The Effects of Environmental Insults on Presumptive and Confirmatory Tests and DNA Degradation

By

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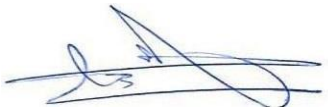

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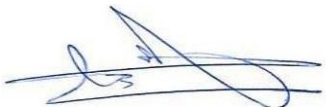

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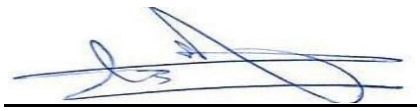
Signature of Candidate 

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ABSTRACT

It is widely understood that environmental insults such as ultra violet (UV) radiation, high temperatures and humidity affect the efficacy of presumptive and confirmatory tests and can have a direct impact on the quality and quantity of amplifiable DNA present. However, little published data exists detailing the empirical effects of environmental insults on presumptive and confirmatory tests. Some studies have assessed the persistence of DNA in biological samples, but none of the published research was based in environments that are routinely exposed to extremely high temperature and levels of UV irradiation.

Initially, data were collected from the Police Forensic DNA Unit in Ras Al Khaimah, in the United Arab Emirates. Analysis of forensic samples received from the year 2012 to 2014 identified items containing body-fluids to constitute over 95% of the total casework samples. The success rate of obtaining STR genetic profiles from these body-fluid samples was over 80% for blood samples, 72% for semen and 46%forsaliva samples. These percentages were lower than data published in the literature and illustrated the potential impact of environmental insult on body fluids, especially semen and saliva.

A series of experiments have been undertaken to empirically investigate the effect of the local environment on biological fluids commonly found at crime scenes. Samples were deposited on cotton, glass, and metal and exposed to the environment in direct sunlight. They were then collected over a period of 51 days in the summer of 2014 at intervals of 48 to 72 hours. Each collected sample was subjected to presumptive and confirmatory tests and DNA was extracted and assessed. The four presumptive tests were: Kastle-Mayer and Hemastix® (Bayer Diagnostics) for blood, Phosphatesmo KM® (Macherey-Nagel) for semen and Phadebas® Tablet Test (Megal LifeSciences) for saliva. The four confirmatory tests were Hexagon® OBTI (Gesellschaft fur Biochemica und Diagnostic) and RSID™-Blood(Independent Forensics) for blood, RSID™-Semen and RSID™-Saliva.

Before undertaking the main experiment the DNA extraction methods available were assessed so that the most appropriate method could be used. These were Chelex-100,

Phenol/chlorophorm, QIAamp® DNA Investigator Kit (Qiagen), InnuPREP® forensic extraction kit (Analytikjina), EZ1® system using the investigator card (Qiagen), AutoMate Express™ System using the PrepFiler™ Express kit (Applied Biosystems), Maxwell® 16 Forensic instrument with the DNA IQ™ chemistry (Promega)and InnuPure® C16 using the innuPREP forensic DNA kit-IPC16 (Analytikjina).One-way analysis of variance (ANOVA) showed significant differences in DNA yields when comparisons were conducted ($p < 0.05$). Pairwise analysisusing the Tukey's (HSD) test identified the AutoMate Express™ System and the Chelex-100 method as giving significantly higher DNA yields. However, DNA quality was compromised when extracting blood samples using the Chelex-100 (mean IPC= 39 C*t*). Based on these data Chelex-100 was chosen as an adequate method, when also taking cost into consideration.

Following evaluation of screening tests; presumptive tests for blood and saliva were shown to be more sensitive in identifying their respective body-fluids than their counterpart confirmatory tests. However, theconfirmatory RSID™-Semen test was shown to be twice as sensitive as the presumptive Phosphatesmo KM® test. In addition, all the screening tests were able to identify their respective body-fluids even when the DNA amounts present were less than recommended for STR analysis.

Following environmental exposure, ANOVA analysis showed that material types did not play a significant role in the identification of environmentally insulted body-fluid samples ($p> 0.05$).

DNA persistence varied widely between different sample types. Saliva was the most susceptible to degradation, with no DNA detected after nine days. DNA recovered from blood samples varied widely between the material types, full STR profiles were still present at day 51. However, partial profiles were seen from day 30 and day 18 for blood on glass and metal, respectively.Allelic drop-outs began to appear in all semen samples after day 9.

Comparisons of DNA quantification results between three different DNA quantification methods, Quantifiler® Human (Applied Biosystems), Quantililer® Trio (Applied Biosystems) and Quantus™ Fluorometer (Promega) showed no significant differences when applying the techniques to environmentally insulted body-fluid samples (p

>0.05). However, some differences were observed when using the Quantus™ where DNA quantities were either over- or underestimated.

Environmental insults were observed to have diverse effects on both presumptive and confirmatory tests, as well as to DNA quantities available for down-stream DNA analysis. The level of these effects was more profound in the DNA quantity and quality than presumptive and confirmatory tests.However, an in depth knowledge of the behavior of such techniques under environmental insults will help with the appropriate selection of screening tests, extraction techniques and accurate quantification methods will improve the recovery of valuableinformation which will ultimately lead to better success rate in the process of STR genetic profiling.

