantities recovered. Although organic methods are generally robust and widely accepted they tend to be rather crude when compared to many of the commercial extraction kits that are available. Other specialised techniques exist such as laser micro dissection, which would also enable the number of cells to be countered. This technique has in the past been used for isolating single cells from hair follicles (Martino *et al*, 2004). Such options should be considered in future research where sensitivity is an issue.

It was thought that the amount of RNA recovered may be dependent on the donor's characteristics such as gender and age. It is known that the rate of hair growth decreases with age (Goldman and Schaefer, 2011) and females are more likely to use hair treatments which may have an effect on the cellular component of the follicular tag. For these reasons a comparison was made between males and females and the two different age groups but the differences observed were not statistically significant. Statistical results are shown in Table 60.

Table 60: Statistical Comparison of the Quantity of RNA Recovered Between Groups. Statistical data concerning the differences observed between male and females and between the two age groups (20-25; 40-45).

	Male v Females		**Age groups**	
	B-actin (males; females)	**18S (males; females)**	**B-actin (20-25; 40-45).**	**18S (20-25; 40-45).**
Mean	28.02; 28.22	21.53; 21.56	28.22; 28.03	21.56; 21.52
Std Deviation	2.15; 2.15	1.52; 1.53	2.24; 2.06	1.64; 1.41
Df	178	178	178	178
P value (ANOVA)	0.53	0.91	0.55	0.84

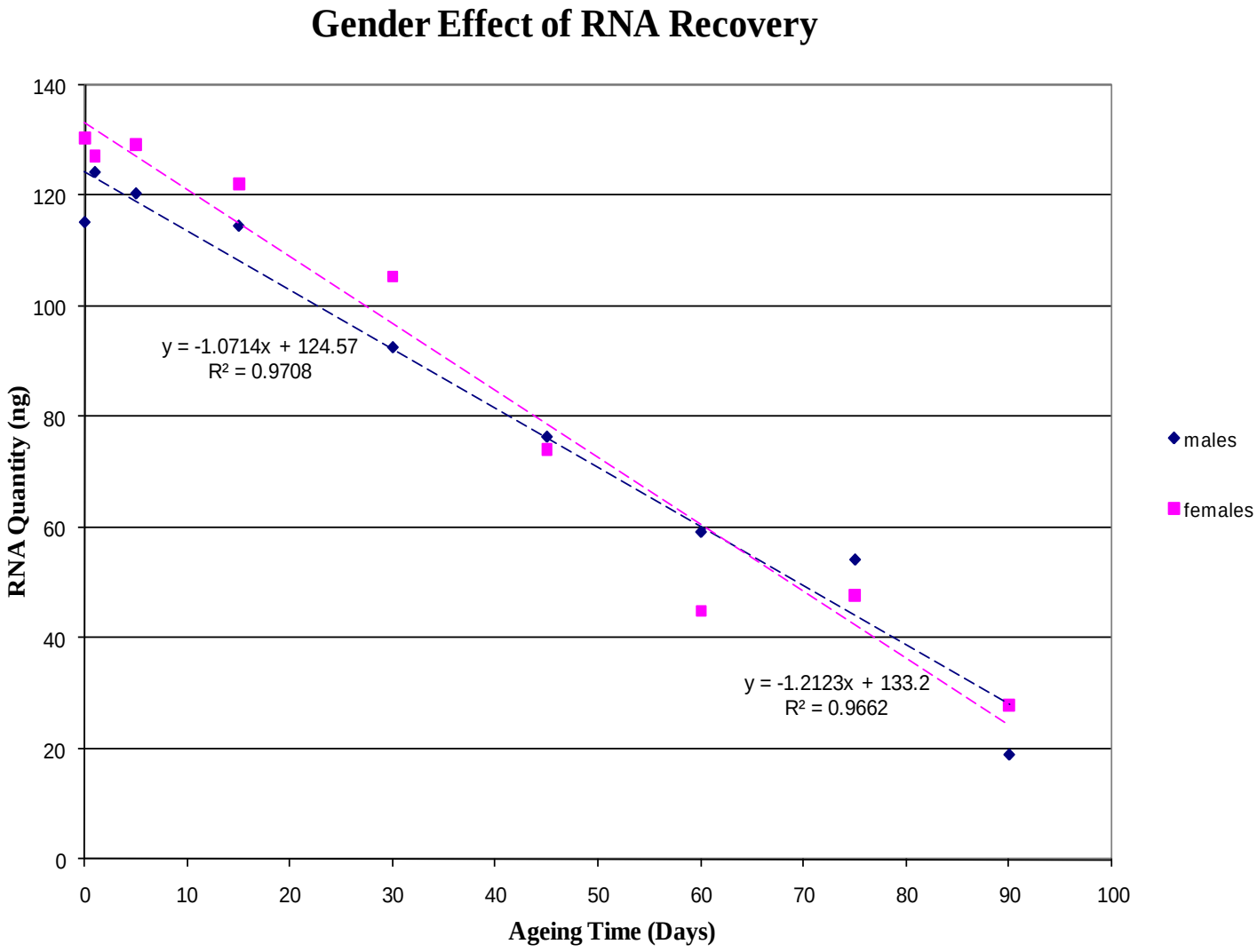


Figure 77: Gender Effect of RNA Recovery Rate. Graphical representation of the mean quantities of RNA recovered between male and female samples. Statistical analysis confirmed there was no significant difference observed.

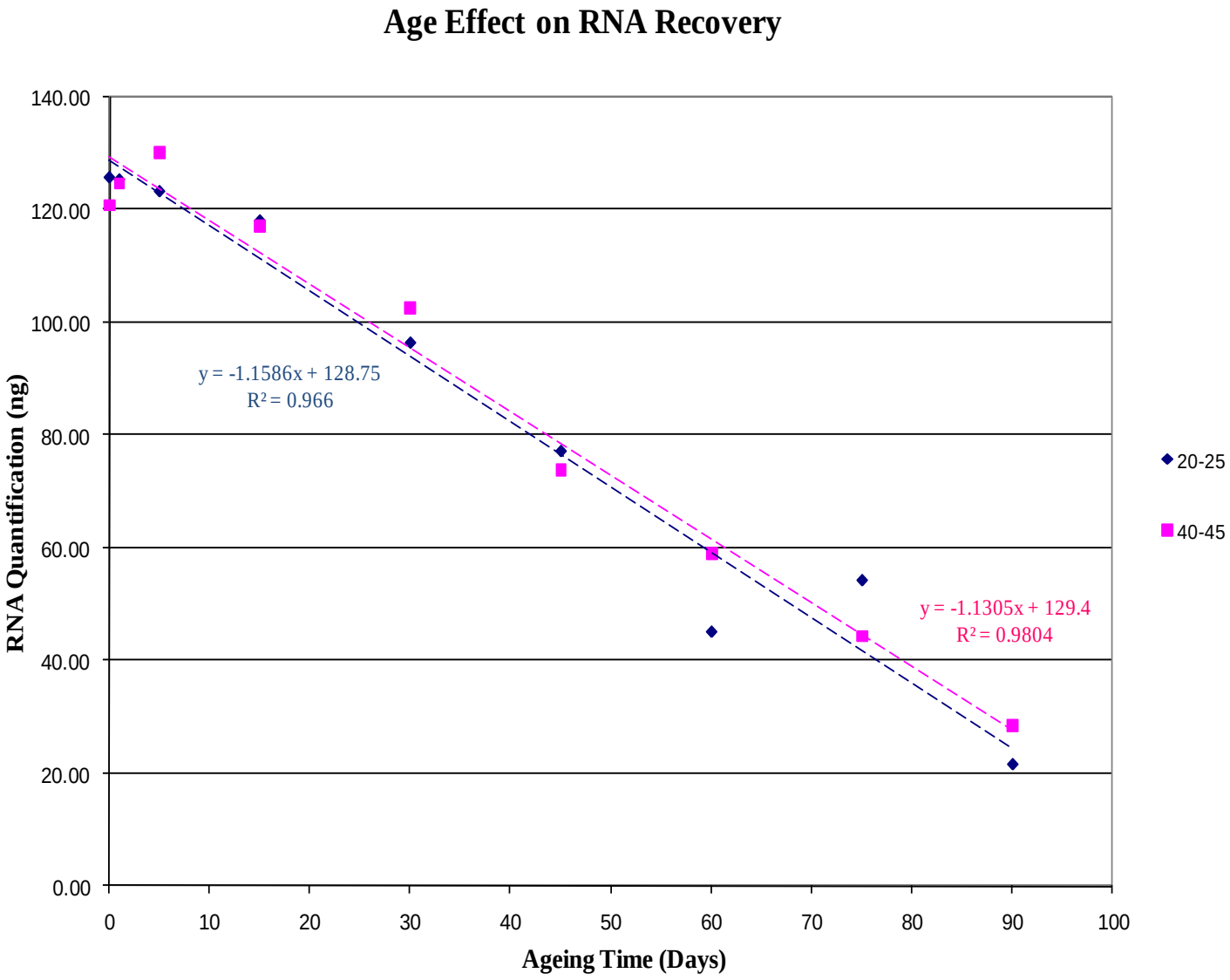


Figure 78: Age Effect on Recovery Rate. Graphical representation of the mean quantities of RNA recovered between the different age groups measured in this study. Statistical analysis confirmed there was no significant difference observed.

The quantification method is a further consideration when optimising an assay of this nature. Implen's Nanophotometer was used according to manufacturer's instructions, but it was noted throughout the study that the precision of the instrument was not ideal. A pilot study was conducted (results not shown here) which showed that the nanophotometer displayed greater accuracy and precision at higher RNA concentrations. Obtaining accurate nucleic acid measurements has posed difficulties for the scientific community for some time (Bustin and Nolan, 2004). Nielsen *et al*, (2008) compared five different quantification methods for DNA. The methods examined included UV spectrometry, a fluorescent dye method (SYBR Green), slot blot hybridization and two real time PCR methods. According to Nielson *et al*, (2008), DNA concentrations must be at least 3ng/ul in order to achieve reliable results using UV spectrometry and therefore is generally not suitable for quantification of nucleic acids in forensic samples. The UV method was heavily criticised stating that if reliable results are to be obtained, the DNA must be pure, of a high quality and must be dissolved in a "UV clean" buffer solution (Nielsen *et al*, 2008). The fluorescent dye method offered greater sensitivity but was susceptible to any form of contaminating DNA. The sensitivity and precision of the slot dot hybridisation technique was low and combined with other issues such as high labour demands and underestimation of DNA in degraded samples (Prinz, 1995) this technique was unsuitable. The two qPCR methods worked well, however the measurements were inaccurate, although reproducible (Nielson *et al*, 2008) suggesting problems with the supplied standard (reference) DNA. The UV spectrometry, SYBR Green dye staining, slot blot and real time PCR (RBI) gave 39, 27, 11 and 12% higher DNA concentrations (respectively) than expected based on the information provided by the manufacturers. This study concluded by stating there was a need for internationally agreed standard reference DNA material and improvements of the methods for DNA quantification (Nielson *et al*, 2008).

Different quantification methods of RNA have also been closely scrutinized in the past. One particular study presented at Sigma's Real Time PCR course (2005) examined five different methods, which included the nanodrop, UV spectrophotometer, Ribogreen dye, Experion and the Agilent Bioanalyzer. They concluded there was no stand out method as each one performed differently under different conditions (sample type and concentration). Due to the inconsistency of all methods, the primary recommendation from this study was that all samples in a particular project should be analysed using the same method. Despite these concerns the methodology used in this study does not depend on accurate absolute RNA quantification as it is only used to gauge the amount of template RNA used in the Reverse Transcription and Real

time PCR processes. The mean quantity of RNA extracted from samples that had been aged for 90 days was sufficient for subsequent processing and as such did not inhibit the study. By using a comparative ratio methodology which incorporates qPCR analysis, accurate relative expression could still be achieved.

8.5.3 Assay Sensitivity

The sensitivity of the multiplex assay was examined in order to determine the number of hair strands required for reliable analysis. The number of hairs per sample ranged from ten, down to a single strand. A standard deviation of less than one Ct cycle was the defining limit for acceptable precision.

Both 18S rRNA and B-actin mRNA showed acceptable levels for samples containing six or more hairs (with the exception of the B-actin species at six (1.02 cycles) and eight (1.48 cycles) hairs). As the number of hairs decreased, the variation generally increased. For samples containing between two and five hairs the deviation for 18S rRNA ranged from 1 to 1.5 so although outside the ideal range the variation was not excessive. 18S rRNA presented with a 2.16 cycle variation in single stranded samples. Expressed as the percent coefficient variation (%CV) the variation ranged between 2.2% (8 hairs) to 5.0% (1 hair) with an average of 3.2%. Values for B-actin mRNA were within the acceptable limits for samples containing four and five hairs, but were outside the limit for samples containing two (1.81) and 3 (1.75) hairs. The %CV for B-actin data ranged from 1.3% (10 hairs) to 4.4% (6 hairs) with an average of 2.9%

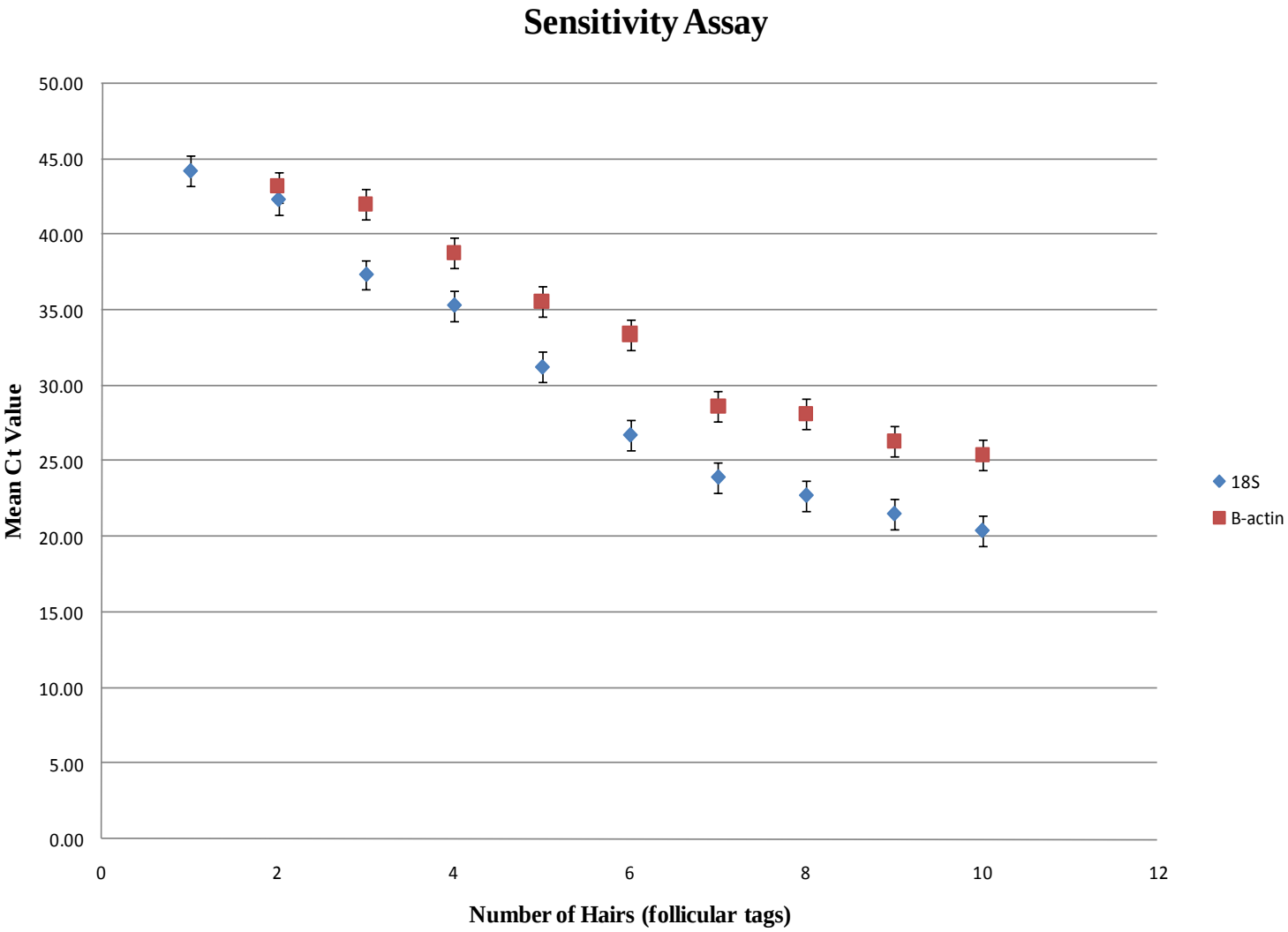


Figure 79: Sensitivity Comparison between 18S rRNA and B-actin mRNA. A Sensitivity assay was performed to evaluate the number of hair strands required for this type of analysis. 18S rRNA was recoverable from a single hair strand although the results were inconsistent. B-actin mRNA could not be recovered at all from samples containing a single hair strand. The error bars represent +/- 1SD.

In terms of sensitivity, 18S rRNA performed slightly better than B-actin mRNA. An amplification signal for 18S rRNA was obtained from every sample containing four or more hairs. 83% of samples containing three hairs produced a result, whilst successful amplification occurred in 66% and 13.3% of samples containing two and a single hair respectively. For B-actin mRNA, all samples containing five hairs produced an amplification signal. Only 73.3% of samples containing four hairs produced results whilst samples consisting of three and two hairs displayed successful amplification in 46.6% and 10% of cases respectively. A complete signal dropout for B-actin mRNA was observed for samples containing a single hair. Though these results appear acceptable in a research environment, they are rather discouraging as a forensic application. Hair evidence is commonly recovered as a single strand therefore the relevant statistics should be considered. In the case of this data set, B-actin mRNA was not detected at all, whilst 18S rRNA was only detected in 13.3% of cases. This poses serious issues regarding the practical use of this technique in the forensic arena.

The reaction kinetics of Real Time PCR suggests that the amount of product should double with every cycle (if operating at 100% efficiency). Therefore, technically, there should only be a one cycle difference between samples containing five and ten hairs (factor of 2). The results obtained (see Figure 80 and 81) show this is clearly not the case as the Ct values differ by almost ten cycles. The difference between expected and actual can in partly be attributed to the fact that the reaction was not operating at 100% efficiency (18S rRNA was estimated to have an amplification rate of 95.2%) however the remaining difference could be attributed to the variable number of cells attached to the follicular tag.

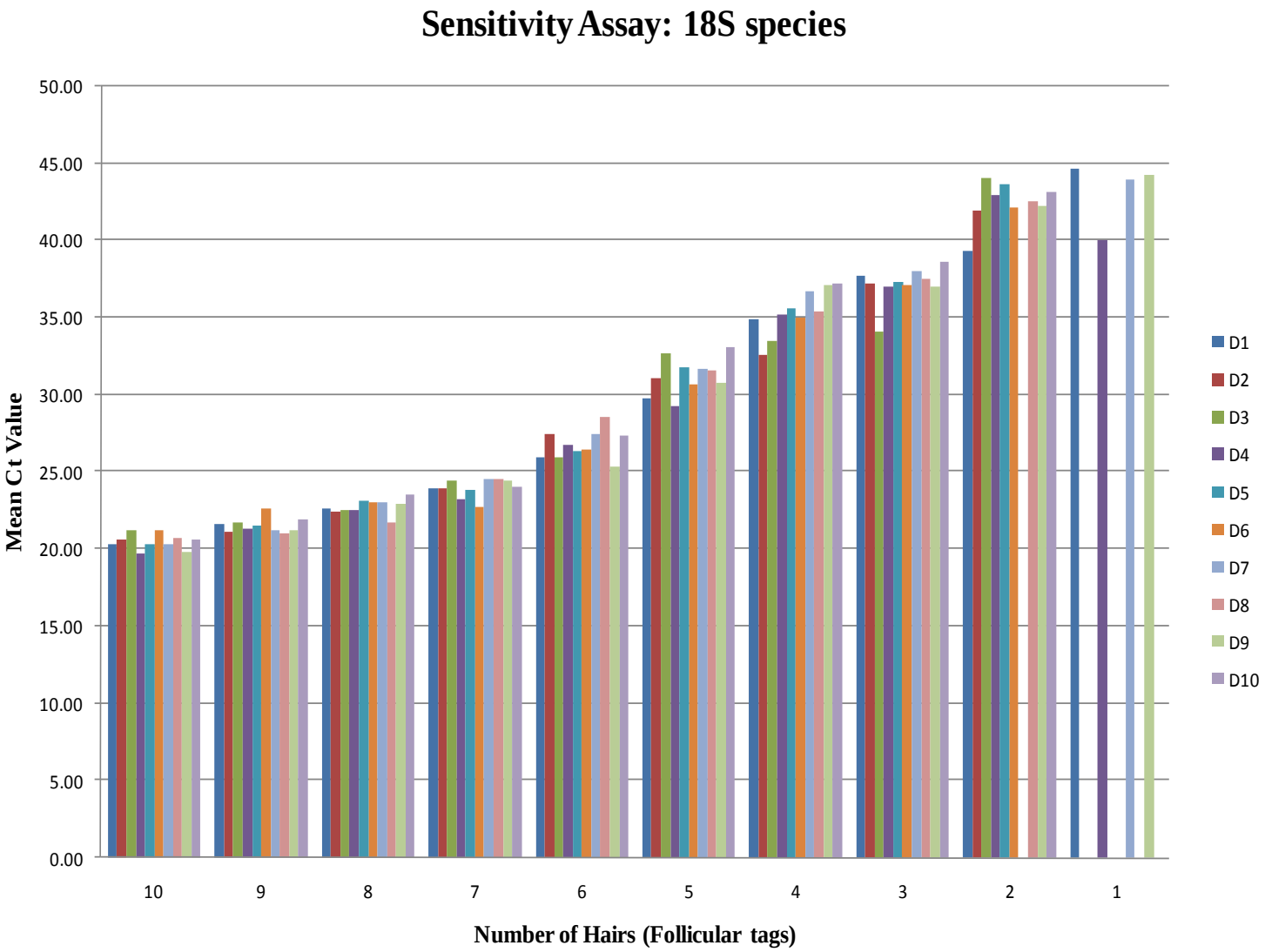


Figure 80: 18S rRNA Sensitivity Assay. The mean Ct values (samples run in triplicate) obtained from the various number of hairs per sample are presented above. 18S rRNA was detected in samples containing as little as one hair, though this was not consistent with all donors. The reaction kinetics suggest that the efficiency rate is not 100% and that there are variation in the number of cells attached to the follicular tag of each hair.

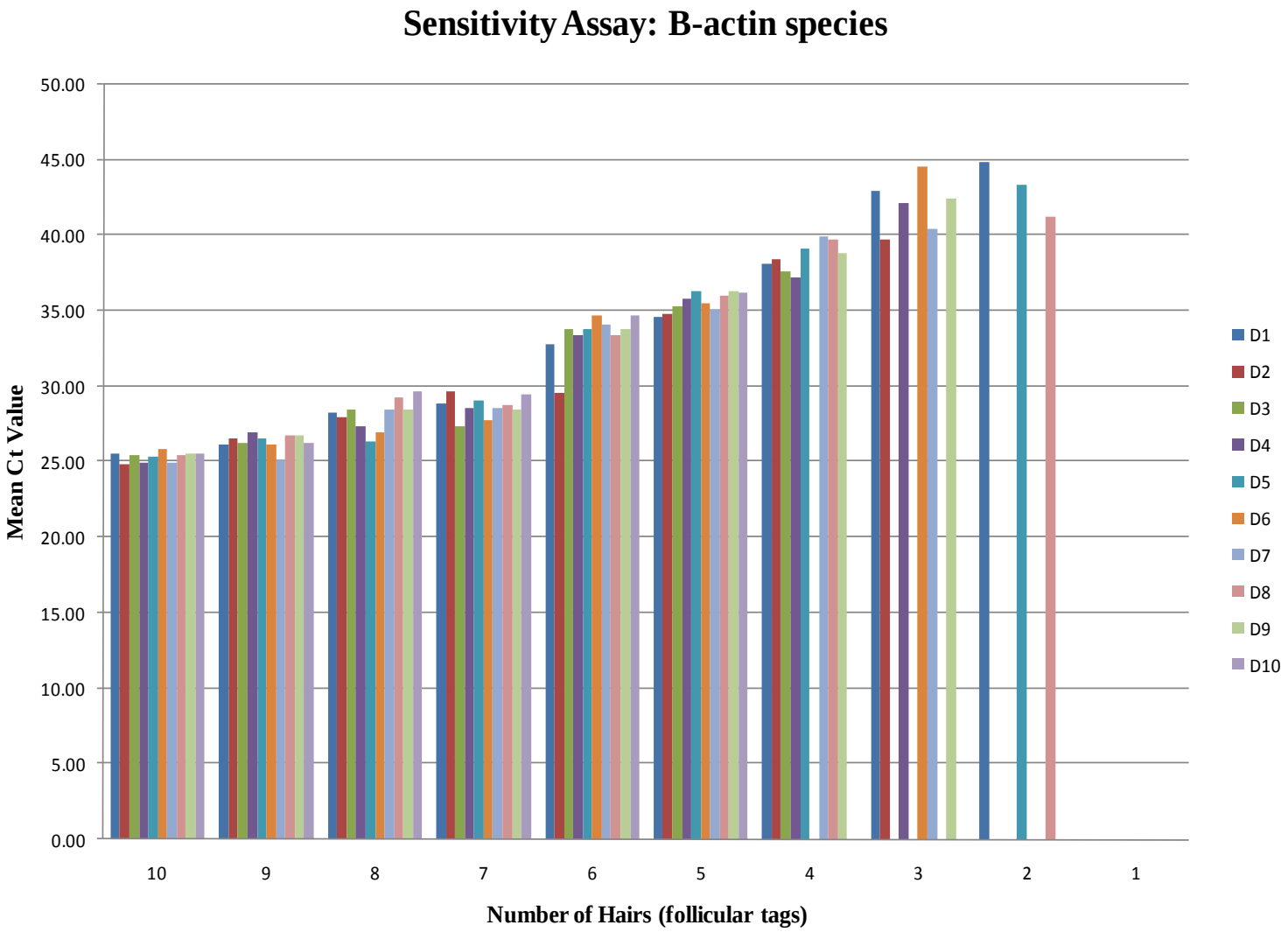


Figure 81: The sensitivity of B-actin mRNA was less than that achieved by 18S rRNA. B-actin mRNA was not detected in any of the samples containing a single hair. There was also a significant dropout in samples containing two and three hairs. B-actin was only consistently recovered from all donors in the samples containing five or more hairs.

