Evaluation of collection protocols for the recovery of biological samples from crime scenes

by

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Abstract

The main focus in forensic genetics in the past 30 years has been either to increase the efficiency of the extraction and identification of DNA from a wide variety of evidence, or to improve DNA profiling technology by making it more sensitive and robust. However, the methods used to recover DNA evidence from crime scenes have seen little development.

This research has developed wetting agents which can be incorporated into most conventional swabbing protocols and has the potential to significantly improve both the recovery rate and stability of the DNA bearing samples. The main objective of this research was to improve the efficacy of the processes of collection and storage up to the point where the evidential material is received at a laboratory. The effect of heat and time post-collection on degradation within collected samples before they reach the laboratory has been assessed.

Three collection methods of biological evidence have been compared: one swab, double swab and pipetting, using distilled water TE buffer and commercial cell lysis (Qiagen) as a wetting agent. An enhancement in quantity and quality of DNA was seen when the double swab collection method was used with the commercial lysis buffer. This led to the development of an in-house detergent based buffer to be used as a wetting agent. In addition, the stability of the DNA post-collection was greatly improved especially at higher temperatures, even with extended periods post-collection. When using ultrapure water as the wetting agent DNA degradation can be seen as early as 6 h at room temperature. However, the detergent-based solution stabilized DNA for up to 48 h, even when the temperature is increased to 50 °C. The impact of this study is likely to be limited in circumstances where crime scene evidence can be kept at temperatures below room temperature until it reaches the laboratory. However, in contexts where this is problematic, the modified method for collection could have a large impact on the preservation of forensic evidence before it reaches the laboratory.

The reliability of the results from analysis of evidential DNA is greatly improved when a careful protocol is observed for the collection, transfer and storage of the original samples. However, there is no published data on the development of protocols particularly suited to collection, transfer and pre-lab storage of samples, especially when there are extreme environmental conditions at the crime scene. The mechanisms of natural degradation of DNA are well understood (Hu *et al.,* 2005) and temperature and

moisture content pay a significant role. In the climatic conditions of places like Saudi Arabia, crime scene evidence can be exposed to extreme levels (high and low) of temperature and humidity before it reaches the laboratory.

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Abbreviations

A: Adenine – A purine base. One of the four bases of DNA

AMP-FLPs: Amplified fragment length polymorphisms.

ANOVA: analysis of variances.

bp: Base pair.

C: Cytosine – a pyrimidine base. One of the four bases of DNA

CE: capillary electrophoresis

CODIS: Combined DNA Index System.

DNA: Deoxyribonucleic acid.

EDTA: Ethylenediaminetetraacetic acid.

FBI: Federal Bureau of Investigation of the USA.

FDA: Food and Drug Administration

FTA: Flinders Technology Associates.

FSS: Forensic Science Service of the UK.

G: Guanine – a purine base. One of the four bases of DNA

kb: Kilobase, a string of a thousand DNA bases.

NaCl: Sodium chloride

NDNAD: National DNA Database.

NP-40: nonyl phenoxypolyethoxylethanol.

PCR: Polymerase chain reaction.

RFU: relative fluorescence units.

RNA: Ribonucleic acid.

SDS: Sodium dodecyl sulphate.

SGM: Second Generation Multiplex.

SNP: Single nucleotide polymorphism.

STR: Short terminal repeat.

T: Thymine – a pyrimidine. One of the four bases found in DNA.

Tween 20: Polysorbate 20.

VNTR: Variable number tandem repeat.

Chapter One

Introduction

The use of Deoxyribonucleic acid (DNA) as evidence has stimulated a revolution in the field of criminal investigation. The recent advances, and in particular, the accessibility of the new and improved technologies, have now made DNA analysis a critical, and important, part of forensic science (Schneider, 2007; Hedman *et al.,* 2010). The original DNA analysis technology was developed by a team at the University of Leicester led by Sir Alec Jeffrey (Jeffreys *et al.,* 1985) who coined the phrase 'DNA Fingerprinting' to describe the process; this term was replaced by DNA profiling, which does not contain the same implications of uniqueness as are associated with the term 'fingerprint'.

What made the DNA profiling particularly influential in forensic science is the high discriminatory power. DNA profiling has the ability to differentiate between individuals through the use of a number of genetic markers, which gives each person a distinctive, possibly unique, DNA profile and we leave a trace of it everywhere we go and with who we've contacted with (Gill *et al.,* 2015). DNA evidence can support an investigation along with other evidence to allow the possibility of including or excluding a suspect from their presence in a crime. DNA discrimination power makes it a powerful tool that can assist an investigation as evidence to be presented in court (Walsh, 2007). Evidence including body fluid (such as blood, saliva, semen, and sweat), tissues from, for example teeth, skin, hair roots and bones (Dissing and Søndervang, 2010) can all be used to produce a DNA profile.

The recent advances in forensic science technology for identifying DNA have played a major role in helping to convict criminals and/or to exclude suspects who might otherwise be falsely charged and convicted (Hedman *et al.,* 2010). DNA Profiling is also important in the identification of victims, particularly in cases where the victim's condition makes them unrecognisable to family or friends (e.g. burn victims, decapitation). In fact, the analysis and characterisation of an individual's DNA has revolutionised the entire field of forensic sciences, and therefore had an impact on the criminal justice system as well (Schneider, 2007).

DNA analysis has been shown to be useful in solving a wide range of criminal investigative cases. These cases may involve crimes and incidents such as homicide, sexual assault, physical assault, hit and run incidents, missing person investigations, identification of human remains, determination of paternity and many others (Castriciano *et al.,* 2010). DNA analysis not only identifies the individuals but can also:

- identify the source of biological evidence found at a crime scene,
- redirect the investigation along a new path,
- link serial crimes together,
- identify the number of assailants,
- identify additional victims,
- Exonerate people who have been wrongfully convicted of a crime.

(Dissing and Søndervang, 2010).

Much effort has been put into the improvement of laboratory-based DNA extraction and analysis techniques (Bogas *et al.,* 2011), but regardless of whatever technology is used, the precursors to extraction and analysis are the sample collection, handling and storage that takes place prior to receipt by the laboratory. Good practice in collection, preservation and storage of samples containing DNA is fundamental in ensuring reliable forensic genetics (Butler, 2009). These steps can have a critical impact on the quality of the sample and the resultant DNA profile. Poor practice at any of these stages can seriously undermine the validity of any results and therefore their potential to be used as evidence in criminal investigations (Bonnet *et al.,* 2010). In the 2013 report of the Forensics Special Interest Group to the Technology Board of the Department for Business Innovation and Skills, a whole range of possible areas for improvement in forensic biology were identified, however, "Total recovery/release of biological material onto/from swabs" as a key requirement and stated that "consistently better swabbing techniques were required".

Once collected, most samples of biological evidence can be protected from contamination and degradation if kept dry and cold to avoid degradation by either base hydrolysis processes or breakdown resulting from DNases. Even with large pieces of evidence, if the biological material is degraded because of carelessness, or ignorance, during sample collection and transport to the laboratory, there will be poor analytical results. It is therefore vital to carefully collect, handle and preserve the sample to international standard protocols to ensure that the results that are generated can be relied upon as evidence in court (Lee *et al.,* 2012).

The dependability of the results from analysis of DNA is significantly enhanced when a well thought out, protocol is carefully observed, thus practicing to international standard protocols when transferring evidence from crime scene to laboratory is essential. Therefore, guidelines have been produced by forensic institutes, for example, the

European Network of Forensic Sciences Institute (ENFSI) and are inspected and examined to its standards (ENFSI, 2015).

Nevertheless, very little published data can be found on the development and use of protocols specifically covering collection, transfer and pre-lab storage of biological samples destined for DNA analysis, particularly when there are extreme environmental conditions at the crime scene. The mechanisms of natural degradation of DNA are well understood (Hu *et al.,* 2005), both temperature and moisture content pay a large role in the process. In places where the climatic conditions are such, crime scene evidence can easily be exposed to extreme levels (high and low) of both temperature and/or humidity before it reaches the laboratory and potentially accelerate the degradation of the evidence.

1.1 Forensic DNA Analysis

Humans share 99.9% of their genetic code with each other (Barbujani *et al.,* 1997). However, there are particular sites on the DNA that shows genetic variation between individuals (Gill *et al.,* 2002). Therefore, for forensic genetics there is no valid reason to analyse the whole genome for human identification, forensic scientists concentrate only on the genetic sites where there is variation and that characterise the individuals.

The hypervariable regions are typically targeted in forensic analysis (Holt *et al.,* 2002) and they include both mini and microsatellites. The original DNA 'profiling' analysed minisatellites and it is often referred to as variable number tandem repeats (VNTRs) (usually 8-100 base pairs (bp) in length) (Jeffreys *et al.,* 1985).

DNA regions with short repeat units (usually 2-6 bp in length) are called Short Tandem Repeats (STR) and the repeats that are (50 - 300 bp) are classified based on their structure as either simple, compound, complex or complex highly variable (Gill *et al.,* 1994). STRs have been shown to be especially suitable for human identification (Kaiser *et al.,* 2008; Walsh *et al,* 2010) because the loci used are tetranucleotide (i.e. having four bp repeats); the variability of these STR regions can be used to discriminate a DNA profile (Alonso *et al.,* 2004).

In the UK, 16 STR loci plus a sex marker are routinely examined and are visualised in a series or peaks on a graph, on which positions corresponds to a length of an STR. Once the STR profile from a crime scene is generated it can be compared to another profile or a compared between laboratories or to profiles stored on a database; the technique is highly sensitive so that it is even possible to obtain results from degraded samples.

Another approach is required when the DNA in a sample of evidence is limited, either in quantity or quality, for example where there are highly degraded samples that have been exposed to environmental insult or inhibitors that may affect analysis, such as temperature, water, oxygen, ultraviolet irradiation and nucleases enzyme (Butler *et al.,* 2003). Analysis of compromised DNA samples often results in dropout of the larger STR loci (not enough template is available to amplify) resulting in only a partial DNA profile being obtained (Gill et al., 2000). One solution to this problem is through the use of mini-STRs. Mini-STRs testing, was developed to specifically increase the success rate when working with degraded human remains as it has been shown provide reliable results with degraded DNA (Alaeddini *et al.,* 2010). It is a testing system that exploits the ability of specially designed primers that preferentially target the larger STR loci. This technology dramatically increases the sensitivity of DNA detection and greatly increases the chances of obtaining a DNA profile from compromised samples (Kleiber, 2001; Butler *et al.,* 2003).

For DNA typing, a common standardised set of markers must be used to allow comparisons between results. The first set of STR markers (four STR loci) which became widely used in forensic genetics laboratories was developed in 1994, by the UK's Forensic Science Service (FSS). Further efforts by the FSS resulted in the development of the second generation multiplex (SGM), which incorporated six polymorphic STRs (THO1, VWA, FGA, D8S1179, D18S51 and D21S11) and the amelogenin marker (Sullivan *et al.,* 1993). Commercial companies' research and development teams' responded by producing a series of multiplexes kits. The SGM Plus produced by Applied Biosystems (AmpF1STR) took over from SGM in the UK (Cotton *et al.,* 2000). In the USA, the Federal Bureau of Investigation (FBI) presently uses a standard set of 13 specific STR regions for the Combined DNA Index System (CODIS). CODIS is a software programme that operates a national database of DNA profiles from convicted offenders, unsolved crimes and missing persons. The odds that two individuals having the same 13 specific STR regions profile is about one in a billion (Piacenza and Grimme, 2004).

Today DNA-17 has taken over SGM Plus as the standard method in the UK. DNA-17 has a further six STR loci to the SGM Plus and the amelogenin marker.

As part of the change the National DNA Database (NDNAD) software was updated in 2014 to be able to store and search full DNA-17 profiles.

1.2 DNA Degradation

DNA degradation is the natural process of breakdown of DNA into smaller fragments. A short while after death, or separation of material from the body, DNA within the biological material starts to degrade. Certain physical environment conditions such as those of heat, humidity, cold or dehydration can all increase the rate of degradation of the biological material and the DNA (Butler *et al.,* 2003).

If the damage to the DNA is extensive, then analysis becomes very difficult. If the DNA is fully degraded there is little that can be done at the extraction stage to improve its quality. Degradation doesn't just happen at the crime or incident scene, once the sample is collected degradation continues and may, under certain conditions, even increase after collection. The chances of obtaining useful information from the DNA profile are greatly enhanced if the maximum amount of DNA is recovered from the crime scene and the degradation of the sample post-collection is minimised (Alaeddini *et al.,* 2010).

After the death of an individual, soft tissues may be lost, while teeth and bone tissues may remain stable. The hard tissues surrounding the bones protect DNA from the action of microorganisms; these tissues also provide a chemical environment that is rich in hydroxyapatite, which has been shown to stabilise DNA (Lindahl, 1993). However, even within bone or tooth materials the DNA continues to breakdown, largely through the process of hydrolysis and to a slighter degree oxidation. DNA can also become denatured at high temperatures, where upon the molecule loses its double helix structure and can literally unzip into two separate strands (Alaeddini *et al.,* 2010).

Several factors can cause degradation of DNA pre and post sample collection; among these factors are environmental such as temperature, ultraviolet irradiation, oxygen, water, and nucleases enzyme. There are numerous mechanisms by which the DNA degrades (enzymatic, physical and chemical process). DNA faces cellular nucleases once an organism dies, becoming vulnerable to environmental insults (such as bacteria and

fungus). Other factors that affect the damage and degradation of DNA are oxidative base damage and hydrolytic cleavage resulting in breakdown of the DNA molecule. In addition, chemical cleaning solutions such as bleach contributes to the DNA degradation process. Studies have also shown that repeated freezing/thawing of short strand DNA samples causes DNA degradation (Davis *et al.,* 2000). Breakdown of regions of the DNA molecule, reduces the efficiency of the amplification process leading to failure to generate a profile.

Assessing the extent of damage is difficult, especially when the DNA is present in a mixture of other biological materials. Qualitative estimates of DNA fragment sizes through gel electrophoresis, followed by visualisation of fragments is one basic approach but it has limited sensitivity (Deagle *et al.,* 2006). Mini-STRs can be employed to maximise the amount of information from the sample when necessary (Gill, 2002). However, DNA excess of 150 base pairs is still required. The best approach is always to do everything possible to avoid DNA degradation during sample collection and transport to the laboratory (Schneider, 2007).

1.3 PCR and PCR inhibitors

The polymerase chain reaction (PCR) process amplifies specific regions of template DNA. It has the potential to amplify a strand to a billion-fold in 30 cycles of amplification. The three stages of PCR are denaturation, annealing and extension (Mullis *et al.,* 1986). The denaturation stage starts by increasing the temperature to 94 °C melting the double strand into two separate strands (hydrogen bonds are weak at this stage). Next is annealing, the temperature is decreased to 50-65 °C allowing primers to anneal to the complementary strand, the two primers must anneal to the two different strands and must extend toward each other. Lastly, Extension; temperature is increased again to 72 °C at this stage allowing the *Taq* enzyme to find free ends of the primer and start to incorporate new nucleotides that are complementary to the strand (Bartlett *et al.,* 2003). The normal range of PCR cycles are 28-32 cycles but at extreme cases of degraded or small samples it may be increased to 34 cycles but it could form artefacts at this stage at this temperature (Goodwin *et al.,* 2007).

When processing forensic samples, it is important to avoid any further degradation whilst at the same time remove inhibitors. Inhibitors may compromise the process of amplification used PCR. The most common PCR inhibitors found in forensic science are

haemoglobin from blood, dyes such as indigo from denim, and melanin from hair (Butler *et al.,* 2003)

These inhibitors bind to the active site of the DNA polymerase enzyme which results in the loss of information therefore compromising the ability to generate a full profile. These PCR inhibitors effects can often be mistaken to severe degradation (Alaeddini *et al.,* 2010).

1.4 DNA and the environment

The natural physical environment such as, temperature, humidity and ultra-violet radiation, can affect the ability to recover DNA from samples. These effects depend upon location and climatic conditions (Barbaro *et al.,* 2008).

Crimes have to be investigated wherever they occur and samples collected from open air crime scenes can be challenging. Once they are collected any degradation will continue and may even accelerate if the physical conditions are demanding and there is a long timeframe for delivery to the laboratory (Lerkin, 2006).

There are many areas of the world where the environment is challenging but the areas which present most difficulties for reducing DNA degradation are locations where there are extremes of temperature and/or humidity. For example, crimes scenes or incidents in remote hot environments can present some of the most challenging situations in which to collect and preserve samples before they are delivered to the forensic laboratory. The development of protocols and processes to improve sample recovery and stability under such conditions could significantly improve the effectiveness of DNA evidence provided to the court. Saudi Arabia is a prime example of a location where improved sample collection and stabilisation prior to reaching the forensic laboratory could be beneficial.

The Kingdom of Saudi Arabia is located in the south west of Asia, the second largest Arabic country and by area, the 13th largest in the world. The country covers 80% of the Arabian Peninsula, a very large land mass with a wide range of climatic conditions. Ranging from the hot humid coastal cities in the West and East to the mountainous regions in the north and the south west to the arid central desserts and the great desert known as the Empty Quarter – the largest sand desert in the world (King Abdul-Aziz City for Science and Technology Annual Report 2013).

The centrally located capital Riyadh, regularly experiences daytime temperatures in excess of 50 °C during the summer along with extremely low humidity average of around

15% relative humidity and night time temperatures drop rapidly by typically 20 °C. By contrast the coastal cities of Jeddah and Dammam have lower summer temperatures but very high humidity typically around 70%. It is not unusual for the air temperature in a vehicle parked in the sun in Riyadh to reach temperatures in excess of 80 °C. The integrity of forensic samples collected in these environments can easily be compromised if the collected samples are not handled and stored correctly. Crime scene samples collected outside of the main cities particularly in rural areas present particular challenges. Not only might samples sit in the crime scene investigation vehicle for hours while other samples are being collected, but it might require many hours of road transport in extreme temperatures before they reach one of the 12 regional forensic laboratories operated by the Ministry of the Interior (Almutairi, 2013).

1.5 Common Protocols and Practices

The protocols for the production of DNA evidence differ from one crime investigation force to another around the world, but they all have common fundamental steps starting at the crime scene with the identification of the biological samples, then collection, labelling, preserving and transfer to the laboratory (Fig 1). Later steps, in the laboratory, include safe storage, DNA extraction, quantification and amplification and the production of a DNA profile (Frumkin *et al.,* 2010). Finally, statistical analysis is undertaken of the data produced from the DNA profile comparing the results with both the victim and the suspect profiles for a direct match that will either exclude or include them in the investigation.

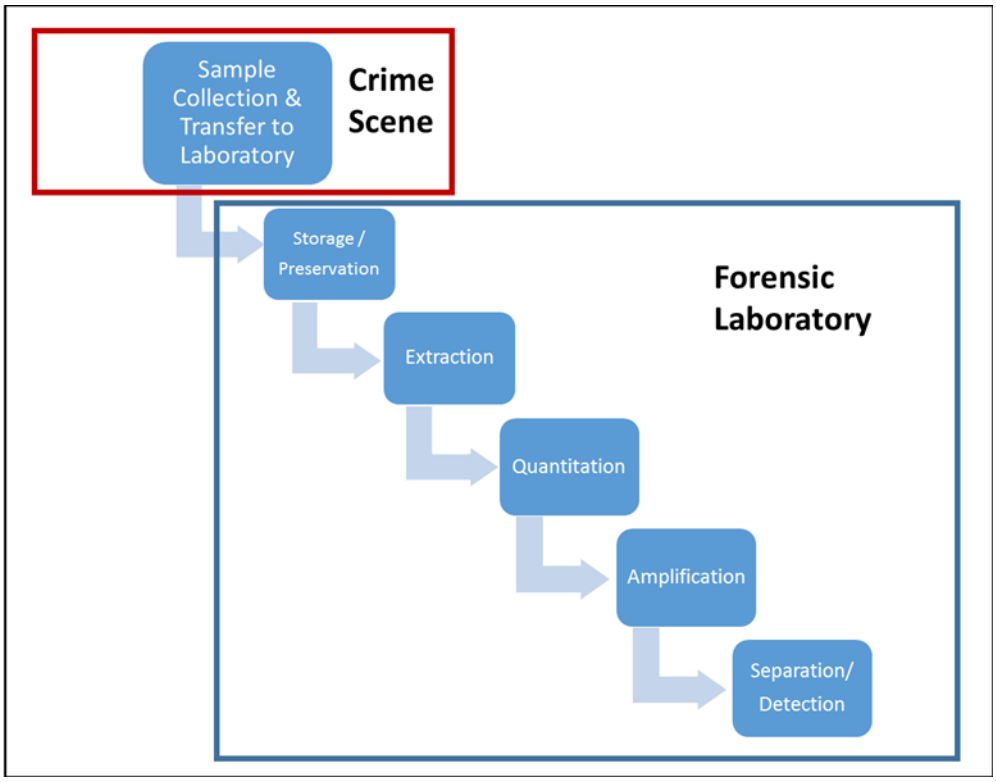

Figure 1 Fundamental steps of forensic practices from the crime scene to the laboratory

Challenges are faced at every step, but all are dependent upon the initial steps which are collection, preservation and handling of the evidence before they reach the laboratory (Lee *et al.,* 1998). It is at these stages, when they are outside laboratory conditions, that the evidence is most at risk of sample contamination or deterioration due to adverse conditions. To avoid this, these initial steps must be undertaken very carefully, following agreed protocols that incorporate the most effective, reliable and reproducible procedures.

1.6 Sources of Samples

Sources of forensic DNA samples are not just biological samples such as tissue fluids and stains. It also includes surfaces, clothing, containers and all items that may have been touched during the incident. Personal items have the most deposits like skin cell, perspiration, hair and oil and are therefore a source of DNA and highly likely to be left at a scene of crime. This could happen either directly by handling objects at the scene such as door handles, windows and surfaces or by leaving items at the scene that have their DNA deposited for example; cigarette butts, masks, drink containers (Van Oorschot *et al.,* 1997).

DNA can be recovered and DNA profiles can be generated successfully from a wide range of biological evidence that can be found at a crime scene. However, the most common biological material used in forensic genetics are blood, saliva, semen, hair and nails, tissues (such as teeth and bones) (Lee and Ladd, 2001).

1.7 Sample Collection and Processing

1.7.1 Sample Collection

Sample collection is one of the important and critical steps in DNA profiling. When collecting a sample for DNA profiling, great care must be taken to avoid contamination or degradation of the samples and the consequential potential to lose evidence. It is essential to wear appropriate personal protective equipment during sample collection in order to minimise the chance of contamination, and the collection and handling of any biological evidence also has to be carefully managed (Lee *et al.,* 2001).

There are various accepted methods of biological evidence collection presently in use. The double swab technique is the most common approach, the process involves moistening swabs with wetting agent, typically distilled water and brushing it over the surface to hydrate and loosen any cells if present. The second swab (the dry swab) is then brushed on the rehydrated stain to maximise the amount of cellular evidence that can be collected (Sweet *et al.,* 1996).

Another commonly practiced stain recovery technique is wetting a stain then collecting the rehydrated material using a pipette. Although investigations have shown that cotton swabs can be used to absorb reasonable volumes of biological fluid they often result in a relatively small amount of DNA being extracted due to the small size of the sample compared with the size of the cotton swab (Sweet *et al.,* 1997).

In a study by Von Wurmb-Schwark et al., 2006; using cell lysis buffer as a wetting agent to moisten the cotton swabs yielded larger amounts of DNA in comparison to swabs moistened with distilled water. The use of such a wetting agent encourages increased cell lysis therefore increasing the amount of DNA recovered.

It needs to be borne in mind, that some of the DNA from the dried body fluid that is collected by a swab becomes bound to the swab material and is difficult to be fully recovered (Van Oorschot *et al.,* 2003). To address this problem, different techniques or materials have been developed like the use of nylon swabs, or peeling off or detaching the swab head from the stick to give better results (Rudin *et al.,* 2010).

Other studies have shown that nylon swabs recover three times the amount of DNA in comparison to rayon swabs (Hedin *et al.,* 2010), and that nylon swabs gives a six-fold increase in DNA recovery in comparison to cotton swabs (Benschop *et al.,* 2010). Despite these facts, cotton swabs are still the most common type of swab that are used by professionals around the world when recovering biological materials and stains from crime scenes.

Alternatively, tape lifting, i.e. applying an adhesive tape to recover cellular materials from surfaces (Hall and Fairley, 2004) has been shown to be useful for trace samples. The tapes are pressed multiple times on evidence such as textiles then placed directly later into the DNA extraction tube (May and Thomsons, 2009).

1.7.2 Storage and preservation

DNA biological samples are collected, packaged and transported to the laboratory in cold conditions; these actions help to prevent mould and bacterial growth, and degradation (Bonnet *et al.,* 2010).

DNA samples are most commonly stored in the laboratories at 4 °C or –20 °C and possibly at –80 °C for long term storage to avoid chemical and enzymatic degradation (Ivanova *et al.,* 2013). On the other hand, it is also possible to store dried DNA. This can be a practical alternative for long-term storage. In addition to reducing molecular mobility, dehydration also removes any water that can participate in the hydrolytic reactions. There are several methods of removing water from liquid preparations that can be used in the laboratory; these include spray drying, spray freeze drying, air drying or lyophilisation. Spraying DNA is the least common option as it has been associated with damage introduced by shear stress (Lee *et al.,* 2012).

Bloodstains should be allowed to air dry and not be heated, whereas for all stained items the best option is to freeze unless it is a metal or glass substrates in which case they are preferably stored at room temperature and submitted to the laboratory as soon as possible. Large objects that cannot be removed from a crime scene with wet bloodstains or pools of wet blood should be transferred by pipettes if possible or onto a clean cotton cloth and allowed to air dry before packaging in a paper container (Lee *et al.,* 2001).

Another option for storage of dried DNA is on blood stain cards; this involves adding little drops of blood on cellulose filter paper then air drying the stain before storing. It is more suitable for reference sample than of crime scene samples. Cells are lysed upon

application to the card and the nucleic acids are immobilized. A study has shown that genomic DNA that has been stored on blood stain cards at room temperature for over 17 years can be successfully amplified by PCR (Kline et al., 2002). The cards are impregnated with reagents which lyse cells, denature proteins and protect nucleic acids from nucleases, oxidation and UV damage whilst enabling high molecular weight DNA to be released from the matrix ready for use in a wide range of molecular biology techniques (Rajendram *et al.* 2006).

1.8 DNA Extraction

Crime scene biological samples contain a number of substances other than DNA. Consequently, the extraction process is a vital step in the production of a DNA profile because it will determine the outcome of other stages. In the process the cells are lysed; the proteins are denatured and then the DNA is isolated. There are many methods to extract both crime and reference samples, often the sample quality and condition determines which extraction method is most suitable (Freeman *et al.,* 2003).