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ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 FORENSIC GENETICS

The term 'forensic genetics' is widely accepted as referring to the use and application of human deoxyribonucleic acid (DNA) in the investigation of crime (Goodwin et al., 2011). The identification of Individual-specific human DNA ‘fingerprints’ by Alec Jeffreys in 1985 (Jeffreys et al., 1985) and the forensic application of these ‘fingerprints’ in the same year by Gill and colleagues have revolutionised criminal investigations (Gill et al., 1985). Today, forensic genetics lies at the intersection of law and science, two forms of institutionalised practice described as the most important contemporary sources and guardians of social order (Williams, 2015).

Forensic DNA procedures may vary slightly between different laboratories and regions, but will generally start at the crime scene where biological samples such as blood, semen and saliva are identified, collected, and transferred to the forensic laboratory. The DNA is then extracted, quantified and amplified to give the final DNA profile results (Frumkin et al., 2010). The resulting DNA profile can then be compared to DNA profiles from both victim and suspect for a direct match that will either exclude or include these reference samples. Statistical analysis normally follows to evaluate the probability of any match. With the type of standard technology applied today this probability is in the magnitude of one in billions for a random match.

1.2 DNA AS FORENSIC EVIDANCE

The use of DNA fingerprints was implemented in an immigration case soon after its first publication (Jeffreys et al., 1985). Soon after in 1986, the power of DNA was highlighted with the exclusion of a man charged with murder, while in the same case identifying the perpetrator through a mass screen (Gill and Werrett, 1987). Evidently, Forensic science has embraced the use of molecular genetics (Budowle and Daal, 2009).

Today forensic DNA analysis routinely deals with materials recovered from crime scenes, paternity testing and the identification of human remains (Jobling and Gill, 2004). The advances in molecular biology techniques in the last decade or so have been unparalleled, including the area of forensic DNA analysis. The ability to retrieve genetic information from the contents of just a few cells has made DNA profiling one

of the most relied upon disciplines in any forensic department. Some may even argue that the implementation of DNA investigation was the single most important event in forensic medicine in the late 20th century (Morling, 2004).

1.3 BACKGROUND: The Local Environment and Forensic Laboratory

1.3.1 United Arab Emirates & Ras Al Khaimah

Ras Al Khaimah (RAK) is one of the seven Emirates that together make up the United Arab Emirates (UAE). The Emirate of Ras Al Khaimah is located on the Arabian Gulf between latitudes 25 and 26 degrees to the north and longitudes 55 and 60 degrees to the east. The total area of the Emirate of Ras Al Khaimah is 2478 square kilometers, just less than 3% of the total area of the country (rasalkhaimahtourism.com accessed on 2-12-2013). Figure 1.1 shows a map of the United Arab Emirates (UAE) illustrating the location of RAK (www.bbc.co.uk, 2-12-2013) and the different Emirates (www.rakinfo.ae, 2-12-2013).

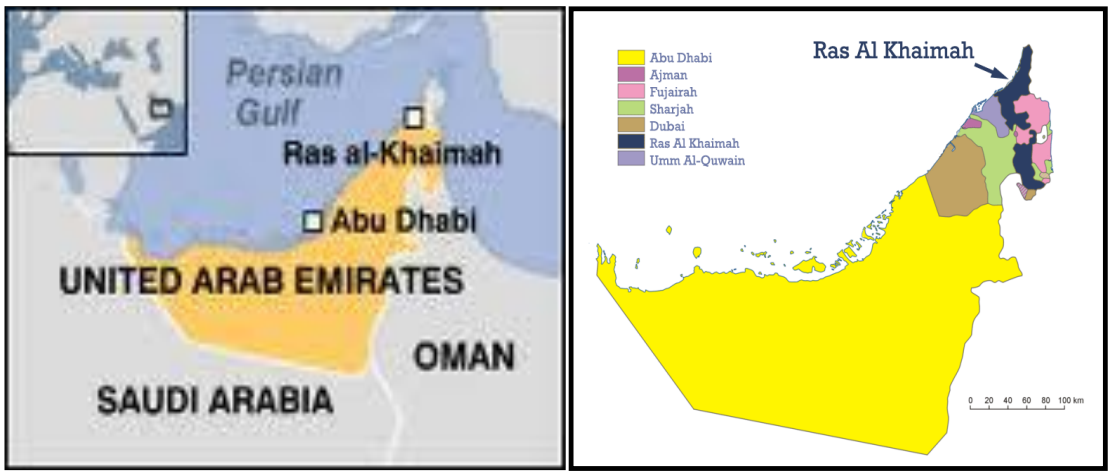


Figure 1.1 Map showing the location of RAK in relation to the region (left) and its relation to other Emirates in the UAE (right).

The Arabian Gulf costal area of the Emirate is a low-lying area of extensive tidal flats, lagoons and sand dunes, whereas the northern part bordering Oman is mountainous. The stronger wind factor compared to the rest of the region and the fine sand is mainly due to the coastal region of the Emirate being unprotected by headlands and directly

facing the length of the Gulf, therefore exhibiting maximum wave fetch (Al Sharhan and Al Sammak, 2004).