8.5.4 Stability of 18rRNA and B-actin mRNA

The purpose of this study was to examine the degradation rates of two different species of RNA to determine whether there was a correlation between the relative expression ratio (18S/b-Actin) and the age of the sample. Anderson *et al,* (2005) established a correlation using blood samples concluding that the lineal trend observed was due to the fact that the B-actin mRNA degraded at a relatively consistent rate, whilst the 18S rRNA did not appreciably change over the course of 150 days. The results from this study, which concerned hair, rather than blood samples, suggest that 18S rRNA is not as stable as suggested by Anderson *et al,* (2005). 18S rRNA was stable in hair samples for approximately 45 days, at which point it began to display a change in the expression levels which was indicative of degradation. Between 60 and 90 days, 18S rRNA appears to degrade at approximately the same rate as B-actin mRNA. This is further supported by the plateau effect seen in the RER's of samples aged for longer than 60 days. These results suggest that 18S rRNA may act as a suitable housekeeping gene for follicular hair samples aged for less than 60 days. The exact reason as to why the 18S rRNA began to degrade around this period of time is uncertain but it may be due to the breakdown of the ribosomal complex, which is primarily responsible for protecting much of the 18S rRNA. Breakdown of this complex would release the 18S rRNA into the same environment as the B-actin mRNA and thus be exposed to the same degradation factors. This would explain the similar changes in Ct values experienced by both species after 60 days. The Ct values for B-actin mRNA continually increased with time suggesting a relative consistent degradation process. A sharp initial increase in the mean values was observed between day 0 and day 1, indicating an accelerated change of expression, when compared to samples aged between one and 60 days. The magnitude of this change is similar to that seen in samples aged for greater than 60 days. This could be a result of the moisture levels within the samples during the early stages of ageing. Enzymatic degradation processes require an aqueous environment (Ballantyne, 2009) and during the first day of ageing the hair samples may maintain the level of moisture required for these processes. Once the samples dry out, the enzymatic activity is likely to decrease, resulting in a decreased degradation rate. The rate over the next 40 or so days remains relatively constant but increases again in samples aged for longer than 60 days. This increase is likely to be due to some external factor rather than any intracellular mechanism.

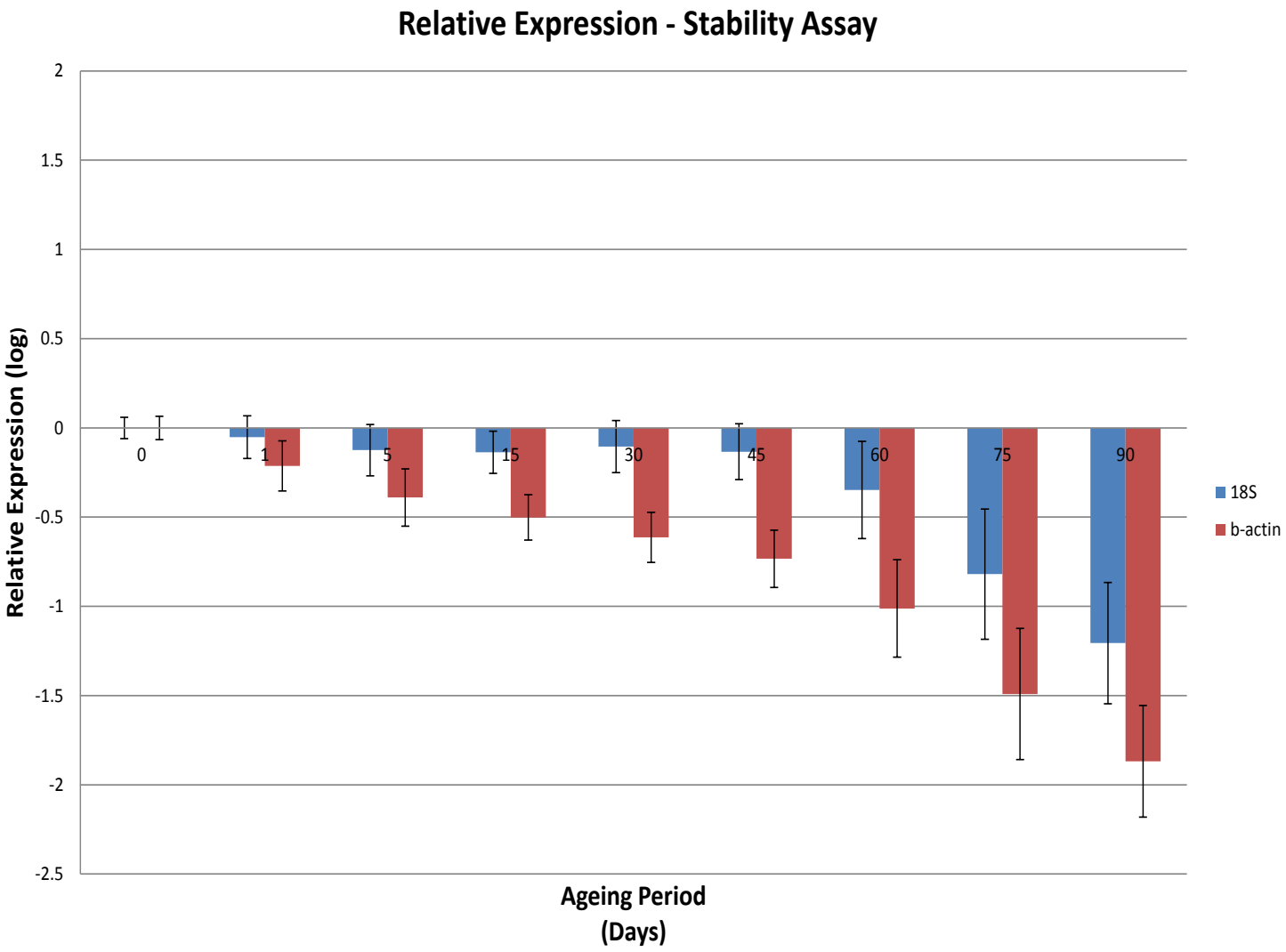


Figure 82: The Relative Expression of B-actin mRNA and 18S rRNA from all donors over the 90 day time course illustrates the stability behaviour of the two target genes. The RE for 18S rRNA remained relatively consistent for the first 45 days, indicating its stability however there was a definitive decrease between 60 and 90 days suggesting 18SrRNA degradation rates increased significantly. The RE for B-actin mRNA continued to decrease in a consistent manner over the 90 days.

8.5.5 Correlation between Relative Expression Ratios and Sample Age

The hair samples used in this experiment were aged over a 90 day period under relatively uncontrolled conditions (room temperature, room humidity and in the presence of daylight), which were designed intentionally to mimic forensic type environments. At each age period, 18S rRNA B-actin mRNA was extracted, quantified and converted to a relative ratio (18S/B-actin). The normalised Ct values and relative 18S/B-actin ratios (RER) for all donors across all time periods can be found in the appendix XV. The mean RERs were plotted against sample age and the correlation is shown in Figure 83. The results show that the ratio changed in a linear manner over time, which meant the 18S/B-actin ratio could be used to estimate the age of a hair sample, albeit within a range. The age of hair samples could be approximated using a second order polynomial ($R^2 = 0.98$):

$$\text{Age} = 3.31RER^2 - 2.85\,RER - 0.54$$

This methodology appears to be more precise with recently deposited samples. The RERs behaved in a linear and reproducible fashion with a relatively strong correlation with samples aged up to 45-60 days but as the time proceeded, the rate of increase plateaued. There was a noticeable decrease in the relative expression of 18S rRNA in samples aged for 60 days or longer and this sudden increase in degradation was comparable to the degradation rate of B-actin mRNA which behaved consistently over the entire 90 days. It is uncertain as to why the 18S species began to degrade around this period of time but as previously suggested it is thought that it may be due to the breakdown of the ribosomal complex. The observed plateau reduced the discriminating ability of this technique, which meant that samples older than 60 days were indistinguishable based on RERs values. The error rate or confidence with any estimation also varied over the 90 day period. Samples aged for less than 30 days could be distinguished within a 10-15 day age range. However, as the time frame increased the ability to distinguish one sample from the other decreased and the estimated age range became larger to the point where samples could not be distinguished at all after 60 days.

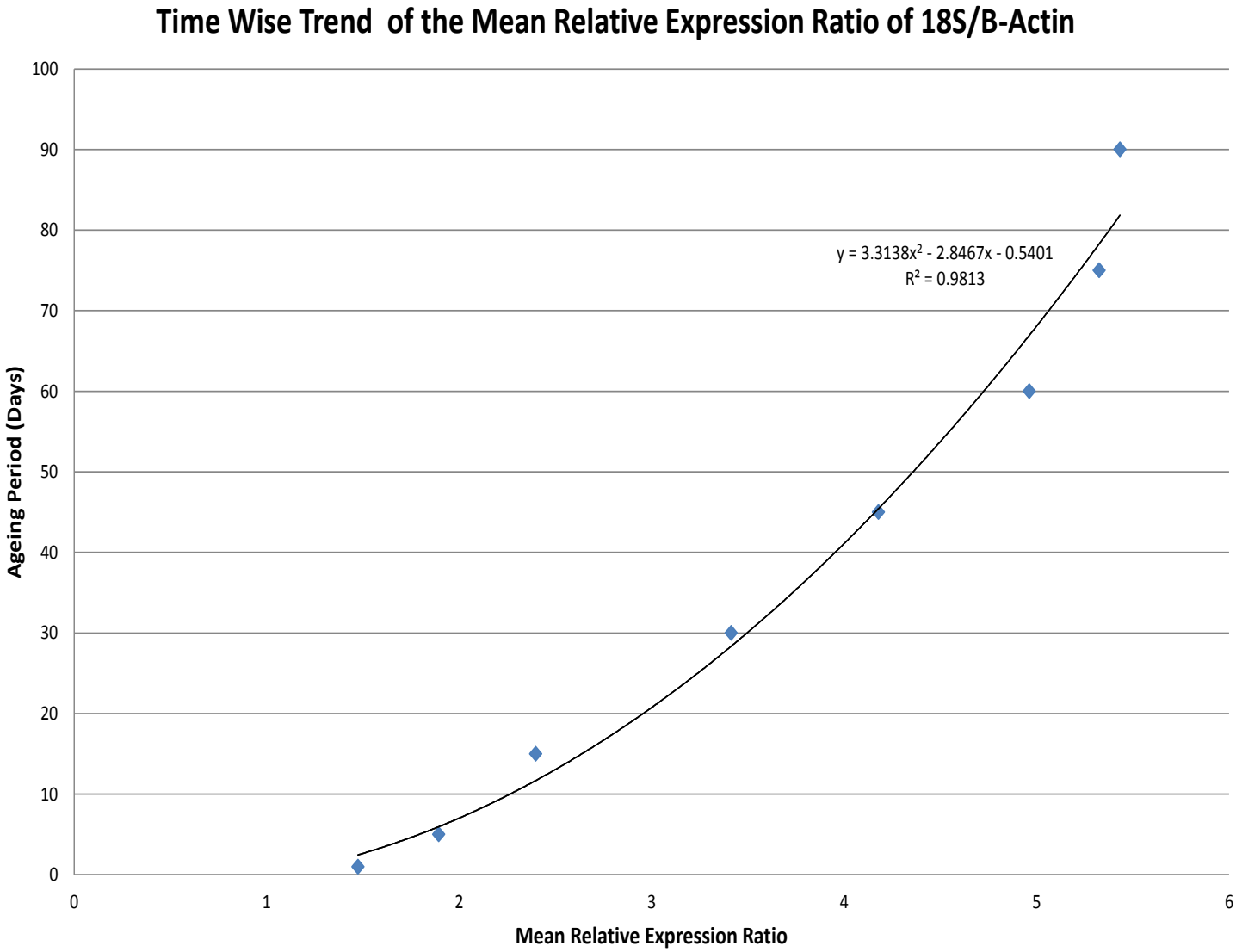


Figure 83: Time wise trend of the Mean Relative Expression. A 2nd order polynomial line of best fit was the most appropriate for this data set. The data shows a strong correlation (0.981) between the mean relative expression ratio and sample age.

8.5.6 Gender and Age effect on the Relative Expression Ratio

It was hypothesised by Anderson *et al,* (2005) that the daily differences observed in the relative degradation ratio (18S/B-actin) between males and females was attributed to the effect of hormonal differences, especially during the female's monthly menstrual cycle. Kim *et al,* (2006) also observed gender differences in the expression of 97 different genes in hair follicles. The differences observed in this experiment were examined to determine whether there was any gender bias. The difference observed between males and females was not significant (ANOVA; p = >0.1).

A comparison of the RERs was made between individuals from different age categories (20-25yrs and 40 -45yrs). The strength of the correlation was greater in the older group (R^2 = 0.98) than in the 20-25 age group (R^2 = 0.93). The difference observed between the two age groups was not significant (ANOVA; p = >0.1).

Statistical analysis suggests there is no gender or age related effects on the mean relative expression ratios from hair samples. These findings must be viewed cautiously given the population size of this study. There were only five males and five females used in this study from which they were categorised into only two age brackets. To increase the confidence in these findings, a greater population set with narrower age group bands should be considered.

8.5.7 Inter-donor Variation

The results and interpretation thus far have concerned the mean data, which ignores inter and intra-donor variation. The fact that hair is biological in nature, one would expect to see a degree of variation between and within each donor. Past studies have confirmed that different tissue types display different degrees of variation [Bas *et al,* (2004); Dheda *et al,* (2004)] therefore it was necessary to determine what degree of variation exists in hair samples using this methodology. The inter-donor variation (%CV) in the RERs was small ranging from 3.5% (day 5) to 8.2% (Day 90). The variation across all ageing periods is presented in Table 59. The variation observed in hair samples was significantly less than the variations observed from blood samples, which ranged from 80% to 190% (determined from earlier work in chapters three to seven). The represents a ten to 20-fold increase. These findings suggest that levels of these housekeeping genes are more consistent in follicular cells, than in blood cells. This is not unexpected given RNA is recovered from a number of different cell types in whole blood

samples, whereas with hair samples of this nature, only a single cell type (follicular cells) is examined.

Table 61: Variation in Relative Expression Values - Illustrates the significance of the standard deviation seen in the data obtained

Ageing Period	Mean Relative Ratio	Standard Deviation	Percentage coefficient of Variation (%)
0	1.474	0.0966	6.55
5	1.894	0.0616	3.25
15	2.398	0.1483	6.18
30	3.414	0.2668	7.81
45	4.178	0.2434	5.82
60	4.963	0.3033	6.11
75	5.327	0.2977	5.59
90	5.435	0.4465	8.22

The variation observed in this experiment increased slightly with sample age. During the earlier phase of the study the range (maximum RER – minimum RER) was 0.307, 0.176 and 0.491 for Day 1, 5 and 15 respectively. By day 60 the difference between the maximum and minimum values had increased to 1.26. The differences are shown in Figure 84. The slight increase in the inter-donor variation observed over the duration of the study is likely due to older samples being exposed longer to the external degradation factors. All samples were aged under similar uncontrolled conditions, in that they were left at room temperature in a glass storage cabinet (exposed to daylight). However, the temperature would not have remained constant throughout the study therefore older samples were likely to experience a greater number of temperature changes, which could have influenced the degradation rate. Other environmental conditions such as exposure to light and humidity were also factors that were not controlled and as such older samples were exposed to longer periods of degradation factors which had the potential to variable.

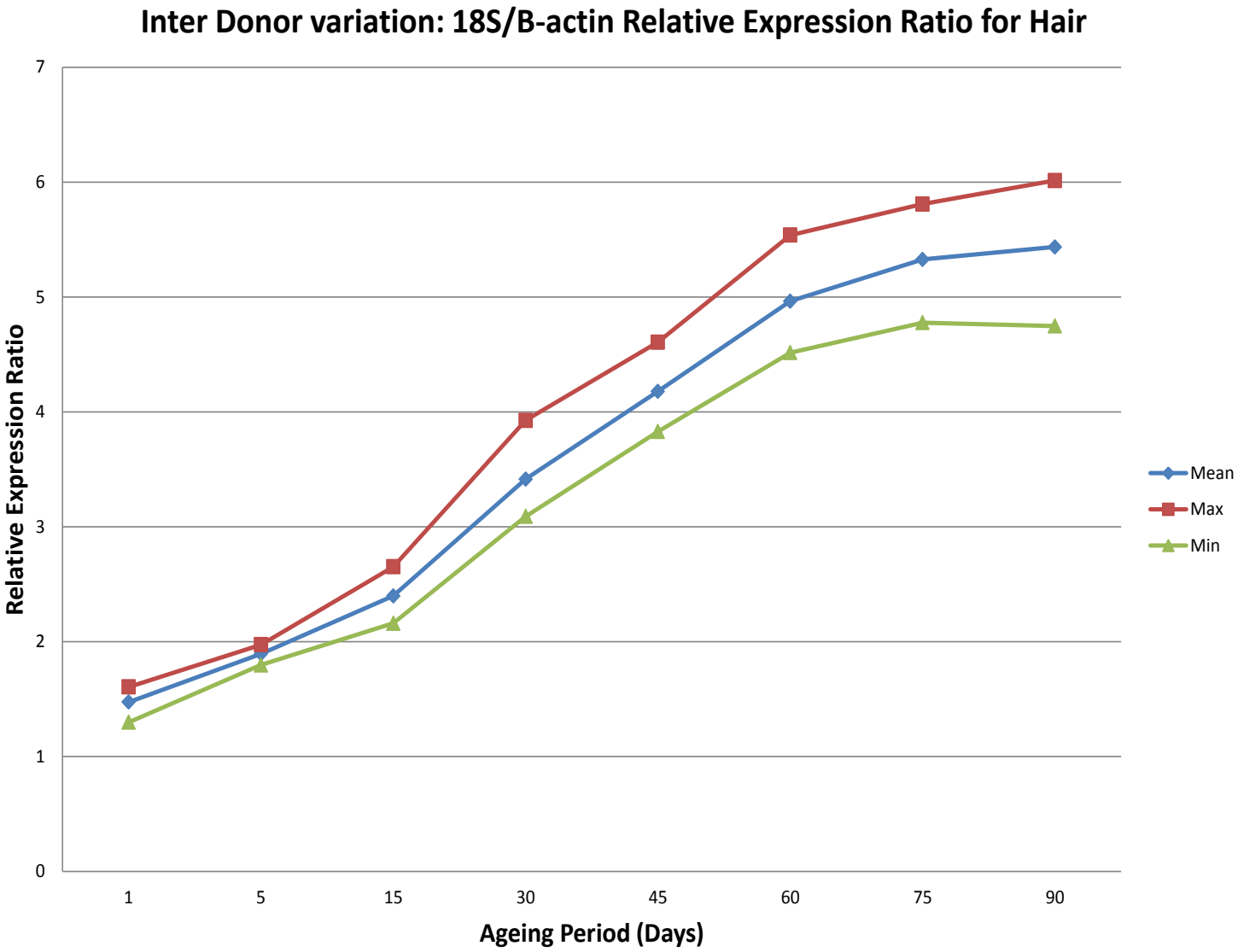


Figure 84: Inter-Donor variation in the relative expression ratios over the ageing period. Expressed as a percentage of the mean the difference ranged from 9.3% (day 5) to 24.5% (day 30). The day five result appears to be an outlier as the variation remained relatively consistent for the other time periods (20.8% - day 1; 20.5% - day 5, 18.6% - day 45; 20.6% - day 60; 19.4% - day 75 and 23.3% - day 90).

Although both 18S rRNA and B-actin mRNA are housekeeping genes their expression under certain conditions and in certain tissue types have been known to change. Variation occurs naturally as well as with certain disease conditions, such as acute respiratory syndrome and autoimmune diseases (McLoughlin *et al,* 2006). Radich *et al,* (2004) also demonstrated that 18S rRNA and B-actin mRNA base levels can vary between individuals, although this research concerned blood, as opposed to hair samples. Kim *et al,* (2006) looked at the expression of 1436 genes in follicular hair cells. The average inter-donor variation (%CV) was 45.31%, which was significantly greater than the average obtained in this study. The variation reported by Kim *et al,* (2006) concerned both housekeeping and non-housekeeping genes explaining the greater variation observed.

There are also a number of internal factors that will degrade RNA, which were previously discussed on page 64. These include the actions of endo and exonucleases as well as degradation by hydrolytic and oxidative means (Gong *et al,* 2006). Oxidative damage prevents cDNA from being synthesised completely, which would effectively act to prevent amplification of the target species. Samples that had been aged for longer periods of time would have been more susceptible to this type of damage. These *ex-vivo* degradation processes are unlikely to be uniform in each sample and therefore could contribute to the inter-donor variation observed.

The degree of inter-donor variation observed with hair samples (although limits the potential use of this technique in the field of forensic science), is encouraging when compared to blood samples. This is not surprising given many blood components, in particular the ones examined in earlier work (chapter six and seven) can undergo major expression changes when necessary. The general trend in RERs along with the increasing variation means that samples aged between 60 and 90 days cannot be distinguished. There is a high degree of uncertainty with samples aged between 30 and 45 days and at best, an age estimation of within ten days is possible for samples that are less than 30 days old. The latter time frame would be relevant to the majority of serious crimes given they are generally discovered within a short period of time though an age estimate (within a ten day range) is likely to be inadequate and not specific enough for these situations. In certain cases this level of detail may have some evidential value.

8.5.8 Intra-donor Variation

There are a number of other factors, other than the inter-donor variation previously mentioned that have the potential to contribute to the overall variation seen in these results. RNA isolation, residual variation which accounts for duplicate assays, experimental error and intra-donor variation are all possibilities. Some of these issues have been addressed through the methodology (normalisation procedures, duplicate samples, using the corrected comparative Ct method for analysis etc) however the intra-donor variation is a factor that could not be controlled by simply manipulating the protocol procedure. Intra-donor variation refers to the differences seen in the replicate samples, within each donor. The differences for each donor have been calculated at each time point, and are shown in Appendix XVI, with mean values presented in Figure 85. The intra-donor variation observed was small. Expressed as percentage coefficient the variation ranged from 0.39% (donor 6) to 1.05% (donor 10) with an overall average of 0.67%. Anderson *et al,* (2005) contributed an average of 3.8% of the total variation to intra-donor variation though the variation related to blood samples.

The data was examined to determine whether there were any significant gender differences in the intra-donor variation in hair samples. There was no significant difference in the variation observed between males and females (Mann-Whitney; $p = >0.1$). The age of the donor was examined to determine whether a person's age affected the size of the intra-donor variation. All volunteers were again categorised into one of two age brackets, 20-25 years or 40-45 years. The average percent variation for the younger age group was 0.63%, whilst the average for the older group was 0.70%. This difference was not considered significant (Wilcoxon; $p = >0.1$) which indicates there is no age related effect on the intra-donor variability of hair samples in tested in this experiment.

There were no consistent patterns in the overall intra-donor variation with respect to time (refer to Figure 85 on page 318). This suggests that the increase in total variation as time progresses is more likely to be a result of external environmental factors, rather than any internal mechanism. Whilst this finding may allow for refinement of this technique (to increase the accuracy and precision) in a laboratory setting where the ageing conditions can be strictly controlled, it poses problems for forensic samples which will be exposed to external environmental factors.