The capability to extract large amounts of DNA from forensic samples for analysing is a critical step in forensic genetics. However, even when large quantities of DNA can be extracted, the sample could be comprised with contaminants such as PCR inhibitors that can considerably obstruct the amplification step resulting in partial profile, false profile or no profiles (Alaeddini, 2012). Therefore, for the best DNA extraction forensic genetics is when you can produce an acceptable amount and quality of DNA for amplification without impurities that can hinder PCR analysis (Alaeddini *et al.,* 2010)

The common practice and method starts by lysing the cells in the sample to release the DNA, the next step is purifying the DNA from other cell contents, i.e. lipids, proteins and PCR inhibitors, as a final step the DNA is isolated (Carpi *et al.,* 2011). Whatever the extraction techniques used all samples must be carefully handled to avoid sample to sample cross-contamination or any other laboratory contaminant introduced during the process. Thus, most laboratories process evidence samples in controlled environments at separate times and in a separate location to reference samples. The first use of cell lysate for DNA isolation process was executed in 1869 by Friedrich Miescher (Dham, 2004).

There are numerous DNA extraction techniques available and the process of deciding which is suitable for your sample must be chosen to fit the process. Commonly for forensic case work it is divided into solution-based, column-based extraction, or recently

the use of magnetic beads has increased in popularity and enormous number of versions of this technology are now commercially available (Tan and Yiap, 2009).

The Chelex 100 is one of the most commonly used in the forensic community. It is an ion exchange resin made up of styrene divinylbenzene co-polymers with iminodiacetate ions, this resin acts like a shield enclosing the DNA during the heating process of the extraction. The chelating properties are assumed to inhibit the amplification process because of the binding magnesium ions ($Mg2^{+}$) (Van Oorschot *et al.,* 2003).

Silica-based extraction protocols are among the most commonly used of the commercially available forensic extraction kits today. The initial step relies on lysing the cell membrane to release the DNA and that is done using a buffer containing detergent such as (SDS, NP-40, and Tween 20) alongside proteinase K. This is followed by the addition of a chaotropic salt to disrupt the protein structure. Several other methods emphasise on the binding properties of the silica. DNA binds to the silica particles and after the washing out steps and all other cellular components are removed the silica particles will suspend the DNA into the solution (Boom *et al.,* 1990).

Phenol-chloroform extraction was widely used but has been gradually phased out because of the toxicity of phenol. The phenol denatures the protein and the DNA is isolated with a phenol-chloroform mixture repeatedly washed for purification (Carpi *et al.* 2011).

FTA paper is the best choice for reference samples and long-term storage. The sample lyses in contact to the FTA card (DNA binds to the paper). The extraction process is simply by washing off non-DNA material leaving only the DNA. Later, the sample is directly ready for PCR (Rockenbauer *et al.,* 2009).

The main aim of DNA extraction in forensic science is to separate DNA from the other materials and/or to remove inhibitors that may influence the analysis, in order to get DNA profiles for a specific investigation (Scherczinger *et al.,* 1997). Each of the methods generate different levels of DNA extraction depending upon the specific type and nature of the sample being analysed. Therefore, choosing the correct extraction process is very important.

1.9 Project Aims

The main objective of this research was to improve the efficiency of the processes of collection and storage up to the point where the evidential material is received at the

laboratory. The effect of the environment on the degradation of the collected samples before they reach the laboratory was assessed and the process were considered.

1.10 Working hypothesis

The working hypotheses were that the collection of biological evidence using swabs is more efficient (i.e. more material is collected) when wetting agents containing detergents are used rather than distilled water. Furthermore, that:

- Post-collection environmental factors will have a significant impact on sample quality.
- The double swabbing technique is the most effective of the ones under consideration.
- A detergent based cell lysis buffer will increase the sample (DNA) recovery.

These hypotheses have been tested using blood and saliva on a variety of substrates with different post-collection time and temperatures.

Chapter Two

Materials and Methods

2.1 Materials

- DNA free forensic cotton swabs (Thermo Scientific).
- Tris-acetate-EDTA (Fisher Scientific).
- Sodium dodecyl sulphate-SDS (Fisher Scientific).
- DNA grade water (Fisher Scientific).
- N-Lauroylsarcosine (Sigma-Aldrich).
- PureGene extraction kit (Qiagen).
- Lambda (λ) DNA (Thermo Scientific).
- Quantifiler Human DNA Quantification Kit (Applied Biosystems).
- Mini 4-plex (in house kit).
- The AmpFlSTR® Identifiler® Plus Kit.
- GeneScan-500 (LIZ) (Thermo Scientific).
- 2X Platinum Multiplex (Thermo Scientific).
- Hi-Di Formamide (Applied Biosystems)
- POP-6 polymer (Applied Biosystems).
- Qubit® dsDNA HS (Thermo Scientific).

2.2 Experimental design

2.2.1 Swabbing techniques

The first part of the research compared three swabbing techniques:

- A single wet/dry swab method; swabbing (one swab) with a wet/dry swab i.e. pipetting the wetting agent at one side of the swab and the other side is dry using both sides to recover the sample;
- the double swab technique (Sweet *et al.,* 1997) using two swabs, one moistened with wetting agent and the other swab dry;
- Directly pipetting the wetting agent up and down of the stain recovering it.

2.2.2 Swabbing buffers

Three wetting agents were used for each of the three methods:

- distilled water (dH_2O),
- Tris-acetate-EDTA (TE Buffer) (10 mM Tris pH 8.0 with HCl and 1 mM EDTA pH 8.0)
- PureGene Cell lysis (Qiagen, Hilden, Germany).

The samples were exposed (post-collection) to various environmental conditions (i.e. -20 °C, room temperature 19-22 °C, 37 °C and 50 °C) and then the DNA was extracted.

2.2.3 Substrates

The experiments undertaken in this research were designed to simulate sample recovery from a crime scene including storage and transfer to the laboratory. Three separate trays were used to hold three types of materials as substrates; glass (domestic window glass), plastic (polypropylene) and metal (aluminium). Grids were drawn with a marker pen on each kind of material. Blood and saliva samples were pipetted into all the squares of the grids, row by row (one for each wetting agent and recovery method). In addition, an extra grid left empty as a negative control (Fig 2).

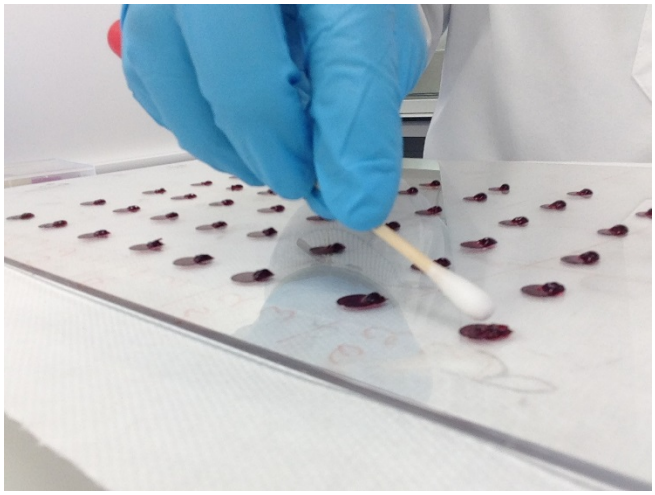

Figure 2 Blood spots on a plastic substrate recovered with a moistened cotton swab

All materials and equipment were cleaned thoroughly to remove any possible contamination before being used in the experiments; the glass, metal and the plastic were first cleaned with 70% (volume/volume) ethanol solution, and then swilled clean with deionised water before being attached to the tray.

2.2.4 Samples

In this part of the research a known sample size of (50 µL, 30 µL and 10 µL) was deposited in the grids for collection.

2.2.4 a) Saliva

Saliva was collected from one person (the researcher). Saliva was collected in a screw capped sterilised tube. Before collecting the saliva, the person chewed the inside of the cheek for around 3-4 min. Care was taken that samples were collected at least one hour after eating and/or brushing teeth.

2.2.4 b) Blood

Blood was collected from one person (the researcher). The blood that was needed was extracted and put into EDTA tubes by the University Phlebotomist in accordance with the University Guidelines on drawing blood and COSHH regulations.

2.3 Collection methods and post-collection treatment of samples

During the initial stage of the research, samples were recovered from the individual squares on the grids of substrates in turn using one swab, double swabbing and pipetting.

With all three techniques, samples were taken comparing all three wetting agents 50 µL of each sample spot was used with dH_2O, TE buffer, PureGene Cell lysis (Qiagen). Samples were produced in triplicate for each of the variables. With the swabbing techniques, once the swabs were laden with the sample, the heads of the swabs were cut off with sterile disposable scalpel and placed immediately in a sterile 1.5 mL Eppendorf tubes® (Eppendorf, Stevenage, UK) and sealed, whereas the pipetted samples were pipetted directly into the same type of tube.

2.4 DNA Extraction

The cotton swab heads were pealed; cutting the swab head longitudinally with a clean cross-linked blade, cross sterilised with 70% ethanol solution, and then all the cotton material detached from the wooden handle before starting the extraction.

The PureGene DNA Extraction kit (Qiagen) was used in this step and the extraction was undertaken according to the manufacturer's recommended conditions and procedures.

A total volume of 300 µL of Cell Lysis solution was added to the sample tubes containing the cotton material from the swab and then 3 µL of PureGene proteinase K (10 mg/mL) (Qiagen) was added to the sample tubes. The samples were then incubated at 56 °C for 2 h, and the samples vortexed every 30 min. After the end of the incubation period, the peeled cotton from the swab heads was removed from the tube by scraping it to the sides of the tube and squeezed with sterilised tweezers to recover as much liquid as possible. To prevent cross-contamination the tweezers were cleaned between each sample using a clean tissue and 70% (volume/volume) ethanol solution. The liquids from the wet and dry swabs for each sample were then combined into one tube. Then an amount of 100 µL of protein precipitation solution (Qiagen) was added to each tube and the tube was vortexed vigorously at high speed for 20 s; the samples were then chilled for 5 min on ice.

Using a digital micro-centrifuge, the chilled samples were centrifuged at full speed (13,300 rpm) for 3 min. The supernatant solution was then added to a new clean and labelled 1.5 mL microfuge tubes each containing 300 µL of isopropanol alcohol. The contents of the tubes were then mixed by inverting gently several times. The resulting samples were then incubated at -20 °C for 20 min. The samples were centrifuged again for 5 min at full speed (13.300 rpm). After centrifugation, the supernatant was carefully discarded; and the tubes were drained by inverting them carefully on a clean piece of absorbent paper taking care that the pellet of DNA remained in the tube. A total volume of 300 µL of 70% ethanol (volume/volume) was then added to the tubes, and inverted several times to wash the DNA pellet. The samples were then centrifuged again for 1 min at full speed (13,300 rpm). Again, the supernatant was carefully removed, the tubes drained on a clean piece of absorbent paper taking care that the DNA pellet remained in the tube, and then the tubes were allowed to dry for 10 min. Finally, 50 µL of DNA Hydration Solution was added to the tubes and vortexed for 5 s. The final DNA solution (Qiagen) of DNA from the samples was then stored either at 4 °C for short-term storage (any period less than a week) or at -20 °C for long-term future use.

2.5 DNA Quantification

In the first part of the research the extracted DNA from each sample was quantified using a Thermo Scientific NanoDrop 2000 Spectrophotometer. After completing the quantitation, the samples were again stored in the fridge either at 4 °C for short-term storage (any period less than a week) or at -20 °C for long-term storage. The same samples were also quantified and visualised using agarose gel electrophoresis to assess

the quantity and quality of DNA. Agarose gel electrophoresis was carried out using 1.5% (weight/volume) agarose gel made from Fisher Scientific (Pittsburgh, USA) agarose powder in a gel tray tank (10 x 8 cm) which was submerged in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA).

The DNA samples were prepared for electrophoresis as follows: 2 µL of the extracted DNA samples were separately placed into PCR tubes, with 3 µL gel loading buffer (loading dye) and 5 µL of dH_2O. These samples were briefly vortexed, centrifuged and loaded in to the wells of the gel. In addition, a serial dilution of 10 ng/uL, 5 ng/uL and 1 ng/uL of Lambda (λ) DNA standard (Thermo Scientific) was prepared from a lambda DNA stock of (500 ng/µL) with TE buffer.

The Lambda (λ) DNA serial dilution (Thermo Scientific) and 3 µL of gel loading buffer (loading dye) were placed into PCR tubes, briefly vortexed and centrifuged and then loaded into the wells. The gel was run at 100 V for 15 to 20 min; gel was removed from the gel tank and visualized using a UV transilluminator (Bio Doc-It imaging system, Hercules, USA).

The amount of DNA extract loaded onto the gel could be visualised by the relative brightness of the resultant bands when compared to the DNA standards.

Extracted DNA samples from blood and saliva were quantified using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Warrington, U.K.) using an ABI 7500 real-time PCR machine (Applied Biosystems). Amplification reactions and amounts used were as recommended by the manufacturer. 1 µL of target DNA was amplified with 11.5 µL of prepared master mix of 5.25 µL of Quantifiler human primer mix and 6.25 µL Quantifiler PCR reaction mix to give a final total volume of 12.5 µL. DNA standards were prepared following the manufacturer's recommended concentrations.

A MicroAmp optical 96-well reaction plate (Applied Biosystems) was placed on its base (MicroAmp splash free 96 well-bases) and 11.5 µL of master mix was loaded separately into the wells. 1 µL of each DNA standard concentration was loaded into its corresponding well in duplicate. 1 µL of the extracted DNA samples were then loaded on the plate into the appropriate wells and the plate was sealed with an optical adhesive cover (Applied Biosystems). The plate was then placed into the ABI 7500, which was already prepared for running DNA quantification. The thermal cycler protocol was performed in accordance with the manufacturer's instructions (Applied Biosystems): Holding stage 1, 50 °C for 2 min and holding stage 2 at 95 °C for 10 min followed by 40

cycles of a two-step cycle; step 1 at 95 °C and step 2 at 60 °C. After completion of amplification, the DNA concentration for each sample was measured in ng/µL.

2.6 DNA Amplification

2.6.1 The mini 4-plex: The DNA was amplified using an in-house assay that amplifies four amplicons 50 bp, 70 bp, 112 bp, and 154 bp amplicons. The primer mix was prepared according to the optimized PCR condition (Table 1).

Table 1 primer concentrations of the mini 4plex PCR reaction

Forward and Reverse Primers (5′-3′)	Concentration in the mix (µM)	Primers length	Amplicon Length (bp)
TGGATTACATGCTGCCCTACT	1.2	21	50
TGGTACCCAAGTGTTGATATCCA	1.2	23	
ACCCAGCCACTTGCACAT T	1.3	19	70
TTTCCCTCCATGGATGATGT	1.3	20	
GAGGGAGCTCAAGCTGCAA	1.2	19	112
GTGCTCATTCCTCGCCCT	1.2	18	
TCGGGGACTCAAGAGGAAGA	1.3	20	154
GCAGTTGGCGATCTTCTTCA	1.3	20	

The multiplex PCR was prepared with a total reaction volume of 10.0 µL; 5.0 µL 2X Platinum® Multiplex PCR Master Mix (Applied Biosystems), 0.6 µL of primers mix, 3.4 µL of dH_2O, and 1 µL of DNA template were added. Four different sets of primer concentrations were prepared to optimise and balance the mini 4-plex peaks heights. The amplification was carried out using the 7500 real-time PCR (Applied Biosystems). The thermal cycler conditions were prepared according to the optimized PCR condition (Table 2). The amplified products were stored at 4 °C for further use.

Table 2 Thermal cycler conditions for the mini 4-plex PCR reaction amplification

PCR Stages		Temperature (°C)	Time
Initial incubation		95	2 min
Denaturation	28 cycles	95	30 s
Annealing		60	1.5 min
Extension		72	60 s
Final extension		60	30 min
Hold		4	∞

2.6.2 The AmpFlSTR® Identifiler® Plus Kit: The PCR Reaction was prepared with a total reaction volume of 25 µL; 10 µL AmpFlSTR® Identifiler® Plus Master Mix and 5 µL AmpFlSTR® Identifiler® Plus as for the test DNA sample. Add 10 µL of the diluted sample to the reaction mix. The amplification was carried out in GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). The thermal cycler conditions were prepared according to the optimized PCR condition (Table 3). The amplified products were stored at 4 °C for further use.

Table 3 Thermal cycler conditions for multiplex PCR reaction amplification

PCR Stages		Temperature (°C)	Time
Initial incubation		95	11 min
Denaturation	28-29 cycles	94	20 s
Annealing/ Extension		59	3 min
Final Extension		60	10 min
Hold		4	∞

2.7 DNA Analysis

2.7.1The mini 4-plex: Each sample was prepared by adding 1.0 µL of PCR product to 8.5 µL of Hi-Di Formamide (Applied Biosystems) and 0.5 µL GeneScan 500 LIZ size standard (Applied Biosystems). The samples were then heated at 95 °C for 3 min and snap-cooled -20 °C for 3 min. DNA fragment analysis was carried out on ABI 3500 Prism® Genetic Analyzer in a 50 cm long capillary using POP-6 polymer (Applied Biosystems). Fragment analysis 50_POP6 run module was used with dye sets DS – 33 (filter set G5): 6 – FAM (blue), VIC® (green), NED (yellow), PET® (red) and LIZ® (orange). The parameters of ABI 3500 POP_6 that were used in this stage are as shown in Table 4.

Table 4 Parameters of ABI 3500 POP_6 module used in the mini 4-plex

Parameters	Values
Run temperature	60 °C
Pre – run voltage	15 kV
Pre – run time	180 s
Injection voltage	1.6 kV
Injection time	5 s
Run voltage	15 kV
Run time	2700 s

2.7.2 The AmpFlSTR® Identifiler® Plus Kit: For each sample was prepared for the AmpFlSTR® Identifiler® Plus (by adding 1.5 µL of PCR product or allelic ladder (one for each injection) to 8.5 µL of Hi-Di Formamide (Applied Biosystems) with 0.5 µL GeneScan 500 LIZ size standard (Applied Biosystems). The samples were then heated at 95 °C for 3 min and snap-cooled -20 °C for 3 min. DNA fragment analysis was carried out on ABI 3500 Prism® Genetic Analyzer (Applied Biosystems) in a 50 cm long capillary using POP-6 polymer (Applied Biosystems). Fragment analysis 50_POP6 run module was used with dye sets DS – 33 (filter set G5): 6 – FAM (blue), VIC® (green), NED (yellow), PET® (red) and LIZ® (orange). The parameters of ABI 3500 POP_6 that were used with this kit are as shown in Table 5.

Table 5 Parameters of ABI 3500 POP_6 module used with the AmpFlSTR® Identifiler® Plus kit.

Parameters	Values
Run temperature	60 °C
Pre – run voltage	15 kV
Pre – run time	180 s
Injection voltage	3 kV
Injection time	7 s
Run voltage	15 kV
Run time	1430 s

2.8 Data Analysis

The data obtained from the capillary electrophoresis (CE) were analysed using ABI 3500 GeneMapper® ID-X Software Version 1.2 (Applied Biosystems). The parameters for the analysis of DNA profiles were kept consistent for every run (Table 6).

Table 6 Parameters of the ABI 3500 GeneMapper® ID-X Software used for the analysis of PCR fragments.

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

Statistical analysis of the DNA concentrations recovered from the samples was carried out to investigate the significant differences between multiple techniques and analyses that were undertaken as well as the quality of the DNA by comparing peak heights measurements. Calculations of averages (avg.) and the standard deviations (S.D.) were obtained using Excel 2010. While R Studio software was used to perform independent sample t-tests used to find out the difference in quantitative variables among two groups and analysis of variances (ANOVA) to analyse the difference among group means depending on the normality of the variables. A value of p-value=0.05 was taken as significant.

Chapter Three

Collection Protocols

3.1 Introduction

An effective Forensic DNA analysis protocol should start with the earliest stage, i.e. with the isolation of the crime scene before the collection process starts. The recovery of biological samples at a scene of a crime can be challenging. When collecting biological samples for DNA analysis, it is important to collect as much of the sample as may be needed in the laboratory for analysis (Rudin and Inman, 2010).

The ability to recover a DNA profile from biological samples is significantly enhanced when a careful, well thought out, protocol for the collection of biological materials is observed. In comparison to the development of PCR methodology, limited attention has been given to the development of protocols that focus on the collection, transfer and pre-lab storage of biological samples that are destined for DNA analysis, especially when there are extreme environmental conditions. The mechanisms of natural degradation of DNA are well understood (Hu *et al.,* 2005) and temperature and moisture content pay a large role.

Several techniques are commonly used to collect biological samples from the crime scene, but in this chapter, we have focussed on the use of cotton swabs, and direct recovery method by pipetting, and the use of different wetting agents for recovery such as buffer fluid to moisten the swab head. The wetting agent can play an important part in enhancing both the recovery and the stabilisation of the DNA (Van Oorschot *et al.,* 2003)

The main aim of this chapter is find the most efficient collection protocol for the recovery of DNA from biological material found at the crime scene.

3.2 The collection process:

In this part, we investigated the effect of various collection protocols for the recovery of biological samples from the crime scene. We included the several swabbing techniques and wetting agents, and used time and temperature as the post-collection variables.

Subsequently, saliva was used as the sample biological material. Saliva samples were collected from one person (the researcher). Care was taken that samples were collected at least one hour after eating and/or tooth brushing. The saliva was collected and stored in a screw capped sterilized tube.

To simulate a crime scene sample collection, the saliva was deposited on various household substrates. Three separate trays were used each holding one of the three different materials that were used as substrates; glass (domestic window glass), plastic (polypropylene) and metal (aluminium). In each tray grids were draw on the substrate with a marker pen. 50 µL of saliva was then pipetted into each of the squares of the grids, row by row (one square for each wetting agent and recovery method and repeated to give triplicate of each). An extra grid was left empty as a negative control.

All materials and equipment were thoroughly cleaned to remove any possible contamination before being used in the experiments; the substrates – i.e. the glass, metal and the plastic were initially cleaned with 70% (volume/volume) ethanol solution, and then rinsed clean with distilled water and air dried before being attached to the tray.

Three collection techniques were used to recover the samples:

single wet/dry swab method; swabbing (one swab) with a wet/dry swab i.e. pipetting the wetting agent at one side of the swab and the other side is dry using both sides to recover the sample.

The double swab technique (Sweet *et al.,* 1997) using two swabs one wet with wetting agent and the other swab is dry, first swab moistens the sample and the second recover the deposited rest from the grid.

Direct collection by pipetting the wetting agent on to and off the stain thus recovering a sample and then directly depositing it into the tube ready for extraction.

When evaluating the three collection methods samples were recovered from the individual squares in turn using a wetting agent. Three different wetting agents were compared:

- Distilled water (dH_2O),
- Tris-acetate-EDTA (TE Buffer) (10 mM Tris pH 8.0 with HCl and 1 mM EDTA pH 8.0)
- PureGene Cell lysis (Qiagen)

Once the swabs were laden with the samples, the effect of temperature and time stored at that temperature was considered. One batch of tubes was stored in the -20 °C freezer, a second batch was left on the laboratory bench (the temperature was monitored every 3 h and was ~19-22 °C), a third batch was stored in an oven at 37 °C and a fourth batch was stored at 50 °C. All the batches were maintained at their temperatures for a range

of set times (6 h, 24 h and 48 h) before extraction. At the end of these set times, the DNA was extracted using PureGene extraction kit (Qiagen).

3.3 The quantification process:

3.3.1 NanoDrop 2000

The extracted DNA from all samples were quantified using Thermo Scientific NanoDrop 2000 Spectrophotometer which was connected to a Toshiba laptop. Samples were thawed to room temperature before quantification. The laptop was switched on and opened to the NanoDrop Program. Nucleic Acid application was selected for this analysis and before measuring the samples, a blank was measured (confirming that the pedestal was clean and the instrument was performing correctly). To measure a blank: the sampling arm was raised and 1 µL of Hydration Solution was pipetted onto the lower measurement pedestal. The sampling arm was closed and a spectral measurement initiated using the operating software on the PC. After measuring the blank, the pedestals were wiped on both sides using a clean wipe. 1 µL of the DNA sample to be measured was placed onto the lower measurement pedestal and the spectral measurement was initiated using the software on the laptop. Pedestals were cleaned using a clean wipe between each sample. After quantitation, the samples were either stored in the fridge (4 °C) for short-term storage (any period less than a week) or in the freezer (-20 °C) for long-term storage. The same samples were also quantified and visualised using the agarose gel electrophoresis.

3.3.2 Agarose gel

The extracted DNA samples were assessed using AGE (agarose gel electrophoresis) to see the quantity and quality of DNA. AGE was carried out using 1.5% (weight/volume) of agarose gel made from Fisher Scientific in a 12 cm x 6 cm gel tray tank which was submerged in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA).

The DNA samples were prepared for electrophoresis as follows: 2 µL of the extracted DNA samples were separately placed into PCR tubes, with 3 µL gel loading buffer and 5 µL of dH_2O. These samples were briefly vortexed, centrifuged and loaded in to the wells of the gel. In addition, a serial dilution of 10 ng/uL, 5 ng/uL and 1 ng/uL of Lambda (λ) DNA standard (Thermo Scientific) was prepared from a lambda DNA stock of (500 ng/µL) with TE buffer.

The Lambda (λ) DNA serial dilution (Thermo Scientific) and 3 µL of gel loading buffer (loading dye) were placed into PCR tubes, briefly vortexed and centrifuged and then loaded into the wells. The gel was run at 100 V for 15 to 20 min; gel was removed from the gel tank and visualized using a UV transilluminator (Bio Doc-It).

3.4 Results:

There were several points and aspects considered for this chapter of the research, the comparison of the swabbing techniques,

- wetting agents,
- substrates

Everything were evaluated at a range of post-collection times and temperatures.

All saliva samples were measured and the average of the triplicate samples for each variable calculated, tabulated and analysed statistically to explain if there was a significant difference resulting from the different sample collection techniques and/or storage conditions.

able 7 Average DNA concentration of samples (in triplicate) using double swab.

Double Swab		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
Cell lysis (Qiagen)	-20	12.01	5.02	13.97
	RT	11.17	3.75	10.98
	37	10.68	3.39	10.09
	50	8.45	2.40	9
TE Buffer	-20	12.44	4.22	11.53
	RT	10.43	3.58	9.98
	37	8.70	2.85	8.13
	50	8.33	2.62	7.54
Distilled Water	-20	10.50	3.97	10.07
	RT	9.54	2.64	9.44
	37	7.81	2.31	2.74
	50	6.70	1.22	6.20

Table 8 Average DNA concentration of samples (in triplicate) using one swab.

One Swab		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
Cell lysis (Qiagen)	-20	11.9	4.53	12.70
	RT	8.99	2.94	10.16
	37	7.65	4.01	5.09
	50	6.78	1.96	6.48
TE Buffer	-20	10.28	4.78	11.02
	RT	8.82	3.04	10.03
	37	6.17	2.60	8.25
	50	3.74	2.83	7.48
Distilled Water	-20	9.70	3.44	8.9
	RT	7.55	3.06	5.25
	37	4.33	1.33	6.01
	50	2.57	0.41	3.26

Table 9 Average DNA concentration of samples (in triplicate) using direct pipetting.