According to the National Bureau of Statistics for 2012 (Table 1.1), the maximum temperature registered in RAK international airport in 2012 was 49.5 °C and the minimum temperature was 6.3 °C with the maximum relative humidity reaching 95% and the minimum relative humidity registered as 9% (www.ded.rak.ae).

Table 1.1 Table showing the maximum and minimum temperatures and humidity recorded in the UAE in 2012 according to the national bureau of statistics.

Climate	Records
Maximum temperature degrees	49.5 °C
Minimum temperature degrees	6.3 °C
Maximum relative humidity	95%
Minimum relative humidity	95%

According to the published report of the National Bureau of Statistics, the mid-year population of RAK as of July 2010 was estimated at 249,000 people. However, the RAK Department of Economic Development (DED) puts the figure at 422,000 by the end of 2012 (DED 2012). This increase in the population is a natural product of the huge economic development in recent years, triggering an influx of a large migrant work force of different nationalities and races. The rapid establishment of RAK as a tourist destination with both its financial services and real estate sectors contributed strongly to the growth of the Emirate. With this increased growth and rapid population influx, a parallel increase in crime rate has followed. The number of registered cases presented at court has increased from 6125 in 2012 to 11430 cases in 2013 (DED 2012).

1.3.2 RAK DNA Unit

The DNA Genotyping Unit at the Forensic Laboratory of Ras Al Khaimah (RAK) Police Head Quarters was established by late 2008. The unit contains four distinct laboratories: an Exhibit Examination Section, an Extraction Laboratory, a Pre-PCR set-up Laboratory and a Post-PCR Laboratory. Although considered as a small throughput Unit (with just over 300 cases per year), the number of cases is increasing steadily (Figure 1.2) and crime and sample types are also becoming more diverse.

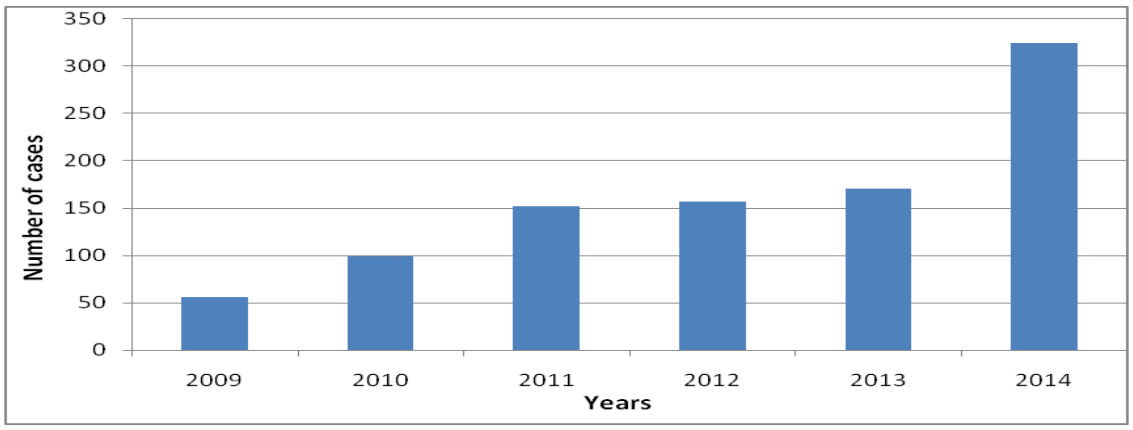


Figure 1.2: The steady increase in the number of samples received by the DNA unit in the past 5 years.

Time delays between sample collection and submission, along with packaging and storage capabilities together with the effects of the local environmental insults, are the main factors influencing the success of genetic analysis in RAK DNA Unit.

1.4 DNA AND THE LOCAL ENVIRONEMENT

An insight into the climate of Ras Al Khaimah was discussed earlier (Section 1.2.1). The effects of environmental insults on the ability to obtain intact DNA for forensic purposes have been studied to some extent over the last 25 years. These included the controlled effects of temperature, humidity, ultra violet radiation and even soil have been well documented in the literature (McNally et al., 1989; Barbaro et al., 2008; Toothman et al., 2008; Dissing et al., 2010). However, the effects of environmental insults on the ability to recover meaningful DNA profile results may differ drastically from one local environment to another (Barbaro et al., 2008). It was shown by Larkin in

2006 that DNA degradation rate will fluctuate according to environmental or seasonal changes (Larkin, 2006).

In parallel, a sound assumption could be drawn by extending the knowledge of the effects of environmental insults on DNA to include the presumptive and confirmatory tests of body-fluids. It is noteworthy that to date there are no studies conducted to evaluate such effects on presumptive and confirmatory testing.