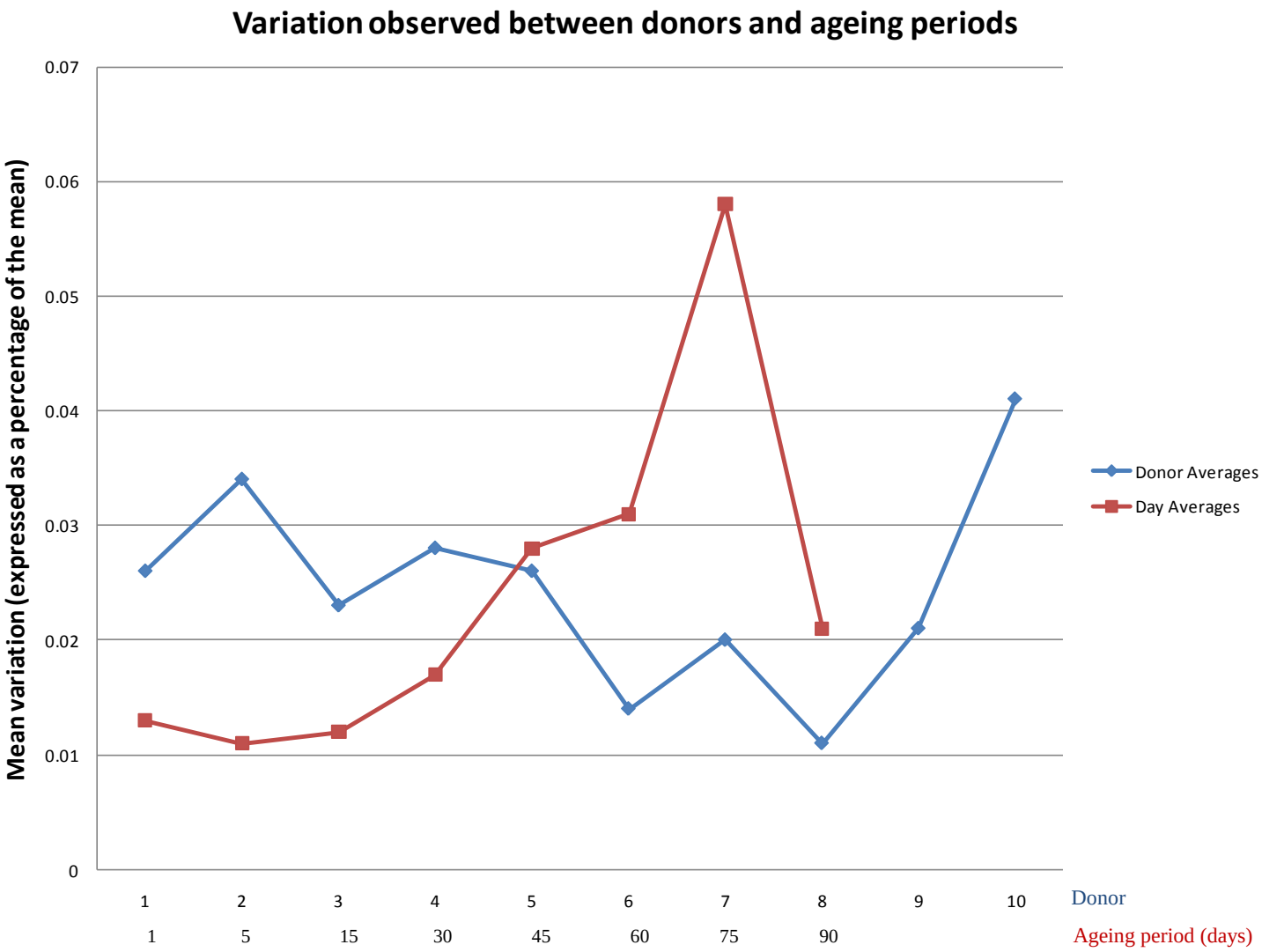


Figure 85: Variation Trends. The mean intra-donor variation did not differ significantly between donors as indicated by the blue trend line. Donor 8 had the smallest variation with donor 10 having the largest, although overall the variation was very small. There was a general pattern of increasing variation with ageing time up until day 75 however at day 90 the variation was significantly reduced, as indicated by the red trend line.

8.6 Limitations of the 18S/B-Actin RNA methodology for ageing hair samples

A number of these issues have already been mentioned during the discussion however it is important to highlight the areas that need to be addressed in future studies. Sample preparation, numbers and ageing conditions are all variable factors that are likely to have affected the results of this study. The population size was relatively small as it was difficult finding volunteers who were willing to have a considerable amount of their hair forcibly removed. Though enough samples were obtained to be useful, a larger population with a greater age range is needed to address some of the areas of uncertainty within this study. The conditions in which the hair samples were aged were not specifically controlled. This was done intentionally to increase the realism between the tested samples and samples likely to be encountered in a forensic environment. Samples were simply left to age on the laboratory bench at room temperature and humidity. Though the fluctuations in temperature were not severe, they may have contributed to the variation seen in the results. Obviously, samples aged for 90 days were subjected to more of these fluctuations than samples aged for only 1, 5 or 15 days. Controlling some of these aspects may provide a clearer understanding of the influential factors involved in the limitations observed.

There were also a number of issues relating specifically to the methodology itself. In theory, Real Time PCR is robust and predictable however in reality variations in reaction components, thermal cycling conditions and mispriming events can lead to large changes in the overall amount of amplified product (Wong and Medrano, 2005). There are two methods of measuring the amount of product from a PCR reaction. There are absolute and relative methods. This thesis has been based around the relative comparative method. The problem with this method is that the Ct values obtained are arbitrary values and are only applicable to the samples run within the same PCR. A degree of variability may be introduced when samples are assayed in different runs on different days and slight variations in reaction mix constituents within each well have the potential to affect the efficiency rates of each target species. Though many of the normalisation processes account for these variations (inter-plate calibrators, ROX dye etc), they are only statistical corrected (post experimental processing) with a certain level of confidence.

One element that was not corrected for was the actual efficiency rates within the samples themselves. Five test samples were chosen randomly and the amplification efficiency rates were measured and compared to the efficiency rates obtained from the optimised multiplex

assay. Despite the fact that differences were observed, the values from the multiplex assay were used to analyse all samples. These differences were small and it was decided that any bias or inaccuracy caused by these differences was not significant enough to warrant measuring the individual efficiency rates of all 180 samples but in doing so has meant that a degree of uncertainty has been introduced into the calculations.

A two-step method was used in this study and despite already discussing potential problems in the second Real time PCR step, there are also issues concerning the initial reverse transcription stage. This stage is extremely important given there needs to be complete conversion of all RNA to cDNA if subsequent analysis is to be accurate and representative of the initial amount of RNA. If the reverse transcription efficiency is not ideal (100%) the value of the resulting Ct data will be limited. Both the reverse transcriptase enzyme and dithiothreitol (DTT) are known PCR inhibitors and any carry over steps can affect the reaction kinetics of the PCR process. This inhibitor carryover can be avoided by introducing a cDNA precipitation protocol but this was not performed during this study. Possible temperature fluctuation of the reverse transcription procedure is another issue that needs to be considered. The reactive step was carried out at 42^{o}C as stipulated by the manufacturer's guidelines. However, Bustin (2002) has stated this temperature can lead to the reverse transcriptase enzyme losing its function and dissociating from the RNA if the target sequence is G/C or secondary structure rich obviously resulting in less cDNA for amplification by RT-PCR. This could potentially contribute to an inaccurate determination of the RNA ratios.

One oversight of this study was that at no stage during the analysis was the efficiency of the reverse transcription (RT) step measured. Failure to do so meant that any rate other than ideal (100%) will result in an underestimation of the RNA quantity. This would not be significant to expression ratios if all species of RNA were "under" transcribed at the same rate but if this was not the case then the resulting ratio estimations would be inaccurate. Priming was obtained using random hexamers but it has been shown that this method of priming does not result in equal efficiencies of reverse transcription for all targets in the sample and therefore there will not be a lineal correlation between template RNA and cDNA yield when specific targets are measured (Nolan *et al*, 2006). Because there are a number of uncertainties with the RT step it has been suggested (TATAA Biocentre) that replicate samples be run in the reverse transcription stage. Using replicate samples is considered an essential approach and therefore commonly performed with Real Time PCR assays, but it is rarely considered for the RT step,

though its importance has been demonstrated. Replicates for the RT step were not carried out in this study. Both reaction steps were carried out using kits from Applied Biosystems, which have been rigorously tested and optimised reducing the amount of laborious testing for users but Bustin (2002) reported a significant Ct value difference from a single template assayed with two different batches of the same kit. This translated into a 2.5-fold difference in the mean mRNA copy number. This demonstrated how variations can occur even when using products from the same manufacturer.

Another issue has been raised regarding the potential issue of primer specificity. There is always a level of uncertainty regarding the amplification of potential pseudo-genes (Harper *et al*, 2003). Unfortunately this issue could not be clarified due to the fact that the primers used were Applied Biosystems patented primers and as such literature regarding primer and target sequences is not readily available. Without knowing the primer and target sequences, it was not possible to check if the primers and probes had the potential to bind and amplify any pseudo-genes. Because we were unable to investigate this issue, it remains as a possibility in this study.

The final limitation of this study involves human error. Peirson (2003) noted that when three different people used the same pipettes, reagents, equipment and laboratory to quantify the same target, the values for the initial copy number ranged from 8.7×10^5 to 2.7×10^3. Even the most careful pipetting may have a 1% error rate and this error will be expressed or even exaggerated with further processing. The same pipettes, batches of enzymes, and operator (the author) were used to reduce experimental variation however different batches of the same master mix (same manufacturer) had to be used because sample numbers and hence reagent consumption. ROX dye which is incorporated into the PCR Master mix can be used as a pipetting/evaporation control and was checked after each run to ensure there were no significant variations.

8.7 Conclusion

Recent studies have shown that the degradation rates of RNA species can be a potentially useful tool in estimating the age of bloodstains [Anderson *et al*, (2005); Bauer *et al*, (2005)]. This theory was applied to hair samples, another type of biological evidence commonly found at crime scenes (Saferstein, 2006). The results presented here demonstrate the potential ability to estimate the age of hair samples for forensic purposes using a polynomial equation and although it may fall short of a robust method for determine the exact age of a sample it may be accurate and precise enough to be used as a useful investigative tool.

The results obtained from this study have demonstrated a number of differences between blood and hair in terms of stability and a gender effects which given the nature of these two tissue types, is not surprising. Blood is a dynamic tissue type and its many constituents are affected by the various biochemical and physiological processes that occur within the body which is likely to contribute to the large inter-donor variations observed. The more predictable and less diverse hair samples presented with considerably smaller variations however they were still large enough to limit the capabilities of this technique, especially with older samples. The results presented in this paper do, however, provide evidence to support the hypothesis raised by various other authors regarding the different rates of RNA decay in various biological samples. It also suggests that B-actin mRNA is less stable and degrades more rapidly than 18S rRNA in hair samples, a finding that correlates to Anderson's research on bloodstains however this appears to be time dependant.

CHAPTER 9: GENERAL CONCLUSION

With current scientific technologies, a significant amount of genetic information can be obtained from biological evidence found at a crime scene (Norrgard, 2008). Not only is it possible to identify the donor of the evidence through routine DNA profiling techniques, but new RNA based methods are being developed to determine body fluid origin as well as to predict physical characteristics such as eye (Branicki *et al,* 2008), skin and hair colour (Rees, 2003), height (Lettre *et al,* 2008) and ethnicity (Spielman *et al,* 2007). Despite the wealth of new information being obtained from biological evidence, the ability to determine the age or time since deposition of biological stains has been something that has eluded the forensic community thus far. The issue of timing is critically important as it could help police define the time frame in which the individual depositing the evidence was present at the crime scene. This type of information could also have exclusionary power by establishing that particular samples were irrelevance to a case. This would save time, resources and money as it would significantly reduce the number of samples requiring examination.

There have been numerous attempts to solve this problem using a wide range of applications, ranging from colourmetric techniques to atomic force microscopy with the most recent trend focusing on nucleic acids [Anderson *et al,* (2005); Bauer *et al,* (2005)]. Thus far, there has been limited success due to the lack of an accurate method for measuring degradation rates and the large inter-donor variation that is associated with biological samples. Furthermore assays are rarely optimised by determining the degradation kinetics of the specific environmental conditions that samples are aged under.

The body of work in this thesis sought to investigate whether the degradation of nucleic acid macromolecules could serve as molecular clocks for age estimations. In order to do so, experiments were performed to gain a better understanding of the degradation products produced from blood samples aged in an internal urban environment. Studies were conducted to examine the presence and effect of loci susceptibility and to identify the optimal target sizes required to resolve blood samples aged in an internal urban environment. Optimal assays were developed accordingly. Experiments were also conducted to quantify the effect of moisture and bacteria on the integrity of a sample. A number of different RNA based techniques for ageing blood samples were examined and despite some encouraging results, variation was a common issue and this was thought to be a result of the dynamic nature of blood tissue. The difficult

nature of this tissue type lead us to investigate the use of RNA based technique for ageing hair samples, which according to a thorough literature review, had never been attempted.

qPCR and qRT-PCR were the methods of choice for quantifying the nucleic acids throughout this work. Though these methods are generally referred to as the gold standard for quantification there are a number of variables that require consideration by the user. Each issue should be addressed appropriately (but are often ignored) if reliability of results is to be achieved. These are primarily concerned with (i) template quantity, quality and optimal assay design, (ii) standardisation of the reverse transcription reaction (iii) and appropriate data analysis. The importance of optimisation was highlighted throughout this body of work and all relevant data was included in each results section so although at times it became repetitive it was essential to demonstrate all steps had been addressed and that data interpretation could be made with confidence.

A common method for determining the degradation state of a sample is to measure the quantity of two difference sized targets. These assays are primarily designed around amplicon size but it was identified that testing loci stability was an essential requirement to the optimisation process. The results presented in this thesis suggest that when selecting two targets it is best practice to choose two that are located on the same loci to avoid any loci susceptibility. In previous research the target sizes used varied greatly and there has been very little comment regarding whether there were optimal sizes for any particular set of samples or conditions. A common setting found in forensic situations is an internal urban environment therefore it was decided to identify the optimal sized targets for this scenario. For blood samples aged up to 60 days in an internal urban environment, the greatest resolution was achieved by using two targets that differed in size by 170 to 240 base pairs with one of the target amplicons being between 200 and 300 base pairs in length.

After identifying TH01 as a robust and suitable target for degradation studies, we examined the effect that moisture has on the integrity of a sample. Using a carefully designed sealed dry swab we were able to remove moisture from the ageing environment and inhibit the growth of DNA consuming micro-organisms. It was determined that bacteria alone can cause a two-fold increase in the degradation rate of a sample aged at room temperature over 90 days. In terms of integrity, storing samples at room temperature in a moisture free environment was equivalent to storing standard samples (exposed to normal humidity levels) in refrigerated conditions. It was also determined that the effect of bacterial degradation can be halved by lowering the

storage temperature from room temperature to 4°C. The effect of moisture was also quantified and it was determined that a dry environment can increase the preservation of DNA by up to 4-fold. These findings could have implications as to how police and forensic personnel recover and store biological samples. The stability of an internal environment was also tested using samples aged at different times of the year. We found that an internal "urban" environment is not as stable as predicted and that seasonal temperature variation can have a large effect on degradation rates. This natural variation makes developing a robust, precise and accurate forensic technique difficult, if not impossible. Given the integrity of DNA was largely influenced by environmental conditions, and these conditions being so variable in real case work, the likelihood of DNA providing a good estimation of a samples age is considered by the author as remote. Most laboratories do not routinely check the quality of a sample before analysis and this is mainly due to the absence of a reliable method. Though there have been many studies that have tried to address this issue (Swango *et al*, (2006); Siwoski *et al*, (2002)), the majority of which are based on the ratio of relative quantities of different sized DNA targets, this work has demonstrated such approach is problematic for various reasons. Results presented in this thesis suggest an approach based on DNA fragment sizes cannot accurately and more importantly precisely, gauge the level of degradation therefore ageing studies based on these theorems will suffer the same unreliability and uncertainty.

For these reasons RNA was tested, using samples that had aged for only ten days. This was done in attempt to reduce the large variations that had inhibited previous attempts. IL-6 and TNF-α were chosen because of their rapid change in expression levels but their dynamic roles in a number of haemopoietic processes meant that levels were highly variable, unpredictable and therefore not suitable for this type of analysis. The methodology used here was sound and it is believed the main contributing factor to the poor results was the selected targets themselves. There are literally thousands of potential RNA transcripts that remain untested and therefore there is still optimism regarding its possible use as a forensic tool. Future research should focus on identifying suitable targets that degrade in a consistent manner over time and independent of external environmental conditions.

Nolan *et al* (2008) suggested that a novel 3'/5' RNA assay was a more accurate method of estimating the RNA integrity of blood samples and given the questionable precision and accuracy of other methods tried thus far it was decided to use this approach to analyse samples that had been aged over 80 days. Targets were chosen based on their existence in certain cell

lineages. It was hypothesised that inter-donor variation could be reduced by using targets confined to the granulocytic cell lineage. Unfortunately AMICA1 and MNDA performed poorly as did CASP1 which was also analysed to test the cell lineage theory. A fourth target, GAPDH, initially incorporated into the study for normalisation purposes proved to have the strongest correlation between 3'/5' ratio and sample age. These results were encouraging though stability over the initial 20 days and inter-donor variation was still an issue. Unfortunately, due to time and financial constrictions, complete optimisation was not possible for this body of work but it is recommended for future research. The focus of any future work should be on optimising the spatial distance between the 3'and 5'amplicons as well as the amplicon sizes. This could be applied to a number of potential mRNA targets. The author remains optimistic that such work will result in more conclusive findings and a stronger correlation between sample age and target ratio.

Aged blood samples proved difficult to deal with. Blood is a dynamic tissue type, in that many of its RNA species have the ability to change expression levels quickly under a range of different conditions. It was thought this dynamic nature, contributed to the poor results obtained. As a result, an RNA method described by Anderson *et al,* (2005) was used to determine whether it was possible to age another forensically important type of biological evidence, namely hair. Using a reverse transcription quantitative PCR (RT-qPCR) assay, the relative expression ratio (RER) of two different RNA species (18S and B-actin) in samples that were aged naturally over a period of three months were examined. The results suggested the age of hair samples containing follicular tags can be approximated using a second order polynomial ($Age = 3.31RER^2 - 2.85RER - 0.54$), although with limitations. No gender or age-of-donor biases were observed and lineal correlations were observed up to 60 days. After this period the results were more variable and gave unreliable estimates of time since deposition. These results were encouraging and should be the basis for future work. The possibility of applying an optimised 3'/5' assay to hair samples could result in a more accurate and precise methods for ageing forensic hair samples and therefore should also form the basis for future work.

Despite continual efforts, there is still no practical method that is applied to ageing blood stains at a crime scene. Perhaps as technology continues to advance more opportunities will become available and a robust forensic application for ageing biological samples will one day become routinely available.

CHAPTER 10: REFERENCES

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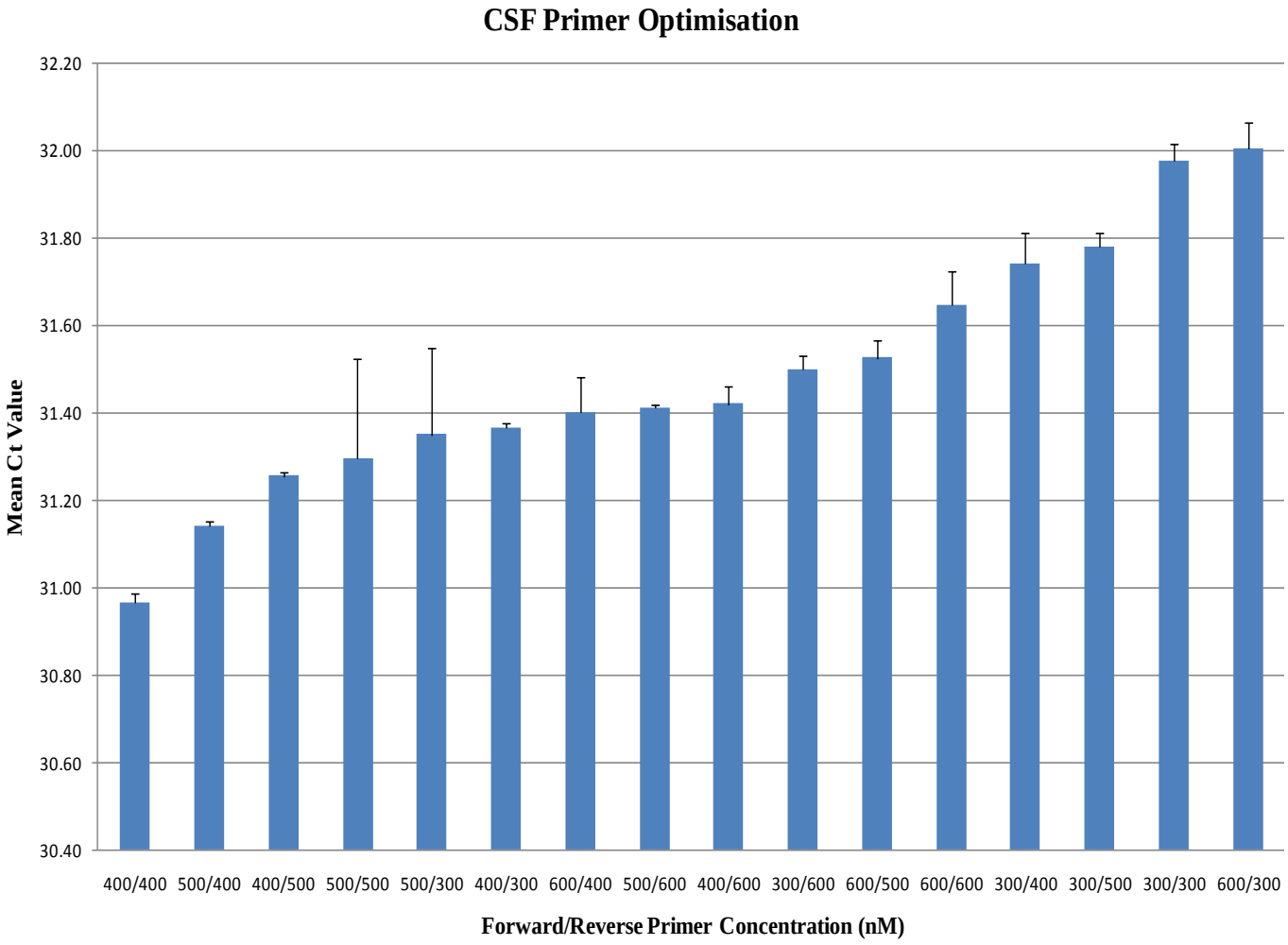
CHAPTER 11: APPENDICES

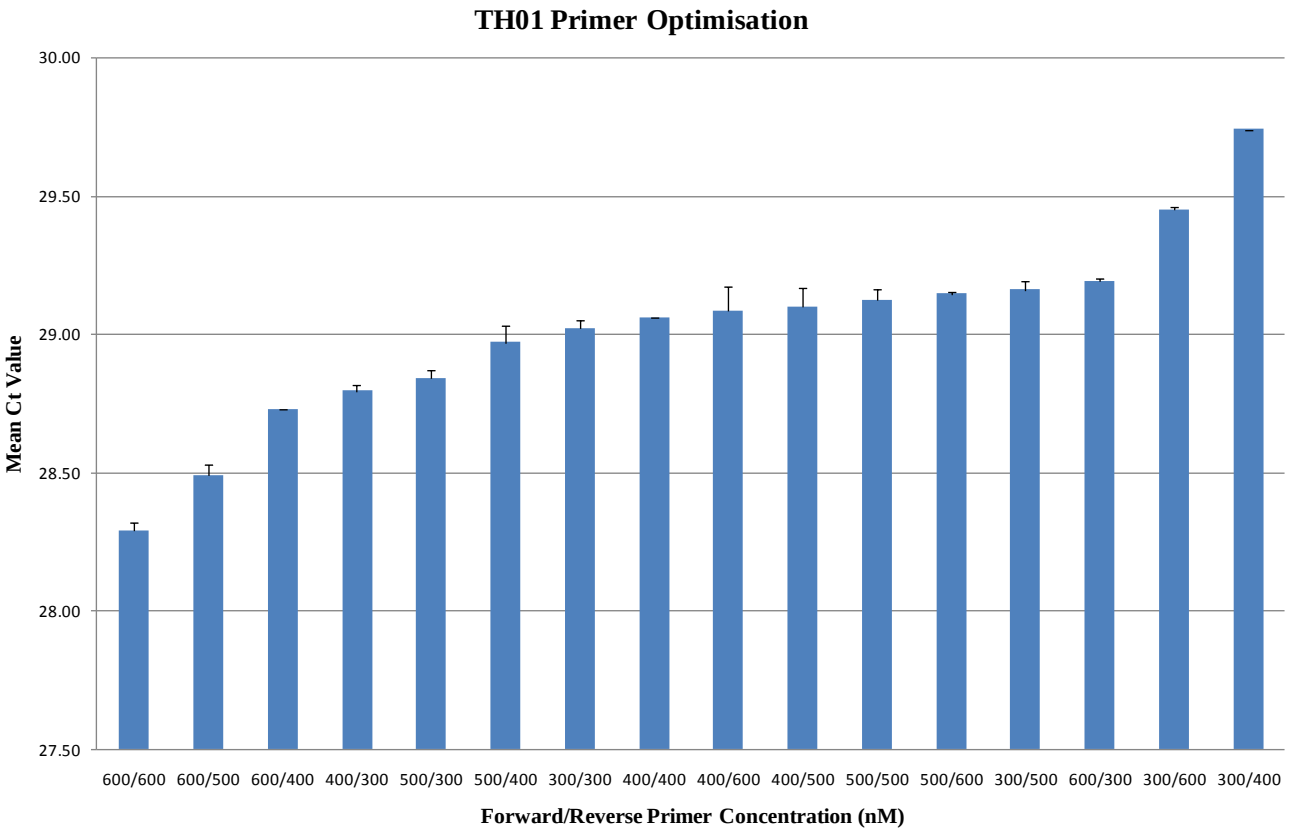
Appendix I: Chemicals, Enzymes, Kits and Reagents