Direct pipetting		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
Cell lysis (Qiagen)	-20	11.76	5.80	10.86
	RT	9.78	2.74	9.68
	37	7.38	2.04	8.18
	50	4.13	1.1	3.83
TE Buffer	-20	10.26	3.81	12.49
	RT	10.61	2.40	9.99
	37	8.83	1.79	7.21
	50	5.56	1.36	4.24
Distilled Water	-20	9.19	2.17	11.92
	RT	6.74	0.8	3.37
	37	3.87	0.02	2.89
	50	2.63	0.04	1.25

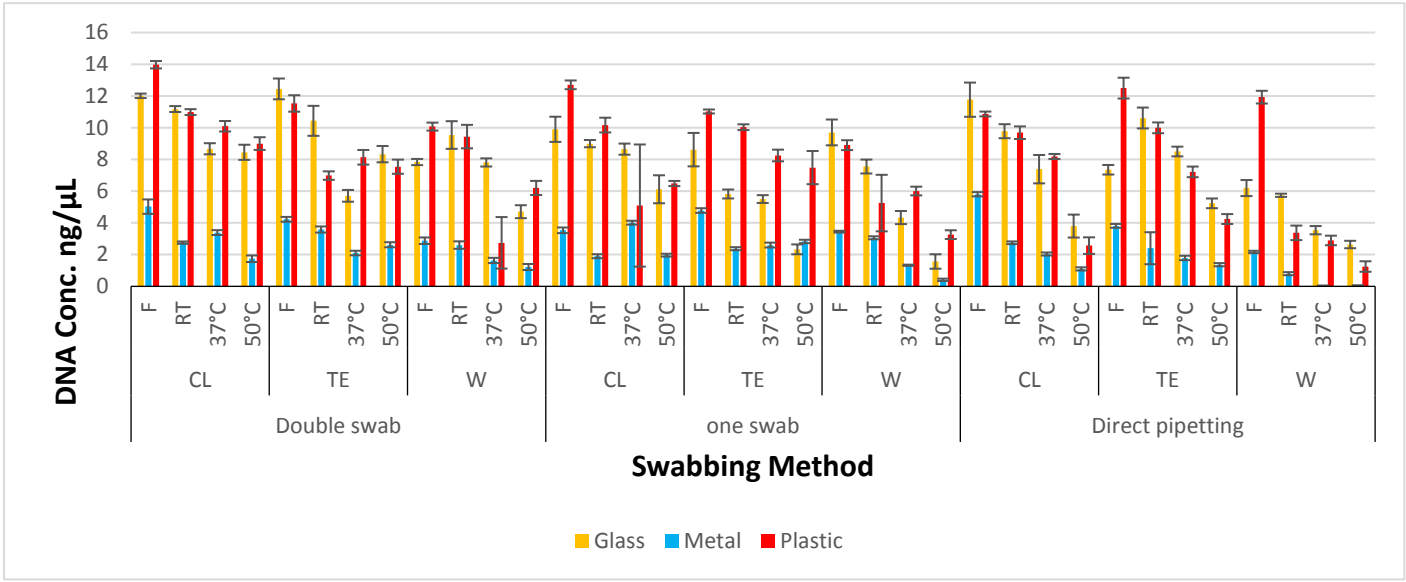

Figure 3 The comparison of the swabbing methods against wetting agents and Post-collection temperatures.

Based upon DNA concentration measured in ng/µL of DNA recovered from the saliva samples as shown in Table 7, it is evident that the double swab technique is consistently the best collection method when compared to the single swab technique or the direct pipetting method. The other two methods were eliminated from the research at this point and hereafter only the double swab technique as a collection method in this research.

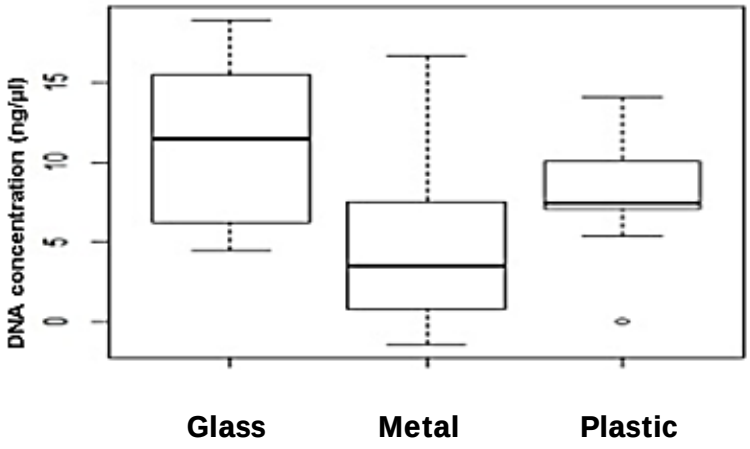

Figure 4 Substrate comparison of the average DNA concentration of saliva samples (in triplicate).

When comparing the results of the average DNA concentration of saliva samples (in triplicate) from the different substrates, (Fig 4). The highest concentrations of DNA were recovered from glass substrate however was the least consistent while the plastic (polypropylene) substrate showed more consistent results, while samples taken from the aluminium substrate had the lowest concentrations of DNA.

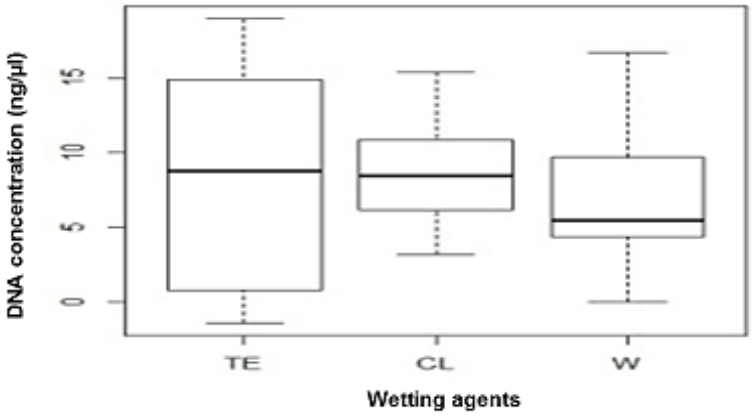

Figure 5 Comparing the effect of three wetting agents (TE buffer, Cell lysis, distilled water) on DNA concentration comparing the average DNA concentration of saliva samples (in triplicate).

When looking at the wetting agents used, Cell Lysis (Qiagen) was the most consistent in comparison to water and TE buffer. On the other hand, the use of TE buffer gave a wider range of results with different post-collection temperatures (Fig 5).

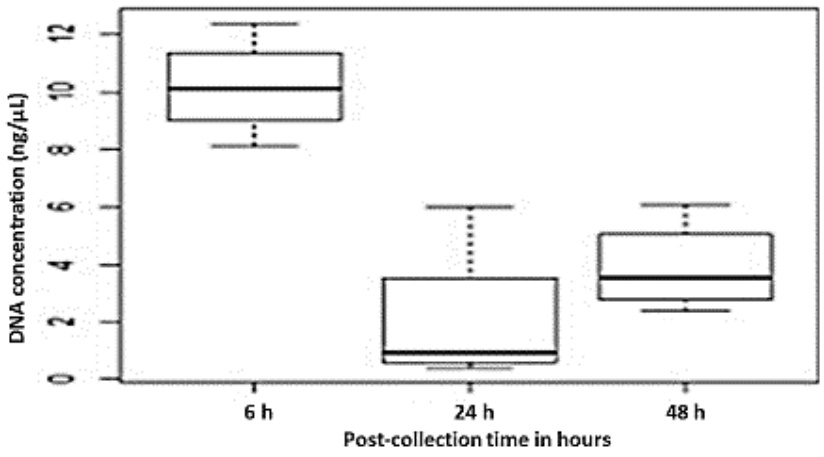

Figure 6 Comparing the effect of post-collection time on the average DNA concentration of saliva samples (in triplicate) recovered.

When testing the effect of post-collection time on sample recovery, 6 h storage time gave the highest DNA recovery rates in comparison to 24 h and 48 h (Fig 6). Surprisingly, 48 h gave higher results than 24 h however this could be because the NanoDrop measurement of DNA is non-human specific and could reflect the microbial growth in the sample. Therefore, measuring bacterial and human DNA yielding higher concentration.

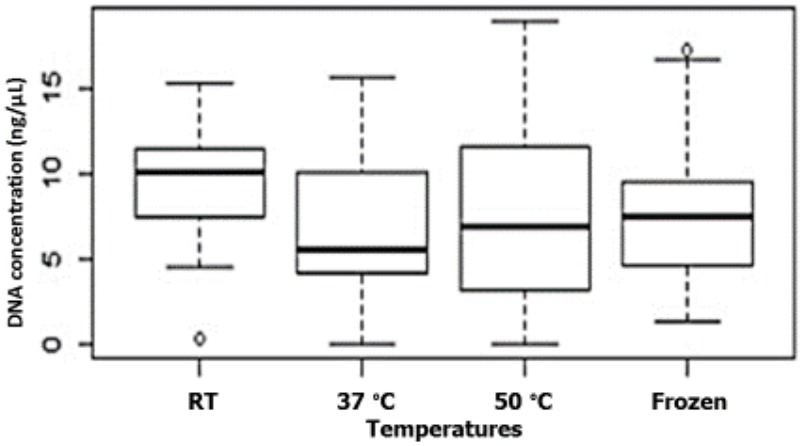

Figure 7 Comparing the effect of post-collection temperatures of ~22 °C (RT), 37 °C, 50 °C, -20 °C (Frozen) on the average DNA concentration of saliva samples (in triplicate).

Fig 6 shows the effect of post-collection temperatures of ~22 °C (RT), 37 °C, 50 °C, -20 °C (Frozen). Room temperature and -20 °C showed most post-collection stability of the samples as far as concentration of DNA recovered room temperature and -20 °C gave the best results for post-collection sample stability during storage.

Analysis of variance of the results (ANOVA) was carried out, using Excel (2013) and R the statistical computing software (version 3.1.1), on the DNA quantification data from the NanoDrop, to see if there was a significant difference between the different groups (p-value<0.05). The ANOVA showed that, the DNA quantity was significantly affected by the different substrates ($F_{2,33}$=64.38, p =.00000407), however, it was less significant with the wetting agents ($F_{2,33}$=0.809, p=0.454), and by temperatures ($F_{3,32}$=1.79, p=0.169).

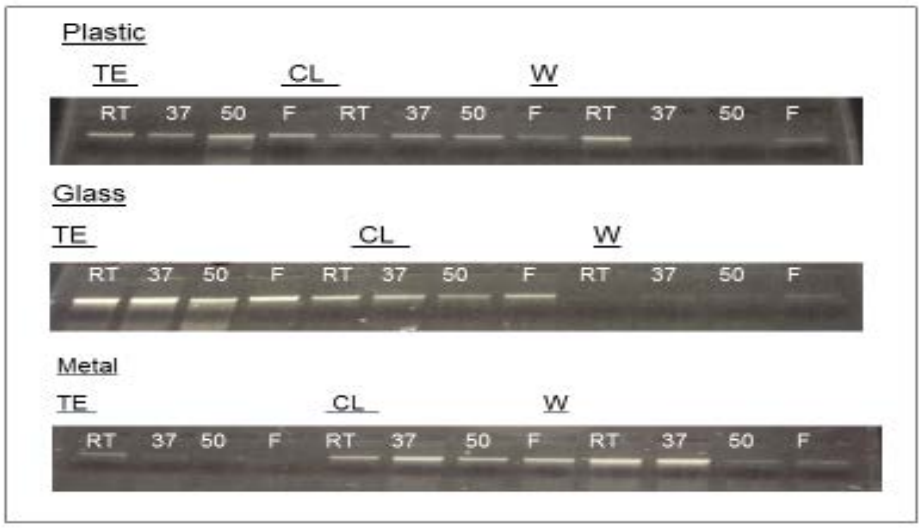

Figure 8 1.5% (weight/volume) agarose gel showing post-collection temperatures of ~22 °C (RT), 37 °C, 50 °C and -20 °C (F) and the use of cell lysis buffer (CL), TE buffer (TE) and distilled water (W).

The initial results using conventional sampling solutions such as distilled water, demonstrated that it is sometimes difficult to obtain high levels of DNA from the sample. Moreover, the downstream stability of the DNA samples can easily be affected by high storage at temperatures above room temperature (~22 °C).

3.5 Discussion

The results of this part of the research show that post-collection environment factors have a significant impact on DNA recovery rates.

However, the NanoDrop results gave such a wide range of concentrations that it raised questions about its suitability studies that are human specific. NanoDrop measures both human and bacterial DNA therefore where bacterial DNA is present higher than expected DNA concentrations can be measured. The samples used in this part of the study were saliva therefore we must accept that the normal flora bacteria present in the mouth would affect the reliability of the NanoDrop results as the technique is non-human specific. Further investigation was undertaken to demonstrate the extent of this effect and is reported in the next chapters.

This research did show that the recovery of biological material using the detergent-based PureGene Cell lysis buffer as a wetting agent improved the quantity of DNA recovered

and that the stability post-collection was greatly improved in comparison to using ultrapure water as a wetting agent. DNA degradation was seen after approximately 6 h at room temperature when ultrapure water was used as a wetting agent. While the detergent-based solution (PureGene Cell lysis) stabilised the collected DNA longer even when the temperature was increased to 50 °C. The impact of this is likely to be limited in circumstances where crime scene evidence can be kept at low temperatures until it reaches the laboratory. However, in contexts where maintaining low temperatures is problematic, a modified method for collection using a detergent-based solution could have a large impact on the preservation of forensic evidence before it reaches the laboratory.

Furthermore, the results showed that, of the collection techniques evaluated, the use of the double swab technique was the best recovery method; this is probably due to the fact that the first wet swab loosens the epithelial cells and then the second dry swab picks loosen epithelial containing the DNA, consequently having a positive impact on the quantity of DNA recovered and the quality of the DNA profile generated. Given that the second dry swab provides sufficient DNA to generate a profile, the processing of both the wet and dry swabs together in the extraction gives even better results.

Finally, using the cell lysis at the initial stage of collection isolates the DNA at an early stage and stabilises the sample at the earliest possible time therefore minimising degradation before extraction.

Chapter Four

Buffer Development

4.1 Introduction:

Improving the DNA quality and quantity in biological samples received from crime scenes is of critical importance for forensic laboratories, especially when dealing with compromised samples where limited quality and quantity of evidential material is available.

The most common practice in crime scene investigations involves the collection of samples from surfaces using a swab, most frequently moistened with sterile water, which sometimes may be followed by a second, dry swab (Sweet *et al.,* 1997) to retrieve cells left at the crime scene. This technique has been used on all types forensic samples, however, water is not necessary the optimum wetting agent for DNA recovery. Researchers have suggested that it would be possible to use cotton swabs moistened as an alternative to water, a special developed buffer or wetting agent designed to loosen and solubilize cells and thereby increase the DNA yields (Thomasma, and Foran, 2013).

The main aim for this chapter was to develop an effective collection buffer with the swabbing technique to recover the highest DNA yield of the sample.

4.2 Buffer recipe comparison:

Results of using lysis buffer as a wetting showed that while the recovery of biological material using the detergent-based PureGene cell lysis buffer (Qiagen) as a wetting agent is better and the stability post-collection is greatly improved in comparison to when using ultrapure water as a wetting agent, DNA degradation can be seen after approximately 6 h at room temperature. However, the detergent-based solution (PureGene Cell lysis) stabilized DNA longer when the temperature was increased. In an attempt to improve the results of the first stage of this research, it was decided to investigate if it was possible to develop a new wetting agent to improve the recovery of the DNA from samples.

The fact that the use of PureGene cell lysis (Qiagen) as a wetting agent worked well in the first stage was used as a basis for the development. Six different recipes were developed (Table 10) based upon research of the literature; first four recipes were prepared with different concentrations of sodium dodecyl sulphate SDS (0.5% and 2%) (weight/volume) from Fisher Scientific with the addition of Tris-HCl and EDTA from Sigma-Aldrich (Thomasma *et al.,* 2013); solutions were prepared with and without sodium chloride. The final two recipes included the anionic surfactant n-lauroylsarcosine

with Tris-HCl and EDTA, one with sodium chloride the other without sodium chloride. These materials were chosen because they are relatively inexpensive and freely available in most laboratories.

Table 10 The six lysis buffer recipes developed

Solution	Component
Lysis buffer 1	SDS 0.5% (w/v) Tris-HCl 10 mM EDTA 0.1 mM
Lysis buffer 2	SDS 0.5% (w/v) Tris-HCl 10 mM EDTA 0.1 mM NaCl 50 mM
Lysis buffer 3	SDS 2% (w/v) Tris-HCl 10 mM EDTA 0.1 mM
Lysis buffer 4	SDS 2% (w/v) Tris-HCl 10 mM EDTA 0.1 mM NaCl 50 mM
Lysis buffer 5	1% n-lauroylsarcosine (w/v) Tris-HCl 10 mM EDTA 0.1 mM
Lysis buffer 6	1% n-lauroylsarcosine (w/v) Tris-HCl 10 mM EDTA 0.1 mM NaCl 50 mM

4.3. Materials and methods used:

Saliva and blood from the researcher were used in this part biological samples and polypropylene as the substrate, because the results of the previous stages had shown it to be the substrate that gave the most consistent data. A plastic (polypropylene) board was placed on a tray. The plastic board was cleaned thoroughly before being used; it was cleaned with 70% ethanol solution (volume/volume), and then rinsed clean with deionised water before being fixed to the tray. Once fixed in the tray grids were drawn, with a marker pen, on the plastic clip board. Blood and saliva samples were pipetted into all the squares of the grids (sample size was 50 µL), row by row (one for each wetting agent, post-collection time and temperature in triplicate). In addition, an extra grid was left empty to represent a negative control.

Samples were recovered from the individual squares on the grids of substrates in turn using the double swab technique (Sweet *et al.,* 1997). Samples were used to compare all six buffers developed as a wetting agent, with distilled water dH_2O, and PureGene cell lysis (Qiagen). A volume of 120 µL of each wetting agent was used to recover the spot. As in the previous stage of the research, one swab moistened with the wetting agent and the other swab was dry, the first swab moistens the sample and the second recovers the rest of the deposited sample from the grid.

One batch of tubes was stored in the -20 °C freezer, a second batch was left on the laboratory bench (~ 20°C), a third batch was stored in an oven at 37 °C and a fourth batch was stored at 50 °C. All the batches were maintained at their temperatures for a range of set times (3 h, 6 h, 24 h and 48 h) before extraction.

Samples were later extracted with PureGene Extraction kit (Qiagen) and quantified with Quantifiler Human DNA Quantification kit. Later, the DNA was amplified using an in-house assay that amplifies four amplicons 50 bp, 70 bp, 112 bp, and 154 bp amplicons.

4.4 Buffer development results:

4.4.1 Real-Time PCR quantitation results:

All saliva samples were quantitated using (Quantifiler), the average of the triplicate samples for each variable was tabulated (Table 11 and 12) and compared (Fig 9). All detergent base lysis buffers gave an overall higher DNA concentration result in

comparison to water. However, lysis buffer 6 gave the highest concentration for all the post-collection temperatures used.

Table 11 Average DNA concentration of samples (in triplicate) of extracted saliva samples after 6 h.

Saliva		
Wetting agent	Temperature (°C)	DNA Conc. in ng/µL
Cell lysis (1)	-20	2.34
	RT	2.10
	37	1.12
	50	0.32
Cell lysis (2)	F	2.54
	RT	2.20
	37	1.33
	50	0.52
Cell lysis (3)	F	2.34
	RT	2.00
	37	1.00
	50	0.22
Cell lysis (4)	F	2.87
	RT	2.30
	37	1.300
	50	0.32
Cell lysis (5)	F	3.15
	RT	2.70
	37	1.80
	50	0.59
Cell lysis (6)	F	3.65
	RT	3.00
	37	2.00

	50	0.71
Cell lysis (Qiagen)	F	2.00
	RT	1.62
	37	1.32
	50	0.32
Distilled Water (Water)	-20	1.65
	RT	1.00
	37	0.88
	50	0.08

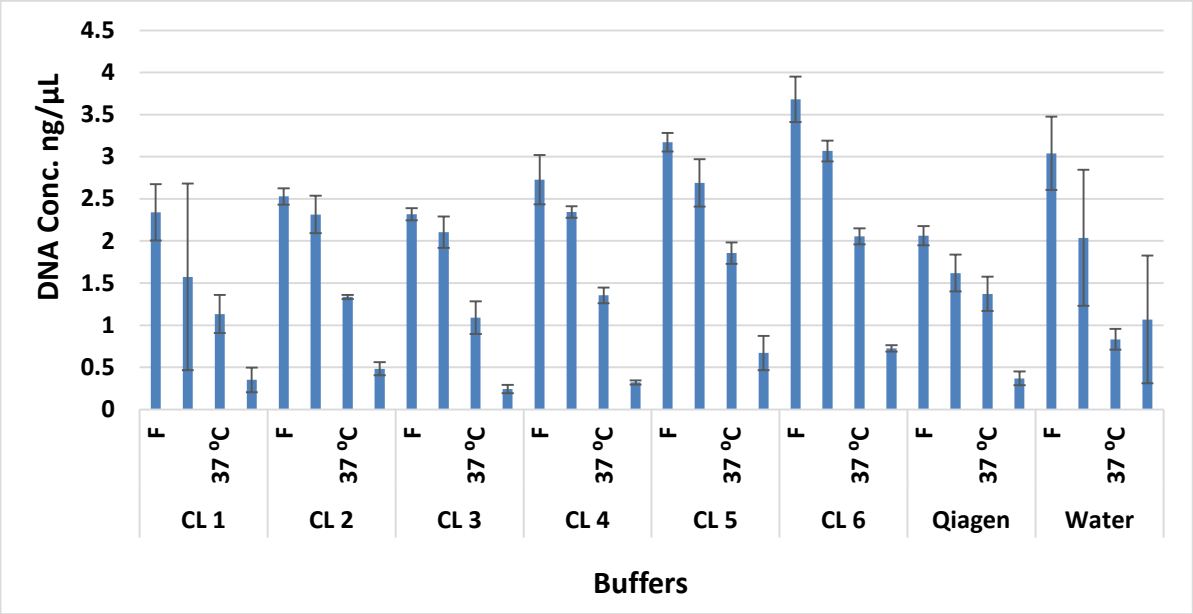

Figure 9 Average concentration of saliva samples (in triplicate) measured using real-time PCR to compare wetting agents used for recovery (6 h post-collection time).

On the other hand, blood samples were quantified as well, using (Quantifiler) the average of the triplicate samples for each variable were tabulates and compared in (Fig 10). All detergent base lysis buffers gave an overall higher DNA concentration results in comparison to water. However, lysis buffer six gave the highest concentration for all the post-collection temperature used.

Table 12 Average DNA concentration of samples (in triplicate) of extracted blood samples after 6 h.

Blood		
Wetting agent	Temperature (°C)	DNA Conc. in ng/µL
Cell lysis (1)	-20	2.90
	RT	2.30
	37	1.70
	50	1.02
Cell lysis (2)	F	3.10
	RT	2.50
	37	1.90
	50	1.22
Cell lysis (3)	F	3.07
	RT	2.60
	37	1.90
	50	1.32
Cell lysis (4)	F	3.17
	RT	2.70
	37	2.00
	50	1.52
Cell lysis (5)	F	3.45
	RT	3.00
	37	2.50
	50	1.99
Cell lysis (6)	F	4.09
	RT	3.79
	37	2.50
	50	1.91

Cell lysis (Qiagen)	F	2.62
	RT	2.12
	37	1.42
	50	0.92
Distilled Water (Water)	-20	2.23
	RT	1.90
	37	1.21
	50	0.62

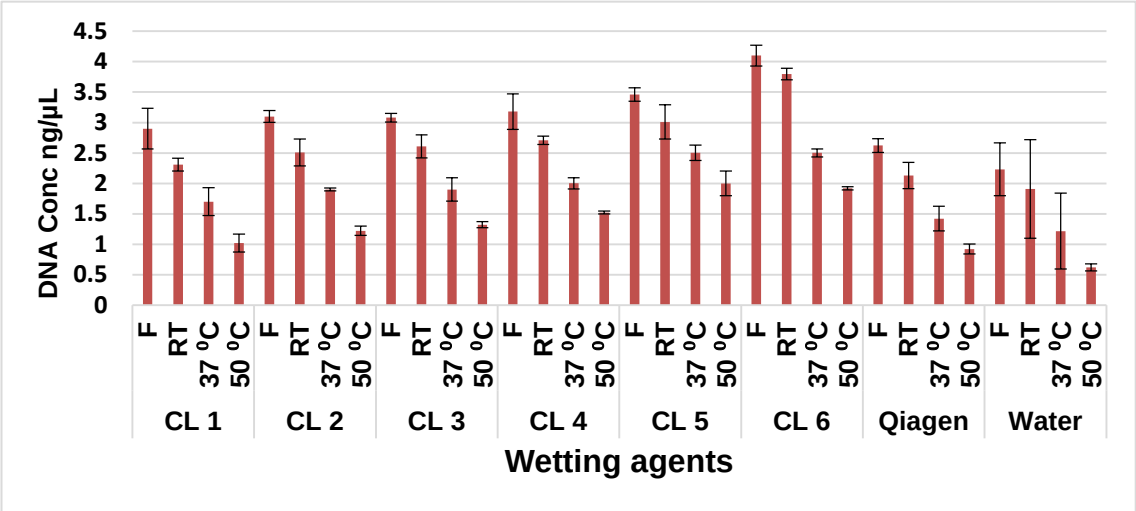

Figure 10 Average concentration of blood samples (in triplicate) measured using real-time PCR to compare wetting agents used for recovery (6 h post-collection).