1.5 ACCUMULATED DEGREE DAYS (ADD)

In a study conducted by Megyesi in 2005 it was found that over 80% of the observed variation in human decomposition could be accounted for by the combination of elapsed time and temperatures (Megyesi, 2005). Temperature fluctuation can also contribute to DNA degradation. It is therefore essential to account for both time and temperature when calculating the rate of DNA degradation. It should be noted that this may not hold true for dry stains containing biological material, however, to enable comparisons of degradation/persistence between different locations, ADD is a useful measure.

Accumulated degree-days (ADD) are the cumulative total of the average daily temperatures, allowing temperature changes to be taken into account when calculating DNA degradation rates (Larkin et al., 2010). ADD was established in the fields of forensic entomology and anthropology as a way to standardize the DNA yield in accordance with temperature (Larkin et al., 2010) and it is a popular method in estimating Post-Mortem Interval (Marhoff et al., 2015). ADD can be calculated in its simplest form using the rectangular method using the following formula:

(Maximum temperature - minimum temperature) / 2

1.6 DNA DEGRADATION

Forensic laboratories will frequently encounter degraded samples which contain either no or highly fragmented DNA molecules, resulting in the decreased ability to gain complete DNA profiles (Hughes-Stamm et al., 2011; Bogas et al., 2015). Once the average DNA fragment length is reduced to a size smaller than 300 bp, a loss of genetic

information occurs due to the lack of suitable template DNA for PCR and the subsequent failure of STR amplification (Bender et al., 2004).

A number of environmental insults will have a direct effect on DNA degradation. However, high temperatures and ultra violet (UV) irradiation are key factors in the rate of which DNA degrades (Barbaro et al., 2008). The complex process of DNA thermal degradation involves a progressive transformation of its chemical structure due to the vibration of the bases' bonds and substantial changes occur in all the various DNA building blocks with increasing DNA temperatures (Alongi et al., 2015). The effects of UV radiation on DNA degradation were recently reviewed by Cadet and co-workers in which they discussed the oxidative reactions to cellular DNA as the result of direct interactions of UV photons, photosensitized pathways and biochemical responses (Cadet et al., 2015).

Enzyme-mediated reactions also play a major role in the extent of DNA degradation when subjected to outside environments. Upon cell death, endogenous enzymes such as lipases and different classes of proteases are activated, which facilitate the initial process of DNA cleavage by endonucleases. DNA fragments are then subject to degradation by endogenous nucleases released by host cells or exogenous nucleases released by microorganisms in the surrounding environment, eventually reducing all nucleic acids to mononucleotides (Alaeddini et al., 2010).

Non-enzymatic processes are also known to affect the integrity of DNA structure and chemical composition. Oxidation, as well as the direct and indirect effects of background radiation, will modify the nitrous bases and the sugar-phosphate backbone of the DNA. Furthermore, deamination, depurination and other hydrolytic processes will lead to destabilization and breaks in DNA molecules (Hofreiter et al., 2001). However, changes mediated through these non-enzymatic pathways are on a much longer time-scale and may not be relevant to short-term studies.

Ultimately, the quality of genotyping depends largely on the degradation processes the DNA molecule has been exposed to. Degrading processes accumulate with time while environmental conditions (temperature, humidity, pH, soil chemistry) modify the rate and aggressiveness of degradation (Fondevila et al., 2008).

1.7 BODY-FLUIDS AND SCENCE OF CRIME

The correct identification and characterisation of body fluids in a crime scene has important implications in the solving of many crimes. It can give an important insight into crime scene reconstruction by supporting a link between sample donors and actual criminal acts (An et al., 2012). Moreover, it allows the sample to undergo further laboratory testing including DNA analysis (Virkler and Lednev, 2009) and it can be used as an indicator of the sequence of events in which the crime has occurred or point the investigation in a new direction (Tobe et al., 2007).

Body-fluid identification is usually focused on – but not restricted to – the search for blood, semen or saliva. In crime scenes, the most commonly found body fluid is blood (Tobe et al., 2007). Incidents concerning the search for semen are also common and are on the rise (Khaldi et al., 2004; Redhead and Brown, 2013) and saliva (along with touch samples) represent the most common DNA source in volume crime (Hedman et al., 2011). Traditionally, the identification process starts by conducting presumptive tests to give an indication of the identity of the substance. This is usually carried out with either chemical or catalytic tests that rely on either the colour change of a particular reagent, or an enzyme activity which catalyses the reaction to produce visible colour changes (An et al., 2012). Presumptive tests suffer from limitations including; low specificity, lack of sensitivity and sample destruction (Virkler and Lednev, 2009). On the other hand, confirmatory tests conclusively identify the presence of a certain body fluid; they are usually immunological reactions based on antigen-antibody interactions that are substance and typically species-specific (Virkler and Lednev, 2009).

Emerging techniques for the identification of body fluids have been developing in recent years. These include the use of messenger RNA profiling (Bauer, 2007), microRNA profiling (Wang et al., 2012) and DNA methylation profiling methods (Lee et al., 2012). Some of these methods promise great potential, but their wide use and application in forensic laboratories is still far from established.