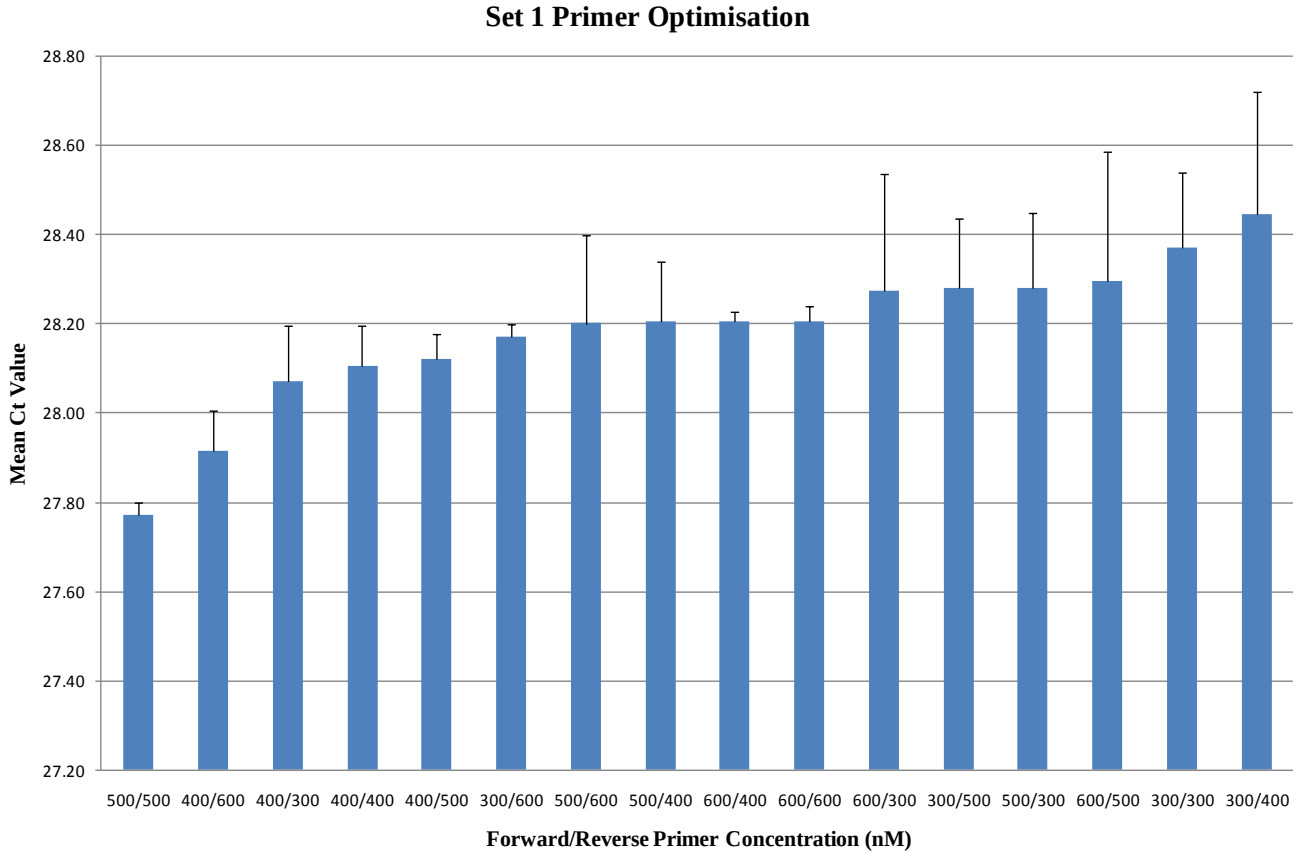
Manufacturer	Item
Invitrogen	Trizol LS Solution Proteinase K dNTP mix Superscript II Reverse Transcription Kit DNase 1 Reaction Kit • DNase 1 Reaction Buffer • DNase Amp Grade Enzyme • EDTA Random Hexamers Reverse Transcription Kit • Random Primers • dNTP mix • 5 X Strand Buffer • RT II Enzyme Oligo dT Reverse Transcription Kit • Oligo Primers • dNTP mix • 5 X Strand Buffer • RT II Enzyme
Ambion	RNAzap RNAqueous Micro
Eppendorf	PCR Grade Water
Bioline	Polyacryl carrier Control DNA
Sigma – Aldrich	DEPC Chloroform 2-propanol 100% Ethanol B-mercaptoethanol TRI Reagent BD 1-bromo-3-chloropropane 10% Sodium Dodecyl Sulphate

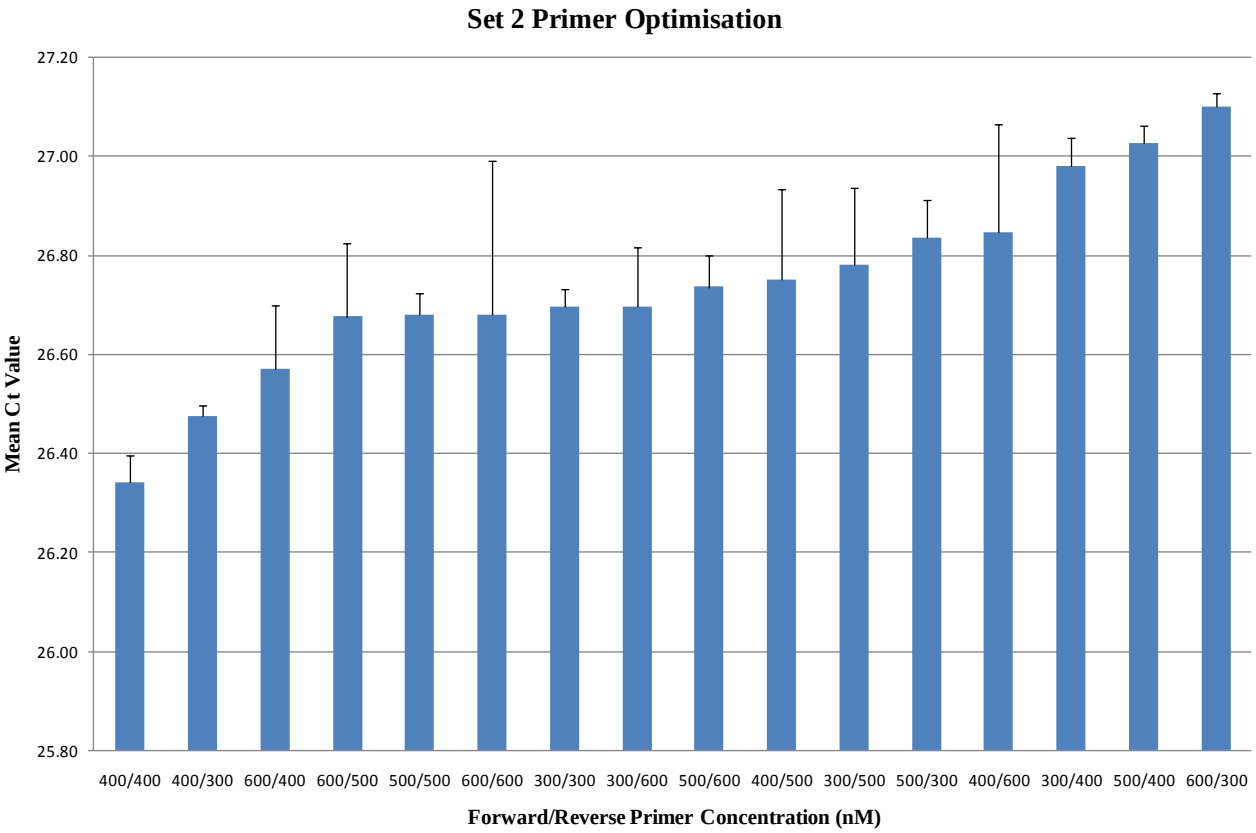
	dNTP mix Tris Boric Acid EDTA Primers and probes • CSF • TH01 • Set 1 • Set 2 • Set 3 • Set 4 • Set 5 • 3’ AMICA • 5’ AMICA • 3’ MNDA • 5’ MNDA • 3’ CASP • 5’ CASP • 3’ GAPDH • 5’ GAPDH
MWG	• TNF • IL-6
Applied Biosystems	Control RNA 2 x Universal PCR Master Mix SYBR Green PCR Master Mix Taqman B-Actin Control Reagent kit Taqman Ribosomal RNA Control Reagent Kit 18S Primers B-actin Primers
Qiagen	QIAamp RNA Blood Mini Kit

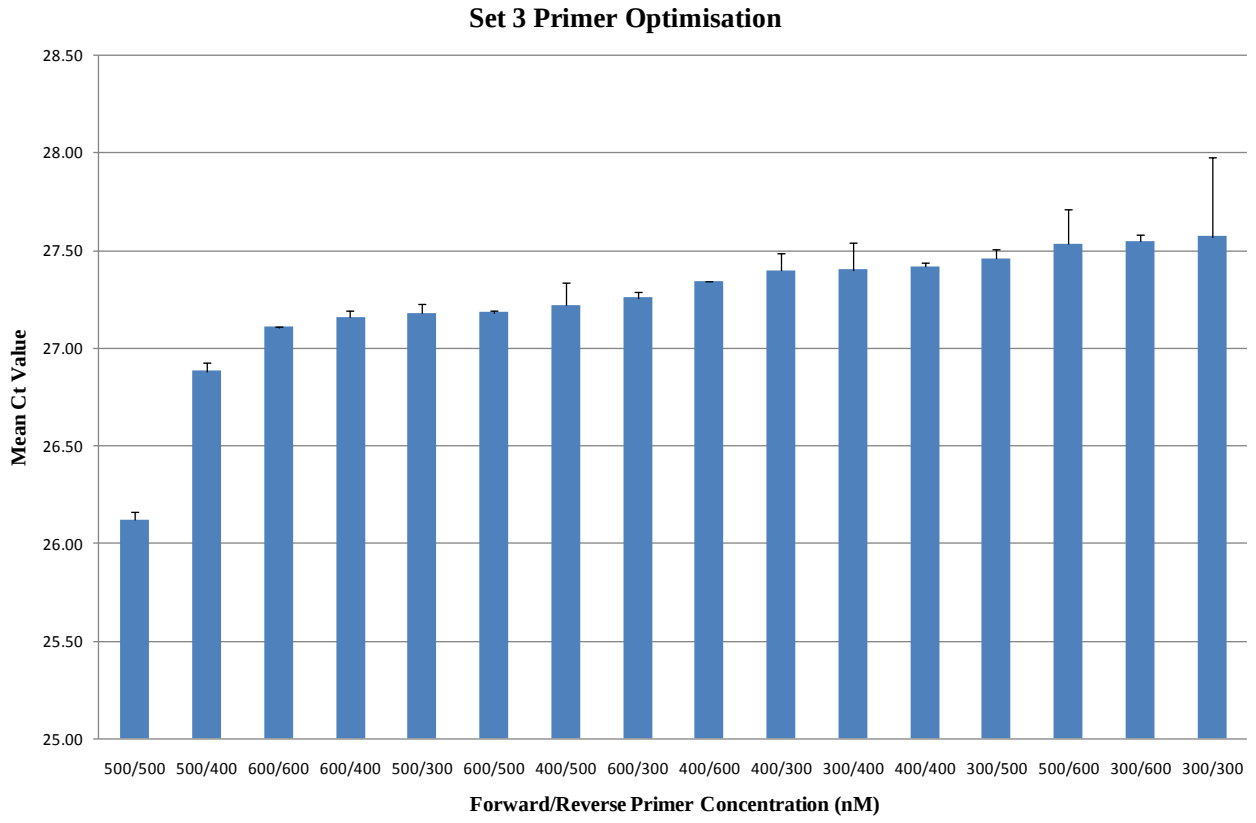
Appendix II: Primer optimisation details for CSF, TH01 and Set 1-5.

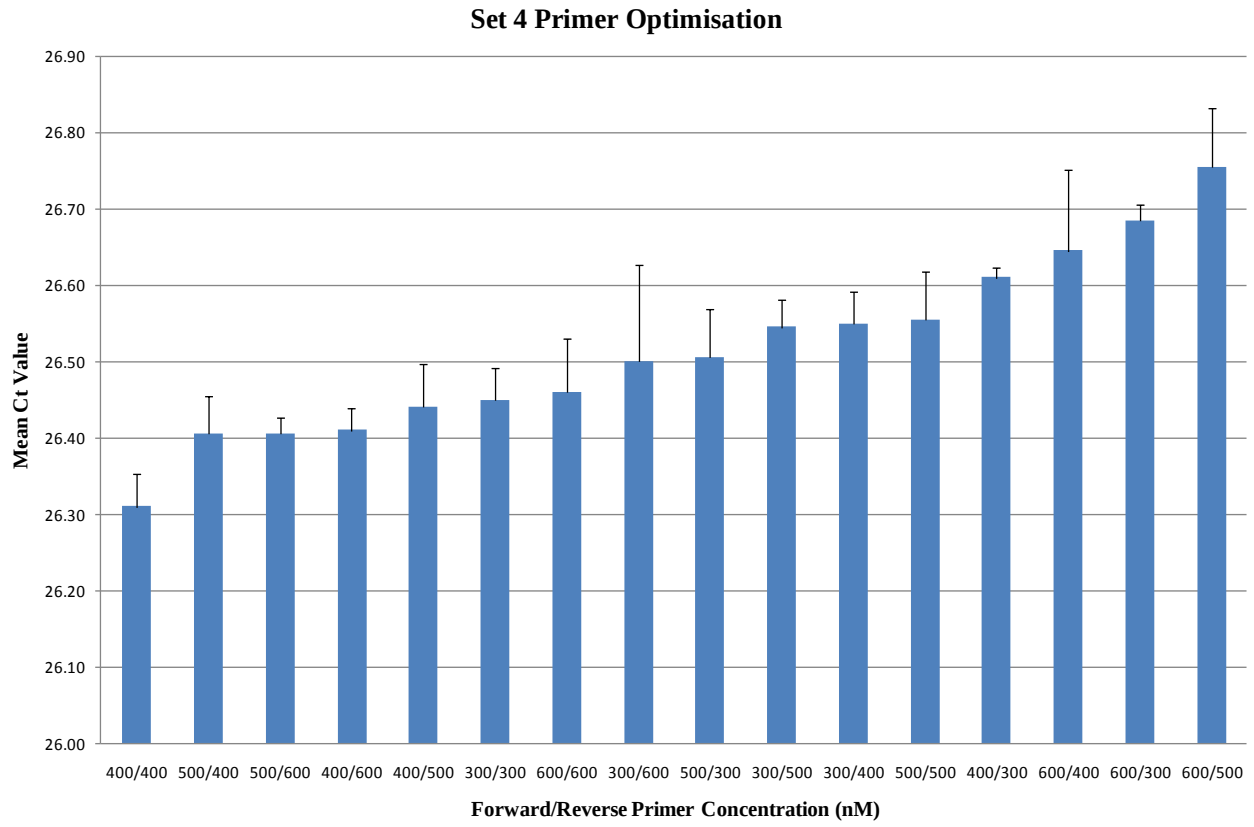


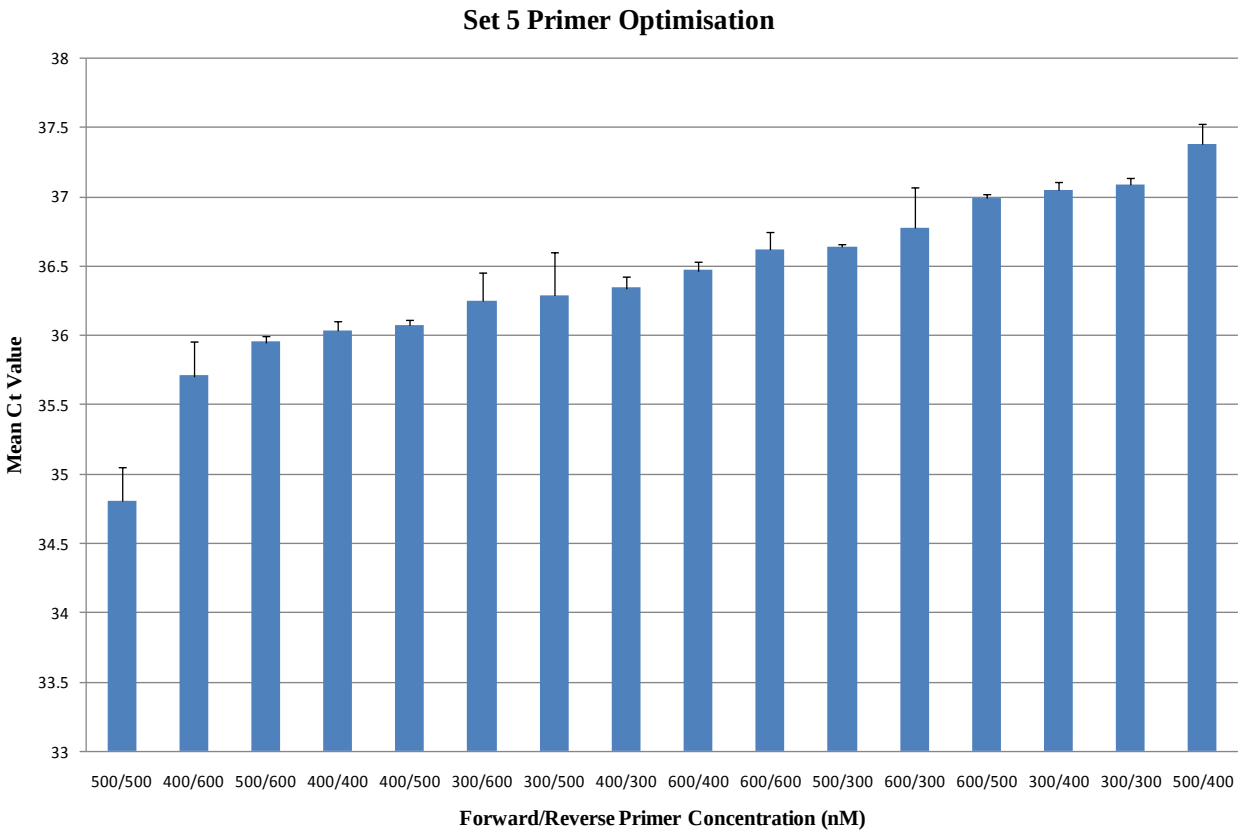

TH01 Primer Optimisation
30.00
29.50
29.00
28.50
28.00
27.50
Mean Ct Value
600/600 600/500 600/400 400/300 500/300 500/400 300/300 400/400 400/600 400/500 500/500 500/600 300/500 600/300 300/600 300/400
Forward/Reverse Primer Concentration (nM)


Set 1 Primer Optimisation
28.80
28.60
28.40
28.20
28.00
27.80
27.60
27.40
27.20
Mean Ct Value
500/500 400/600 400/300 400/400 400/500 300/600 500/600 500/400 600/400 600/600 600/300 300/500 500/300 600/500 300/300 300/400
Forward/Reverse Primer Concentration (nM)


Set 2 Primer Optimisation
Mean Ct Value
27.20
27.00
26.80
26.60
26.40
26.20
26.00
25.80
400/400 400/300 600/400 600/500 500/500 600/600 300/300 300/600 500/600 400/500 300/500 500/300 400/600 300/400 500/400 600/300
Forward/Reverse Primer Concentration (nM)


Set 3 Primer Optimisation
Mean Ct Value
28.50
28.00
27.50
27.00
26.50
26.00
25.50
25.00
500/500 500/400 600/600 600/400 500/300 600/500 400/500 600/300 400/600 400/300 300/400 400/400 300/500 500/600 300/600 300/300
Forward/Reverse Primer Concentration (nM)


Set 4 Primer Optimisation
Mean Ct Value
26.90
26.80
26.70
26.60
26.50
26.40
26.30
26.20
26.10
26.00
400/400 500/400 500/600 400/600 400/500 300/300 600/600 300/600 500/300 300/500 300/400 500/500 400/300 600/400 600/300 600/500
Forward/Reverse Primer Concentration (nM)


Set 5 Primer Optimisation
Mean Ct Value
38
37.5
37
36.5
36
35.5
35
34.5
34
33.5
33
500/500 400/600 500/600 400/400 400/500 300/600 300/500 400/300 600/400 600/600 500/300 600/300 600/500 300/400 300/300 500/400
Forward/Reverse Primer Concentration (nM)

Appendix III: Mean Ct Values from the Naturally Degraded Samples

0 Day Samples		***Amplicon Size***				
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	Mean	26.43	26.47	26.73	27.31	27.43
	Std Dev	0.44	0.21	0.43	0.54	0.54
Donor 2	Mean	25.96	26.03	26.19	26.68	27.27
	Std Dev	0.35	0.39	0.18	0.41	0.12
Donor 3	Mean	25.25	25.90	25.88	27.16	26.97
	Std Dev	0.33	0.45	0.61	0.13	0.52
Donor 4	Mean	24.88	25.64	25.19	26.46	27.75
	Std Dev	0.62	0.44	0.99	0.29	0.40
Donor 5	Mean	25.47	25.67	26.26	26.42	27.04
	Std Dev	0.46	0.30	0.05	0.50	1.03

10 Day Samples		***Set 1 (64)***	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	Mean	27.53	27.84	28.28	29.28	30.98
	Std Dev	0.46	0.36	0.28	0.28	0.76
Donor 2	Mean	27.01	27.66	28.21	29.22	30.95
	Std Dev	0.59	0.32	0.41	0.50	0.86
Donor 3	Mean	27.18	27.24	28.50	29.98	30.66
	Std Dev	0.27	0.28	0.51	0.46	0.52
Donor 4	Mean	27.25	27.91	27.79	30.32	31.09
	Std Dev	0.32	0.65	0.56	0.26	0.68
Donor 5	Mean	25.91	26.85	28.20	29.09	31.06
	Std Dev	0.24	0.30	0.81	0.78	0.83

20 Day Samples		***Amplicon Size***				
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	27.62	27.74	28.72	31.17	31.71
	Std Dev	0.43	0.45	0.24	0.44	0.85
Donor 2	Mean	27.29	27.60	27.86	30.58	32.03
	Std Dev	0.32	0.46	0.39	0.51	1.23
Donor 3	*Mean*	27.04	27.92	30.02	32.26	32.80
	Std Dev	0.73	0.27	0.29	0.02	0.78
Donor 4	*Mean*	27.10	27.86	28.19	31.72	32.53
	Std Dev	0.11	0.12	0.13	0.27	0.46
Donor 5	*Mean*	27.12	27.56	28.52	31.04	30.06
	Std Dev	0.78	0.41	0.56	0.30	0.44
30 day Samples						
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	27.76	28.70	29.22	32.07	32.97
	Std Dev	0.54	0.47	0.52	0.96	0.89
Donor 2	*Mean*	27.05	27.88	29.15	30.65	32.27
	Std Dev	0.11	0.30	0.20	0.32	0.59
Donor 3	*Mean*	27.64	28.26	29.21	32.68	33.88
	Std Dev	0.56	0.09	0.12	0.59	0.15
Donor 4	*Mean*	27.75	28.92	29.12	32.81	34.00
	Std Dev	0.51	0.66	0.22	0.23	0.05
Donor 5	*Mean*	27.35	29.02	29.42	31.81	33.87
	Std Dev	0.22	0.03	0.42	0.96	0.60
40 Day Samples						
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	28.01	28.92	31.20	33.20	33.67
	Std Dev	0.76	0.27	0.27	0.74	1.46
Donor 2	*Mean*	28.12	29.01	29.83	32.89	34.22
	Std Dev	0.83	0.57	0.46	0.41	0.55
Donor 3	*Mean*	28.22	29.09	31.46	34.10	35.19
	Std Dev	0.36	0.55	0.63	0.18	0.16
Donor 4	*Mean*	27.93	29.00	29.96	34.18	35.43
	Std Dev	0.04	0.62	0.43	0.78	0.27
Donor 5	*Mean*	28.25	28.47	30.03	33.39	34.90
	Std Dev	0.28	0.48	0.23	0.54	0.54