As the lysis buffer six gave the highest results for concentration of DNA recovered from blood and saliva samples the other variables were investigated using only two of the wetting agent's lysis buffer 6 and distilled water. Despite its poor performance in the earlier tests water was included again in this stage for comparison in addition, it is the most common used wetting agent. We can see that with the lysis buffer DNA stability is maintained up to 48 h while when distilled water was used the concentration decreased with time (Figs. 11 and 12)

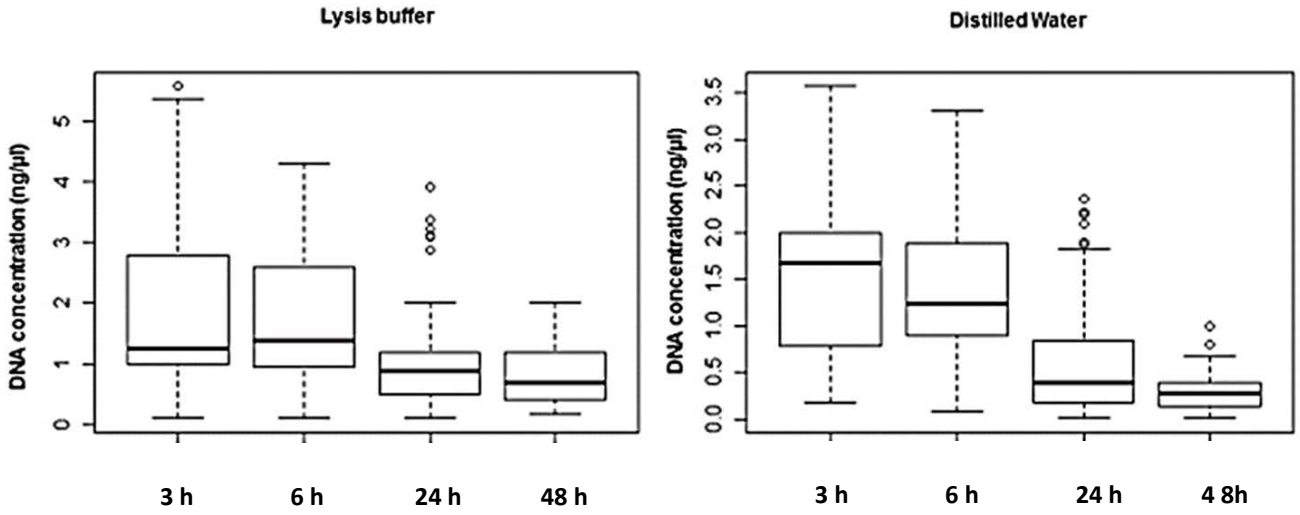

Figure 11 Compares the effect of post-collection time between lysis buffer 6 and distilled water from blood samples on the average DNA concentration in triplicate (Real-time quantitation results in ng/µL)

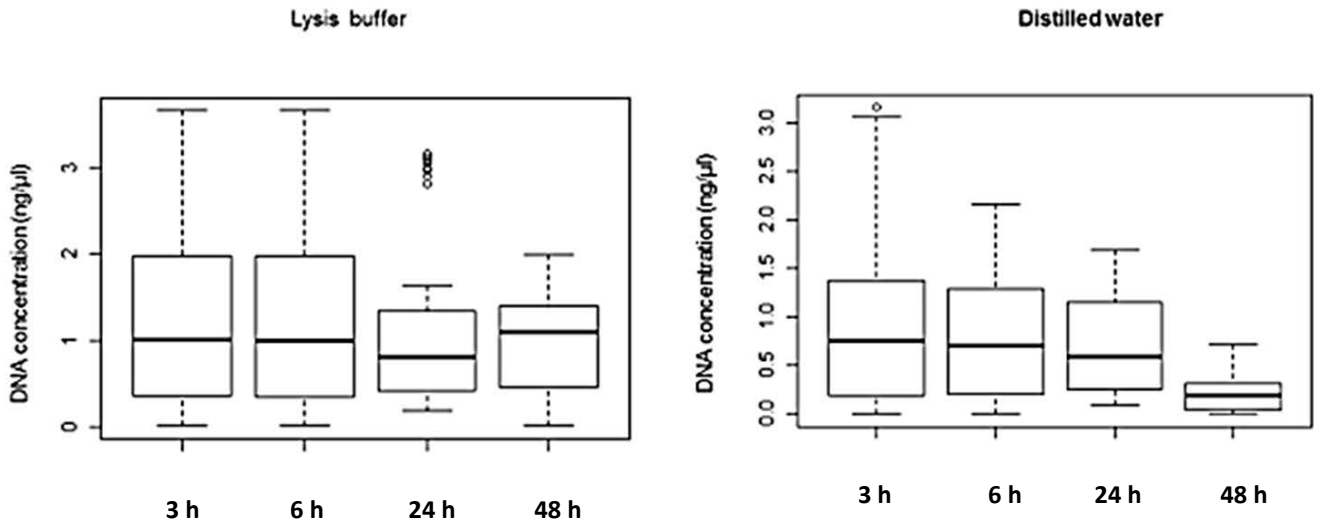

Figure 12 Compares the effect of post-collection time between lysis buffer 6 and distilled water from saliva samples on the average DNA concentration in triplicate (Real-time quantitation results in ng/µL).

When considering post-collection storage temperature, the average of the blood and saliva samples quantified (in triplicate) a significant difference could be seen between lysis buffer six and distilled water at 3 h post-collection and 48 h. Distilled water showed a big drop in DNA concentration after 48 h at all different temperatures whereas the detergent based lysis buffer showed stability after 48 h at 50 °C.

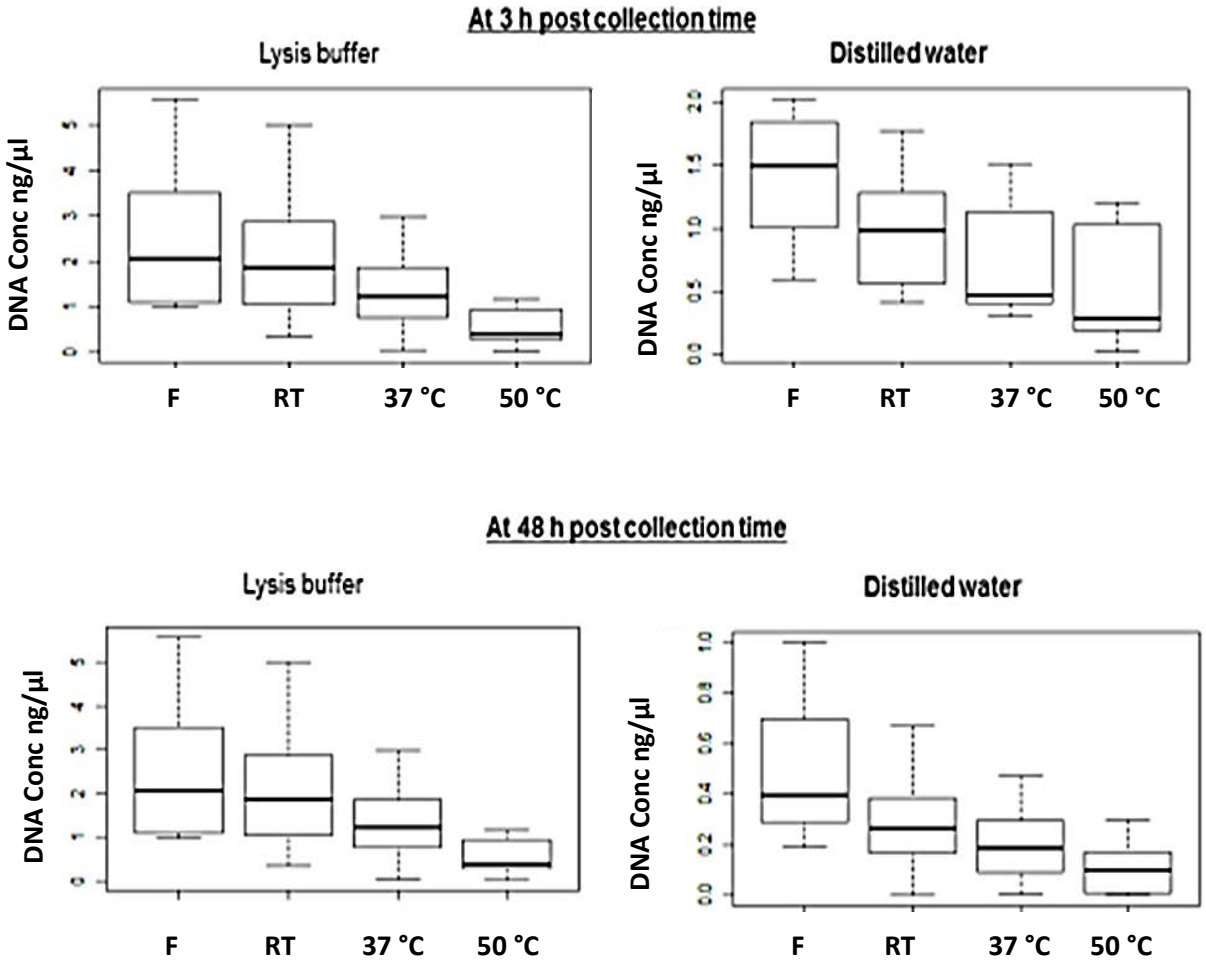

Figure 13 Comparison of the effect of post-collection temperature of both lysis buffer 6 and distilled water on the average DNA concentration from extracted saliva samples in triplicate (Real-time quantitation results in ng/µL)

Saliva and blood samples of known quantities of 50 µL, 25 µL and 5 µL were deposited in triplicate on the grids and then recovered with lysis buffer 6. Using plastic as a substrate with the four post collection storage temperatures, -20 °C direct freezing (appropriate storage temperature), room temperature (the temperature was monitored~19-22°C) average storage temperature, 37 °C (average ambient temperature in some countries) and 50 °C (extreme temperature in some areas), for 24

h. We can see from (Tables 13 and 14) that it was possible to recover a substantial amount of DNA after 24 h at 50 °C, even with as small amount of sample as 5 µL.

Table 13 Average DNA concentration of saliva samples (in triplicate) 24 h post-collection.

Saliva		
Sample quantity	Temperature (°C)	DNA Conc. in ng/µL
50	-20	3.6
	RT	2.8
	37	1.9
	50	0.9
25	-20	2.0
	RT	1.6
	37	1.09
	50	0.3
5	-20	1.0
	RT	0.7
	37	0.6
	50	0.2

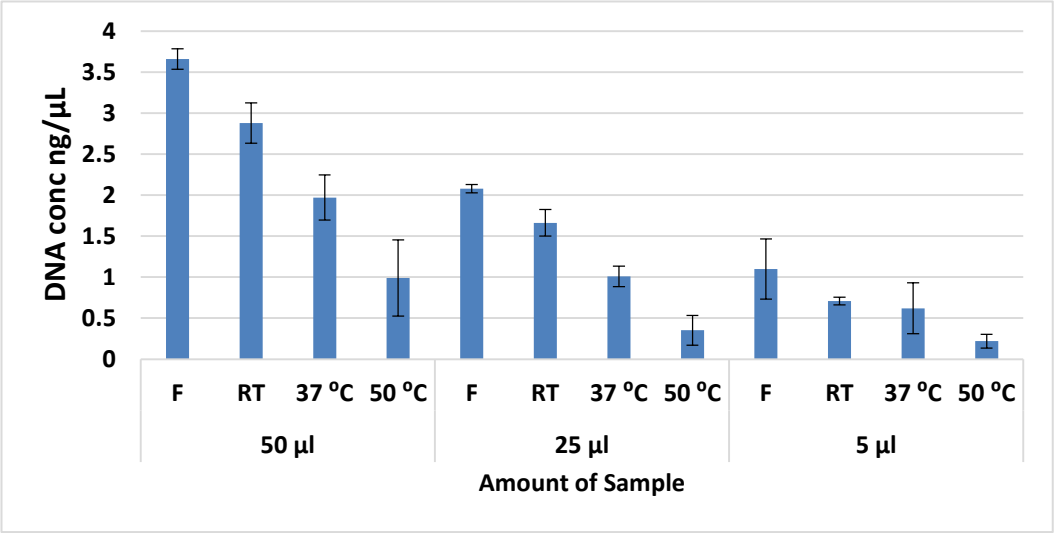

Figure 14 Comparison of saliva sample quantities recovered in relation to the concentration measured (sample deposited to recover 50 µL, 25 µL and 5 µL) 24 h post-collection.

Table 14 Average DNA concentration of blood samples (in triplicate) 24 h post-collection.

Blood		
Sample quantity	Temperature (°C)	DNA Conc. in ng/µL
50	-20	4.0
	RT	3.7
	37	2.2
	50	1.8
25	-20	2.8
	RT	1.9
	37	1.4
	50	0.7
5	-20	1.0
	RT	1.0
	37	0.9
	50	0.3

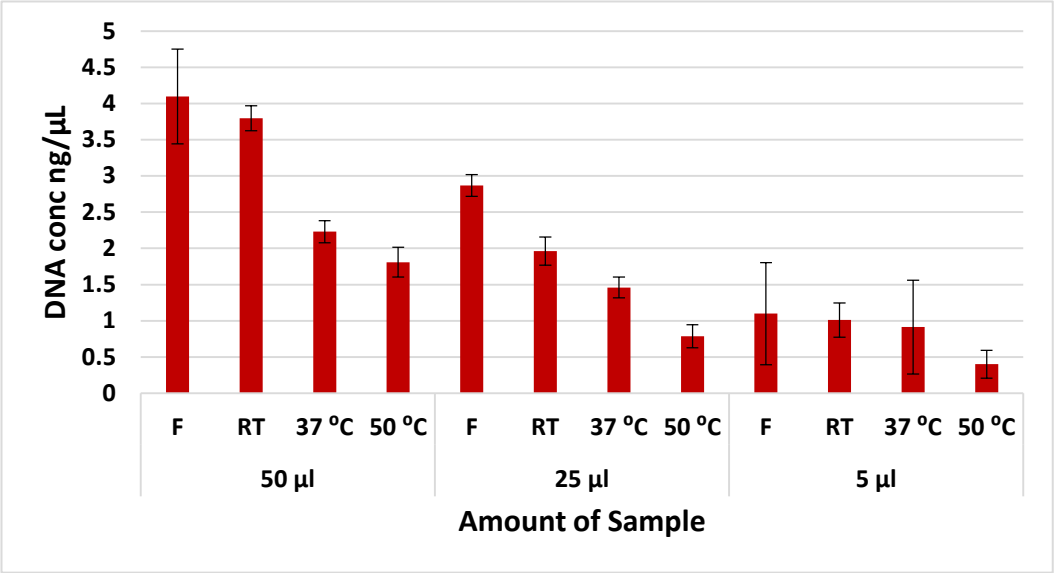

Figure 15 Comparison of blood sample quantities recovered in relation to the concentration measured (sample deposited to recover 50 µL, 25 µL and 5 µL) 24 h post-collection.

4.4.2 Genetic analyser results

In addition to the quantity of DNA recovered, it was important to also investigate the quality. Extracted saliva and blood samples produced during the research were amplified using the in-house mini 4-plex kit amplifying four amplicons 50 bp, 70 bp, 112 bp, and 154 bp to measure the quality of DNA recovered when using lysis buffer and compared it to when water was used as a wetting agent. The variables of post-collection time and temperature of storage were also considered.

It can be seen with the saliva samples shown in (Fig 16 and 17) that when distilled water was used as a buffer when swabbing, the quality of the recovered DNA deteriorated post-collection with both time and temperature. Whereas in (Fig 18 and 19) show that the use of the lysis buffer stabilised the DNA for 48 h after collection and at to temperatures of 50 °C.

Scaling in the electropherograms were adjusted in some figures to show the smaller peaks that cannot be seen at a higher scale.

The results show the same effects with respect to the quality of DNA recovered from blood samples (Fig 20 and 21). With the lysis buffer concentrations of DNA recovered were stable and consistent whereas when water was used as a buffer (Fig 22 and 23) there was a decrease in stability and quality with both time and temperature.

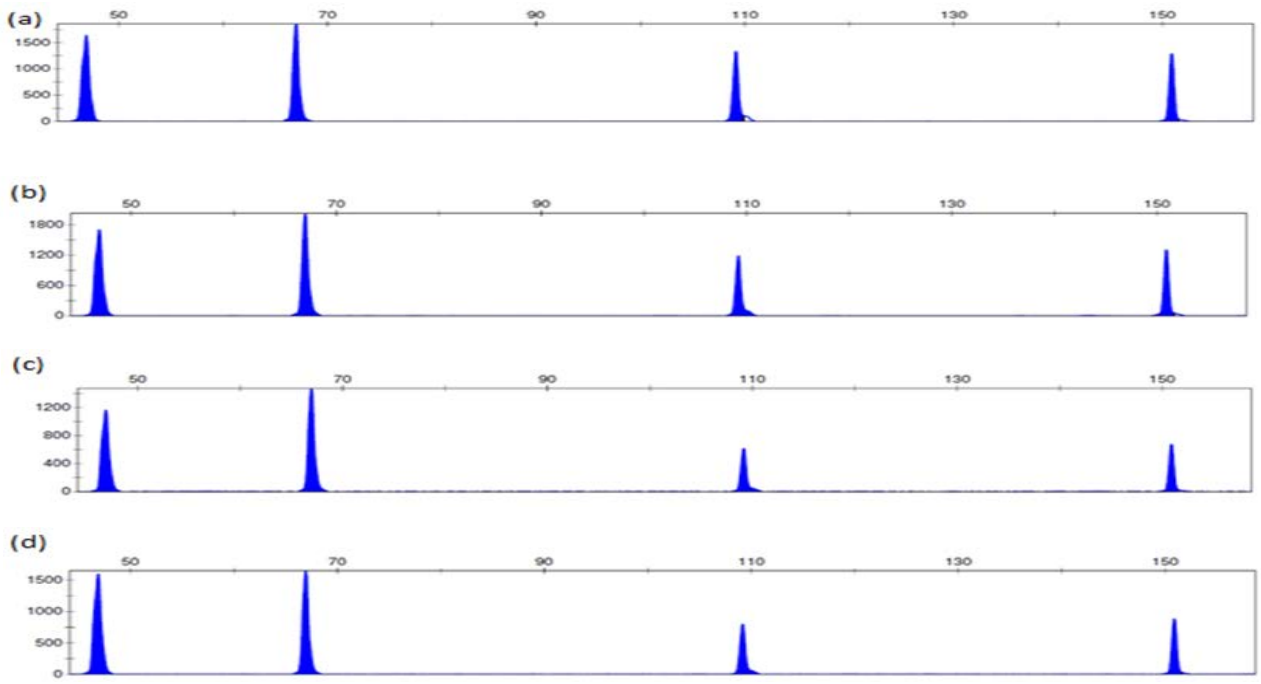

Figure 16 Electropherograms above shows the mini 4-plex amplification of extracted DNA of saliva samples after 3 h collection with water at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

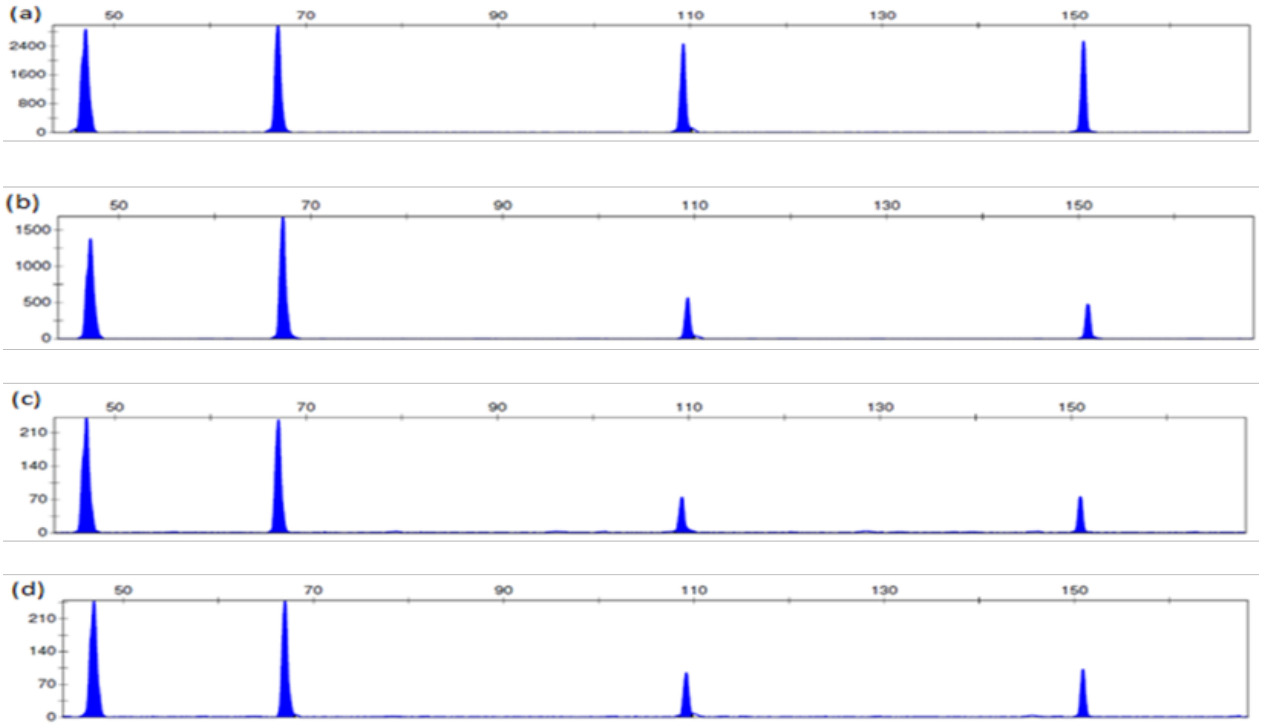

Figure 17 Electropherograms above shows the mini 4-plex amplification of extracted DNA of saliva samples after 48 h collection with water at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

The electropherogram scale was adjusted accordingly to illustrate the differences in peak heights for an improved visual of the results to distinguish when compared.

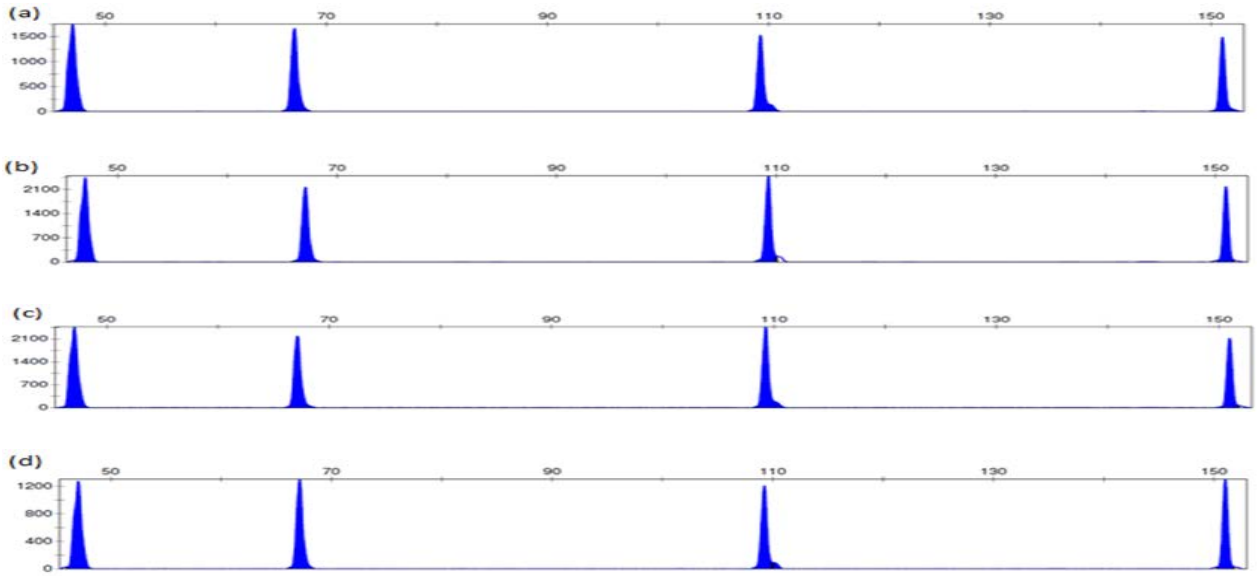

Figure 18 Electropherograms above shows the mini 4-plex amplification of extracted DNA of saliva samples after 3 h collection with lysis buffer at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

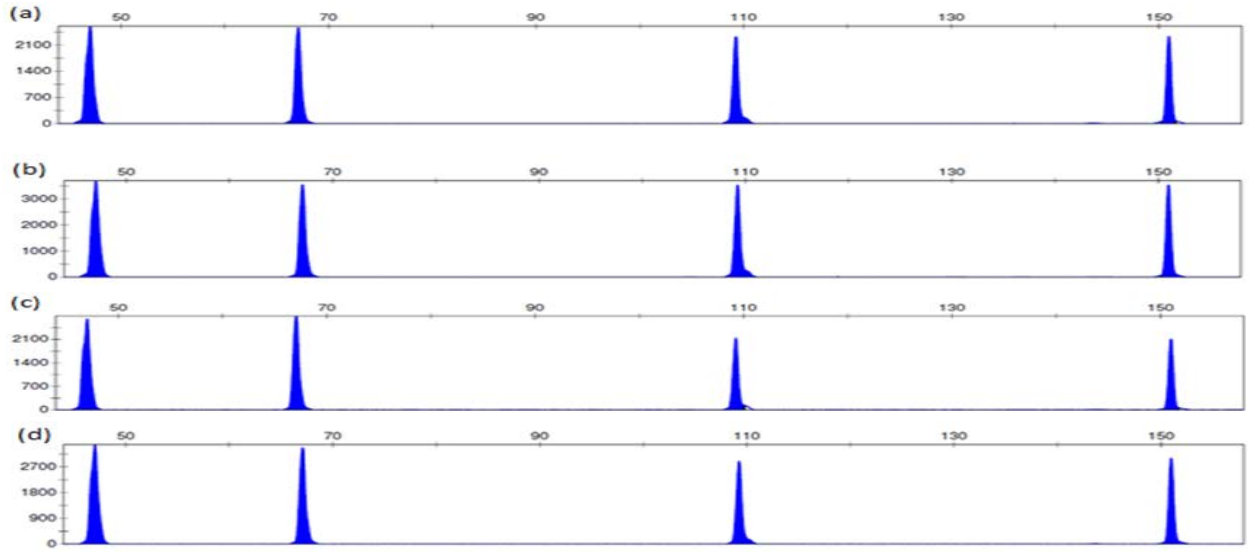

Figure 19 Electropherograms above shows the mini 4-plex amplification of extracted DNA of saliva samples after 48 h collection with lysis buffer at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

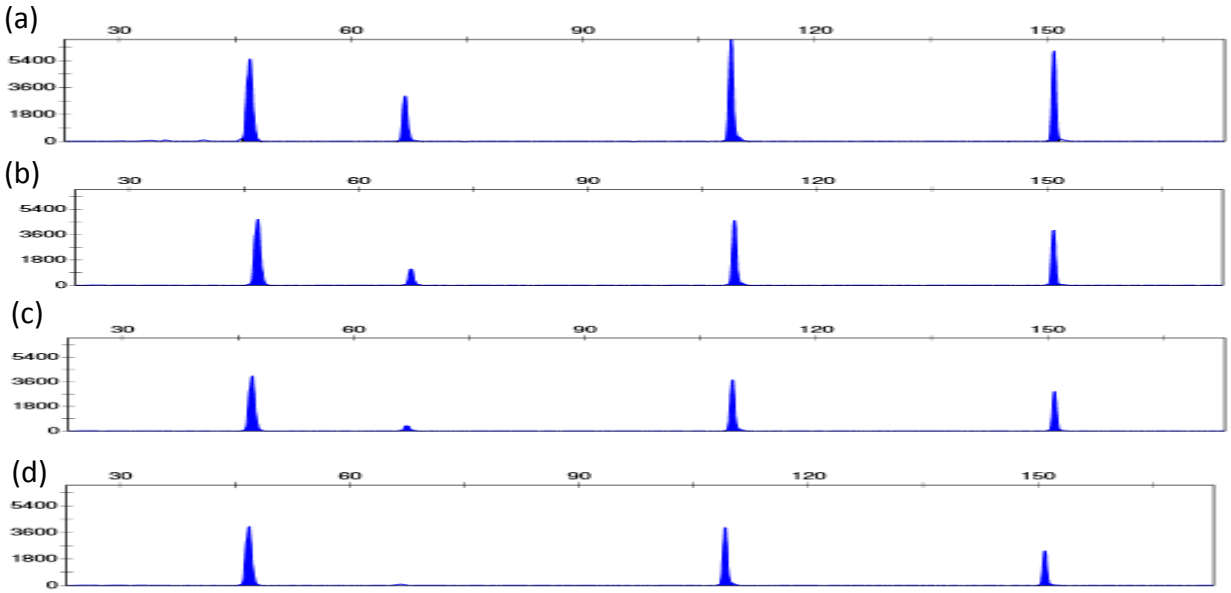

Figure 20 Electropherograms above shows the mini 4-plex amplification of extracted DNA of blood samples after 3 h collection with lysis buffer at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