1.8 Presumptive and Confirmatory Testing

In the Arabian Gulf region, commonly used presumptive tests include the use of the Kastle-Meyer (Spex Forensics, USA) and Hemastix® tests (Bayer Diagnostics, Germany) for blood, the Phosphatesmo KM® (Macherey-Nagel, Germany) as an acid phosphatase test for semen and the Phadebas® (Megal LifeSciences, Sweden) amylase test for saliva. The RSID™ kit for blood, semen and saliva (Independent Forensics, USA) are examples of confirmatory tests currently used for their respective body fluids. The Hexagon® OBTI test (Gesellschaft fur Biochemica und Diagnostic, Germany) is also a common test for the confirmative presence of human blood.

1.8.1 Phenolphathalein Test (Spex Forensics, USA)

Also known as the Kastle-Meyer test, the phenolphathalein test is a presumptive test for the presence of blood that takes advantage of the peroxidase-like activity of the haem group present in haemoglobin (Spalding, 2003). When phenolphathalein is added to dry blood in the presence of hydrogen-peroxide, a change in colour of the phenolphathalein reagent from colourless to pink occurs. The peroxidase-like activity of the haem group catalyses the breakdown of the hydrogen-peroxide, which in turn oxidises the reduced state phenolphthalin into phenolphthalein producing a pink colour (An et al., 2012).

Since the test depends on the oxidation of the phenolphathalein reagent, any chemical oxidants and vegetable peroxidases can give false positive results when tested (Virkler and Lednev, 2009). However, the Kastle-Meyer test has gained popularity due to its relatively high sensitivity (Johnston et al., 2008) and the non-destructive nature of the method for subsequent downstream DNA analysis (Webb et al., 2006).

1.8.2 Hemastix® Test (Bayer Diagnostics, Germany)

Originally developed for the detection of blood in urine (Poon et al., 2009), the high sensitivity of the Hemastix® strip test was quickly utilized in forensic investigations as a presumptive test for blood. Like most presumptive tests for blood, the Hemastix® test relies on the peroxidase activity of haem, using diisopropylbenzene dihydroperoxide as the substrate and 3,3´,5,5´-tetramethylbenzidine (TMB) as the reporting dye (Poon et

al., 2009). In its oxidised state, the (TMB) dye turns from orange to a deep green colour.

The ease of use and transport of the Hemastix® reagent strips are some of the advantages of this method, although its main advantage over other presumptive tests for blood is its high sensitivity (Webb et al., 2006). On the other hand, the Hemastix® test tends to show more false positives than other popular tests (Virkler and Lednev, 2009) and suffers from the lack of specificity common to all other presumptive tests. Generally, the Hemastix® test was not reported to effect downstream DNA analysis. However, a thorough investigation was carried out by Poon et al. in 2009 in which they demonstrated that DNA recovery may be prevented when using magnetic bead-based DNA extraction techniques (Poon et al, 2009).

1.8.3 Hexagon® OBTI Test (Gesellschaft fur Biochemica und Diagnostic, Germany)

The Hexagon OBTI test is an immuno-chromatographic method that was first developed for the determination of blood in stool samples (Hochmeister et al., 1999). Unlike catalytic presumptive tests, the OBTI kit contains anti-human haemoglobin (Hb) antibodies as means of detection for the presence of human and primate Hb (Johnston et al., 2008). A second anti-human haemoglobin (Hb) antibody is immobilised at the test region of the strip, which reacts with the sample forming an antibody-antigen-antibody complex indicated by a red line in the test region.

Although not as sensitive as presumptive tests, the advantage of this test is its specificity. The test is marketed as "primate specific" after the identification that some primates and ferrets share a common amino acid sequence (TNAVAHV) in the alpha chain of hemoglobin; the sequence responsible for the production of the monoclonal antibodies used for the test (Misencik and Laux, 2007).

1.8.4 The RSID™-Blood (Independent Forensics, USA)

The Rapid Stain Identification–Blood (RSID™-Blood) system is an immuno-chromatographic method that is not hemoglobin-based. The test is unique as it targets the red blood cell membrane protein Glycophorin-A, unlike the hemoglobin-based lateral flow strip tests (Turrina et al., 2008). Since Glycophorin-A does not exhibit cross reactivity with other primates (Schweers et al., 2008), this test is the only true confirmatory immuno-chromatographic method in the market. The sample pad on the strip contains colloidal gold conjugated to anti-human glycophorin-A monoclonal antibodies which will bind to glycophorin-A in blood samples, and react with the immobilised anti-glycophorin-A antibodies present at the detection window of the strip to form a gold-conjugated antibody–antigen complex (Schweers et al., 2008).

The sensitivity of the RSID™-Blood kit might be the only drawback of the method, which ranges between 1:20 as stated by Schweers et al. in 2008 and 1:400 according to Castello et al. (2011). Although well within the manufacturers' claims (1 µl of blood), the sensitivity is at least a magnitude of 100 times less sensitive than Hb-based kits such as the Hexagon® OBTI Test. On the other hand, the specificity and robustness of the method are its main strengths (Schweers et al., 2008; Turrina et al., 2008).