50 Day Samples		***Amplicon Size***				
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	28.44	29.42	32.07	34.09	34.50
	Std Dev	0.41	0.22	0.13	0.15	0.68
Donor 2	*Mean*	28.25	30.50	31.03	34.31	33.31
	Std Dev	0.33	0.19	0.13	0.45	0.42
Donor 3	*Mean*	29.08	29.88	32.39	36.21	36.71
	Std Dev	0.50	0.15	0.43	0.27	1.00
Donor 4	*Mean*	27.94	29.28	31.75	34.85	36.09
	Std Dev	0.29	0.29	0.88	0.91	0.09
Donor 5	*Mean*	28.61	29.59	31.90	34.16	35.99
	Std Dev	0.43	0.41	0.16	0.67	0.09

60 Day Samples						
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	28.87	31.55	34.53	34.34	36.84
	Std Dev	0.50	0.61	0.57	1.14	0.18
Donor 2	*Mean*	29.11	29.80	32.75	35.10	37.03
	Std Dev	0.98	0.26	1.48	0.83	0.39
Donor 3	*Mean*	30.22	31.64	35.43	35.97	38.02
	Std Dev	0.87	0.22	1.07	1.62	0.62
Donor 4	*Mean*	28.42	32.00	34.38	36.04	38.02
	Std Dev	0.56	0.58	1.18	1.38	0.81
Donor 5	*Mean*	29.10	31.69	33.94	36.15	36.88
	Std Dev	0.07	0.74	0.57	0.84	0.35

70 Day Samples						
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	***Mean***	31.16	33.68	36.22	37.21	39.71
	Std Dev	0.42	1.08	0.25	0.50	0.68
Donor 2	***Mean***	30.89	31.81	35.78	36.87	38.73
	Std Dev	0.56	0.68	0.58	0.32	0.77
Donor 3	***Mean***	30.29	33.24	36.18	38.09	39.83
	Std Dev	0.26	0.26	0.35	0.52	0.38
Donor 4	***Mean***	31.31	33.83	35.29	38.31	39.55
	Std Dev	0.33	0.58	0.56	0.11	0.90
Donor 5	***Mean***	31.97	33.86	34.29	38.25	40.02
	Std Dev	0.88	0.33	0.51	0.23	0.13

80 Day Samples		***Amplicon Size***				
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	34.28	37.33	38.21	40.41	43.18
	Std Dev	0.54	0.83	1.96	0.54	1.57
Donor 2	*Mean*	35.61	35.67	37.71	39.68	40.91
	Std Dev	0.41	0.98	0.44	0.66	0.37
Donor 3	*Mean*	33.81	36.07	39.45	41.07	42.79
	Std Dev	0.45	0.25	0.91	1.42	0.42
Donor 4	*Mean*	34.68	36.97	38.94	39.67	43.91
	Std Dev	0.81	0.24	1.25	0.56	0.61
Donor 5	*Mean*	32.80	35.57	37.70	41.83	43.83
	Std Dev	0.64	0.51	0.91	0.52	0.76

90 Day Samples						
		Set 1 (64)	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
Donor 1	*Mean*	36.15	38.32	42.01	42.04	43.83
	Std Dev	0.33	0.31	1.56	0.53	0.38
Donor 2	*Mean*	35.94	36.27	38.76	42.08	43.39
	Std Dev	0.32	0.84	0.67	1.32	1.49
Donor 3	*Mean*	35.65	38.37	41.78	43.19	44.25
	Std Dev	0.31	0.36	0.46	0.44	
Donor 4	*Mean*	34.85	37.09	39.32	42.03	44.28
	Std Dev	0.34	1.22	1.12	1.58	0.02
Donor 5	*Mean*	33.77	36.59	39.89	40.48	43.31
	Std Dev	0.66	1.06	0.98	0.47	1.45

Appendix IV: The Relative Expression Values from the Naturally Aged Blood Samples

Donor 1	Relative Expression (RE)				
Ageing Period (Days)	*Set 1 (64)*	*Set 2 (136)*	*Set 3 (198)*	*Set 4 (239)*	*Set 5 (308)*
0	1.00000	1.00000	1.00000	1.00000	1.00000
10	0.47487	0.37104	0.33680	0.24081	0.08852
20	0.44680	0.39889	0.24729	0.06144	0.05362
30	0.40639	0.19913	0.17408	0.03206	0.02266
40	0.34312	0.18257	0.04335	0.01417	0.01404
50	0.25645	0.11826	0.02354	0.00745	0.00796
60	0.19168	0.02532	0.00418	0.00622	0.00161
70	0.04067	0.00542	0.00128	0.00078	0.00023
80	0.00492	0.00039	0.00032	0.00008	0.00002
90	0.00139	0.00019	0.00002	0.00002	0.00001

Donor 2	Relative Expression (RE)				
Ageing Period (Days)	*Set 1 (64)*	*Set 2 (136)*	*Set 3 (198)*	*Set 4 (239)*	*Set 5 (308)*
0	1.00000	1.00000	1.00000	1.00000	1.00000
10	0.49011	0.30815	0.24157	0.15989	0.08063
20	0.40548	0.32027	0.30959	0.05969	0.03862
30	0.47702	0.26216	0.12544	0.05688	0.03278
40	0.23221	0.11572	0.07764	0.01124	0.00864
50	0.21169	0.03937	0.03343	0.00830	0.00298
60	0.11933	0.06533	0.00999	0.00228	0.00127
70	0.03544	0.01529	0.00119	0.00063	0.00040
80	0.00509	0.00093	0.00031	0.00009	0.00009
90	0.00116	0.00060	0.00015	0.00001	0.00002

Donor 3	Relative Expression (RE)				
Ageing Period (Days)	***Set 1 (64)***	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
0	1.00000	1.00000	1.00000	1.00000	1.00000
10	0.27073	0.37827	0.15927	0.13029	0.08008
20	0.29697	0.23237	0.05465	0.02508	0.01859
30	0.19783	0.18169	0.09652	0.01851	0.00888
40	0.13389	0.09941	0.01989	0.00663	0.00363
50	0.07480	0.05612	0.01035	0.00144	0.00128
60	0.03457	0.01582	0.00122	0.00083	0.00052
70	0.03297	0.00493	0.00072	0.00037	0.00015
80	0.00373	0.00064	0.00007	0.00004	0.00002
90	0.00088	0.00012	0.00001	0.00001	0.00001

Donor 4	Relative Expression (RE)				
Ageing Period (Days)	***Set 1 (64)***	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
0	1.00000	1.00000	1.00000	1.00000	1.00000
10	0.20098	0.19392	0.16076	0.06144	0.10172
20	0.22247	0.20009	0.12140	0.02234	0.03810
30	0.14294	0.09291	0.06334	0.01649	0.01395
40	0.12712	0.08790	0.03512	0.00378	0.00525
50	0.12597	0.07178	0.00999	0.00233	0.00334
60	0.09102	0.01003	0.00158	0.00098	0.00089
70	0.01289	0.00267	0.00083	0.00019	0.00031
80	0.00131	0.00027	0.00006	0.00007	0.00002
90	0.00117	0.00012	0.00005	0.00001	0.00001

Donor 5	Relative Expression (RE)				
Ageing Period (Days)	***Set 1 (64)***	***Set 2 (136)***	***Set 3 (198)***	***Set 4 (239)***	***Set 5 (308)***
0	1.00000	1.00000	1.00000	1.00000	1.00000
10	0.74238	0.42574	0.25673	0.14555	0.12688
20	0.32797	0.25468	0.20459	0.03548	0.03189
30	0.28068	0.08854	0.10875	0.02034	0.00938
40	0.15261	0.13151	0.07070	0.00649	0.00464
50	0.11906	0.05847	0.01902	0.00372	0.00220
60	0.08564	0.01282	0.00455	0.00088	0.00120
70	0.01227	0.00266	0.00176	0.00019	0.00014
80	0.00700	0.00077	0.00032	0.00008	0.00004
90	0.00363	0.00037	0.00007	0.00004	0.00001

Appendix V: Raw Ct values for the 18S stability assay

18S rRNA												
Ageing Period (Days)	**Donor 1**		**Donor 2**		**Donor 3**		**Donor 4**		**Donor 5**		**Donor 6**	
0	17.26	17.57	17.21	17.35	18.05	17.85	17.59	17.59	17.52	17.21	16.79	16.89
10	18.01	17.89	17.35	17.59	17.69	17.68	18.14	18.65	17.25	16.89	17.12	17.54
20	17.68	17.89	17.89	17.24	17.72	18.11	18.42	18.4	17.49	17.67	17.26	17.56
30	18.05	18.01	18.25	18.24	18.09	18.15	18.67	18.59	17.89	18.25	17.69	17.68
40	18.12	18.04	18.00	18.03	18.69	18.78	18.69	18.95	18.26	18.01	18.61	18.26
50	18.51	18.24	17.98	18.52	18.88	19.02	18.69	18.75	18.49	18.64	18.63	19.02

Appendix VI: Ct values of TNF-α, IL-6 and 18S from all donors across the ten day ageing period

Donor 1

Time	TNF a	TNF b	Mean	Std Dev	IL6 a	IL6 b	mean	Std Dev	18S a	18S b	Mean	Std Dev
0	23.25	23.58	**23.42**	0.23	25.29	25.16	**25.23**	0.09	15.80	15.73	**15.77**	0.05
1	22.14	22.30	**22.22**	0.11	23.05	23.14	**23.10**	0.06	14.93	15.04	**14.99**	0.08
2	24.49	24.48	**24.49**	0.01	24.07	24.15	**24.11**	0.06	16.20	16.18	**16.19**	0.01
3	26.06	25.44	**25.75**	0.44	25.47	25.31	**25.39**	0.11	17.88	17.48	**17.68**	0.28
4	29.25	29.46	**29.36**	0.15	28.26	28.41	**28.34**	0.11	16.78	16.99	**16.89**	0.15
5	34.21	34.02	**34.12**	0.13	33.88	32.02	**32.95**	1.32	22.13	21.19	**21.66**	0.66
6	31.33	31.64	**31.49**	0.22	27.50	27.40	**27.45**	0.07	17.23	17.26	**17.25**	0.02
7	35.80	35.98	**35.89**	0.13	33.53	36.28	**34.91**	1.94	21.62	21.13	**21.38**	0.35
8	34.58	34.91	**34.75**	0.23	33.58	33.80	**33.69**	0.16	16.05	15.56	**15.81**	0.35
9	33.52	33.17	**33.35**	0.25	33.54	33.62	**33.58**	0.06	15.94	16.09	**16.02**	0.11
10	35.77	35.95	**35.86**	0.13	36.17	37.59	**36.88**	1.00	18.03	17.99	**18.01**	0.03
Donor 2												
0	25.05	24.58	**24.82**	0.33	27.12	28.25	**27.69**	0.80	18.88	19.10	**18.99**	0.16
1	24.28	24.31	**24.30**	0.02	25.21	24.94	**25.08**	0.19	17.24	16.60	**16.92**	0.45
2	26.27	26.78	**26.53**	0.36	25.83	25.33	**25.58**	0.35	17.50	17.75	**17.63**	0.18
3	28.13	28.41	**28.27**	0.20	26.73	26.28	**26.51**	0.32	17.83	17.71	**17.77**	0.08
4	27.46	27.89	**27.68**	0.30	27.56	26.89	**27.23**	0.47	16.87	16.92	**16.90**	0.04
5	28.97	29.52	**29.25**	0.39	27.36	27.06	**27.21**	0.21	17.70	17.58	**17.64**	0.08
6	29.80	30.05	**29.93**	0.18	28.52	27.34	**27.93**	0.83	18.76	18.14	**18.45**	0.44
7	31.08	31.19	**31.14**	0.08	34.61	33.80	**34.21**	0.57	19.09	19.11	**19.10**	0.01
8	35.49	36.05	**35.77**	0.40	36.11	38.13	**37.12**	1.43	21.38	21.33	**21.36**	0.04
9	34.51	34.60	**34.56**	0.06	34.31	33.93	**34.12**	0.27	17.31	18.33	**17.82**	0.72
10	33.28	34.41	**33.85**	0.80	35.12	34.77	**34.95**	0.25	18.21	18.39	**18.30**	0.13

Donor 3

Time	TNF a	TNF b	mean	Std Dev	IL6 a	IL6 b	mean	Std Dev	18S a	18S b	Mean	Std Dev
0	23.91	24.25	**24.08**	0.24	27.92	28.32	**28.12**	0.28	18.74	17.15	**17.95**	1.12
1	21.89	22.31	**22.10**	0.30	24.02	23.94	**23.98**	0.06	15.52	16.58	**16.05**	0.75
2	28.29	26.98	**27.64**	0.93	26.40	26.34	**26.37**	0.04	17.49	17.05	**17.27**	0.31
3	28.11	27.98	**28.05**	0.09	27.96	27.24	**27.60**	0.51	17.57	17.88	**17.73**	0.22
4	29.30	28.25	**28.78**	0.74	27.68	27.00	**27.34**	0.48	16.70	16.90	**16.80**	0.14
5	28.67	29.50	**29.09**	0.59	28.14	27.96	**28.05**	0.13	16.22	16.33	**16.28**	0.08
6	31.18	30.25	**30.72**	0.66	28.16	27.32	**27.74**	0.59	17.39	18.02	**17.71**	0.45
7	31.65	32.25	**31.95**	0.42	27.68	28.66	**28.17**	0.69	15.75	16.28	**16.02**	0.37
8	31.61	32.69	**32.15**	0.76	29.31	28.91	**29.11**	0.28	14.10	14.35	**14.23**	0.18
9	32.57	32.87	**32.72**	0.21	30.66	30.74	**30.70**	0.06	15.56	16.67	**16.12**	0.78
10	34.81	34.55	**34.68**	0.18	36.22	35.10	**35.66**	0.79	21.34	21.07	**21.21**	0.19
Donor 4												
0	24.09	24.57	**24.33**	0.34	23.90	24.31	**24.11**	0.29	15.15	15.26	**15.21**	0.08
1	25.71	25.57	**25.64**	0.10	25.55	25.59	**25.57**	0.03	15.86	16.03	**15.95**	0.12
2	26.50	25.76	**26.13**	0.52	24.43	25.23	**24.83**	0.57	16.66	16.35	**16.51**	0.22
3	26.78	27.01	**26.90**	0.16	24.89	24.95	**24.92**	0.04	15.88	16.21	**16.05**	0.23
4	26.67	26.78	**26.73**	0.08	26.01	26.85	**26.43**	0.59	17.25	17.01	**17.13**	0.17
5	28.95	28.15	**28.55**	0.57	26.02	26.85	**26.44**	0.59	16.21	16.62	**16.42**	0.29
6	30.05	29.95	**30.00**	0.07	28.95	28.81	**28.88**	0.10	17.97	16.83	**17.40**	0.81
7	32.89	34.87	**33.88**	1.40	27.88	27.02	**27.45**	0.61	16.92	16.86	**16.89**	0.04
8	31.31	31.90	**31.61**	0.42	25.27	25.24	**25.26**	0.02	14.62	13.44	**14.03**	0.83
9	32.10	31.92	**32.01**	0.13	30.29	30.17	**30.23**	0.08	19.00	18.41	**18.71**	0.42
10	35.56	34.81	**35.19**	0.53	32.22	32.86	**32.54**	0.45	17.85	17.87	**17.86**	0.01

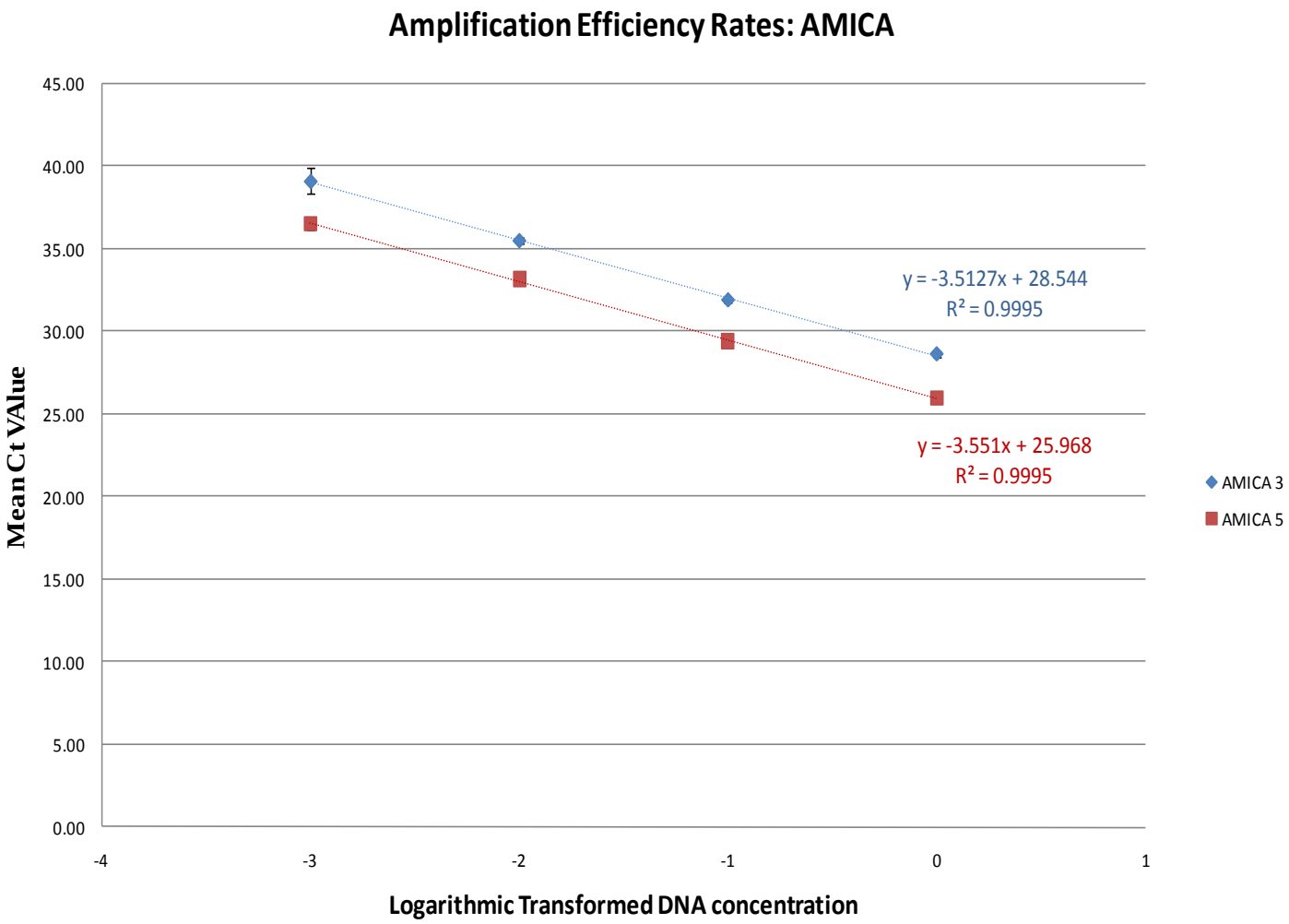
Donor 5

Time	TNF a	TNF b	mean	Std Dev	IL6 a	IL6 b	mean	Std Dev	18S a	18S b	Mean	Std Dev
0	24.63	24.11	**24.37**	0.37	23.29	24.01	**23.65**	0.51	16.25	16.08	**16.17**	0.12
1	24.93	24.97	**24.95**	0.03	24.91	25.23	**25.07**	0.23	17.86	17.32	**17.59**	0.38
2	25.53	25.51	**25.52**	0.01	25.19	25.23	**25.21**	0.03	16.82	16.82	**16.82**	0.00
3	25.89	26.57	**26.23**	0.48	27.89	26.43	**27.16**	1.03	16.99	16.87	**16.93**	0.08
4	28.97	28.12	**28.55**	0.60	27.46	26.87	**27.17**	0.42	17.12	17.19	**17.16**	0.05
5	28.99	29.60	**29.30**	0.43	29.75	28.29	**29.02**	1.03	18.17	18.05	**18.11**	0.08
6	33.68	33.82	**33.75**	0.10	34.31	33.36	**33.84**	0.67	25.25	19.61	**22.43**	3.99
7	31.26	31.95	**31.61**	0.49	30.93	30.17	**30.55**	0.54	19.08	19.07	**19.08**	0.01
8	36.69	35.98	**36.34**	0.50	36.56	37.79	**37.18**	0.87	22.27	22.29	**22.28**	0.01
9	31.00	32.87	**31.94**	1.32	32.68	32.83	**32.76**	0.11	16.42	16.57	**16.50**	0.11
10	37.54	37.00	**37.27**	0.38	35.28	34.58	**34.93**	0.49	21.34	20.25	**20.80**	0.77
Donor 6												
0	24.57	24.70	**24.64**	0.09	24.28	24.03	**24.16**	0.18	16.42	17.61	**17.02**	0.84
1	23.89	24.01	**23.95**	0.08	23.12	23.68	**23.40**	0.40	16.25	16.01	**16.13**	0.17
2	26.27	24.76	**25.52**	1.07	24.26	26.66	**25.46**	1.70	16.82	16.63	**16.73**	0.13
3	25.41	25.67	**25.54**	0.18	24.40	24.46	**24.43**	0.04	16.49	15.21	**15.85**	0.91
4	29.36	28.98	**29.17**	0.27	26.67	27.01	**26.84**	0.24	17.25	17.36	**17.31**	0.08
5	30.02	31.00	**30.51**	0.69	30.67	29.83	**30.25**	0.59	18.88	19.11	**19.00**	0.16
6	32.03	32.42	**32.23**	0.28	28.50	27.81	**28.16**	0.49	17.41	17.57	**17.49**	0.11
7	32.75	33.40	**33.08**	0.56	28.00	28.03	**28.02**	0.02	17.72	17.47	**17.60**	0.18
8	35.50	34.71	**35.11**	0.46	32.48	33.36	**32.92**	0.62	17.17	17.23	**17.20**	0.04
9	33.86	34.06	**33.96**	0.14	33.25	33.96	**33.61**	0.50	18.59	18.26	**18.43**	0.23
10	36.36	36.28	**36.32**	0.06	35.68	35.81	**35.75**	0.09	20.12	20.51	**20.32**	0.28