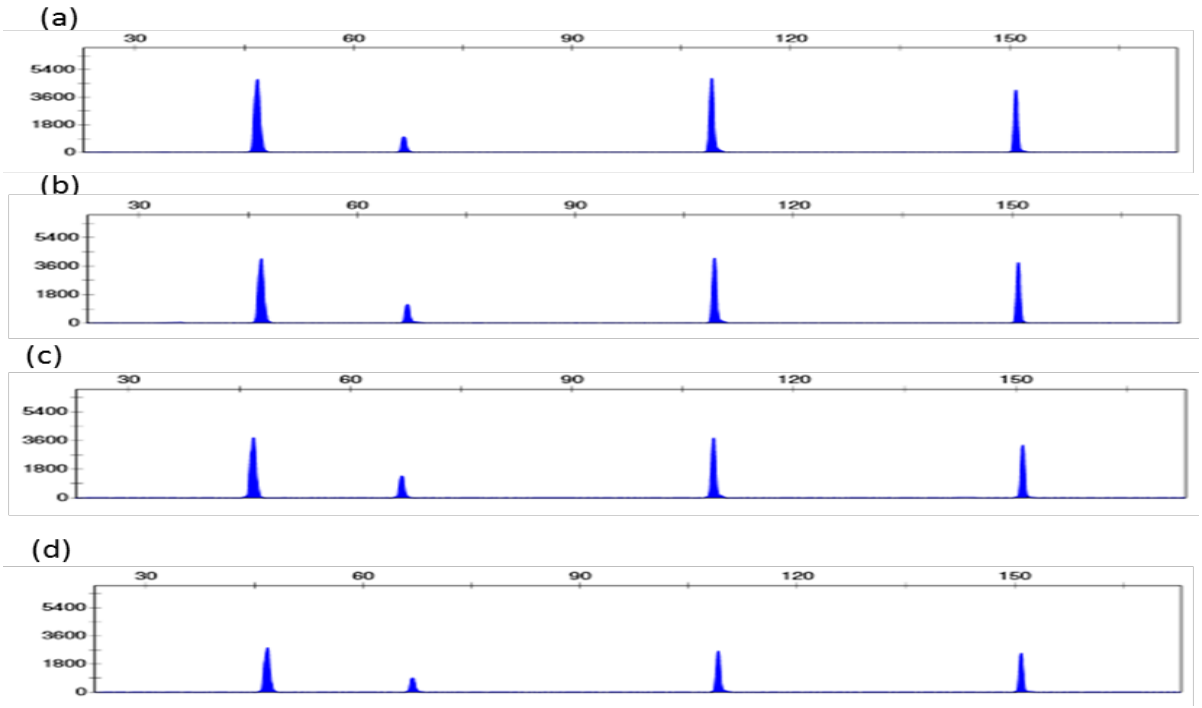

Figure 21 Electropherograms above shows the mini 4-plex amplification of extracted DNA of blood samples after 48 h collection with lysis buffer at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

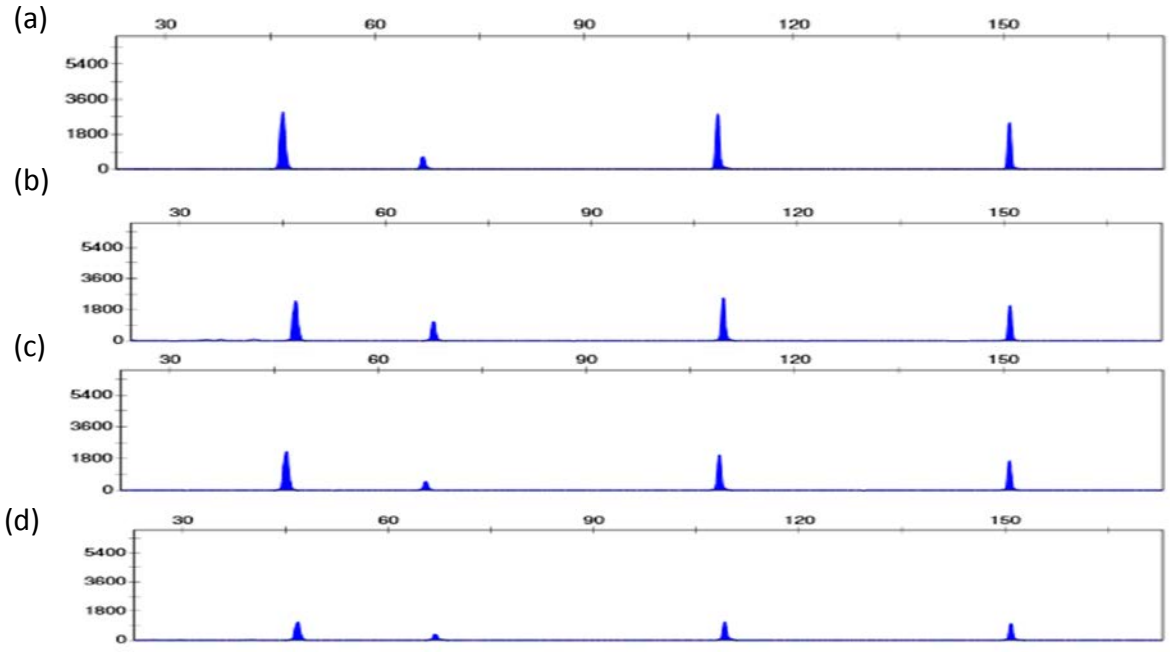

Figure 22 Electropherograms above shows the mini 4-plex amplification of extracted DNA of blood samples after 3 h collection with water at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

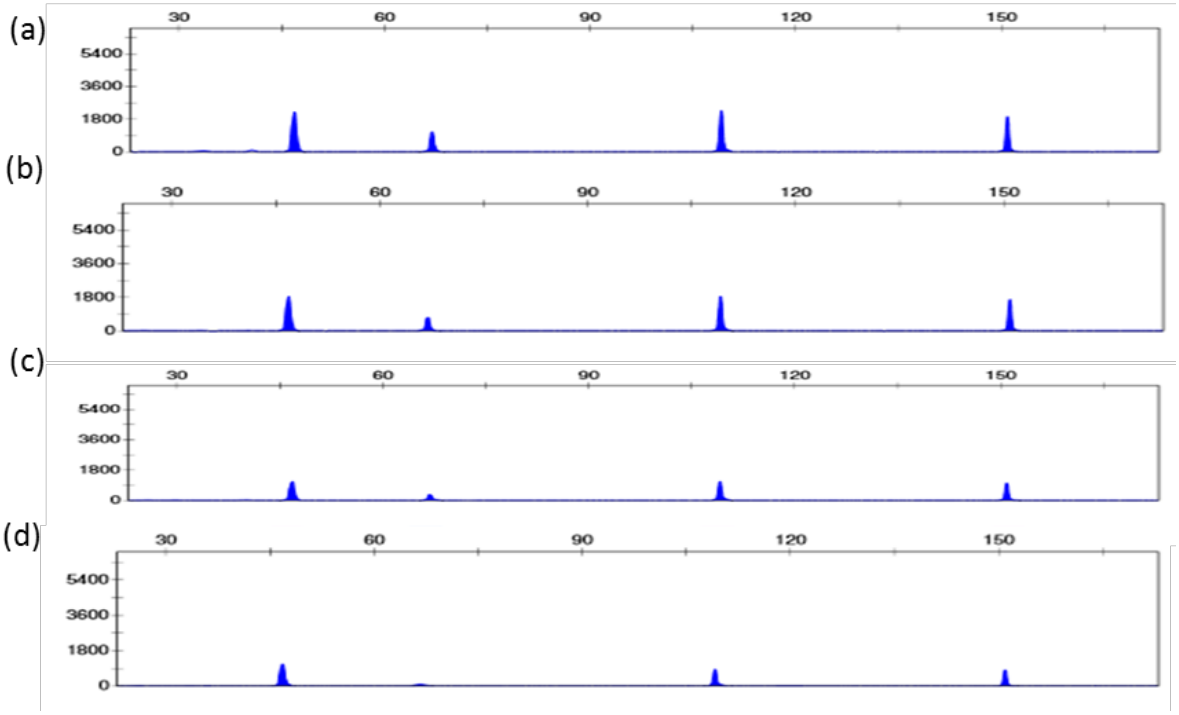

Figure 23 Electropherograms above shows the mini 4-plex amplification of extracted DNA of blood samples after 48 h collection with water at different temperatures (a) -20 °C, (b) Room Temperature, (c) 37 °C, (d) 50 °C.

The tables below show the average, standard deviations and relative standard deviation of DNA samples of blood and saliva peak heights measured by the Genetic Analyser.

Table 15 The average (avg.), standard deviation (S.D) and relative standard deviation (R.S. D%) Peak height (RFU) of extracted DNA from saliva samples collected by using water after 3 h and 48 h, amplified by mini-4plex.

At 3 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	2844	2938	2445	2491	2679.5	247.9	9.25
RT	1375	1656	533	475	1009.7	595.6	58.9
37	315	309	106	145	218.7	108.8	49.7
50	247	243	93	102	171.2	82.2	49.7

At 48 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	1640	1824	1311	1274	1512	265	17.5
RT	1672	1964	1174	1293	1525	361.7	23.6
37	1162	1454	607	674	974.2	404.3	41.5
50	1600	1606	792	872	1217.5	446.3	36.6

Table 16 The average (avg.), standard deviation (S. D.) and relative standard deviation (R.S. D%) Peak height (RFU) of extracted DNA from saliva samples collected by using cell lysis after 3 hand 48 h, amplified by mini-4plex.

At 3 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	3139	2964	3424	3315	3210.5	201.9	6.29
RT	3424	3296	3238	3186	3286	102.3	3.12
37	2686	2774	2114	2083	2414	366.5	15.1
50	3389	3075	2866	2947	3069	229.8	7.5

At 48 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	2530	2305	2540	2179	2388.5	176.8	7.4
RT	2403	2145	2392	2141	2270.2	147	6.4
37	2388	2176	2432	2120	2279	154	6.7
50	2207	2035	2182	1724	2037	222	10.9

Table 17 The average (avg.), standard deviation (S. D) and relative standard deviation (R.S. D%) Peak height (RFU) of extracted DNA from Blood samples collected by using water after 3 hand 48 h, amplified by mini-4plex.

At 3 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	3432	848	3129	2773	8102.25	1163.2	14.35
RT	1642	449	1682	1561	4163.25	591.8	14.21
37	1629	102	1292	1102	3298.5	656.7	19.9
50	755	120	681	4693	2729.2	2106.3	77.1

At 48 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	3312	838	3139	2653	7952.2	1133.20	14.25
RT	1592	443	1581	1493	3989.2	557.9	13.9
37	745	112	661	5781	2963.2	2652.3	89.5
50	771	155	679	634	1763.5	275.7914369	15.63886798

Table 18 The average (avg.), standard deviation (S. D) and relative standard deviation (R.S. D%) Peak height (RFU) of extracted DNA from Blood samples collected by using cell lysis after 3 h and 48 h, amplified by mini-4plex.

At 3 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	4775	1058	4834	4064	11683	1784.5	15.27
RT	3323	835	3136	2643	7954.7	1136.2	14.28
37	3321	831	3134	2633	7944.2	1136.8	14.31
50	2349	1117	2488	2040	6464	616.7	9.54

At 48 h		Peak height (RFU)					
Temperature (°C)	50	70	112	154	Avg.	S. D	R.S. D%
-20	4194	958	4158	3502	10185.5	1530	15.02
RT	2206	1121	2289	1951	6103.7	533.5	8.74
37	2241	524	2014	1684	5200	762.9	14.6
50	1864	785	1863	1695	4935.7	517.3	10.4

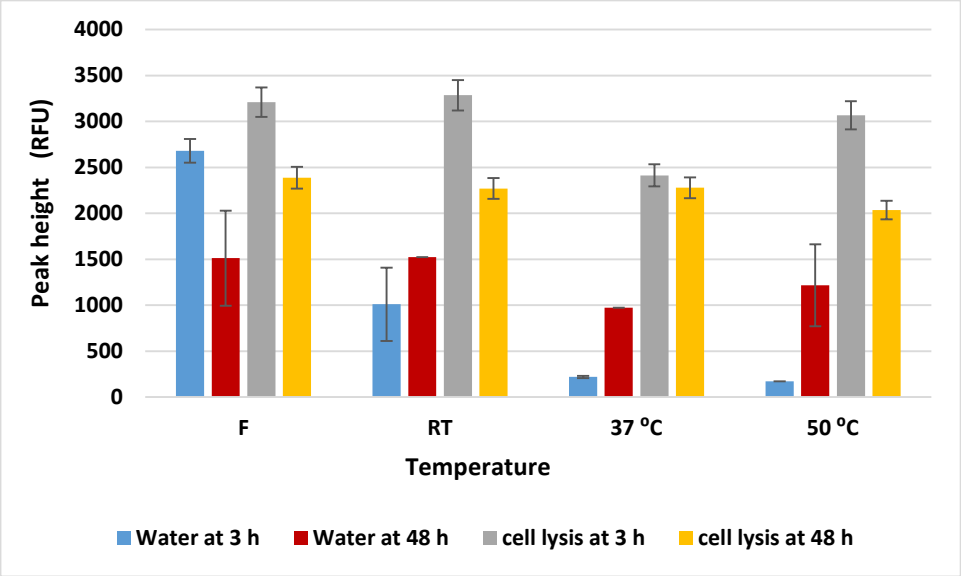

Figure 24 Average peak heights (RFU) of saliva samples recovered comparing the use of lysis buffer and distilled water at 3 h and 48 h and various storage temperatures.

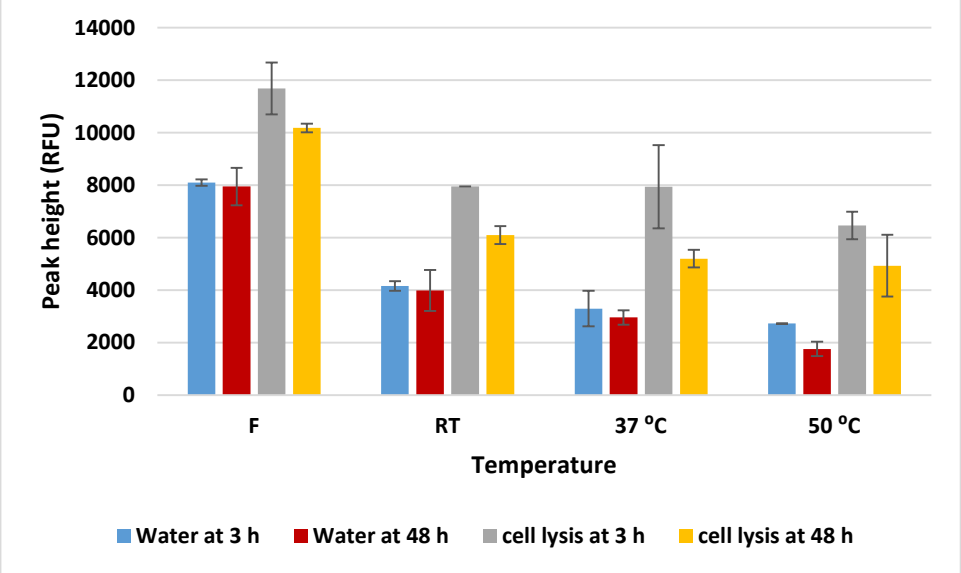

Figure 25 Average peak heights (RFU) of blood samples recovered comparing the use of lysis buffer and distilled water at 3 h and 48 h and various storage temperatures.

4.4.3 Statistical analysis

Statistical Analysis of Variance (ANOVA) was carried out using R Studio software to study the F value and to compare the differences of the peak heights of saliva samples that were collected after different times, (i.e. 3 h and 48 h) using either water or the lysis

buffer, and amplified by mini 4-plex multiplex PCR. The ANOVA results showed that there was no significant difference in height of the peaks of the saliva samples collected using water as the wetting agent after 3 h ($F_{3, 12}$ =1.976, *P*=0.171), but there was a significant difference of the samples collected after 48 h by water ($F_{3, 12}$ =50.43, *P*=4.47e^{-07}) as the *P*-value was less than the significance level (*p*-value<.05). The ANOVA results for the saliva samples were collected using the lysis buffer also showed that there was no significant difference of the samples collected after 3 h ($F_{3, 12}$ =2.78, *P*=0.0867), but the difference was statistically significant differences for the samples collected after 48 h by the lysis buffer ($F_{3, 12}$ =10.6, *P*= 0.001) as the *P*-value is less than 0.05 (Aloraer *et al.*, 2015).

4.5 Sterilisation of Saliva

Several methods are available for the collection of DNA for forensic genetics from biological fluids including the collection of blood; urine; saliva; semen. Each approach has distinct advantages and disadvantages. Obtaining high quality genomic DNA is a critical factor in forensic genetics for achieving a DNA profile. Blood samples are an excellent source of large amounts of genomic DNA, it is the preferred source of DNA with respect to both quality and quantity compared to saliva because of the microbial contamination that is characteristic of saliva. However, saliva is one of the most common body fluid left at crime scenes and incidents and is a valuable sources DNA evidence.

Microbial flora from the (mouth) oral cavity consists of over 700 bacterial species (Aas *et al.,* 2005). The different configuration of the oral microbial flora depends upon by many factors, for example our diet, our body's immune system, induced antibiotic treatment and many other factors (Ruby and Barbeau, 2002). Most of the bacteria in the mouth are from epithelial cells shed into the saliva, and the degree at which bacteria vanishes into the saliva compared to the amount swallowed is the same degree at which they are being exfoliated from the oral mucosa or/and teeth into saliva (Dawes, 2003). Typically, more than 70% of the DNA from a human saliva sample from a normal person is from bacteria (Hu *et al.*, 2012).

Exfoliated buccal epithelial cells found in saliva are a very promising alternative source of DNA because they can be obtained using self-administered, noninvasive, and relatively inexpensive techniques. Buccal swabs and mouthwash protocols are the most commonly used protocols for buccal cell collection. Studies using different types of buccal swabs,

i.e., cotton swabs or cytobrushes, have obtained similar DNA yields and PCR success rates (Calvano *et al.*, 2010). Earlier studies using mouthwash to collect DNA for PCR-based assays used saline rinses that were processed or frozen immediately after collection (Hayney et al., 1996). The study evaluated the stability of saline mouthwash samples stored for 7 days at temperatures to which samples are likely to be exposed if collected and then sent by mail to the laboratory. This study indicated that samples stored at 25 °C and 37 °C tended to have higher amounts of high molecular weight DNA than similar samples stored at lower temperatures (-20 °C and 4 °C), suggesting the presence of DNA of bacterial origin. Similarly, a study conducted by (Walsh et al., 1992) suggested that the DNA on cotton swabs of saliva samples stored for 4 days at 3 °C was predominantly of bacterial origin. In a further study, it was proposed that the use of an alcohol-containing mouthwash would be more appropriate in epidemiological studies for self-collection of samples that are sent by mail. Because the alcohol content is likely to reduce bacterial growth during mailing. The results of their work indicated that buccal swabs treated with alcohol-containing mouthwash could be stored at room temperature or at 37 °C for 7 days without affecting the DNA yields or the ability of the PCR to amplify the DNA in the samples when compared with samples stored at -20 °C (García-Closas *et al.,* 2011).

Some companies that produce home kits for saliva sampling were concerned about the possible presence of bacteria in saliva samples shipped under routine conditions. To address this question and to further prove the robustness and reliability of their products, experiments were conducted by DNAGenotek Inc (Ottawa, Canada) to demonstrate that their Oragene/saliva samples can be “super-pasteurized” (i.e. treated for up to 3 h at 72°C) with no effect on the quality and quantity of human DNA recovered. The Oragene self-collection kit is a non-invasive method for collecting large amounts of DNA. The kits’ ability to release and stabilize DNA from saliva for long periods of time at ambient temperature makes it an ideal collection method. The kit is increasingly being used to collect DNA samples around the world which has led to questions being asked regarding potential pathogens in oral samples.

Pasteurisation the process of killing bacteria from food and drink invented by Louis Pasteur during the nineteenth century. The process is widely used today in the food and drink industry to kill any bacteria and to prolong the shelf-life of the products. The process of pasteurisation involves heating and per the United States of America Food

and Drug Administration (FDA). The requirement is to heat up the product to 71.7 °C for at least 15 s (Penn State University, 2010).

4.5.1 The effect of sterilisation process:

This part of the research investigated the effect of pasteurization on the recovery of DNA from Saliva samples. Saliva was collected from one person (the researcher) and stored in a screw capped sterilized tube. Before collecting the saliva, the person chewed the inside of the cheek for around 3-4 min. Care was taken that samples were collected at least one hour after eating and/or brushing of teeth.

Microscope slides were used as the substrate for this experiment. A tray was used to hold the glass slides, the glass slides were cleaned thoroughly before being used; first with 70% ethanol solution (volume/volume), and then rinsed clean with distilled water before being fixed to the tray.

Once fixed in the tray, grids were drawn, with a marker pen, on the glass slides dividing it into squares. Saliva samples were pipetted into all the squares of the grids on the slides (sample size was 50 µL), with a slide for each wetting agent (water and cell lysis buffer), sterilisation temperature (~77 °C and ~90 °C), post-collection time (6 h, 24 and 48 h) and temperature (-20 °C, room temperature, 37 °C and 50 °C) all in triplicate for each slide. In addition, an extra slide was left empty to use as a negative control. Pasteurisation was achieved by incubating the tray containing the saliva samples on the slides at ~77 °C for 30 min before recovering and another tray for ~90 °C also for 30 min before recovering the samples.

Samples were recovered from the individual squares on the glass slides in turn using the double swab technique (Sweet et *al.,* 1997). When the samples were taken the use of the detergent based buffer developed for the earlier experiments was compared with the use of distilled water (dH_2O) as a wetting agent. A volume of 120 µL of each wetting agent was used to recover the spots. once the swabs were laden with the sample, one batch of 54 tubes was stored in the -20 °C freezer, a second batch of 54 tubes was left on the laboratory bench (the temperature was monitored 20 °C to 22 °C), a third batch of 54 tubes was stored in an oven at 37 °C and a fourth batch of 54 tubes was stored in an oven at 50 °C. All the batches were maintained at their temperatures for a range of set times (6 h, 24 h and 48 h) before extraction.

The DNA was then extracted using the PureGene extraction kit (Qiagen).

4.5.2 Real-time PCR quantification results

The extracted DNA samples from the Saliva were quantified using the Quantifiler Human DNA Quantification Kit using the ABI 7500 real-time PCR machine (Applied Biosystems). Amplification reactions and amounts used were as recommended by the manufacturer. A 1 µL of target DNA was amplified with 11.5 µL of a prepared master mix consisting of 5.25 µL of Quantifiler human primer mix and 6.25 µL Quantifiler PCR reaction mix to give a final total volume of 12.5 µL. The DNA standards were prepared following the manufacturer's recommended concentrations.

MicroAmp optical 96-well reaction plate (Applied Biosystems) was placed on its base (MicroAmp splash free 96 well-bases) and 11.5 µL of master mix was loaded separately into each of the wells. 1 µL of each DNA standard concentration was loaded into its corresponding well in duplicate. 1 µL of the extracted DNA samples were then loaded on the plate into the appropriate wells and the plate was sealed with an optical adhesive cover (Applied Biosystems). The plate was then placed into the ABI 7500, which was already prepared for running DNA quantification. The thermal cycler protocol was performed in accordance with the manufacturer's instructions (Applied Biosystems): Holding stage 1, 50 °C for 2 min and holding stage 2 at 95 °C for 10 min followed by 40 cycles of a two-step cycle; step 1 at 95 °C and step 2 at 60 °C. After completion of amplification, the DNA concentration for each sample was measured in ng/µL.

4.5.3 Results of sterilisation process

All pasteurised saliva samples (in triplicate) were quantified using Quantifiler to compare the effect of pasteurisation on the quality of the saliva samples obtained for forensic genetic process. We can see the result show an increase in DNA concentration when saliva was treated up to ~70 °C (Fig 26) and an even higher increase when treated to at ~90 °C (Fig 27)

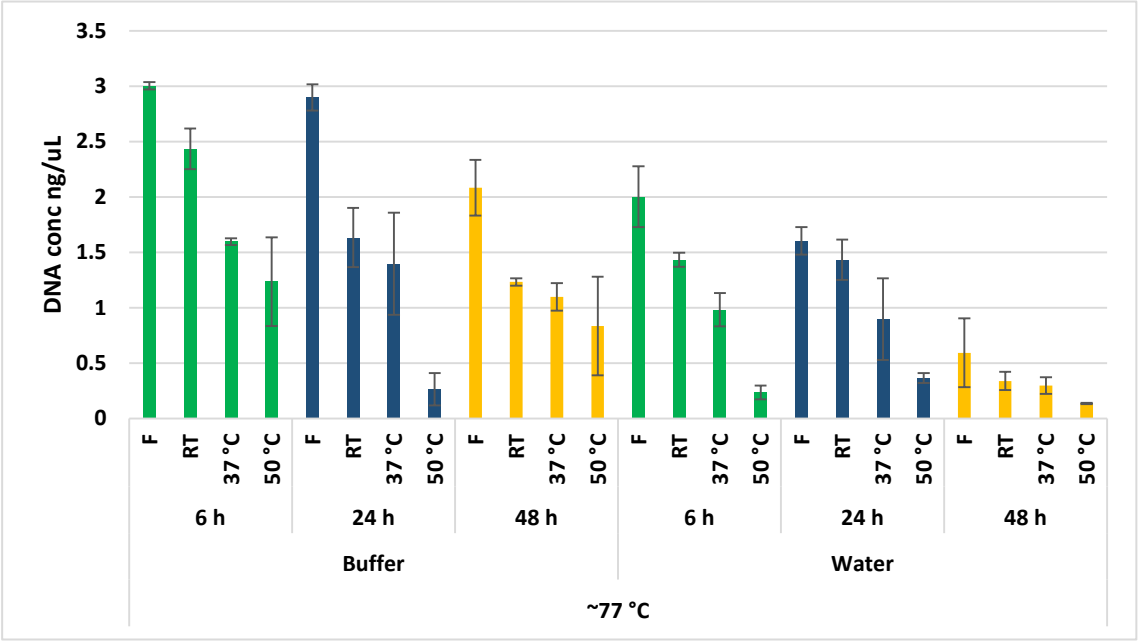

Figure 26 Average DNA concentration recovered in triplicate from saliva treated at ~77 °C, after being stored at various temperatures for 6, 24 and 48 h.