1.8.5 Phosphatesmo KM® (Macherey-Nagel, Germany)

The Phosphatesmo KM® kit is a rapid qualitative enzymatic staining paper test for the identification of semen. Although the product producers' do not disclose the specific nature of the substrate nor the colour developer of the kit (personal communication), the components of the kit are hinted at by Keil et al. (1996) in reference to the 1984 paper by Kloosterman and co-workers, in which the *p*-nitrophenolphosphate was used as a substrate to give off *p*-nitrophenol as an indicator, a process catalysed by the enzyme acid phosphatase.

Acid phosphatase is an enzyme secreted by the prostate gland that is present in larger amounts in seminal fluid than any other body fluid (An et al, 2012). The enzyme catalyses the hydrolysis of phosphates, which results in the formation of a product that will react with a colour developer and it is used in a forensic context to identify potential seminal stains (Greenfield and Sloan, 2003).

Despite the test for the seminal acid phosphatase being the most accepted presumptive test for semen due to its sensitivity and ease of use (Virkler and Lednev, 2009), it suffers from major limitations in terms of specificity. Acid phosphatase is present in many body fluids including vaginal secretions that give positive results in prolonged test exposures. In addition to body fluids, false positive results have been reported when the enzyme came into contact with certain plant extracts, as well as various vegetables, such as cauliflower, potatoes and green peas (Evers et al., 2009). In a study by Evers et al. in 2009, the researchers found that 30% of all 786 samples of alleged sexual assaults examined gave false positive results when tested with acid phosphatase (Evers et al., 2009). Acid phosphatase is an excellent indicator of the presence of seminal fluid but it cannot serve as confirmatory evidence.

1.8.6 The RSID™-Semen (Independent Forensics, USA)

The Rapid Stain Identification – Semen (RSID™-semen) is the first available test kit for the detection of semenogelin; a protein originating in the seminal vesicles and accounts for about 40% of the total seminal proteins (Sato et al., 2004). Positive results on the RSID™-semen kit are considered confirmatory for the presence of human semen.

The test comprises immunochromatographic strips that use two mouse monoclonal antibodies specific for human semenogelin; one form of the antibodies is conjugated to colloidal gold present in the sample pad of the kit, whilst the other form of the antibodies is present on the test region of the kit. If human semenogelin is present, an antigen-antibody conjugated to the colloidal gold complex will form. As this complex migrates down the kit test strip the immobilised anti-semenogelin antibodies on the test region bind the semenogelin-antibody gold complexes, producing a red coloured band (Old et al., 2012)

The RSID™-semen kit has gained immediate popularity due to its high specificity, accuracy and reliability (Boward and Wilson, 2013). Studies have shown that the kit does not exhibit high hook effects (Old et al., 2012) and is highly sensitive (Pang and Cheung, 2007). More significantly, it was suggested by Sato and co-workers (2004) that

the natural degradation of semenogelin to smaller peptides will still allow antigen detection of the resulting fragments (Sato et al, 2004).

1.8.7 Phadebas® Tablet Test (Megal LifeSciences, Sweden)

The Phadebas® test is one of the well-established tests and it is the most commonly used commercial kit currently available. It has been used for nearly four decades for the presumptive identification of saliva (Willott, 1974). Like other presumptive tests for saliva, the Phadebas® tablet test relies on the enzymatic activity of α-amylase which is present in high concentrations in saliva (Nelson and Kirk, 1963). The Phadebas® amylase test consists of starch microspheres with a blue dye (procion red amylopectin) cross-linked to the starch. In the presence of amylase, the starch is digested, releasing the water-soluble dye into solution (Virkler and Lednev, 2009).

Phadebas® is a catalytic test which in the presence of α-amylase will produce a colour change from clear to blue. However, there are two different forms of α-amylase in the human body; AMY1 found in saliva, breast milk and perspiration and AMY2 found in the pancreas, semen and vaginal secretions. The two variants are almost indistinguishable in terms of their enzyme activity. Therefore, the test is not specific for saliva (An et al., 2008). In addition, the test is not human specific as high concentrations of salivary amylase are found in other primates and animals (Willott, 1974). False positive results were also observed in hand cream, face lotion, urine, and faeces (Martin et al., 2006). Generally, the test is relatively cheap, quick, and highly sensitive. The test has also shown a higher specificity than other presumptive tests for saliva (Myers et al., 2008). In 2007, the manufacturers of the Phadebas® developed a press form of the kit using the same chemistry, with the advantage of enabling large surface area search for saliva on materials (Hedman et al., 2008).

1.8.8 RSID™-Saliva (Independent Forensics, USA)

The RSID™-Saliva kit is an immunochromatographic strip that targets α-amylase present in human saliva. The RSID™-saliva test employs the same principle used in other RSID™ kits which uses two anti-human salivary amylase monoclonal antibodies (a mobile and a stationary) that form a visible red line in the presence of an antigen (Casy and Price, 2010).