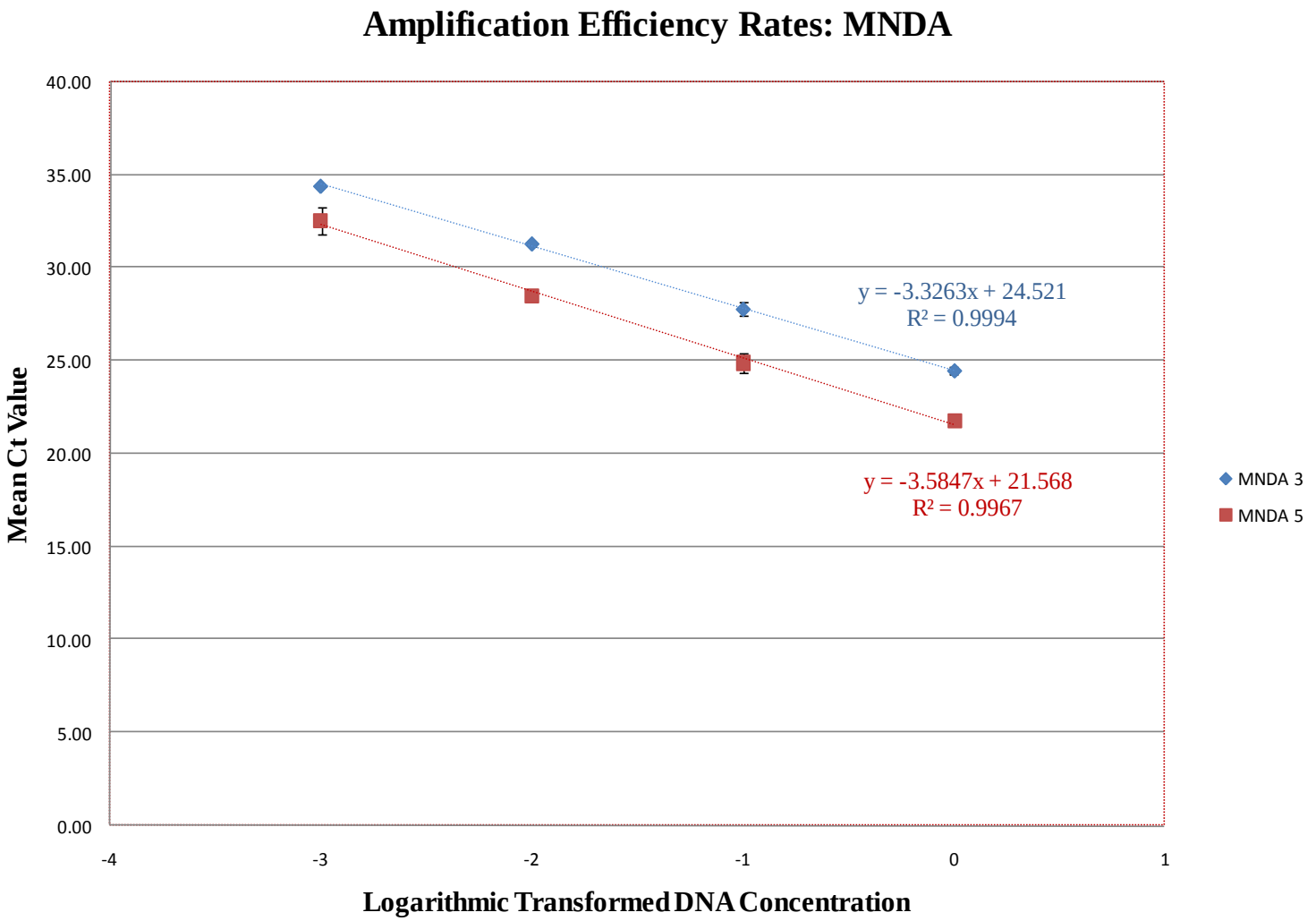
Donor 7

Time	TNF a	TNF b	mean	Std Dev	IL6 a	IL6 b	mean	Std Dev	18S a	18S b	Mean	Std Dev
0	24.37	23.95	**24.16**	0.30	25.01	25.59	**25.30**	0.41	17.45	17.19	**17.32**	0.18
1	24.08	24.07	**24.08**	0.01	24.69	24.69	**24.69**	0.00	16.45	16.61	**16.53**	0.11
2	25.76	25.98	**25.87**	0.16	25.32	25.44	**25.38**	0.08	16.88	17.02	**16.95**	0.10
3	28.89	28.53	**28.71**	0.25	25.55	25.79	**25.67**	0.17	16.18	16.08	**16.13**	0.07
4	30.49	29.89	**30.19**	0.42	25.69	26.94	**26.32**	0.88	16.68	16.79	**16.74**	0.08
5	33.68	33.89	**33.79**	0.15	30.11	29.56	**29.84**	0.39	20.52	20.07	**20.30**	0.32
6	32.26	32.35	**32.31**	0.06	31.25	31.68	**31.47**	0.30	17.34	17.18	**17.26**	0.11
7	29.84	30.20	**30.02**	0.25	30.86	30.77	**30.82**	0.06	16.22	16.28	**16.25**	0.04
8	32.19	30.82	**31.51**	0.97	32.49	31.58	**32.04**	0.64	17.24	17.39	**17.32**	0.11
9	33.75	33.15	**33.45**	0.42	34.19	34.52	**34.36**	0.23	19.73	20.43	**20.08**	0.49
10	35.95	37.07	**36.51**	0.79	37.41	37.11	**37.26**	0.21	21.72	21.37	**21.55**	0.25
Donor 8												
0	24.02	24.00	**24.01**	0.01	26.14	26.37	**26.26**	0.16	17.48	17.56	**17.52**	0.06
1	23.88	23.13	**23.51**	0.53	24.49	24.69	**24.59**	0.14	16.16	16.43	**16.30**	0.19
2	23.65	23.78	**23.72**	0.09	24.17	24.14	**24.16**	0.02	16.19	16.12	**16.16**	0.05
3	25.67	25.96	**25.82**	0.21	24.82	24.89	**24.86**	0.05	16.69	16.24	**16.47**	0.32
4	27.88	27.59	**27.74**	0.21	25.49	26.48	**25.99**	0.70	16.94	16.28	**16.61**	0.47
5	28.89	29.64	**29.27**	0.53	28.28	28.37	**28.33**	0.06	17.91	17.86	**17.89**	0.04
6	31.77	31.83	**31.80**	0.04	33.79	34.04	**33.92**	0.18	19.87	18.66	**19.27**	0.86
7	33.20	33.96	**33.58**	0.54	36.33	36.12	**36.23**	0.15	18.81	18.74	**18.78**	0.05
8	31.98	31.48	**31.73**	0.35	34.91	35.37	**35.14**	0.33	17.60	17.98	**17.79**	0.27
9	31.78	31.00	**31.39**	0.55	33.47	33.51	**33.49**	0.03	16.57	17.06	**16.82**	0.35
10	35.81	34.80	**35.31**	0.71	38.14	37.75	**37.95**	0.28	20.59	21.14	**20.87**	0.39

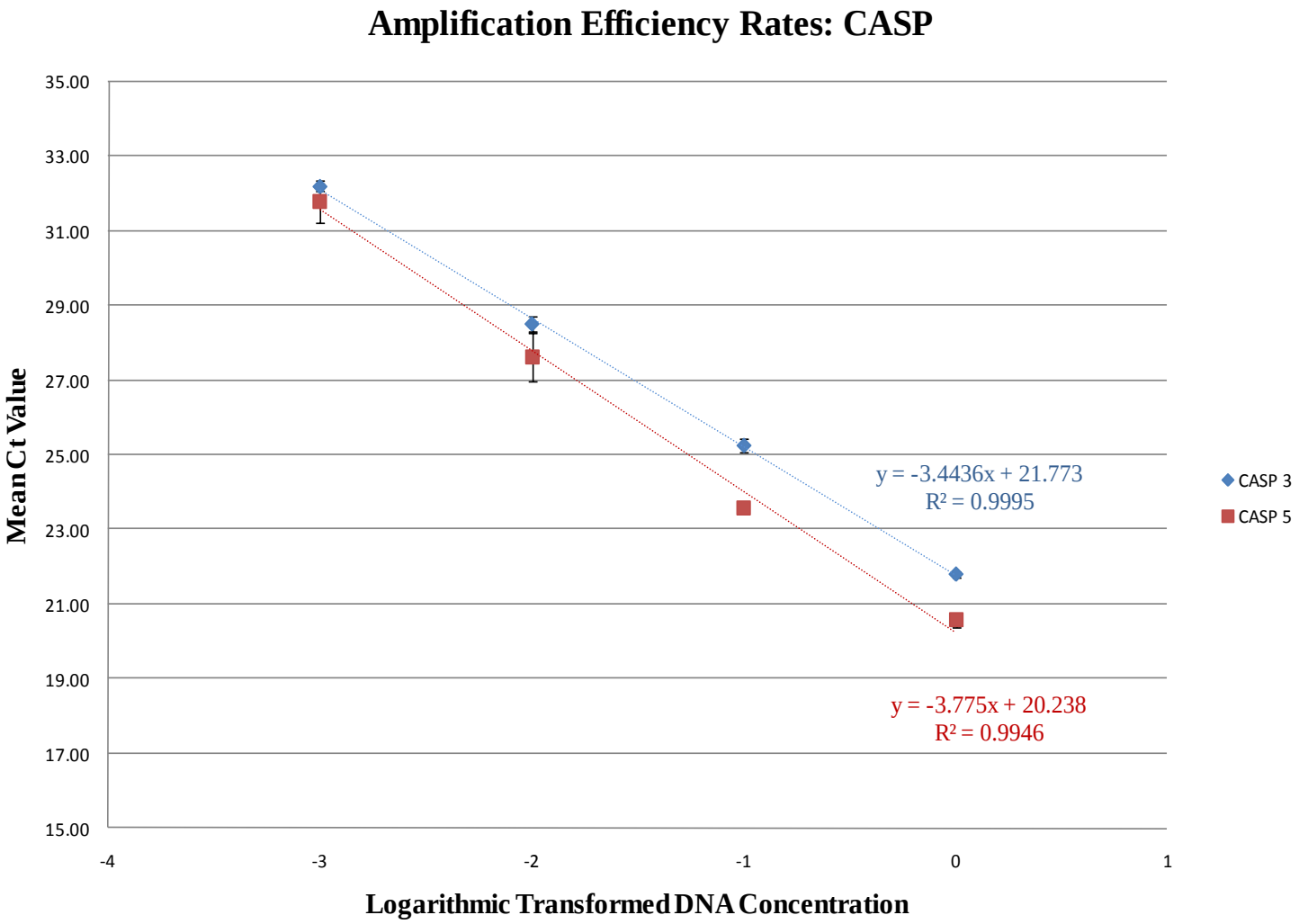
Appendix VII: Amplification Efficiency Data for AMICA, MNDA, CASP1 and GAPDH



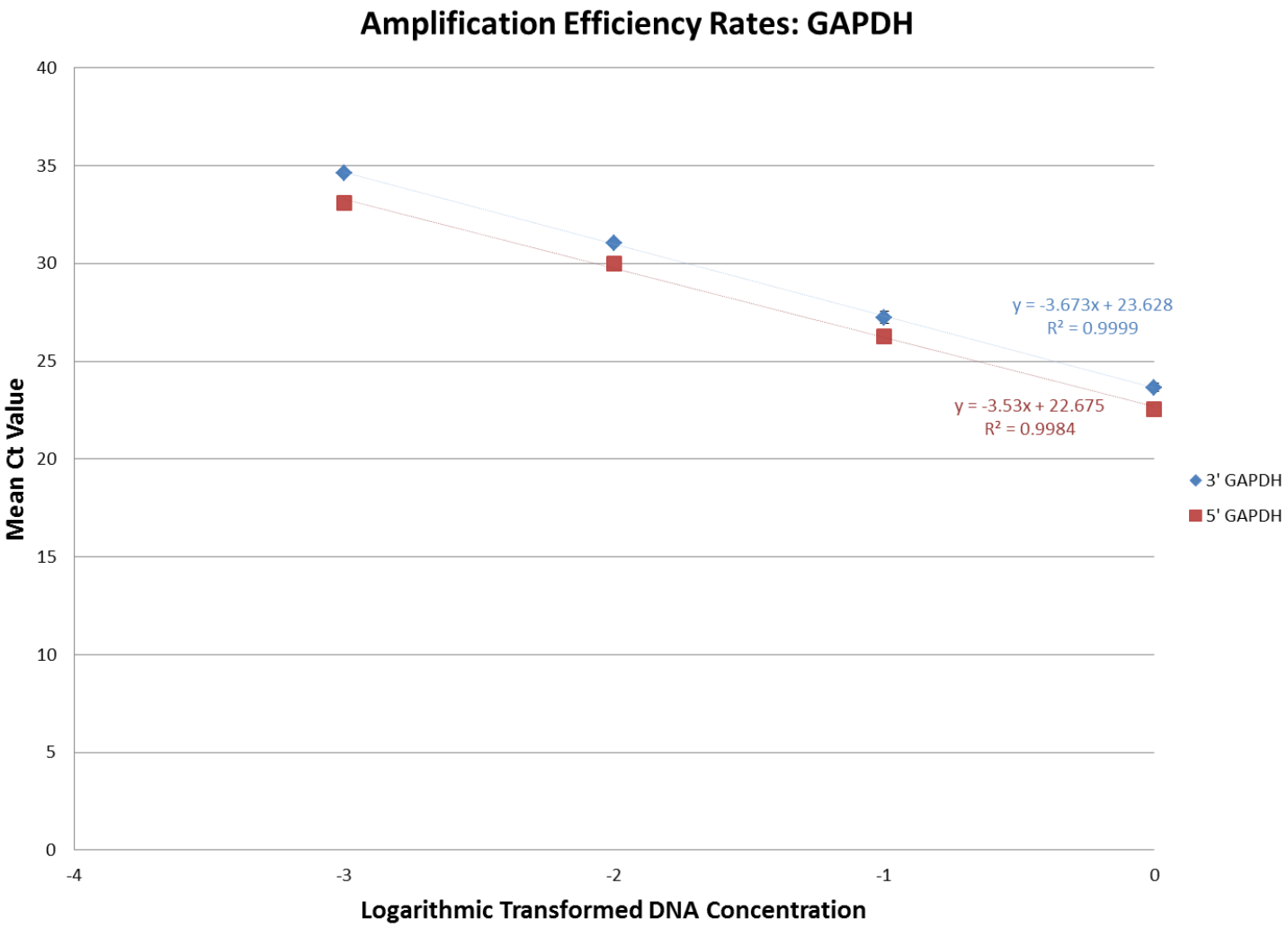
Amplification Efficiency rate for AMICA was determined using a 10 fold serial dilution assay. The data generally presented with high levels of reproducibility with the only concern being the 3' target at a concentration of 1/1000. The variation observed between runs in this case was greater than 1 cycle.



Amplification Rate of MNDA. Using the method previously described the rate for each target was determined. The variation in this data set was good with the only variation outside the acceptable range being observed with 5' target at a dilution of 1/1000.



Amplification Efficiency rates. Using the standard serial dilution method the amplification rate of CASP was calculated. The 3' target performed as sample variation was minor however the 5' target did not behave as well. The variation observed in the 1/100 and 1/1000 samples were greater than 1 cycle which is not ideal.



Using the data from a 10 fold serial dilution assay, the amplification efficiency rate of GAPDH was determined. The data presented with a high level of reproducibility and all points fell within the acceptable variation range.

Appendix VIII: Mean Ct values, Relative Quantities and 3'/5' Ratios for AMICA1

DONOR 1

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	24.33	26.90	1.189E-07	2.666E-08	**4.46**
10	37.70	39.50	1.855E-11	7.545E-12	**2.45**
20	38.09	40.69	1.436E-11	3.48E-12	**4.12**
30	38.24	40.73	1.302E-11	3.391E-12	**3.84**
40	n/a	n/a	n/a	n/a	**n/a**
50	38.99	42.69	7.966E-12	9.536E-13	**8.35**
60	40.34	n/a	3.288E-12	n/a	**n/a**
70	n/a	n/a	n/a	n/a	**n/a**
80	n/a	n/a	n/a	n/a	**n/a**

DONOR 2

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	24.09	26.89	1.391E-07	2.683E-08	**5.18**
10	37.15	41.07	2.666E-11	2.720E-12	**9.80**
20	38.34	42.91	1.222E-11	8.277E-13	**14.76**
30	39.67	42.93	5.090E-12	8.171E-13	**6.23**
40	38.69	42.92	9.697E-12	8.180E-13	**11.85**
50	38.22	40.32	1.319E-11	4.415E-12	**2.98**
60	40.27	n/a	3.450E-12	n/a	**n/a**
70	44.13	n/a	2.748E-13	n/a	**n/a**
80	n/a	n/a	n/a	n/a	**n/a**

Donor 3

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	23.95	26.72	1.522E-07	2.983E-08	**5.10**
10	37.50	39.02	2.120E-11	1.028E-11	**2.06**
20	39.15	41.28	7.173E-12	2.374E-12	**3.02**
30	n/a	n/a	n/a	n/a	**n/a**
40	39.90	41.03	4.397E-12	2.792E-12	**1.57**
50	39.77	41.54	4.777E-12	2.006E-12	**2.38**
60	42.57	n/a	7.607E-13	n/a	**n/a**
70	n/a	n/a	n/a	n/a	**n/a**
80	n/a	n/a	n/a	n/a	**n/a**

Donor 4

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	25.66	28.48	4.962E-08	9.570E-09	**5.18**
10	37.64	39.42	1.929E-11	7.913E-12	**2.43**
20	38.06	41.27	1.468E-11	2.384E-12	**6.16**
30	38.85	41.90	8.712E-12	1.585E-12	**5.49**
40	38.76	40.75	9.242E-12	3.348E-12	**2.76**
50	41.22	n/a	1.851E-12	n/a	**n/a**
60	44.05	n/a	2.899E-13	n/a	**n/a**
70	n/a	n/a	n/a	n/a	**n/a**
80	n/a	n/a	n/a	n/a	**n/a**

Donor 5

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	24.02	26.66	1.450E-07	3.115E-08	**4.65**
10	37.57	39.53	2.024E-11	7.400E-12	**2.73**
20	39.29	41.07	6.558E-12	2.726E-12	**2.40**
30	39.25	41.35	6.718E-12	2.264E-12	**2.96**
40	41.85	44.45	1.219E-12	3.04E-13	**4.01**
50	41.85	n/a	1.219E-12	n/a	**n/a**
60	n/a	n/a	n/a	n/a	**n/a**
70	n/a	n/a	n/a	n/a	**n/a**
80	n/a	n/a	n/a	n/a	**n/a**

Appendix IX: Mean Ct values, Relative Quantities and 3'/5' Ratios for MNDA

Donor 1

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	21.31	21.21	1.12E-06	4.07896E-07	**2.74**
10	22.84	24.62	4.18E-07	3.84417E-08	**10.88**
20	24.45	26.73	1.48E-07	8.89552E-09	**16.65**
30	28.47	30.21	1.12E-08	7.95851E-10	**14.07**
40	30.14	34.58	3.82E-09	3.84045E-11	**99.58**
50	32.44	37.22	8.71E-10	6.15304E-12	**141.58**
60	33.66	39.63	3.97E-10	1.15634E-12	**343.75**
70	35.19	43.38	1.48E-10	8.54872E-14	**1732.46**
80	35.97	n/a	8.97E-11	n/a	**n/a**

Donor 2

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	19.99	20.16	2.62E-06	8.4794E-07	**3.09**
10	22.95	25.67	3.89E-07	1.85564E-08	**20.94**
20	24.79	29.40	1.19E-07	1.39586E-09	**85.53**
30	26.63	35.98	3.66E-08	1.45424E-11	**2513.88**
40	28.55	35.95	1.06E-08	1.47968E-11	**716.33**
50	30.51	39.92	3E-09	9.42359E-13	**3188.51**
60	34.50	44.25	2.32E-10	4.67537E-14	**4953.13**
70	36.79	n/a	5.29E-11	n/a	**n/a**
80	38.54	n/a	1.72E-11	n/a	**n/a**

Donor 3

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	19.93	20.07	2.72E-06	9.02562E-07	**3.01**
10	22.33	25.13	5.79E-07	2.68943E-08	**21.53**
20	25.34	29.28	8.38E-08	1.51177E-09	**55.44**
30	27.00	32.64	2.88E-08	1.46992E-10	**196.04**
40	29.11	35.45	7.39E-09	2.0931E-11	**353.24**
50	31.40	38.30	1.7E-09	2.90899E-12	**582.72**
60	33.61	41.60	4.1E-10	2.93847E-13	**1396.93**
70	35.24	43.82	1.43E-10	6.32206E-14	**2268.51**
80	36.54	n/a	6.22E-11	n/a	**n/a**

Donor 4

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	20.35	20.39	2.08E-06	7.229E-07	**2.87**
10	23.73	28.65	2.36E-07	2.34843E-09	**100.52**
20	25.10	33.68	9.75E-08	7.14493E-11	**1364.43**
30	27.64	37.01	1.91E-08	7.11789E-12	**2682.32**
40	29.30	38.40	6.56E-09	2.70465E-12	**2427.01**
50	31.23	40.16	1.89E-09	7.97842E-13	**2370.14**
60	35.20	44.29	1.48E-10	4.54743E-14	**3246.40**
70	36.41	n/a	6.78E-11	n/a	**n/a**
80	36.52	n/a	6.3E-11	n/a	**n/a**

Donor 5

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	22.73	23.18	4.49E-07	1.04376E-07	4.30
10	22.95	28.46	3.9E-07	2.66998E-09	146.01
20	24.91	32.51	1.11E-07	1.60863E-10	687.01
30	27.41	35.77	2.21E-08	1.67645E-11	1320.43
40	29.53	38.69	5.66E-09	2.21951E-12	2550.84
50	33.56	42.39	4.24E-10	1.70468E-13	2486.67
60	35.35	44.95	1.34E-10	2.87702E-14	4659.36
70	38.65	44.29	1.6E-11	4.54743E-14	351.83
80	40.14	n/a	6.14E-12	n/a	n/a

Appendix X: Mean Ct values, Relative Quantities and 3'/5' Ratios for CASP1

Donor 1

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	22.70	22.49	9.53E-07	2.90139E-07	**3.28**
10	34.11	25.45	8.99E-10	4.01465E-08	**0.02**
20	34.44	36.37	7.33E-10	2.68795E-11	**27.26**
30	33.95	41.19	9.92E-10	1.06744E-12	**928.96**
40	34.33	44.52	7.84E-10	1.14532E-13	**6843.84**
50	36.65	n/a	1.9E-10	n/a	**n/a**
60	36.49	n/a	2.1E-10	n/a	**n/a**
70	36.88	n/a	1.65E-10	n/a	**n/a**
80	37.13	n/a	1.42E-10	n/a	**n/a**

Donor 2

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	22.25	22.16	1.25E-06	3.63065E-07	**3.45**
10	33.29	28.95	1.48E-09	3.84427E-09	**0.38**
20	34.18	35.73	8.59E-10	4.11153E-11	**20.89**
30	35.86	40.05	3.08E-10	2.28939E-12	**134.48**
40	36.27	44.21	2.4E-10	1.40941E-13	**1705.86**
50	36.98	n/a	1.55E-10	n/a	**n/a**
60	35.71	n/a	3.37E-10	n/a	**n/a**
70	36.69	n/a	1.85E-10	n/a	**n/a**
80	37.24	n/a	1.33E-10	n/a	**n/a**

Donor 3

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	21.99	22.30	1.47E-06	3.3059E-07	**4.44**
10	35.54	24.85	3.75E-10	5.97863E-08	**0.01**
20	33.37	36.56	1.41E-09	2.35906E-11	**59.90**
30	34.83	43.75	5.78E-10	1.92399E-13	**3001.93**
40	36.71	n/a	1.83E-10	n/a	**n/a**
50	37.03	n/a	1.51E-10	n/a	**n/a**
60	35.39	n/a	4.12E-10	n/a	**n/a**
70	36.89	n/a	1.64E-10	n/a	**n/a**
80	37.59	n/a	1.07E-10	n/a	**n/a**

Donor 4

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	22.24	22.15	1.27E-06	3.64282E-07	**3.47**
10	33.09	25.19	1.68E-09	4.76179E-08	**0.03**
20	33.20	35.44	1.56E-09	4.9923E-11	**31.31**
30	33.02	n/a	1.74E-09	n/a	**n/a**
40	35.24	n/a	4.5E-10	n/a	**n/a**
50	36.82	n/a	1.72E-10	n/a	**n/a**
60	34.91	n/a	5.5E-10	n/a	**n/a**
70	36.82	n/a	1.72E-10	n/a	**n/a**
80	36.50	n/a	2.09E-10	n/a	**n/a**

Donor 5

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	23.33	22.64	6.5E-07	2.63304E-07	**2.47**
10	33.45	27.33	1.35E-09	1.13688E-08	**0.12**
20	33.65	37.97	1.19E-09	9.21157E-12	**129.29**
30	35.81	n/a	3.18E-10	n/a	**n/a**
40	36.48	n/a	2.11E-10	n/a	**n/a**
50	37.15	n/a	1.4E-10	n/a	**n/a**
60	35.43	n/a	4E-10	n/a	**n/a**
70	36.94	n/a	1.59E-10	n/a	**n/a**
80	37.58	n/a	1.08E-10	n/a	**n/a**

Appendix XI: Mean Ct values, Relative Quantities and 3'/5' Ratios for GAPDH

Donor 1

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	21.12	21.04	1.04E-06	1.85623E-06	**0.56**
10	26.72	26.47	2.7E-08	6.13432E-08	**0.44**
20	31.31	31.61	1.35E-09	2.44705E-09	**0.55**
30	32.59	35.13	5.88E-10	2.68877E-10	**2.19**
40	33.48	35.89	3.29E-10	1.66908E-10	**1.97**
50	33.80	36.73	2.66E-10	9.82297E-11	**2.71**
60	34.08	36.00	2.22E-10	1.5529E-10	**1.43**
70	32.70	36.90	5.45E-10	8.82923E-11	**6.18**
80	33.75	38.41	2.75E-10	3.43446E-11	**8.01**

Donor 2

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	19.68	19.56	2.66E-06	4.69769E-06	**0.57**
10	26.63	27.57	2.87E-08	3.08613E-08	**0.93**
20	31.06	31.78	1.6E-09	2.19949E-09	**0.73**
30	34.33	35.46	1.88E-10	2.18595E-10	**0.86**
40	33.53	35.01	3.18E-10	2.89901E-10	**1.10**
50	33.24	36.81	3.84E-10	9.34211E-11	**4.11**
60	34.77	36.60	1.42E-10	1.06577E-10	**1.33**
70	32.55	37.16	6.04E-10	7.50035E-11	**8.05**
80	33.04	37.02	4.37E-10	8.18892E-11	**5.34**

Donor 3

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3'/5' Ratio
	3'	5'	3'	5'	
0	19.47	19.02	3.06E-06	6.57135E-06	**0.47**
10	26.54	26.70	3.04E-08	5.32674E-08	**0.57**
20	30.22	30.09	2.76E-09	6.35029E-09	**0.43**
30	33.59	34.39	3.06E-10	4.27741E-10	**0.72**
40	36.41	36.68	4.85E-11	1.01678E-10	**0.48**
50	33.80	36.43	2.66E-10	1.18572E-10	**2.24**
60	34.70	36.16	1.48E-10	1.40459E-10	**1.05**
70	32.55	36.15	6.02E-10	1.41343E-10	**4.26**
80	32.25	36.79	7.34E-10	9.46007E-11	**7.76**

Donor 4

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	19.61	19.47	2.79E-06	4.97057E-06	**0.56**
10	24.54	24.61	1.12E-07	1.9766E-07	**0.57**
20	30.56	30.21	2.21E-09	5.87131E-09	**0.38**
30	33.13	35.75	4.12E-10	1.82232E-10	**2.26**
40	33.89	37.42	2.51E-10	6.37147E-11	**3.94**
50	33.34	37.18	3.59E-10	7.40682E-11	**4.85**
60	32.32	36.89	6.99E-10	8.8848E-11	**7.87**
70	35.41	35.64	9.31E-11	1.95252E-10	**0.48**
80	34.06	36.34	2.25E-10	1.2546E-10	**1.79**