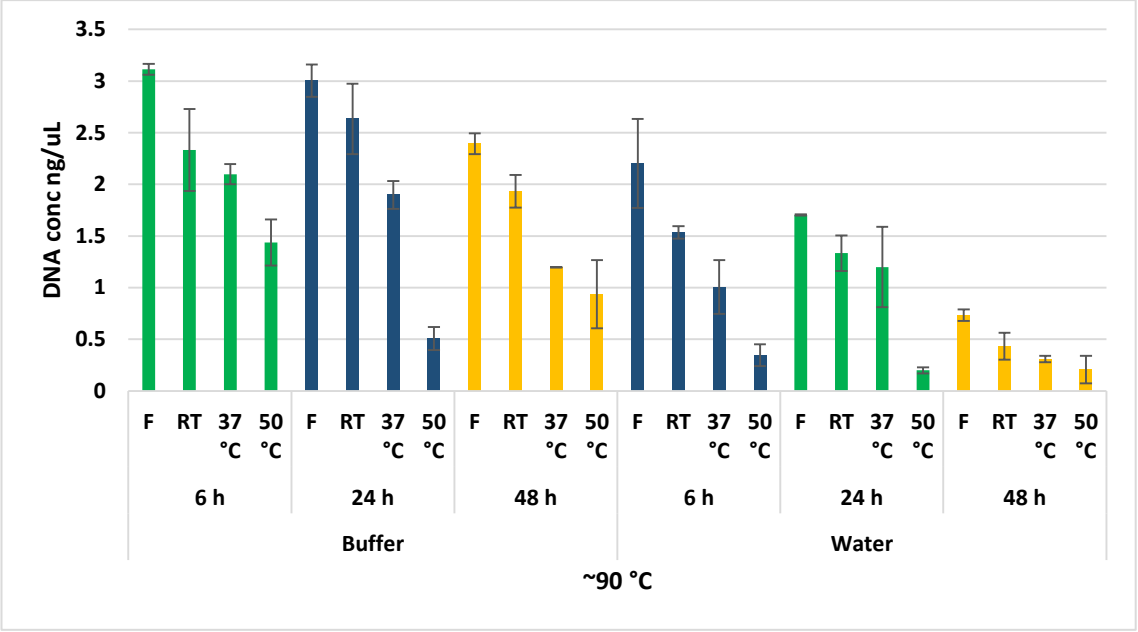

Figure 27 Average DNA concentration recovered in triplicate from saliva treated at ~90 °C, after being stored at various temperatures for 6, 24 and 48 h.

The Quantifiler Human DNA Quantification Kit gave demonstrably reliable results as it is human DNA specific unlike the NanoDrop which resulted in inconsistencies due to it also measuring non-human DNA. The results of the research showed that the sterilisation process enhanced of the quality of the DNA extracted and quantified from saliva samples. Samples treated at ~77 °C showed more stability and consistency than the previous untreated saliva samples. While the real-time PCR results of treated saliva at ~90 °C showed even more stability and consistency than the ~77 °C saliva samples. In both cases the use of the detergent based buffer gave significantly higher concentrations of DNA than when distilled water was used. This was true at all temperatures tested in this research.

4.6 Discussion

The research results reported in this chapter have shown that, the use of swabs moistened with a detergent base lysis buffer yields larger amounts of DNA compared to using swabs that had been moistened with distilled water. The probable cause of the increase is that the process of cell lysis increases the amount of recoverable DNA in the sample.

Interestingly it was noted that the new detergent based buffer, developed in this research, also gave significantly improved the stability of the recovered DNA in the samples after 48 h with environmental temperatures as high as 50 °C in comparison to distilled water. The combination of anionic surfactant in a solution which also contains a chelating agent, sodium chloride and Tris buffer had the extra beneficial effect of greatly improving the stability of the DNA in the recovered samples, particularly at temperatures above room temperature such as 37 °C and 50 °C. The practical impact of this development is likely to be limited in circumstances where crime scene evidence can be kept at low temperatures until it reaches the laboratory; however, in contexts where maintaining low temperatures is problematic, the modified method for collection could have a large impact on the preservation of forensic evidence before it reaches the laboratory. Specifically, this development could be of considerable importance in countries with high ambient temperatures and where refrigerated facilities to store samples during transportation to laboratories, is not always available.

As well, that sterilisation process enhanced of the quality of the DNA extracted compared to the untreated saliva samples. However, it wasn't significant enough to make a large

impact on the results in comparison to the impact of the use of detergent based wetting agent.

Overall has been shown that the recovery of biological material using the detergent-based wetting agent in the double swabbing technique is significantly better than when distilled water is used and the stability post-collection is greatly improved. When using ultrapure water as the wetting agent DNA degradation can be seen after approximately 6 h even at room temperature, compared to the use of the detergent-based solution which stabilized DNA for up to 48 h, even when the temperature is increased to 50 °C.

Chapter Five

Touch DNA

5.1 Introduction

As humans, we shed in large numbers of skin cells each day, some of us shed more than others (Lowe *et al.,* 2002). Therefore, at a scene of a crime thousands of skin cells will be deposited, either when handling items or touching surfaces. Many of the cells when shed are not nucleated, however, DNA is still deposited through touch. Hence, recovery of touch DNA has the potential to link offenders to the scenes.

Van Oorschot first reported in 1997 that DNA profiles could be generated from touched objects that led to the prospects of retrieving DNA from a varied range of items from tools, clothing, vehicles, firearms, bedding, wallets, jewellery, glass, skin, paper and doors (Wickenheiser, 2002). This type of evidence is known as Touch DNA, Trace DNA or even Epithelia DNA samples. This has resulted in a broadening of the application of DNA profiling in investigations to far more offences, such as theft, homicide and sex offences. In such crimes, when the commonly collected forensically relevant biological samples like blood and saliva are absent at such crime scenes, however, touched evidence is often present. Sometimes DNA profile generated from these touched objects might be the only source of evidence in an investigation (Van Oorschot *et al.,* 2010).

However, touch samples are by their nature, small samples and the DNA containing cells are not present in large quantities, especially compared to evidence in the form of blood, semen or saliva. Thus, a more precise technique to identify suitable sample and a more careful recovery method are required in order generate a DNA profile (Aditya et al., 2011). All samples are susceptible to degradation and/or contamination either by environmental conditions or improper handling of objects during recovery. When you have such small samples as you get from Touch DNA samples, it is even more critical to ensure that degradation and contamination are minimised otherwise there will be difficulties in obtaining a meaningful profile that can stand up in court (Templeton *et al.,* 2015).

Touch DNA analysis has now become an essential part of the armoury of the forensic scientists and an important tool for investigators. The growth in the significance of touch DNA is a result of a great deal of significant research investigating the characteristics of trace DNA and the best methods to improve its collection, amplification and interpretation.

The main aim of this part of this research is to determine whether detergent-based wetting agents significantly increase DNA yields from touch samples when compared to swabs moistened with water.

5.2 DNA recovery

5.2.1 Substrates

Plastic (polyethylene terephthalate), metal (aluminum) and glass bottles were used in this investigation as typical objects from which touch DNA could be recovered in an investigation. The bottles were cleaned thoroughly before being used; first with 70% ethanol solution (volume/volume), and then any unwanted DNA and DNase was eliminated from the objects by washing with DNA away solution (Thermo Scientific). Once objects were cleaned the researcher washed their hands before handling objects. Five minutes after handwashing, the researcher handled all three bottles depositing the sample. Contact was for 30 seconds using medium pressure (to ensure consistency). All sample deposited were clearly visible.

5.2.2 Collection

All visible marks were collected using a double swab technique (Sweet et *al.,* 1997). Samples were collected comparing the in-house buffer developed (lysis buffer 6) as a wetting agent and distilled water dH_2O as a buffer, 120 µL of each wetting agent was used to recover the spots. Samples were collected in triplicate for each variable.

5.2.3 Post collection

Five batches of samples were collected from each object. One batch of samples was extracted immediately after collection, the other four batches were stored at four different temperatures (-20 °C, at room temperature (the temperature was monitored), at 37 °C and at 50 °C) for 24 h before extraction).

All samples were extracted using a PureGene Extraction kit (Qiagen)

5.2.4 Quantification

Three quantification methods were compared in this section of the research, Quantifiler Human DNA Quantification kit; Thermo Scientific NanoDrop 2000 Spectrophotometer and Qubit® dsDNA HS (High Sensitivity) Assay Kit (the latter is designed specifically for use with the Qubit® 3.0 Fluorometers (Thermo Scientific).

5.2.5: amplification

All touch samples were amplified using an in-house assay that amplifies four amplicons 50 bp, 70 bp, 112 bp, and 154 bp amplicons.

5.3 Results

5.3.1 Comparison of quantification methods:

All touch samples recovered were quantified (in triplicate for each variable) using the three quantification methods.

We observed from Fig 28 that when samples were extracted straight after recovery, that the use of the lysis buffer gave an overall higher DNA concentration in comparison to the distilled water. The Qubit® gave the lowest results inclusively. The metal substrate gave very low DNA yields.

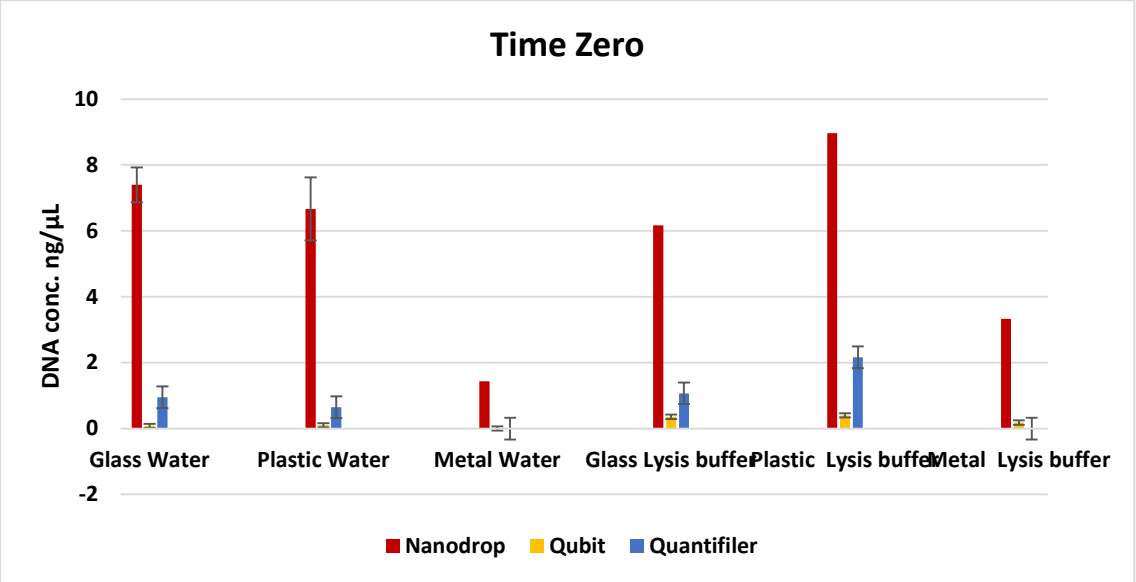

Figure 28 Results of different quantification methods used to compare the wetting agents used to recover touch samples from different substrates.

When post-collection temperature was considered we can still see a significant difference between lysis buffer and distilled water as a wetting agent however NanoDrop gave the most inconsistent results. The concentrations of DNA in all touch samples were measured, tabulated and analysed statistically to explore if there were any significant differences in the data obtained (the tables show the average of the triplicate samples for each variable).

Table 19 Average DNA concentration of samples (in triplicate) using NanoDrop 24 h post-collection.

NanoDrop		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
CL	-20	10.13	5.7	9.9
	RT	9.7	0.6	1.7
	37	8.9	15.4	2
	50	8.4	1.1	0
W	-20	7.5	2.3	4.4
	RT	6.2	11.4	5.07
	37	9.1	8.8	7.3
	50	1.7	0	2.4

Table 20 Average DNA concentration of samples (in triplicate) using Qubit 24 h post-collection.

Qubit		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
CL	-20	0.12	0.14	0.17
	RT	0.22	0	0.03
	37	1.26	0.2	0
	50	0.4	0.2	0.4
W	-20	0.04	0.035	0.03
	RT	0	0.25	0.06
	37	0.07	0.4	0.4
	50	0.5	0.3	0.7

Table 21 Average DNA concentration of samples (in triplicate) using Quantifiler 24 h post-collection.

Quantifiler		DNA Conc. in ng/µL		
Wetting agent	Temperature (°C)	Glass	Metal	Plastic
CL	-20	3.5	0.2	4.4
	RT	1.4	0.07	3.9
	37	0.9	0.03	1.8
	50	0.85	0	1.2
W	-20	1.8	0.07	2.6
	RT	0.9	0	1.7
	37	1.5	0	0.19
	50	0.06	0	0.06

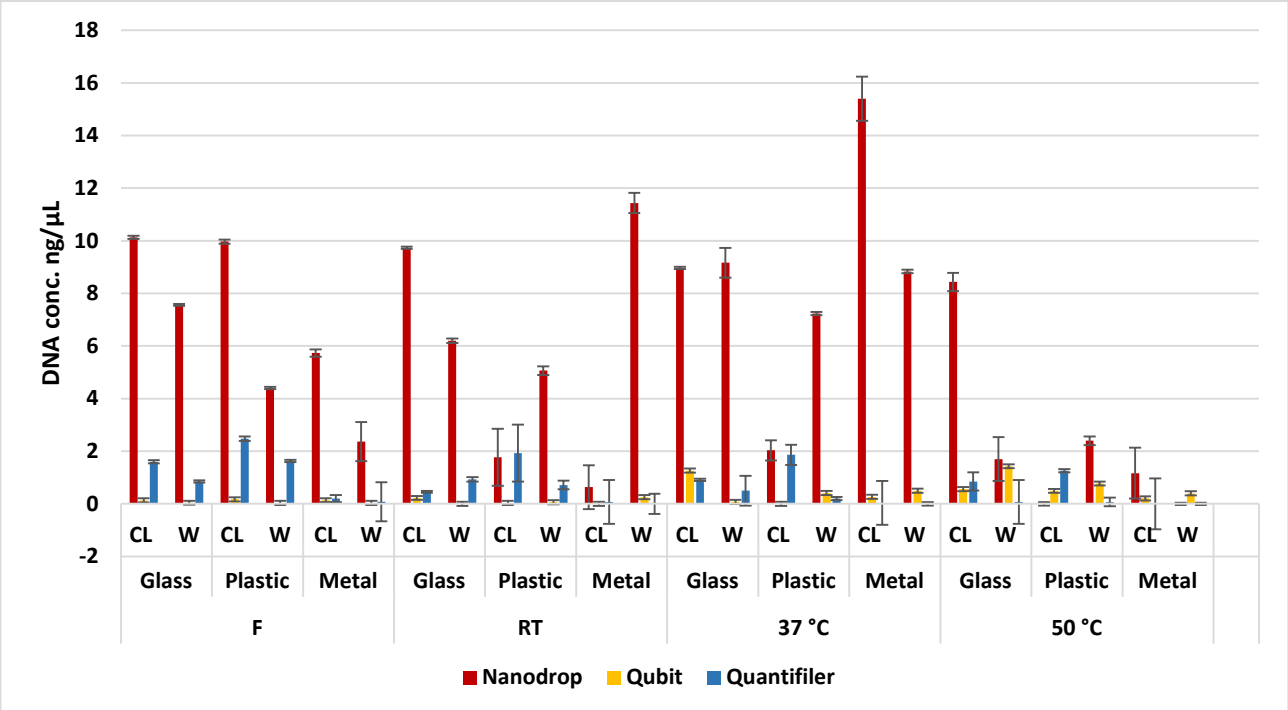

Figure 29 Concentrations of recovered DNA (24 h post-collection) measured using different quantification methods to compare the wetting agents, distilled water (W) and lysis buffer (CL) and the different post-collection temperatures.

When post-collection touch samples were stored for 24 h at a range of temperatures it was observed (Fig 29) that there was increasing loss in the DNA concentration as the temperature increased. The samples recovered using distilled water as a wetting agent deteriorated the most. Consistent with the earlier work in this research, the use of the detergent based buffer generally gave better results showing less degradation. Again, the inconsistencies of the results from the NanoDrop were visible and the results from the Qubit® were very poor.

5.3.2 Amplification results:

As well as looking at the quantity of DNA in the recovered samples it was important to investigate the quality and eliminate the possibility of contamination with non-human DNA. To evaluate the quality of the DNA in the extracted touch samples they were amplified using the in-house mini 4-plex kit amplifying four amplicons 50 bp, 70 bp, 112 bp, and 154 bp to compare the quality of DNA recovered using the detergent based buffer with that recovered using distilled water as a wetting agent.

The results of the amplification of the DNA in the touch samples are shown in (Fig 30 and 31). It can be seen from the results that when distilled water was used the quality of the DNA deteriorated with time and temperature post-collection, whereas detergent based buffer stabilised the DNA for up to 24 h and temperatures of up to 50 °C.

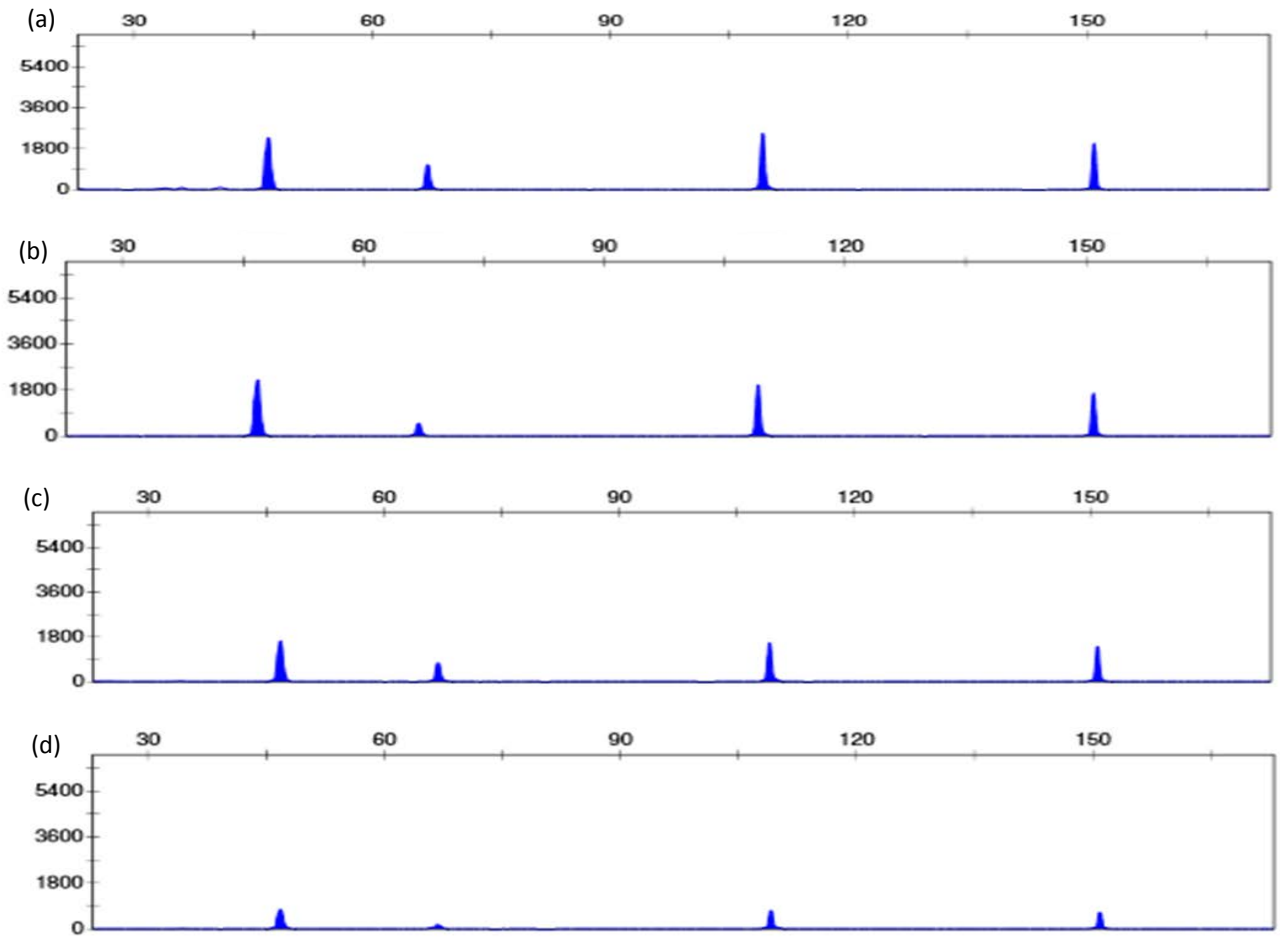

Figure 30 Electropherograms showing results of using mini 4-plex amplification with extracted DNA from touch samples collected with lysis buffer after 24 h post collection storage at different temperatures (a -20 °C, b Room Temperature, c 37 °C and d 50 °C).

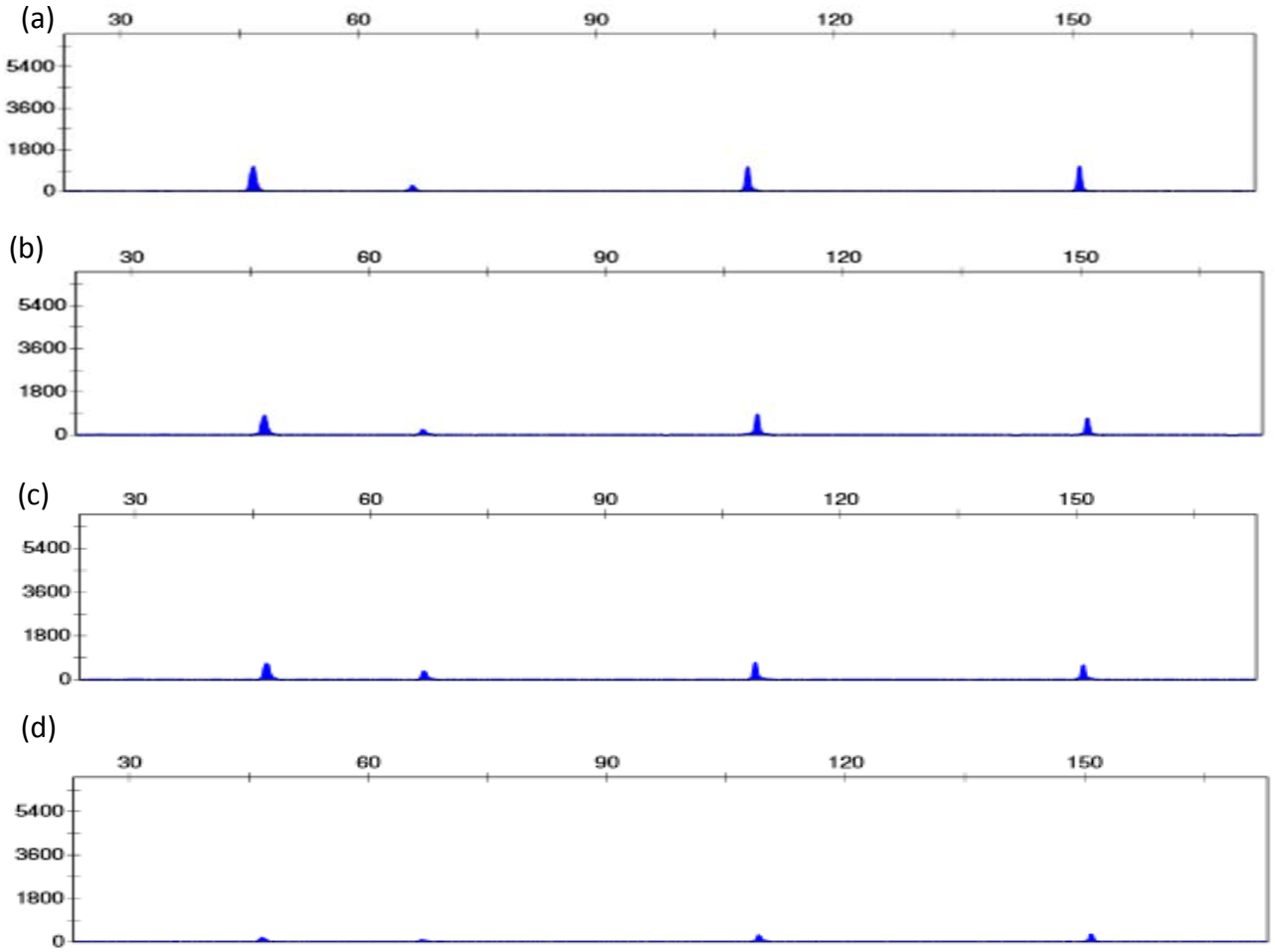

Figure 31 Electropherograms showing results of using mini 4-plex amplification with extracted DNA from touch samples collected with distilled water after 24 h post collection storage at different temperatures (a -20 °C, b Room Temperature, c 37 °C and d 50 °C).

5. 4 Statistical analysis

Statistical Analysis of Variance (ANOVA) was carried out using R Studio software to study the F value to see the concentration differences (Real-time PCR quantitation) of touch samples that were collected using water and lysis buffers after 24 h of post collection storage. The ANOVA results show that there is difference but that it is not that significant ($F_{1, 12} = 1.175$, $P = 0.29$), as the P-value is higher than the significance level (p-value<.05).

5.5 Discussion

DNA profiling is one of the most reliable forms of forensic identification, and the potential to generate a DNA profile from a touched item means that maximizing DNA yield when swabbing such evidence is vital. The small sample size resulting from the collection of touch samples makes it critically important to ensure that the DNA in the samples does not degrade before it reaches the forensic laboratory and undergo genetic analysis. It had been shown in the earlier stages of this research that the use of a detergent based lysis buffer gave increased yields and DNA stability when collecting samples such as blood and saliva, compared to that collected using the conventional buffer – distilled water. The main aim of this part of the research was to determine whether the lysis buffer developed in the research had a similar effect of significantly increasing the DNA yield and stability from touch samples when compared to swabs moistened with water.