Although the test is not strictly considered as confirmatory for the presence of human saliva, research has demonstrated a high level of specificity and sensitivity (Pang and Cheung, 2008; Casey and Price, 2010). The test showed positive results when rat saliva was tested (Pang and Cheung, 2008) and the products technical information admits reactivity with gorilla saliva. In terms of sensitivity, the product manufacturers claim that the RSID™ kit can detect up to 1 µl of saliva but both Pang and Cheung (2008), and Casey and Price (2010) put the figure at 2 ng/µl and 10 ng/µl respectively, claiming it to be more sensitive than other presumptive tests for saliva.

1.9 DNA EXTRACTION TECHNIQUES

The ability to extract maximum quantities of DNA from forensic samples for downstream processing is one of the most crucial steps in Forensic Genetics. In addition, large quantities of DNA containing PCR inhibitors can hinder the process of amplification resulting in partial or no profiles for analysis (Alaeddini, 2012). An appropriate DNA extraction method ensures not only the efficient extraction of target DNA from the substrate, but it also allows for the removal of any potential inhibitor(s) which will interfere with subsequent downstream processing (Ip et al., 2015).

The process of DNA extraction usually starts by lysing the cells present in the sample followed by purification of the DNA from other cell contents such as proteins, lipids and inhibitors (Carpi et al., 2011). Friedrich Miescher performed the first crude DNA isolation in 1869 by adding acid to cell lysate (Dham, 2005). Currently, there are many specialised methods of extracting DNA which are generally divided into solution-based or column-based protocols (Tan and Yiap, 2009). More recently, the use of magnetic beads to purify DNA molecules has gained popularity and many magnetic carriers are now commercially available (Tan and Yiap, 2009). Since its first isolation by Miescher, improvements to existing DNA extraction methods have been continuously conducted, and the search for the ideal extraction method has been likened to the quest for the Holy-Grail.

1.9.1 Chelex®-100 Extraction

The Chelex®-100 resin is composed of styrene divinylbenzene co-polymers containing paired iminodiacetate ions, which act as chelating groups (Walsh et al., 1991). Chelex was first adapted in forensic DNA extraction by Singer-Sam and colleagues in 1989, they proposed that the presence of Chelex during boiling prevents the degradation of DNA (Singer-Sam et al., 1989). Sweet and co-workers further modified the technique in 1996 by an addition of a pre-extraction preparation step that involves heating and then boiling the sample without the presence of Chelex. This step is then repeated with the presence of Chelex (Sweet et al., 1996). Since the Chelex®-100 extraction produces single-stranded DNA molecules, the procedure was not compatible with Restriction Fragment Length Polymorphism (RFLP) analysis. Later, the application of PCR in forensic laboratories led to the technique becoming one of the most widely used DNA extraction techniques in forensic science (Verdon et al., 2011). However, Chelex extractions are not without their problems, since they are well known to be inefficient at removing PCR inhibitors and can potentially hinder the amplification process by binding magnesium ions (Mg^{2+}) in the amplification master mix (van Oorschot et al., 2003).

1.9.2 Phenol (organic) Extraction

The Phenol-Chlorophorm extraction is probably the most widely used DNA extraction technique in forensic genetics (Carpi et al., 2011). The initial step of cell lysis is achieved by adding proteinase K and a protein-denaturing agent such as SDS to the sample. Isolation of nucleic acid is then carried out by adding Phenol: Chlorophorm (1:1) to the sample. A biphasic emulsion is formed upon centrifugation in which the upper phase contains DNA diluted in water, while the lower phase is composed of the organic solvents along with the hydrophobic cellular compounds such as proteins, lipids, carbohydrates and cell debris (Tan and Yiap, 2009; Carpi et al., 2011). DNA precipitation is carried out by either the addition of ethanol and high concentration salts, or through filtration.

Phenol is a corrosive, flammable and toxic carbolic acid with associated health hazards (Wang et al., 2011) and although this method produces acceptable results for samples

of diverse origins, it is time consuming and requires the use of reagents that can also chemically contaminate the extracted DNA (Cawthorn et al., 2011).

1.9.3 Silica Columns Solid-Phase Adsorption

Silica columns are among amongst the most widely used DNA purification systems and can be found in many of the commercial extraction kits (Tan and Yiap., 2009). Two examples of such kits are the InnuPREP® forensic kit (Analytika Gena, Germany) and the widely used QIAamp® Investigator kit (Qiagen, Germany). Silica columns solid-phase adsorption was previously reported to have a higher and purer DNA yield than conventional techniques across a wide range of DNA extraction applications (Cler et al., 2006; Guo et al., 2009; Cawthorn, 2011; Babaei et al., 2011). The principle of silica-based extraction relies on four main steps including: cell lysis; in which the contents of the cell are degraded by means of a lysis buffer and then presented to the surface of the column. The second step involves the selective binding of DNA to the silica column, which is influenced by hydrogen-bond interactions and ionic exchange between the DNA molecules and a pre-conditioned silica matrix. Binding of DNA is dependent on the pH and salt concentration of the buffer in addition to the size of the silica particle itself (Melkaz et al., 1996). A final wash step aims to remove contaminants such as proteins by means of a buffer containing a competitive agent. For the elution step, TE buffer or water is introduced to release the desired nucleic acid from the column. The binding of DNA molecules in the presence of a chaotropic salt solution, such as guanidinium hydrochloride (GuHCl) to the surface of silica particles, is the basis of many bench-scale DNA purification procedures (Poeckh et al., 2008).It is the adsorption and elution characteristics of DNA on silica surfaces that determine the level of performance of the kit. Silica-based extraction allows quick and efficient purification compared to conventional methods. In addition, it prevents many of the problems that are associated with conventional extraction (organic and Chelex) techniques such as incomplete phase separation (Esser et al., 2005).