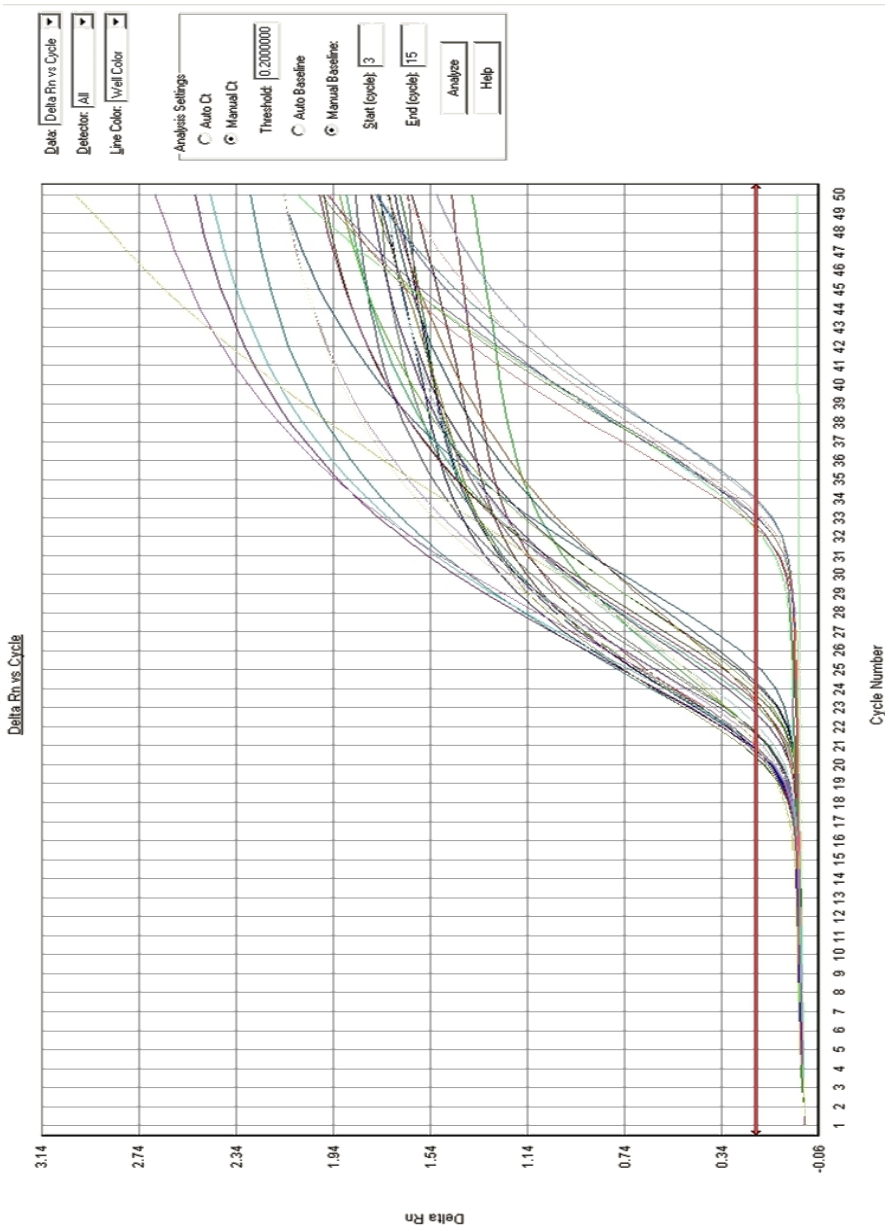
Donor 5

Ageing Time (Days)	Mean Ct Values		Relative Quantities		3’/5’ Ratio
	3'	5'	3'	5'	
0	20.17	19.88	1.94E-06	3.83117E-06	**0.51**
10	25.32	25.11	6.74E-08	1.43987E-07	**0.47**
20	30.73	29.65	1.97E-09	8.34291E-09	**0.24**
30	35.16	35.20	1.1E-10	2.57325E-10	**0.43**
40	33.69	36.45	2.87E-10	1.17462E-10	**2.44**
50	31.98	35.19	8.75E-10	2.58133E-10	**3.39**
60	32.37	35.72	6.79E-10	1.85112E-10	**3.67**
70	32.59	36.34	5.88E-10	1.25854E-10	**4.67**
80	33.49	37.53	3.26E-10	5.96527E-11	**5.46**

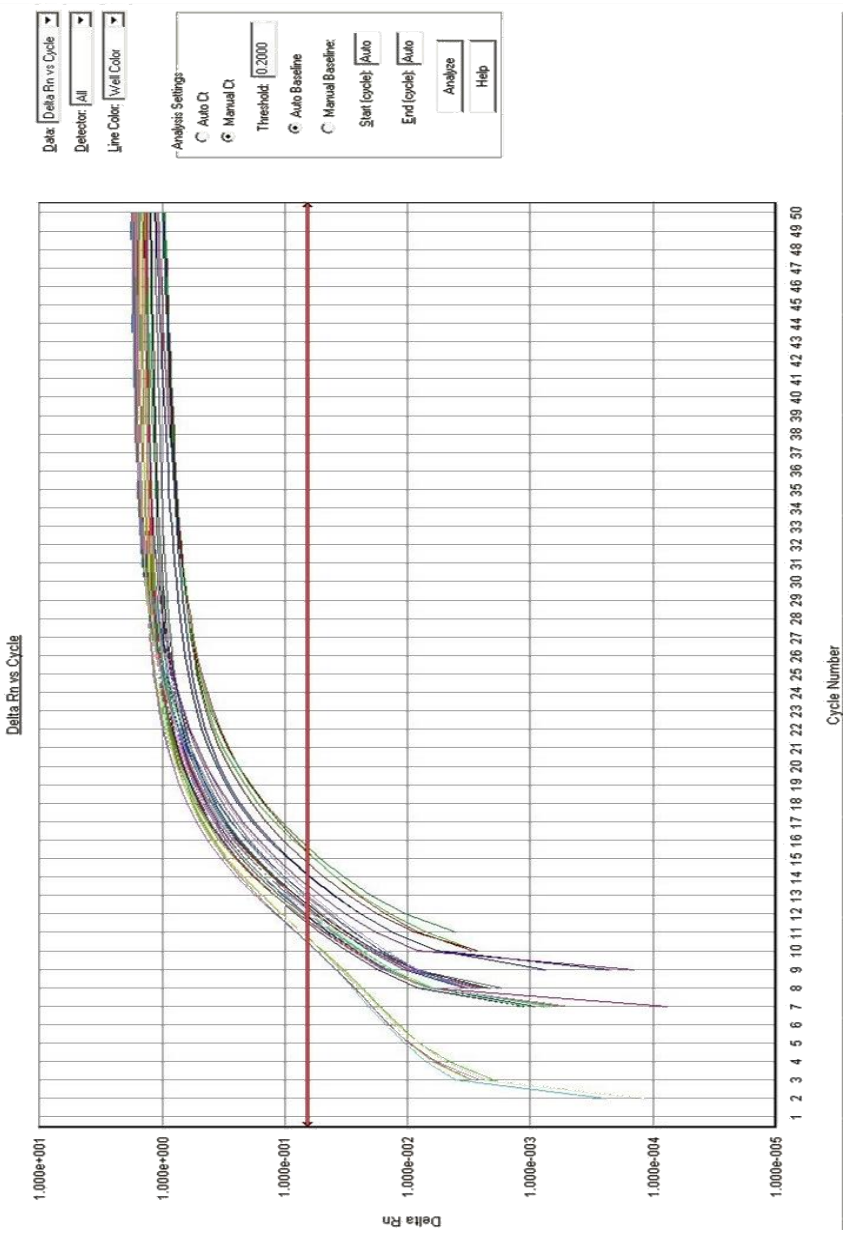
Appendix XII: Quantification of Total RNA for All Hair samples (all results are in ng)

Sample	0 Day	1 Day	5 Days	15 Days	30 Days	45 Days	60 Days	75 Days	90 Days
1 A	122.4	138.5	132.3	128.8	104.3	68.5	80.4	38.4	40.3
1 B	107.5	137.9	121.3	137.6	112.4	43.4	68.8	53.2	27.4
2 A	138.7	124.2	141.4	104.3	132.1	79.4	68.3	38.5	47.7
2 B	135.6	127.9	113.9	126.5	102.5	94.4	69.1	59.0	12.8
3 A	109.4	128.9	154.4	107.3	93.4	83.0	57.7	43.3	29.3
3 B	129.0	105.4	123.6	103.2	101.3	73.6	58.3	28.4	38.3
4 A	114.3	135.6	122.4	103.6	84.3	74.3	70.0	38.4	9.83
4 B	118.0	109.0	128.6	114.3	94.0	79.0	81.7	49.0	12.3
5 A	127.0	115.2	127.3	118.6	103.6	73.3	12.4	56.3	37.3
5 B	107.0	125.6	138.2	127.3	99.3	69.4	23.6	38.0	28.4
6 A	124.8	132.2	127.3	106.4	81.9	66.5	54.3	28.4	24.8
6 B	122.2	111.2	128.3	121.0	106.4	96.3	37.5	40.5	27.4
7 A	157.4	124.2	136.2	128.3	116.3	70.0	23.5	57.3	14.3
7 B	162.4	135.1	127.5	121.9	95.8	79.3	12.0	68.3	18.3
8 A	128.9	138.9	126.0	106.3	93.6	85.3	58.9	71.3	42.3
8 B	125.1	105.1	125.3	127.3	104.4	80.2	36.6	70.3	21.3
9 A	115.9	136.3	123.6	125.3	81.5	65.6	68.3	30.3	15.4
9 B	100.7	128.8	108.3	NR	83.4	60.6	46.4	48.0	7.3
10 A	108.4	120.6	126.3	115.0	97.7	70.1	56.3	69.0	20.0
10 B	111.7	121.0	103.9	111.0	103.0	97.0	56.0	58.0	23.4
Control 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix XIII: Real Time Amplification Curve: B-Actin and 18S Primer Matrix Optimisation



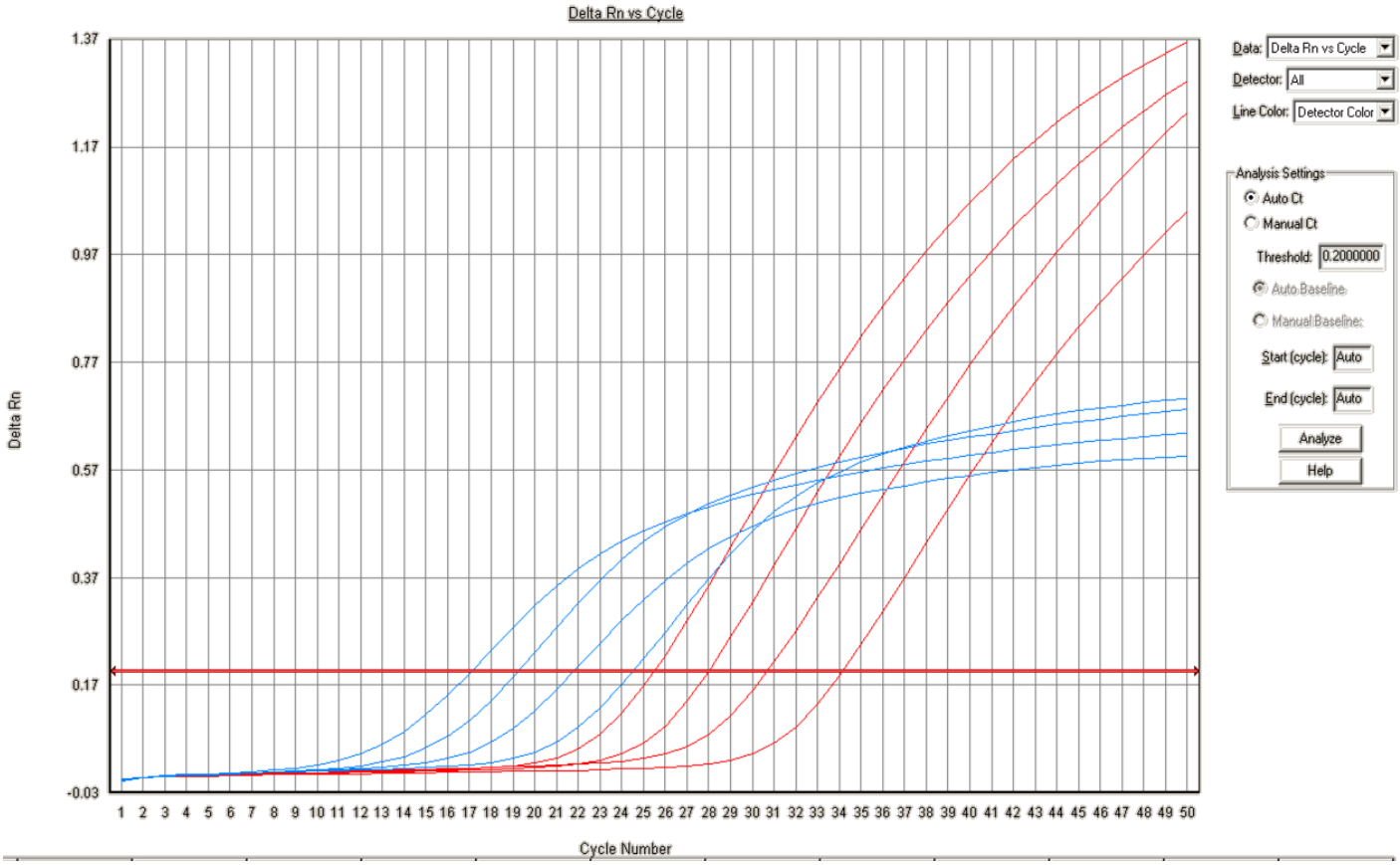
B-ACTIN		FORWARD PRIMER CONCENTRATION (MEAN CT VALUES)					
		100	**200**	**300**	**400**	**500**	**600**
REVERSE PRIMER CONCENTRATION (MEAN CT VALUES)	**100**	23.23	21.44	25.15	24.15	34.12	34.26
	200	22.62	21.00	22.67	22.65	31.12	36.49
	300	20.94	21.19	20.85	21.56	23.05	23.45
	400	22.15	24.31	22.99	22.64	25.68	31.25
	500	24.69	24.25	24.01	23.65	32.29	33.16
	600	33.46	23.52	23.30	30.59	32.89	34.52



18S		FORWARD PRIMER CONCENTRATION (MEAN CT VALUES)					
		25	**50**	**100**	**150**	**200**	**250**
REVERSE PRIMER CONCENTRATION (MEAN CT VALUES)	**25**	16.25	15.67	14.68	14.65	14.22	15.68
	50	15.34	13.41	13.58	14.26	15.19	15.77
	100	14.49	14.21	13.29	14.09	14.88	14.96
	150	15.02	14.34	14.79	15.02	15.34	17.87
	200	14.89	15.10	14.60	15.45	17.21	18.79
	250	15.34	14.64	16.21	17.34	18.45	18.83

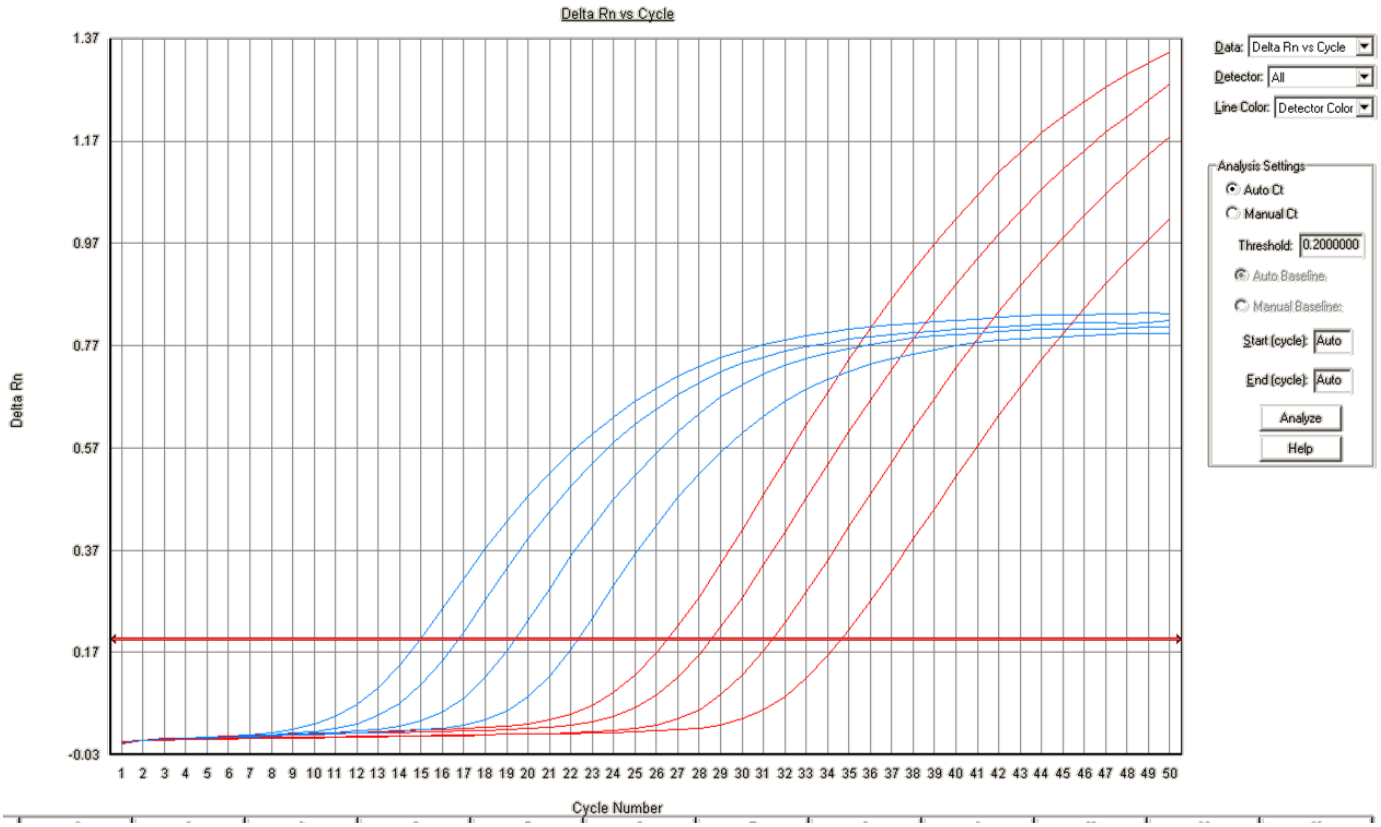
Appendix XIV: Real Time Amplification Curves: Multiplex Optimisation

Assay 1: 50nM 18S ribosomal forward, 50nM 18S ribosomal reverse and 300nM β –actin forward, 300nM β –actin reverse primers



Line Number (counting from top to bottom)	cDNA dilution
1	β-actin neat
2	β-actin 1/10
3	β-actin 1/100
4	β-actin 1/1000
5	18S ribosomal 1/1000
6	18S ribosomal 1/10
7	18S ribosomal neat
8	18S ribosomal 1/100

Assay 2: 100nM 18S ribosomal forward, 100nM 18S ribosomal reverse and 300nM β –actin forward, 300nM β –actin reverse primers



Line Number (counting from top to bottom)	cDNA dilution
1	β-actin neat
2	β-actin 1/10
3	β-actin 1/100
4	β-actin 1/1000
5	18S ribosomal neat
6	18S ribosomal 1/10
7	18S ribosomal 1/100
8	18S ribosomal 1/1000

Appendix XV: Hair Analysis - Ct Values and relative 18S/B-actin ratios for all donors across the entire ageing period

0 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	20.34	25.49	n/a
1 B	20.39	25.51	n/a
2 A	20.22	25.31	n/a
2 B	20.57	25.89	n/a
3 A	20.62	25.51	n/a
3 B	20.67	25.50	n/a
4 A	20.76	25.15	n/a
4 B	20.09	25.11	n/a
5 A	20.55	25.37	n/a
5 B	20.60	25.30	n/a
6 A	20.35	25.81	n/a
6 B	20.36	25.64	n/a
7 A	20.53	25.64	n/a
7 B	20.41	25.34	n/a
8 A	20.64	25.83	n/a
8 B	20.39	25.17	n/a
9 A	20.04	25.22	n/a
9 B	20.10	25.24	n/a
10 A	20.51	25.46	n/a
10 B	20.52	25.61	n/a
Mean	**20.43**	**25.45**	**n/a**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	26.05	26.60	1.463
Positive Control	26.28	26.79	1.424

1 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	20.97	26.80	1.598
1 B	21.41	27.22	1.606
2 A	20.32	25.99	1.485
2 B	20.66	26.59	1.507
3 A	19.71	25.32	1.595
3 B	20.69	26.23	1.605
4 A	20.80	25.63	1.341
4 B	21.09	26.54	1.355
5 A	20.61	26.03	1.492
5 B	20.61	25.92	1.504
6 A	20.37	26.46	1.521
6 B	21.02	26.92	1.525
7 A	20.55	26.23	1.455
7 B	20.42	25.92	1.463
8 A	19.97	25.57	1.299
8 B	20.50	25.69	1.321
9 A	21.08	26.73	1.395
9 B	20.12	25.79	1.417
10 A	20.60	26.16	1.500
10 B	20.61	26.31	1.504
Mean	**20.61**	**26.20**	**1.470**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	26.00	26.41	1.439
Positive Control	26.21	26.71	1.432

5 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	21.53	27.58	1.868
1 B	20.58	26.65	1.890
2 A	20.29	26.35	1.923
2 B	21.49	27.77	1.927
3 A	20.84	26.61	1.805
3 B	19.97	25.70	1.797
4 A	20.96	26.35	1.945
4 B	21.13	27.13	1.953
5 A	20.80	26.52	1.825
5 B	20.75	26.36	1.837
6 A	20.52	26.86	1.800
6 B	20.53	26.69	1.810
7 A	21.70	27.78	1.940
7 B	20.63	26.56	1.958
8 A	21.05	27.22	1.933
8 B	20.59	26.35	1.933
9 A	20.21	26.38	1.955
9 B	21.28	27.42	1.973
10 A	21.64	27.52	1.891
10 B	20.68	26.74	1.911
m**ean**	**20.86**	**26.83**	**1.894**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	24.84	25.41	1.481
Positive Control	25.22	25.76	1.452

Undetermined = No result obtained

15 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	20.53	27.09	2.573
1 B	21.25	27.76	2.561
2 A	21.29	27.64	2.37
2 B	20.78	27.40	2.386
3 A	20.84	27.04	2.402
3 B	20.85	27.00	2.414
4 A	20.96	26.50	2.160
4 B	21.31	27.46	2.164
5 A	22.01	28.19	2.531
5 B	20.75	26.84	2.537
6 A	21.05	27.70	2.245
6 B	20.53	27.02	2.253
7 A	20.70	27.09	2.342
7 B	20.63	26.83	2.350
8 A	21.13	27.76	2.635
8 B	20.59	26.83	2.651
9 A	20.21	26.68	2.386
9 B	21.28	27.71	2.410
10 A	20.65	26.84	2.292
10 B	20.68	27.02	2.306
Mean	**20.90**	**27.22**	**2.398**
CONTROL 1	Undetermined	undetermined	0.00
CONTROL 2	Undetermined	undetermined	0.00
Negative Control	Undetermined	undetermined	0.00
Positive Control	25.48	26.05	1.481
Positive Control	26.29	26.85	1.473

Undetermined = No result obtained

30 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	19.67	26.88	3.924
1 B	21.54	28.68	3.926
2 A	20.29	27.20	3.380
2 B	20.78	27.93	3.400
3 A	20.08	26.84	3.455
3 B	20.85	27.55	3.479
4 A	20.96	27.06	3.138
4 B	21.31	28.03	3.162
5 A	20.80	27.35	3.175
5 B	21.05	27.47	3.181
6 A	20.52	27.85	3.506
6 B	20.53	27.68	3.492
7 A	20.70	27.80	3.780
7 B	20.63	27.54	3.754
8 A	21.16	28.11	3.262
8 B	20.59	27.14	3.272
9 A	20.21	27.22	3.409
9 B	21.28	28.23	3.393
10 A	21.65	28.27	3.090
10 B	20.68	27.46	3.104
mean	**20.76**	**27.61**	**3.414**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	25.42	25.97	1.464
Positive Control	26.11	26.68	1.481

Undetermined = No result obtained

45 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	21.53	28.78	4.162
1 B	20.58	27.84	4.192
2 A	21.29	28.41	3.960
2 B	20.78	28.17	3.996
3 A	19.84	27.01	4.525
3 B	20.85	27.95	4.537
4 A	20.96	27.36	3.829
4 B	22.31	29.30	3.869
5 A	20.80	27.77	4.215
5 B	20.75	27.61	4.245
6 A	20.52	28.26	4.590
6 B	21.02	28.57	4.606
7 A	20.70	27.86	3.921
7 B	20.63	27.60	3.931
8 A	20.82	28.15	4.178
8 B	20.59	27.51	4.190
9 A	21.21	28.52	4.235
9 B	20.28	27.59	4.257
10 A	20.65	27.69	4.025
10 B	21.62	28.80	4.101
Mean	**20.89**	**28.04**	**4.178**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	25.58	26.16	1.488
Positive Control	25.68	26.21	1.438

Undetermined = No result obtained

60 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	20.53	28.08	4.998
1 B	21.58	29.10	5.044
2 A	20.29	27.68	4.670
2 B	22.78	30.37	4.700
3 A	21.84	29.04	4.782
3 B	22.01	29.17	4.812
4 A	20.96	27.79	5.095
4 B	23.18	30.57	5.113
5 A	21.61	28.91	5.321
5 B	20.75	27.96	5.367
6 A	22.52	30.32	4.941
6 B	20.53	28.20	4.951
7 A	23.03	30.34	4.516
7 B	21.63	28.80	4.550
8 A	20.82	28.39	4.892
8 B	20.59	27.75	4.906
9 A	21.98	29.67	5.530
9 B	21.28	28.96	5.538
10 A	23.00	30.25	4.812
10 B	21.68	29.07	4.716
mean	**21.63**	**29.02**	**4.963**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	25.67	26.25	1.498
Positive Control	26.01	26.57	1.477

Undetermined = No result obtained

75 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	22.36	29.92	5.163
1 B	24.52	32.15	5.223
2 A	23.23	30.69	5.689
2 B	23.59	31.47	5.809
3 A	21.65	28.96	5.105
3 B	24.95	32.11	5.044
4 A	21.67	28.63	5.628
4 B	24.09	31.63	5.728
5 A	23.79	31.04	5.326
5 B	24.68	31.81	5.398
6 A	25.64	33.45	5.221
6 B	22.89	30.59	5.237
7 A	22.56	30.22	5.631
7 B	23.24	30.70	5.661
8 A	22.67	30.34	5.392
8 B	21.40	28.68	5.382
9 A	23.68	31.21	5.115
9 B	22.10	29.65	5.135
10 A	21.87	29.16	4.869
10 B	24.54	31.88	4.777
mean	**23.26**	**30.71**	**5.327**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	25.06	25.61	1.461
Positive Control	26.37	26.93	1.473

Undetermined = No result obtained

90 Day Samples

Donor	18S Ct value	B-actin Ct value	Relative ratio
1 A	23.39	31.14	5.989
1 B	25.44	33.13	6.013
2 A	22.25	29.78	5.27
2 B	23.65	31.39	5.290
3 A	23.71	31.10	5.583
3 B	24.76	32.08	5.607
4 A	24.87	31.84	5.961
4 B	23.10	30.73	5.975
5 A	22.63	30.09	5.989
5 B	25.68	32.94	5.967
6 A	26.31	34.00	4.850
6 B	24.41	31.97	4.882
7 A	25.60	33.08	5.256
7 B	24.47	31.80	5.278
8 A	24.73	32.36	5.400
8 B	25.44	32.63	5.403
9 A	25.05	32.44	4.747
9 B	26.11	33.46	4.787
10 A	25.58	32.89	5.230
10 B	24.59	32.06	5.216
Mean	**24.59**	**32.04**	**5.435**
CONTROL 1	Undetermined	undetermined	n/a
CONTROL 2	Undetermined	undetermined	n/a
Negative Control	Undetermined	undetermined	n/a
Positive Control	25.75	26.18	1.431
Positive Control	24.74	25.30	1.469

Undetermined = No result obtained

Appendix XVI: Variation between replicate hair samples for each donor at each time point.