The results shown in figures 30 and 31 show that the use of the detergent-based lysis buffer led to greater DNA recovery from the fingerprints than when distilled water was used. Such detergents are amphiphilic in nature allowing their solubility in both water and nonpolar solvents, consequently the organic molecules that make up cells, including fats, lipids, and proteins, become suspended in solution. Water itself does not have these properties and therefore is less effective at producing a suspension of cellular components. Therefore, the incorporation of detergents, into the recipe of a cell lysis buffer for use in the collection DNA sample collection should cause the epithelial cells present in a fingerprint to become suspended in the aqueous solution, hence enhancing cellular recovery during swabbing.

The results presented in this chapter confirm that inclusion of a detergent in the swabbing solution can significantly increase DNA yields from samples of fingerprints collected from different substrates even when stored at high post-collection temperatures for up to 24 h.

Chapter Six
Commercial multiplex kit vs in house

6.1 Introduction

Today there are numerous multiplexes kits available on the market, they range between 10 and 15 autosomal STR systems including the sex-specific amelogenin locus. Although these multiplexes have been developed at first to address the system requirements of the US database it has now been combined with all European core systems (Martin et al., 2001). Today DNA-17 has taken over SGM Plus as the standard method in the UK. DNA-17 has a further six STR loci to the SGM Plus and the amelogenin marker.

As part of the change the National DNA Database (NDNAD) software was updated in 2014 to be able to store and search full DNA-17 profiles. In response, commercial companies such as Applied Biosystems and Promega Corporation, has developed multiplex systems as well has improved the buffer systems (Welch *et al.,* 2012).

The resulting use of the multiplex STR kits has quickly spread to laboratories around the world and is fast becoming the accepted standard methodology. Over the last decade similar national DNA databases have been established in countries around the world. Thus, these STR markers are ideal for designing new primers that generate smaller PCR products (Dieffenbach *et al.,* 2011). Commercial multiplex STR kits used in forensic DNA typing can generate amplicons in the size range of 100 to 450 bp (Gill *et al.,* 2006).

The main aim of this section of the research was to identify the multiplex kit best suited when trying to generate a DNA profile from small quantities of samples and/or degraded samples.

6.2 The AmpFlSTR® Identifiler® Plus PCR Amplification Kit

The AmpFlSTR® Identifiler® Plus PCR Amplification Kit is an STR multiplex assay that amplifies 15 tetranucleotide repeat loci and the amelogenin sex-determining marker in a single PCR amplification.

It includes the thirteen loci of the required CODIS loci for known-offender data basing in the United States (Budowle *et al.,* 1998), plus two additional loci, D2S1338 and D19S433. These loci are consistent with the AmpFlSTR SGM Plus PCR Amplification Kit (Thermo Scientific).

The blend of the 15 loci are compliant with several worldwide database recommendations. The AmpFlSTR Identifiler Plus Kit conveys a 16-locus multiplex with the same power of discrimination with more sensitivity and improved robustness than

the earlier AmpFlSTR Identifiler Kit. The modified PCR cycling conditions enhance the sensitivity and a new buffer formulation has improved performance when samples are inhibited (Wang et al., 2012). The kit has also improved the development of DNA synthesis and purification of the amplification primers to get much cleaner electrophoresis (Thermo Fisher Scientific, 2015).

6.3 The in-house multiplex PCR

A 4-plex multiplex PCR was developed at the University of Central Lancashire to assess the degradation of DNA that amplifies four regions of the nuclear recombination activating gene 1 (RAG-1), which oversees the somatic (V (D) J) re-arrangement of the (T and B) lymphocytes (Nazir *et al.,* 2013)

It first started by developing two internal amplification controls, IAC90 and IAC410, from several non-homologous regions of the PBR322 plasmid that amplifies 90 bp and 410 bp fragments (Zahra *et al.,* 2011). IACs fragments were created by primer technology, that is designer primers for the first amplification (binding site for the primers of second PCR), then labelled forward primers for the second amplification (nested PCR) to amplify the IACs fragments (Nathalie *et al.,* 2012).

6.4 The comparison of the amplification kits

6.4.1 Samples

Samples used in this part were extracted blood and saliva samples collected for a previous part of the research and used here to compare the in-house kit with the commercially available AmpFlSTR® Identifiler® Plus PCR Amplification Kit.

6.4.2The AmpFlSTR® Identifiler® Plus Kit:

The PCR Reaction was prepared with a total reaction volume of 25 µL; 10 µL AmpFlSTR® Identifiler® Plus Master Mix and 5 µL AmpFlSTR® Identifiler® Plus as for the test DNA sample (a portion of the test DNA sample was diluted with low TE buffer so that 1.0 ng of total DNA would be in the final volume of 10 µL). 10 µL of the diluted sample was added to the reaction mix. The amplification was carried out in GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). The thermal cycler conditions were prepared per the optimized PCR condition (Table 22). The amplified products were stored at 4 °C for later use.

Table 22 Thermal cycler conditions used with the AmpFlSTR® Identifiler® Plus Kit

PCR Stages		Temperature (°C)	Time
Initial incubation		95	11 min
Denaturation	28-29 cycles	94	20 s
Annealing/ Extension		59	3 min
Final Extension		60	10 min
Hold		4	Up to 24 h

6.4.3 The mini 4-plex:

DNA was amplified by the Polymerase Chain Reaction (PCR) using the in-house assay described above that amplifies four amplicons ranging between 50 bp and 154 bp.

The new multiplex was developed using four primers pairs of 4-plex. This multiplex amplifies 50, 70, 112, and 154 bp amplicons. The concentration of primers used in the Mini 4-plex kit are shown in (Table 23).

Table 23 Primer concentrations of the in-house mini 4-plex kit

Forward and Reverse Primers (5′-3′)	Concentration (µM)	Primers length	Amplicon Length (bp)
TGGATTACATGCTGCCCTACT	1.2	21	50
TGGTACCCAAGTGTTGATATCCA	1.2	23	
ACCCAGCCACTTGCACAT T	1.3	19	70
TTTCCCTCCATGGATGATGT	1.3	20	
GAGGGAGCTCAAGCTGCAA	1.2	19	112
GTGCTCATTCCTCGCCCT	1.2	18	
TCGGGGACTCAAGAGGAAGA	1.3	20	154
GCAGTTGGCGATCTTCTTCA	1.3	20	

The Multiplex PCR Reaction was prepared with a total reaction volume of 10.0 µL; 5.0 µL 2X Platinum® Multiplex PCR Master Mix (Life Technologies, UK), 0.6 µL of primers mix, 3.4 µL of dH_2O, and 1 µL of DNA template were added. Four different sets of primer concentrations were prepared to optimise and balance the mini 4-plex peaks heights. The amplification was carried out in GeneAmp® PCR System 9700 thermal cycler (Life Technologies, UK). The thermal cycler conditions were prepared per the optimized PCR condition (Table 24). The amplified products were stored at 4 °C for later use.

Table 24 Thermal cycler conditions of the mini 4-plex kit

PCR Stages		Temperature (°C)	Time
Initial incubation		95	2 min
Denaturation	28 cycles	95	3 s
Annealing		60	1.5 min
Extension		72	60 s
Final Extension		60	30 min
Hold		4	∞

6.4.3 Fragment analysis:

For fragment analysis, each sample was prepared for both kits (by adding 1 µL of PCR product or allelic ladder (one for each injection) to 8.5 µL of Hi-Di Formamide (Life Technologies, UK) with 0.5 µL GeneScan 500 LIZ size standard (Life Technologies, UK). The samples were then heated at 95 °C for 3 min and snap-cooled -20 °C for 3 min. DNA fragment analysis was then carried out on ABI 3500 Prism® Genetic Analyzer in a 50 cm long capillary using POP-6 polymer (Life Technologies, UK). Fragment analysis 50_POP6 run module was used with dye sets DS – 33 (filter set G5): 6 – FAM (blue), VIC® (green), NED (yellow), PET® (red) and LIZ® (orange). The parameters of ABI 3500 POP_6 are as shown in the table (Table 25).

Table 25 The parameters of ABI 3500 POP_6 module used for fragment analysis

Parameters	Mini 4plex	Identifiler® Plus
Run temperature	60 °C	60 °C
Pre – run voltage	15 kV	15 kV
Pre – run time	180 s	180 s
Injection voltage	1.6 kV	3 kV
Injection time	5 s	7 s
Run voltage	15 kV	15 kV
Run time	3000 s	1430 s

The data obtained from the capillary electrophoresis (CE) were analysed using ABI 3500 GeneMapper® ID-X Software Version 1.2 (Life Technologies, UK). The parameters for the analysis of DNA profiles were kept the same for every run (Table 26).

Table 26 Parameters used for the analysis of PCR fragments.

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

6.5 Results:

As reported by the manufacturer the AmpFlSTR® Identifiler® Plus Kit amplifies <360 bp while the in-house mini 4-plex kit amplifies four amplicons 50 bp, 70 bp, 112 bp, and 154 bp. In this research, we concentrated on the loci with the smaller bp.

Table 27 shows the results for the AmpFlSTR® Identifiler® Plus Kit and presents the peak height of extracted saliva samples stored post-collection at different temperatures showing the size of the base pairs on each loci. While Table 28 does the same for the in-house 4-plex kit.

Table 27 Variation of Peak RFU heights at the designated loci and its size according with the different post collection storage temperatures using the AmpFlSTR® Identifiler® Plus Kit.

At 3 h		Peak height (RFU)		
Temperature (°C)	D8S1179 128–172	D21S11 187–243	D19S433 106–140	D18S51154 258-277
-20	9112	2356	196	26
RT	7921	1796	86	9
37	6341	836	32	0
50	3948	375	12	0

Table 28 Variation Peak RFU heights at the designated loci and its size according with the different post collection storage temperatures using the in-house 4-Plex kit.

At 3 h		Peak height(RFU)		
Temperature (°C)	50	70	112	154
-20	3139	2964	3424	3315
RT	3424	3296	3238	3186
37	2686	2774	2114	2083
50	3389	3075	2866	2947

As the AmpFlSTR® Identifiler® Plus Kit overall has a larger base pair range there was a drop in the peak heights at the larger loci and almost no visible peaks at the smaller base pairs. In contrast the mini 4-plex kit showed good peaks at as low as 50 base pair even when the samples had been exposed to higher temperatures.

6.6 Discussion

The AmpFlSTR® Identifiler® Plus Kit is strong robust kit with its improved presentation through an improved master mix formulation, optimized and flexibility PCR cycling providing options for high sensitivity and routine work. However, with samples of degraded nature that may only be visible at a lower size range. The mini 4-plex multiplex PCR with short amplicons of 50 bp, 70 bp is an effective at assessing the degree of DNA degradation and allowing the visualization of fragments in that small size range.

Chapter Seven

Conclusion

In conclusion, the results of this research have shown the following:

- That the use of double swab technique was the best recovery method for biological samples; this is maybe due to the fact that the wet swab loosens the epithelial cells and then the dry swab picks them up, consequently having a positive impact on the quantity of DNA recovered and the quality of the DNA profile generated. In addition, the second dry swab itself provides sufficient DNA to generate a profile, therefore processing the two swabs together in the extraction gives even better results.

- That the recovery of biological material using an in-house developed detergent-based lysis buffer instead of ultrapure water as a wetting agent increased DNA yield even at elevated temperatures. The probable cause of the increase is that the buffer encourages cell lysis and hence increasing the amount of DNA recovered.

- It was also identified that the combination of anionic surfactant in a solution which also contains a chelating agent, sodium chloride and Tris buffer had another beneficial effect. In addition to improving the recovery of DNA samples from substrates it also greatly improved the stability of the recovered sample, even at elevated temperatures of up to 50 °C. This could be of great benefit particularly where maintaining low temperatures is problematic. A modified method for collection using a detergent-based solution could have a large impact on the preservation of forensic evidence before it reaches the laboratory

- That the normal flora bacteria present in the mouth can affect the reliability of the NanoDrop results from the saliva samples quantified as the technique is non-human specific resulting inconsistent results. This research showed that Quantifiler results generated more accurate and consistent data.

- That the use of the detergent-based lysis buffer led to greater DNA recovery from the fingerprints than when distilled water was used. Such detergents are amphiphilic in nature allowing their solubility in both water and nonpolar solvents, consequently the organic molecules that make up cells, including fats, lipids, and

proteins, become suspended in solution. Water itself does not have these properties and therefore is less effective at producing a suspension of cellular components. Therefore, the incorporation of detergents, into the recipe of a cell lysis buffer for use in the collection DNA sample collection should cause the epithelial cells present in a fingerprint to become suspended in the aqueous solution, hence enhancing cellular recovery during swabbing.

- The AmpFlSTR® Identifiler® Plus Kit is strong robust kit with its improved presentation through an improved master mix formulation, optimized and flexibility PCR cycling providing options for high sensitivity and routine work. However, the mini 4-plex multiplex PCR with short amplicons of 50 bp, 70 bp is more effective at assessing the degree of DNA degradation and allows the visualization of fragments in that small size range.

Future work:

- Test the use of the developed lysis buffer for DNA extraction,
- Compare the efficiency of the developed lysis buffer with commercially available wet swabs,
- Determine the extent of the detergent based lysis buffer's ability to stabilise post-collection DNA samples by increasing post-collection incubation time until the sample degrades,
- Optimise the lysis buffer solution developed in the initial research by varying the concentration of each component and the optimum pH of the buffer to find the most effective buffer solution with respect to sample recovery and stabilisation,
- Test the new methodology with different substrates: samples will be recovered from different substrates with newly developed protocol and if necessary the buffer will be modified accordingly,
- Test the new methodology with other biological materials commonly found at crime scenes. The ability to give better recoveries and extended stability means

that samples with lower original DNA counts might become viable sources of DNA evidence,

- Evaluate the buffer's effectiveness in recovering samples on mixtures of biological materials (e.g. Blood with saliva). The new methodology has been shown to significantly improve of the recovery of blood and saliva each on its own, it might also be useful to recover mixed samples.

- Casework efficacy: The positive results from the laboratory testing will be evaluated with the Saudi Authorities to assess the practical application of the new methodology/protocol in casework. This would entail collecting samples at crime scenes using both the conventional technique and the lysis-based collection technique and comparing the quality and quantity of DNA recovered.

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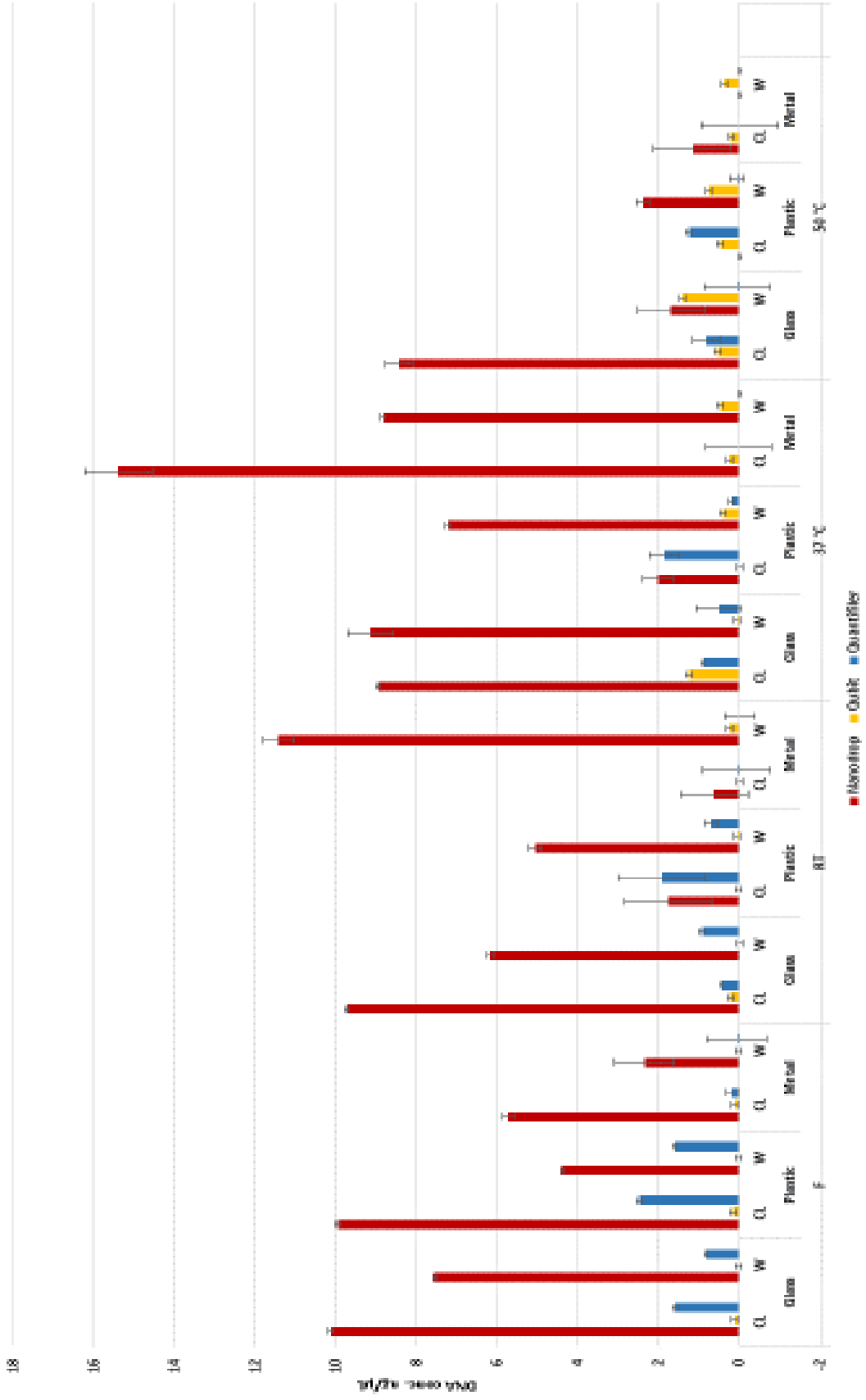
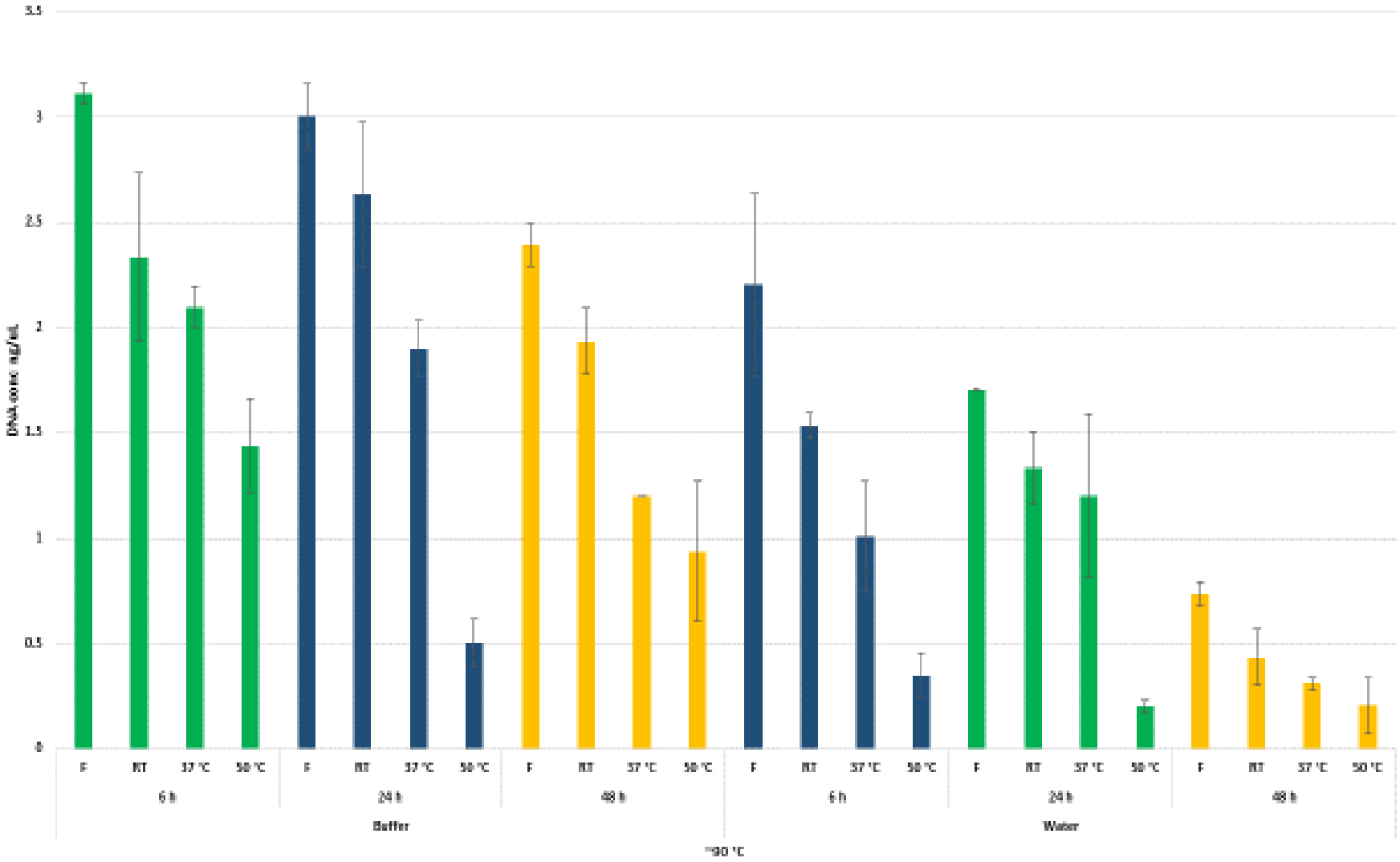
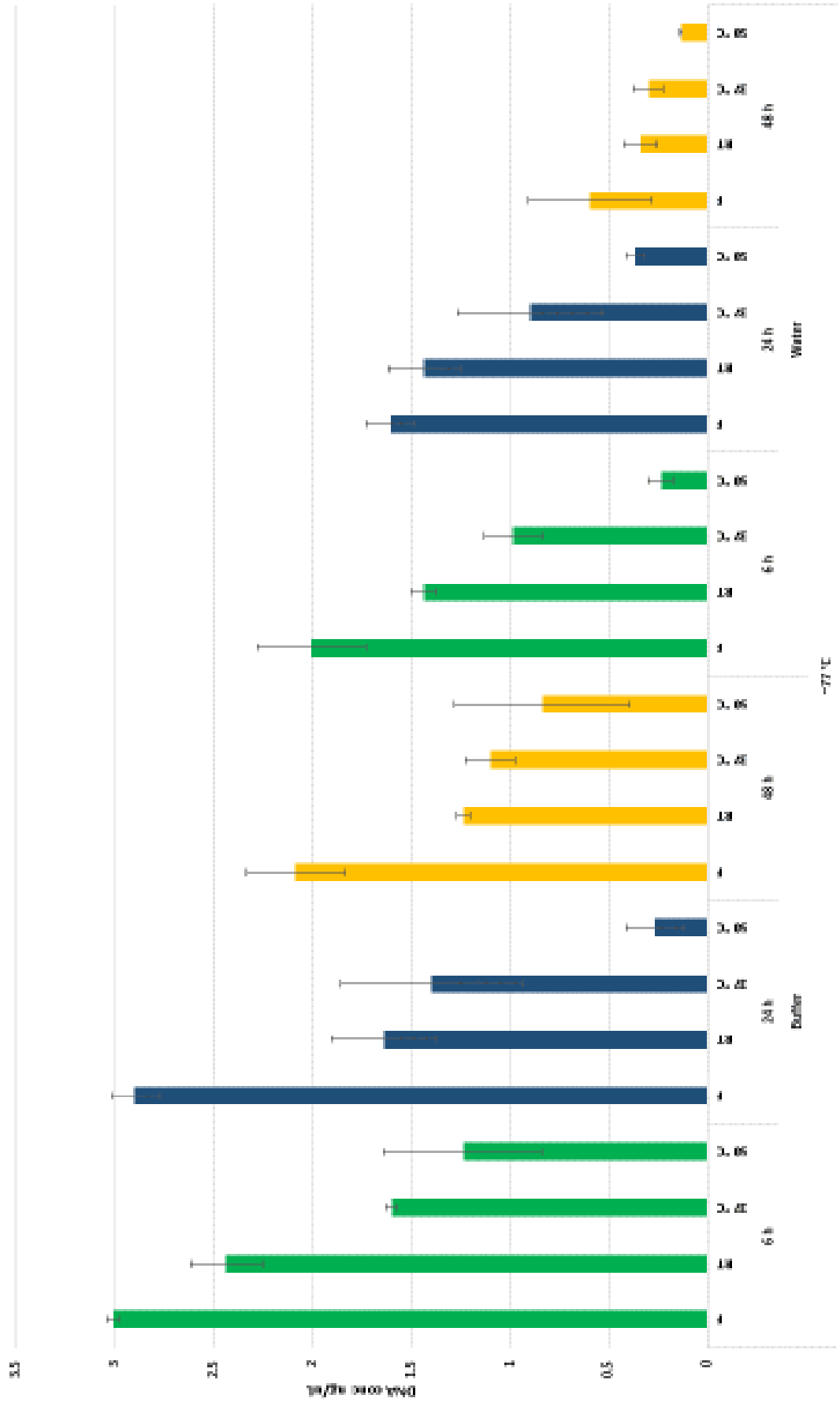
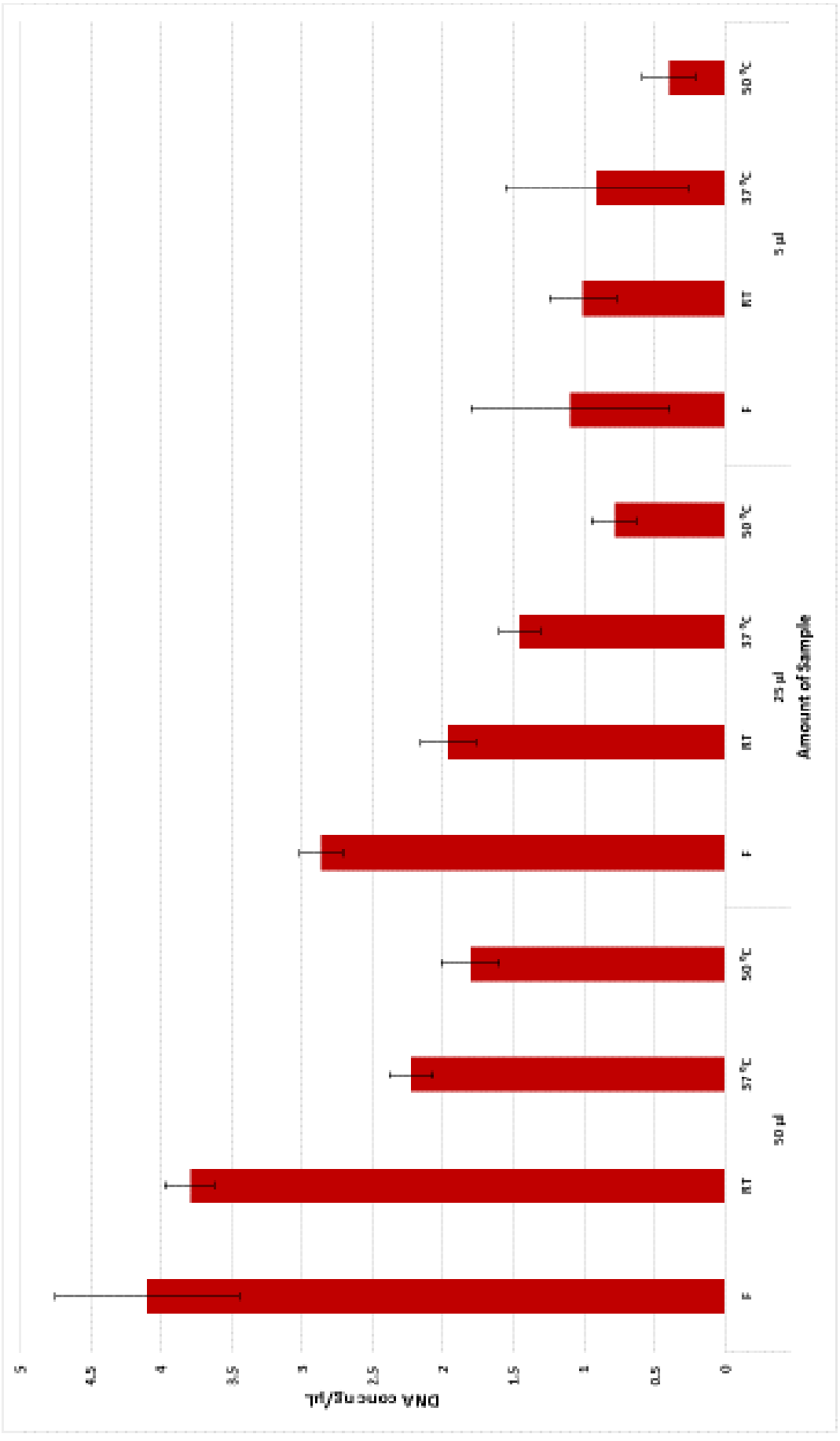
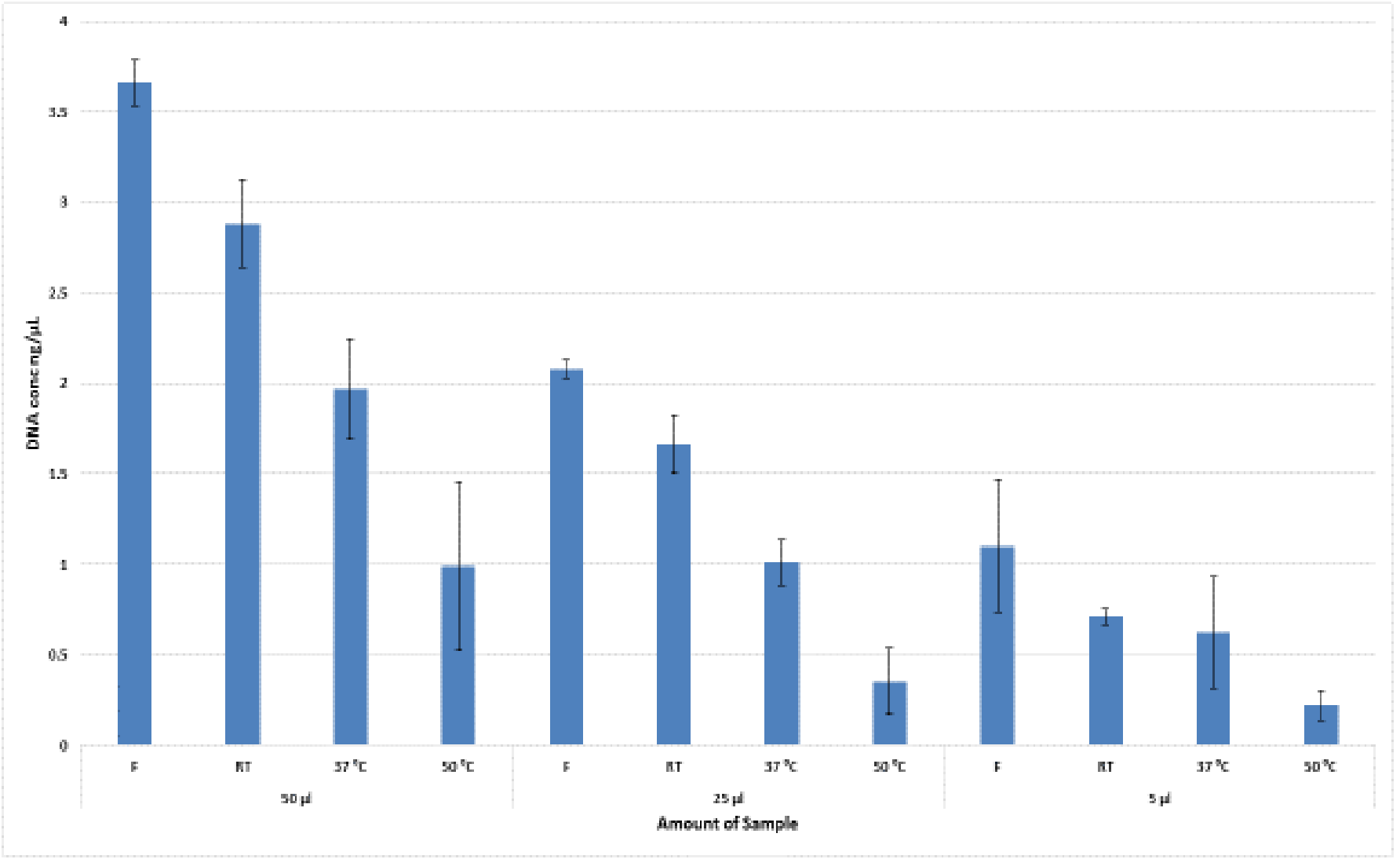
Appendix