Donor	Variation between Replicate Samples at each Time period (days) (expressed as a percentage against the mean)								
	1	**5**	**15**	**30**	**45**	**60**	**75**	**90**	**Average**
1	0.008	0.022	0.012	0.002	0.030	0.046	0.060	0.024	**0.026**
2	0.022	0.004	0.016	0.020	0.036	0.030	0.120	0.020	**0.034**
3	0.010	0.008	0.012	0.024	0.012	0.030	0.061	0.024	**0.023**
4	0.014	0.008	0.004	0.024	0.040	0.018	0.100	0.014	**0.028**
5	0.012	0.012	0.006	0.006	0.030	0.046	0.072	0.022	**0.026**
6	0.004	0.010	0.008	0.014	0.016	0.010	0.016	0.032	**0.014**
7	0.008	0.018	0.008	0.026	0.010	0.034	0.030	0.022	**0.020**
8	0.022	0.000	0.016	0.010	0.012	0.014	0.010	0.003	**0.011**
9	0.022	0.018	0.024	0.016	0.022	0.008	0.020	0.040	**0.021**
10	0.004	0.020	0.014	0.014	0.076	0.093	0.092	0.014	**0.041**
Average	**0.013**	**0.011**	**0.012**	**0.017**	**0.028**	**0.031**	**0.058**	**0.021**	

CHAPTER 12: PUBLICATIONS

1. Hampson, C., Louhelainen, J and McColl, S. 2011. An RNA Expression method for ageing Forensic Hair Samples. *Journal of Forensic Sciences,* vol. 56(2), pp. 359-365.

An RNA Expression method for ageing Forensic Hair Samples

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Abstract

A common limitation to most forensic trace evidence analysis is the ability to determine the time at which the evidence was deposited at the crime scene. This issue of timing is vitally important as it may not only reveal when the crime occurred, but could exclude potential suspects from the investigation. Using a Reverse Transcription Quantitative PCR (RT-qPCR) assay, we monitored the Relative Expression Ratio (RER) of two different RNA species (18S and β-actin) in hair samples that were aged naturally over a period of 3 months. No gender or age-of-donor biases were observed and results were linear up to 60 days. After 60 days the results were more variable and gave unreliable estimates of time since deposition. Overall the results presented in this paper suggest that the age of hair samples containing follicular tags can be approximated using a second order polynomial, although with limitations: Age = $3.31RER^2$ - 2.85RER – 0.54 (R^2=0.98)

Keywords: Forensic science, Hair, Trace Evidence, RNA degradation, Quantitative Polymerase Chain Reaction, Deposition age estimation

Determining the age of physical evidence or more specifically the time it was deposited at a crime scene is a common limitation of many different types of trace evidence. Linking a particular piece of evidence or person to a crime scene is vitally important in any criminal investigation. The time that this interaction occurs is significant and without it, the value of the evidence can be significantly decreased or in some cases, completely lost.

Chemical evidence such as paints, inks and drugs (to list a few) tend to age in chemically predictable ways (1) and therefore developing methods to measure these changes (and age these types of samples) have been relatively successful (2). Ageing biological evidence is more difficult due to the unpredictable degradation processes that are inherently involved with such material. The success rate in developing techniques to age the more common types of biological evidence recovered from crime scenes such as blood (3,4), semen, hair, fingerprints (5) and bone (6,7) have varied considerably. The techniques themselves have been just as diverse ranging from simple visual examinations to more complicated techniques such as GC-MS, X-Ray diffraction and DNA/RNA analysis. In the last few decades there has been a focus on developing methods for determining the age of bloodstains due primarily to the prevalence of blood at major crime scenes. Spectroscopic (8), entomological (9), enzymatic (10) and chromatographic methods (11, 12) have all been tried as well as Electron Paramagnetic Resonance Spectroscopy (13) and even specialised microscopy techniques (Atomic Force Microscopy). Unfortunately the results from these studies have been approximate at best and have failed to pinpoint the age accurately.

Perhaps the most promising work to date has involved RNA degradation studies. Anderson *et al* (2005) developed a RT-qPCR assay to show that the ratio between two different types of RNA changed in a linear manner over the course of 150 days (3). There were a number of advantages to this approach. By examining a ratio of relative quantities, the analysis is independent of the initial sample volume, high sensitivity is achieved through the PCR

amplification step, species specific probes increases the assays specificity and the method allows for the simultaneous extraction of DNA and RNA. Their results also suggest that there is the potential to apply this method to various other tissues as the targeted RNA species are universally expressed in all tissue types. Based on these assumptions we have used a previously untested sample type (hair) to carry out a similar study.

Hair is one of the most common types of trace evidence found at crime scenes owing to the fact that each human loses on average 100 telogen phase hairs a day, it is easily removed in a struggle and hair is generally very persistent, especially on rough fabrics and beneath fingernails (15). Hair can often provide corroborating evidence to link a person or object to a crime scene, provide a drug history of the donor and in some cases it may even identify the source of the evidence via DNA profiling. However to our knowledge there are no available methods to reliably determine the age of hair samples. We have therefore applied the RT-qPCR technique to estimate the age of hair samples based on the degradation rates of two different RNA species, β-actin and 18 s rRNA.

Methods

Hair Collection and sampling

Hair samples were collected from a total of ten individuals, all of who were white Caucasian of either English or Irish ethnic background; five individuals were aged between 20-25 years old (2 females and 3 males) and five individuals where aged between 40-45 years old (3 females and 2 males). The hairs were plucked from the head of each volunteer using tweezers and examined microscopically to ensure that a hair root (follicular tag) was attached. The last one centimetre of each hair (inclusive of the follicular tag) was cut and stored in sterile 1.5 ml Eppendorf tubes. This was repeated so that each tube contained ten hair roots. One tube constituted one sample. A total of twenty hairs (2 samples) were collected from each person for

each time point (0 days (control), 1 day, 5 days, 15 days, 30 days, 45 days, 60 days, 75 days and 90 days), ensuring there were duplicate samples for each time frame. This created a total of 180 samples for all time points. The samples were then left to age at room temperature for the specified time periods to simulate natural aging.

RNA Extraction

RNA was isolated from each sample at the various time periods using an organic extraction method (TRI reagent BD, Sigma-Aldrich, St.Louis, USA) according to the manufacturer's instructions with the exception of a few minor modifications. For each sample, 500 µl of TRI reagent was added to each of the Eppendorf tubes containing the ten hair follicles along with 200 µl of PCR grade water. The tubes were then vortexed for 15 seconds followed by ten minute incubation at 50^{o}C. Instead of chloroform, 100 µl of 1-bromo-3-chloropropane was added to each tube and the samples were again vortexed for 15 seconds. The samples were incubated for 5 minutes at room temperature and then centrifuged at 12,000 x g for 15 minutes at 4^{o}C. The aqueous layer was transferred to a new sterile 1.5 ml Eppendorf tube and 500 µl of cold (4°C) 2-propanol and 3 µl of a polyacrylamide carrier (Bioline, UK) were added. The samples were mixed gently, incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The pellet was washed with 1ml of 75% ethanol vortexed briefly and centrifuged at 12,000 x g for another 5 minutes at 4°C. The supernatant was removed and the wash step repeated. The RNA pellet was allowed to air dry for 5 minutes and resuspended in 20 µl of RNase free ddH_2O. Two negative control samples (containing no hair) were carried out at each time point, and processed in the same manner. All samples were treated with DNase I to avoid genomic DNA carryover. The RNA quantity was measured using Implen's NanoPhotometer (Implen GmbH, Germany).

Reverse Transcription

Reverse transcription was carried out using random priming and Superscript II reverse transcriptase chemistry. Before the actual RT reaction, the random hexamers (Invitrogen, USA), deoxynucleotriphosphate mixture (dNTP) and ddH_2O were mixed to final volume of 7 µl. Five microliters of RNA sample was then added and the mixture incubated for 5 minutes at 65^oC. Samples were immediately chilled on ice and spun briefly before 5X First Stand Buffer (Invitrogen, USA) and DTT were added. The samples were briefly vortexed before incubation at room temperature for 2 minutes. Finally 200 U of Superscript II Reverse Transcriptase (Invitrogen, USA) was added to each tube, giving final volume of 20 µl containing 500 µM dNTPs, 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 200 U of SuperScript II, and 0.1 µg random hexamers. The reaction components were gently mixed by inverting the tubes a couple of times before being incubated for 10 minutes at room temperature followed by 42^oC for 50 minutes. The reaction was terminated by incubating the samples at 70^oC for 15 minutes, and the samples were stored at -20˚C prior to further use.

Quantitative Real-time PCR

A real time PCR master mix was prepared and consisted of: Primers and Probes β-actin control reagent kit, Ribosomal RNA control kit, TaqMan Universal PCR Master Mix with UNG (all from Applied Biosystems, Foster City, USA) and nuclease free ddH_2O. The final optimised concentrations used in the reactions were: 200 nM β-actin probe (FAM dye) and 300 nM for both the forward and the reverse β-actin primers; 200 nM 18s rRNA probe (VIC dye) and 100 nM for both the forward and the reverse 18s rRNA primers. Twenty three microliters of the prepared PCR master mix was added to each well of the MicroAmp® Optical 96-well reaction plate (Applied Biosystems, California, USA). 2 ul of each reverse transcribed RNA sample was added to the appropriate well to make a final reaction volume of 25 ul. Triplicate biological replicates were run for each RNA sample along with negative control and human genomic

DNA as a positive control (Bioline, UK). The reaction plates were centrifuged briefly prior to qPCR analysis.

For qPCR analysis, an AB 7500 qPCR instrument was used according to manufacturer's instructions. The data was analysed using SDS software (version 1.4.0, Applied Biosystems, USA). The fluorescence threshold was set in the middle of the exponential (linear) phase of the PCR reaction. A passive reference dye (ROX) was included in each sample as an evaporation and pipetting control, and an exogenous control (cDNA interplate calibrator) was used to adjust any variation between runs.

Statistical Analysis

Ct values were exported into GenEx statistical software (version 4.3.7, BioEPS GmbH, Germany), which is designed specifically for qPCR data. Values were pre-processed which involves normalising raw fluorescence threshold cycle (Ct) values against interplate calibrators, individual efficiency rates, qPCR repeats and reference controls. This normalisation process addresses the issues raised by Pfaffl (14) and the resulting Ct values were corrected according to his compensating formula, which involved replacing the base number of 2 with 1 + E (where E is the amplification efficiency rate).

Results were tested for normal distribution and upon conformation were subjected to regression analysis (geometric mean, standard deviation, SEM, %CV and independent t-tests) using InStat 3 statistical software (version 3.06, GraphPad, USA). The relative expression ratios were calculated from the normalised data. Due to the differences observed in the target amplification efficiency rates the formula proposed by Pfaffl (14), which compensates for any slight variation was used to calculate the Relative Expression Ratios:

$$\text{Relative Expression Ratio} = (1 + E_{\text{target}})^{\Delta Ct\,(\text{target})} / (1 + E_{\text{reference}})^{\Delta Ct\,(\text{reference})}$$

where E is the efficiency of the individual target species and ΔCt is the change in the Ct values.

To ensure there were no inhibitors present the efficiency rates in a selected number of test samples (actual) were determined to ensure they were similar to those predicted from the standard curve (expected). For the purpose of this study the values were considered similar enough to use the expected rates of 95.2% (18S) and 92.5% (β-actin) to quantify all test samples

Primers and Probes Selection for qPCR

Human specific primers and probes were obtained from commercially available kits (Applied Biosystems, Foster City, USA). 18S primers and probes were obtained from the Taqman Ribosomal RNA Control Reagent Kit (Part Number: 4308329), whilst the B-actin primers and probes originated from the Taqman Human ACTB Endogenous Control Kit (Part Number: 4352935E). The primers and probes were selected based on their success by Anderson *et al* (2005).

Results and Discussion

Assay Optimisation

Prior to any sample analysis it was imperative to ensure that the qPCR assay was working efficiently and any limitations corrected for. This required full primer optimisation (Figures 1 & 2), and complete efficiency testing of the optimised multiplex assay as well as on a selection of random test samples. The primer combinations that had the greatest efficiency (lowest Ct

values) in the singleplex optimisation studies were tested as a multiplex assay. Multiple combinations were tested though only results from the two most efficient multiplex assays are presented here.

In assay 1, primer concentrations were the same as those stipulated by Anderson *et al* (2005) in their analysis of blood samples. Using a standard curve method the efficiency rates for each species were calculated (B-actin: 83% and 18S: 88%). A second multiplex assay (assay 2) was tested using the optimal primer concentrations, as defined in the singleplex optimisation study. The primer concentrations for B-Actin were unchanged (300 nm) however the concentration of both the forward and reverse 18S primers were increased to 100 nm. Efficiency rates of 92% (B-actin) and 95% (18S) were obtained. This particular combination of primers outperformed all other combinations tested (data not shown).

The efficiency rates from 5 hair samples, chosen at random, were determined and compared to the rates obtained from the Multiplex Assay 2. This was essential to ensure there were no inhibitors present in the test samples. The mean efficiency rates of the 5 random samples were 97.2% (18S) and 94.1% (B-actin) and this was comparable to 95.2% (18S) and 92.5% (B-actin) respectively from Assay 2. Detailed results can be found in table 1. The differences observed were small at 2% and 1.6% respectively however the variation in this data set was significant (data not shown).

Stability of 18rRNA and β-actin mRNA

The results presented here suggest the two selected targets are suitable for this type of analysis given the key criteria was to have two targets that degraded at consistent and yet different rates. 18S rRNA appears to be stable for approximately 45 days (excluding the noticeable effects during the first day) therefore may be a suitable candidate gene for this sample type over this particular period of time. After 45 days the 18S species began to show signs of degradation and

this was evident in the increase in Ct values but more accurately described by the decrease in the relative expression (Figure 3). The relative expression of β-actin mRNA continually decreased with time suggesting that the degradation process was relatively uniform. A sharp initial decrease in the expression of both species was observed between day 0 and day 1, indicating an accelerated rate of degradation. This rate was similar to those seen with samples older than 60 days.

RNA Quantification: Sensitivity

One of the prior concerns regarding the extraction of RNA was whether sufficient amounts could be obtained from very small starting quantities (10 follicular tags). Though various authors (3, 15) have claimed to have recovered sufficient quantities of RNA from a single hair, the Figures published regarding the exact quantities vary considerably (average RNA yield was 112.5ng per hair follicle).

The quantity of total RNA in each sample was determined using a Nanophotometer. The average yield of total RNA extracted per sample (10 hair roots) from all 180 samples was 88.25 ng. The mean quantities displayed a discernable trend with a continual decline in the amount of RNA extracted over the 90 days. Spectroscopic analysis of the RNA recovered from hair samples estimated a 79% loss of total RNA from 0 to 90 days however at 90 days there was still a sufficient amount of RNA recovered for subsequent processing using qPCR.

The level of sensitivity was tested by reducing the number of hair strands (follicular tags) used per sample. Real time PCR signals from both the β-actin and 18S species were consistently detected in samples containing as little as 3 follicular tags. 18S was detected in samples containing a single hair strand, however success was inconsistent (3 out of 10 samples) and the Ct values obtained were outside the dynamic range of the assay. This would

have implications in an operational setting where only a single hair strand was recovered from a crime scene.

Inter and Intra-donor Variation

Inter-donor variation was statistically calculated using the normalised qPCR data. A number of features were observed though given the size of the population used in this study further investigation is required to confirm these findings. The inter-donor variation of this data set was determined at each time period and ranged from 3.26% (5 days) to 8.22% (90 days) with a mean value of 5.98% across all ageing times. The inter-donor variation showed a time-wise dependant increase suggesting that external degradation factors are not consistent. The small inter-donor variation observed at the beginning of the study, suggests low levels of natural biological variance *in-vivo*. This is an important feature for this type of forensic analysis. Although the inter-donor variation increased with time the values obtained were comparable to previously published data. Biological samples are often a source of significant variation especially dynamic tissue types such as whole blood (3, 4) where a single sample yields an average expression value from numerous different cell types. With hair samples only a single cell type is analysed which may explain why the variation was less than reported values from studies involving blood tissue.

Intra-donor variation varied from 0.39% (donor 6) to 1.05% (donor 10) with an overall average of 0.67%. The small intra-donor variation may be a direct reflection on the physical nature of hair in that it may be a more consistent entity than other tissue types. There was no evidence to suggest any gender or age bias associated to this variation (based on non-parametric tests).

Relative Expression Ratio

The common occurrence of hair samples found at crime scenes means they are a relevant sample type for aging studies. Our results demonstrate how the relative expression ratio of two different species of RNA, 18S rRNA and β-actin mRNA, extracted from the follicular tag of hair samples, changed in a linear manner over time thus allowing an age estimation to occur. The Relative Expression ratios (RER) obtained from hair samples that had been aged for up to a period of 90 days under relatively uncontrolled conditions (room temperature, room humidity and in the presence of daylight), are presented in Figure 4. Samples were intentionally aged under these conditions to mimic forensic type environments and to assess the robustness of the proposed method.

The RERs behaved in a linear and reproducible fashion with a relatively strong correlation with samples aged up to 45-60 days but as the time proceeded, the rate of increase tapered off and a plateau effect was observed. There was a noticeable decrease in the 18S relative expression in samples older than 60 days. This sudden increase in degradation rate was comparable to that experienced by β-actin mRNA which behaved consistently over the entire 90 days. It is uncertain as to why the 18S species began to degrade around this period of time but it is thought that it may be due to the breakdown of the ribosomal complex, which is thought to be primarily responsible for protecting the 18S rRNA (3) Breakdown of this complex would release the 18S species into the same environment as the β-actin mRNA and thus be exposed to the same degradation factors.

Another notable feature of this study involved the higher rate of degradation experienced by both species of RNA during the first day of ageing. This could be a result of increased intra-cellular enzymatic activity, which is dependent on the natural aqueous environment common to all living cells. This increase rate was consistent across all ten donors.

The error rate or confidence with any estimation varied over the 90 day period. Samples that had aged for less than 30 days could be distinguished within a 10-15 day age range.

However as the time frame increased the ability to be able to distinguish one sample from other decreased and the estimated age range became larger to the point where samples could not be distinguished at all after 60 days

The RER values presented with a normal (Gaussian) distribution and therefore accurate interpretation could be made from the relevant statistical analysis although consideration must be made to the small number of samples tested. Independent paired t-tests and F-tests were performed on the mean relative expression ratios for this data set. The results suggested that the difference in the means between genders were not significant. A similar statistical analysis was performed to determine if there was any evidence to suggest an age bias in the data. We could not detect any age-related effects on the mean relative expression ratios in this sample set. Given the small population size (10) any statistical findings should be viewed with caution but based on the results presented here there is sufficient evidence to warrant further investigation.

Combined data and trend analysis

Figure 5 illustrates the degradation kinetics and based on these RER values, the aging process can be approximated using a second order polynomial (R^2=0.98):

$$A = 3.31RER^2 - 2.85\,RER - 0.54$$

where A is the age of the hair in days and RER is the Relative Expression Ratio.

To provide age estimations with a higher degree of confidence it is suggested that a range rather than an exact Figure be stipulated. Since the analysis is based on a single calibrator (slow degrading housekeeping gene), multiple aging markers combined with geometric means could improve the estimation significantly.

Although a polynomial equation has been proposed to age hair samples using the resulting RER's, the authors acknowledge the limitations associated with this study. The

population tested was small (n = 10), the lineal relationship between sample age and RER only exists for samples younger than 60 days (although could be improved with additional data), there is a limit to the amount of material required (minimum of 3 hairs) and hair sample type is specific (must contain a follicular tag).

Conclusion

This paper provides further support for RNA as a suitable candidate for forensic analysis due to the highly sensitive processing techniques and its stability in forensic type samples. Both 18S and β-actin species have proven to be suitable targets for age estimations of hair samples and this is predominately due to their patterns and rates of degradation, though this may be limited time-wise. The accuracy and precision observed suggests that a more reliable approach would be to provide an estimate range, rather than an exact value. In many criminal cases, especially those involving serious crime where the scene is generally processed immediately, the range will be too large to be of any value. However, in certain situations this level of detail may provide corroborating evidence or merely present as an investigative tool.

As well as using increased sample numbers, future research will focus on determining the cause of the increasing inter-donor variation and the effects of the various environmental factors. Different targets will be examined in an attempt to find suitable candidates for discriminating samples aged for longer than 60 days and we are currently evaluating other suitable housekeeping genes for this type of analysis.

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Additional Information and Reprint Requests

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Table 1: The 18S and B-actin efficiency rates of 5 random test samples (actual rates) were determined and compared to the efficiency rates obtained from the optimised multiplex assay (expected rates).

Random Sample	**Mean Ct Values**		
18S	**Neat**	**1/10**	**1/100**
Sample 1	20.76	23.99	27.35
Sample 2	21.28	24.50	27.84
Sample 3	19.84	23.34	26.57
Sample 4	24.52	27.91	31.49
Sample 5	25.60	29.08	32.68
B-actin			
Sample 1	25.15	28.56	32.34
Sample 2	27.42	30.45	34.29
Sample 3	27.01	30.23	33.85
Sample 4	32.15	35.39	38.84
Sample 5	33.08	35.53	40.24

Efficiency Rate (%)

	Optimised Multiplex Assay	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
18S	95.2	100.9	101.7	98.2	93.6	91.6
B-actin	92.5	89.7	95.5	96.0	99.0	90.2