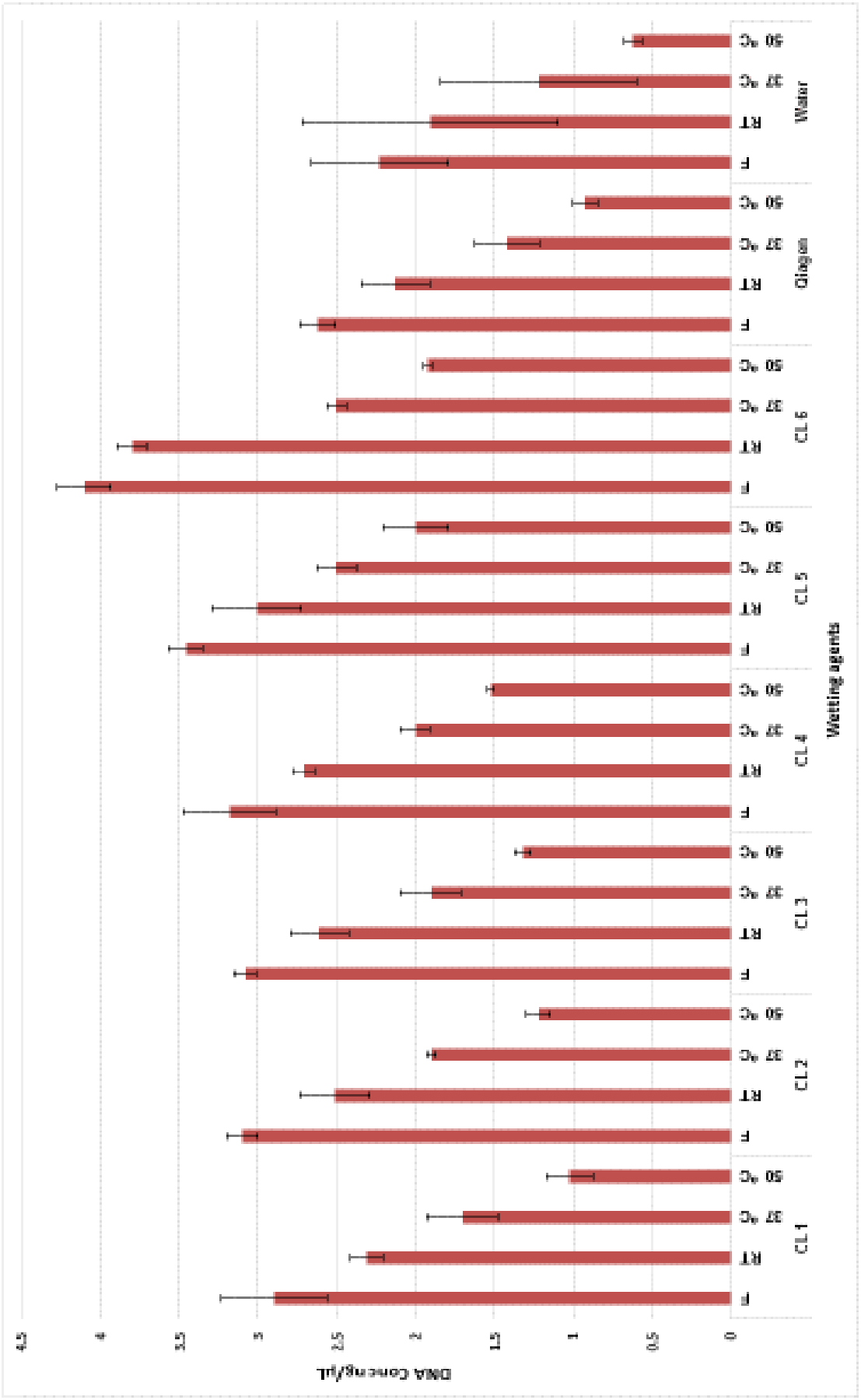

Glass
Plastic
Metal
Glass
Plastic
Metal
Glass
Plastic
Metal
Glass
Plastic
Metal
RT
37 °C
56 °C
CL
W
Nanotrap
Outlet
Quantifier

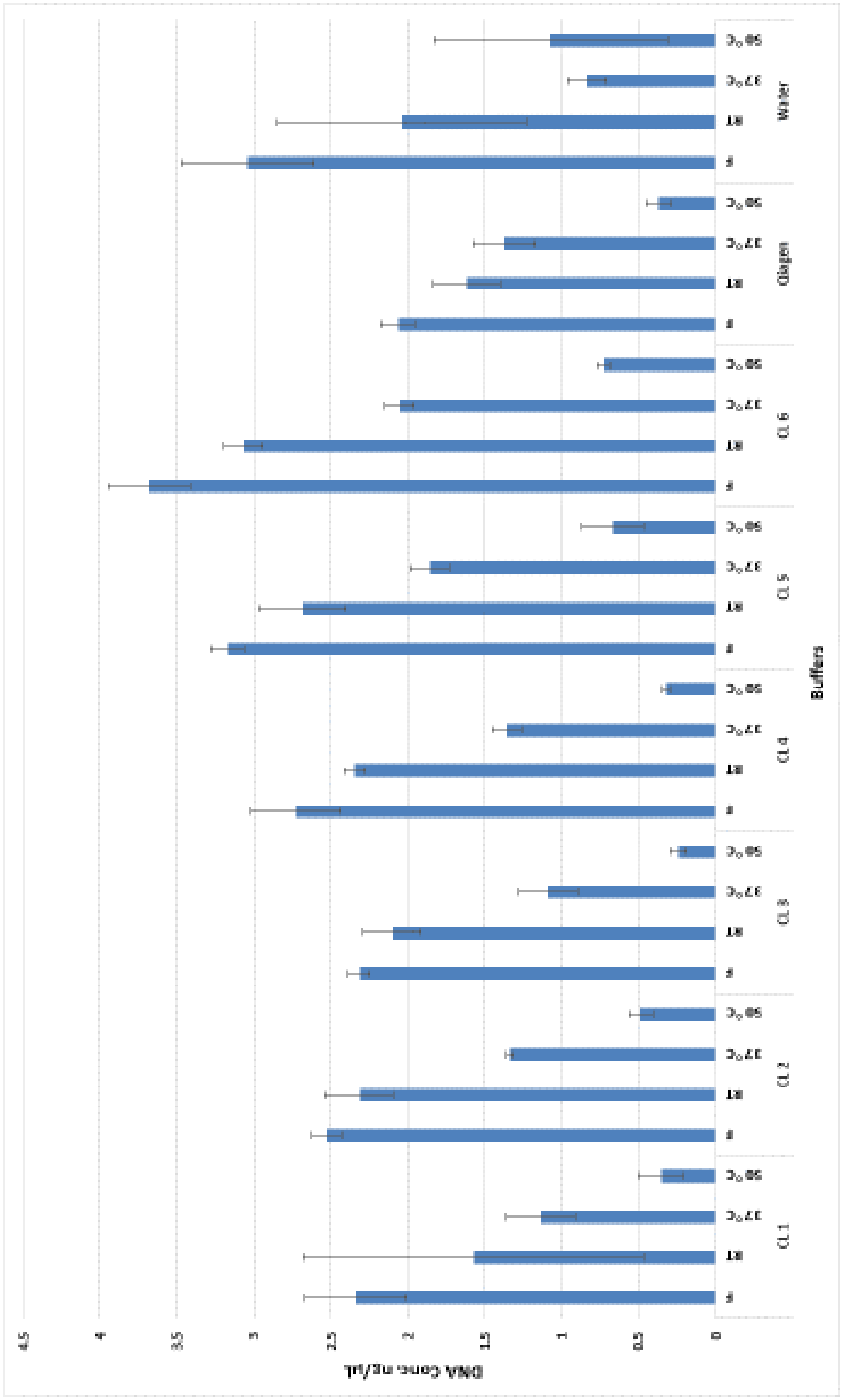

5.5
3
2.5
2
1.5
1
0.5
0
4
RT
37 °C
50 °C
6 h
24 h
48 h
Buffer
Water
°50 °C

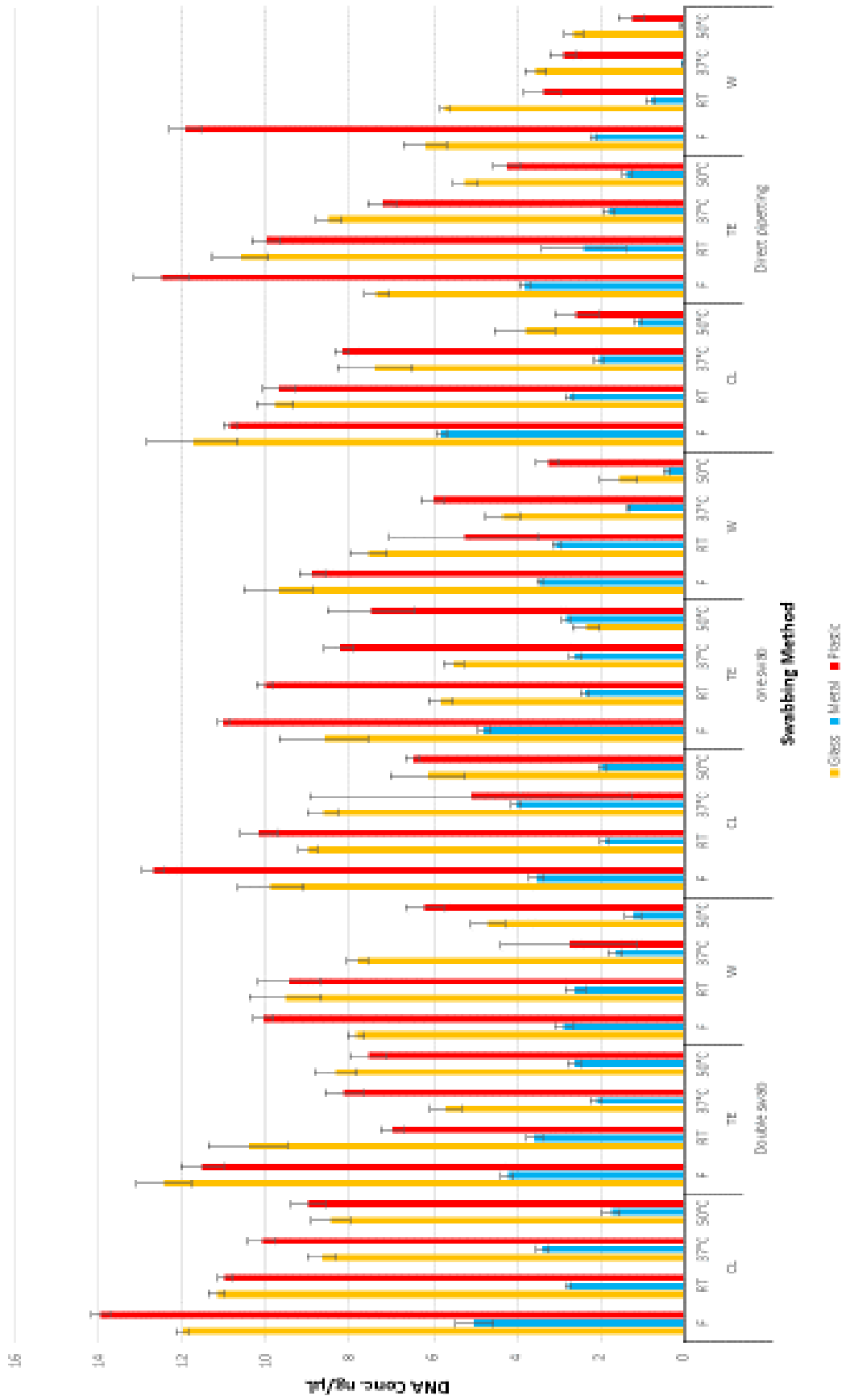

DNA conc. ng/µL
0
0.5
1
1.5
2
2.5
3
3.5
Buffer
Water
6 h
24 h
48 h
4
RT
37 °C
50 °C
−77 °C


DNA conc ng/µL
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
50 µl
25 µl
5 µl
Amount of Sample
4
RT
37 °C
50 °C


4
3.5
3
2.5
2
1.5
1
0.5
0
DNA conc ng/µL
F
RT
37 °C
50 °C
F
RT
37 °C
50 °C
F
RT
37 °C
50 °C
50 µl
25 µl
5 µl
Amount of Sample


DNA Conc ng/µL
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Wetting agents
Water
Qiagen
CL 6
CL 5
CL 4
CL 3
CL 2
CL 1
50 °C
37 °C
RT
F


DNA Conc. ng/µl
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Buffers
Water
Qiagen
CL 6
CL 5
CL 4
CL 3
CL 2
CL 1
4°C
RT
37°C
90°C


DNA Conc. ng/µL
0
2
4
6
8
10
12
14
16
F
RT
37°C
56°C
90°C
CL
TE
W
Double swab
Direct plating
Swabbing Method
Glass
Metal
Plastic

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Collection protocols for the recovery of biological samples

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ABSTRACT

The main focus in forensic genetics for two decades has been to improve the extraction of DNA from a wide variety of evidence and to make the profiling technology more sensitive and robust. In contrast, the recovery methods for biological material have seen little development. This study aims to improve the efficacy of the collection and storage processes, from crime scene to receipt at the laboratory. This study compared the use of ultrapure water as a wetting agent when collecting biological evidence using swabs with a detergent-based buffer. The results show that the stability post-collection greatly improved by using a newly developed buffer. When ultrapure water is used, DNA degradation was seen after 6 h at room temperature. However, the detergent-based buffer stabilized DNA for up to 48 h, even when the temperature was increased to 50 °C. The impact of these findings may be limited where crime scene evidence can be refrigerated until it reaches the laboratory. However, there are many situations/contexts where sample refrigeration is not possible and there is scope to improve the preservation of the genetic forensic evidence before it reaches the laboratory.

1. Introduction

When analyzing biological evidence the precursors to extraction and analysis are the sample collection, handling and storage. Collection, preservation and storage of biological evidence have a fundamental impact on the quality of the sample and the resultant DNA profile. If the sample quality is damaged by poor practices it can undermine the potential for it to be used as evidence [1]. These initial steps must be undertaken carefully, and the most reliable and reproducible protocols should be used for the crime scene collection and preservation of the biological materials before they reach the laboratory. DNA collection procedures can vary, but often start at the crime scene where biological samples such as blood, semen and saliva are identified, collected, and then transferred to the forensic laboratory. Many samples are collected at crime scenes using DNA-free water as a wetting agent with cotton swabs—this basic system does not have any preservative properties.

2. Material and method

Saliva samples were deposited on a plastic, metal and glass substrates and allowed to dry overnight and then recovered using the double swab technique [2,3] using Ultrapure water and a detergent-based wetting agent (PresGene, UK). PureGene extraction (Qiagen) was used after the samples had been exposed (post-collection) to various environmental conditions (−20 °C, room temperature, 37 °C and 50 °C). The quantity of extracted DNA present in samples was measured using real-time PCR, whereas the quality of the extracted DNA present was determined using in-house multiplex PCR.

2.1. DNA quantification

All extracted DNA samples from Saliva were quantified using the Quantifiler™ Human DNA Quantification kit on the ABI7500 real-time PCR machine (Applied Biosystems, UK). Amplification reactions and amounts were carried as recommended by the manufacturer.

2.2. DNA Amplification

DNA (1 μL) was amplified using the polymerase chain reaction (PCR) using an in-house assay that amplifies four amplicons ranging between 50 bp and 154 bp.

2.3. DNA analysis

DNA fragment analysis was carried out on ABI 3500 Prism® Genetic Analyzer.

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1875-1768/

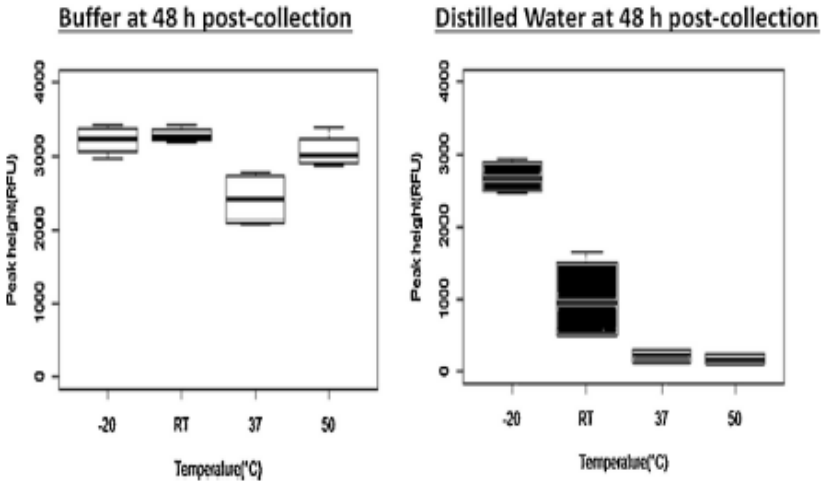

Fig. 1. Boxplots demonstrate the peaks heights of Saliva samples collected by distilled water and buffer separately, at four different temperatures (−20 °C, room temperature (RT), 37 °C and 50 °C) for 48 h post-collection, and amplified by mini 4-plex multiplex PCR.

2.4. Data analysis

The data obtained from the capillary electrophoresis (CE) were analysed using an ABI3500 with GeneMapper® ID-X Software Version 1.2 (Life Technologies™, UK). The peak heights (RFU) of the samples were used to perform the statistical analysis. R-Studio software was used for statistical analyses such as the analysis of variances (ANOVA).

3. Results and discussion

Samples were recovered in triplicates from glass, plastic and metal, results below are of plastic only using the Double Swab technique [2,3] using ultrapure water and a detergent-based buffer and stored post-collection for up to 48 h at different temperatures (−20 °C, room temperature, 37 °C and 50 °C). The quantitation data is shown in Fig. 1 and the profiles generated using an in-house

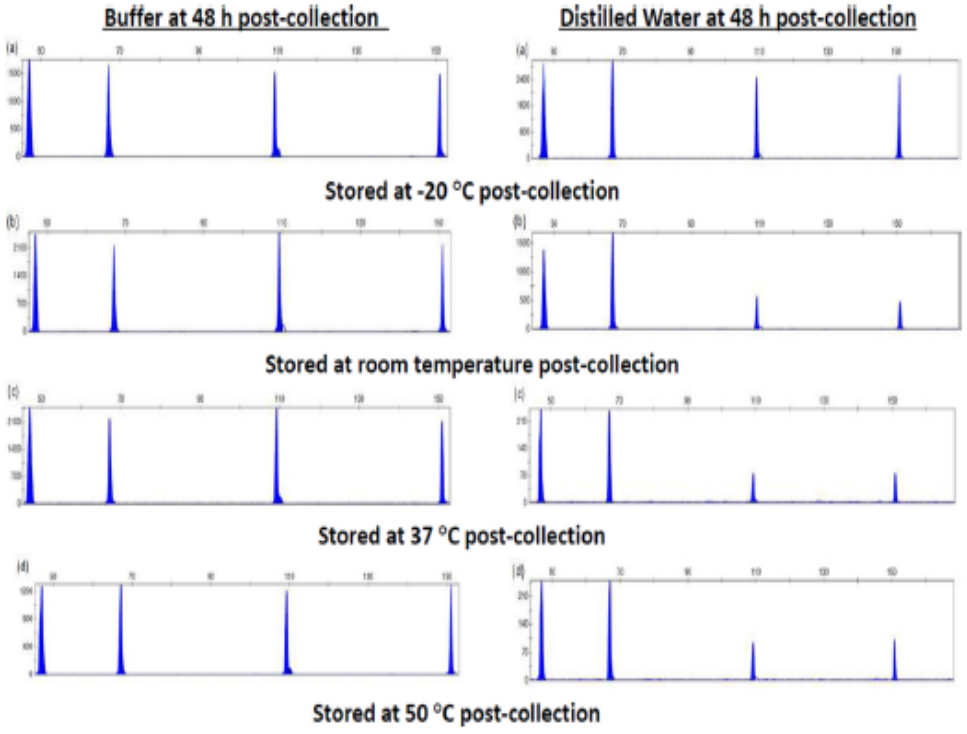

Fig. 2. Electropherograms show the mini 4-plex multiplex PCR amplification of extracted DNA saliva samples after 48 h post-collection by buffer against distilled water in different temperatures (−20 °C, room temperature, 37 °C and 50 °C).

multiplex are shown in Fig. 2. At 48 h there is a statistically significant difference between the amount of DNA using the two collection methods ($F(3, 12) = 50.43$, $P = 4.47e\text{-}07$)).

The data presented demonstrates that post-collection degradation of biological material could be a significant problem when working in environments were optimum storage of the swabbed material is not possible. Using a detergent-based buffer stabilized the DNA on the swabs, as illustrated both by the quantitation and the quality of the DNA profiles, both in terms of peak height and balance.

4. Conclusion

Further work need to be done to examine the efficacy of the detergent-based buffer when collecting biological evidence such as touch DNA. Gaining a better understanding of the key factors in crime scene sample collection and post-collection handling that impact on DNA recovery should give a clear indication of best practices in post-collection sample handling whilst in transit to the laboratory.

Conflict of interest

None.

References

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