1.9.4 Automated DNA Extraction

The lucrative prospect of automating DNA extraction has prompted the development of "bench-top" instruments that have gained popularity since their early introduction to the forensic science field (Kishore et al., 2006). Such systems include the BioRobot EZ1® system and the QIAsymphony® system (Qiagen Inc., Germany), the AutoMate Express™ Forensic DNA Extraction System (Applied Biosystems, USA), the Maxwell® 16 (Promega Corporation, Madison, WI) and the InnuPure® C16 (Analytikjina, Germany). These instruments use a chemistry that involves binding DNA to coated magnetic particles in the presence of chaotropic salts, washing of the particles to remove undesirable compounds, and elution of DNA from the particles in a low-salt solution (Davis et al., 2012).

Bench-top systems are a way of automating the liquid handling steps for various DNA extraction methods. The use of paramagnetic beads coated with a DNA-binding surface renders ‘‘hands-off’’ automated handling possible as the magnetic beads can be collected and re-suspended using a magnetic bead separation device without the need for any manual intervention (Witt et al., 2012). This has the advantage of offering a more rapid extraction time, high throughput and hands-free operation processing of evidence samples (Liu et al., 2012).

1.10 DNA QUANTIFICATION

Following DNA extraction, most forensic casework samples will undergo the process of DNA quantification to estimate the amount and integrity of DNA present in the DNA extract. Gaining information on the quantity and quality of the resultant DNA extract can have a great impact on downstream analysis. This is mainly done by influencing the decision on the type of STR kits to be employed for genetic profiling and permits a more informed interpretation of downstream analytical results (Lee et al., 2014).

Traditionally, several methods have been developed for the estimation of the amount and quality of DNA present in forensic samples. These included the use of UV spectrophotometry, fluorescent assays using intercalating dyes such as PicoGreen™ and the use of agarose gel. All these methods are not human specific and the

sensitivity level was not comparable to the extremely sensitive PCR-based STR analysis (Butler, 2012). Other human specific procedures such as the QuantiBlot® (Applied Biosystems) which utilises the primate specific probe D17Z1, were able to estimate the total human/primate DNA amounts but also failed to provide information on the "quality" or the amount of intact DNA present (Lee et al., 2014).

Currently, Real-time quantitative PCR (RT-qPCR) technology, which employs either the 5'-nuclease fluorogenic or TaqMan® assays is the method of choice in most forensic laboratories. RT-qPCR offers a wide range of dynamic DNA measurements and a very high level of sensitivity (Heid et al., 1996). Many ready to use commercial RT-qPCR kits are available which vary in their chemistries and target products. These include the PowerQuant® kit (Promega, USA) and the Investigator® Quantiplex HYres kit (Qiagen, Germany). The Quantifiler® (Applied Biosystems, USA) is also among the widely used RT-qPCR kits. This assay targets a human telomerase reverse transcriptase gene (hTERT) and a synthetic internal positive control (IPC) that is used to monitor inhibition. The procedure utilized a TaqMan assay resulting in a quantification range from 46 pg to 100 ng (Alonso et al., 2004). Recently, the Quantifiler® Trio kit (Applied Biosystems, USA) was developed which detects a small 80 bp and larger 214 bp PCR target in addition to the 130 bp region of the sex-determining region Y (SRY), and a synthetic 130 bp oligonucleotide Internal Positive Control (IPC). The addition of the small and large PCR targets facilitates the estimation of the level of degradation of the sample DNA (Vernarecci et al., 2015).

In addition to RT-qPCR technologies, recent advances in spectrophotometry have enabled the development of quantification methods that can accurately detect fluorescent dyes attached to DNA molecules. One such technology is the Quantus™ Fluorometer (Promega, USA), which uses a double strand DNA-binding fluorescent (QuantiFluor®) dye system to detect DNA molecules in casework samples. Product information on the Quantus™ Fluorometer claims a sensitivity of 10 pg/µl using 1 µl of sample input per assay (Quantus™ Fluorometer operating manual).

1.11 PCR INHIBITION

Forensic samples will often contain chemical substances that will either limit or inhibit the process of amplification using the polymerase chain reaction (PCR). Some of the more encountered inhibitors include haem from blood (Hudlow et al., 2011), dyes from clothes material (Larkin and Harbison, 1999), humic acid from soil samples (Akane et al., 1994) and calcium from bones (Fisher et al., 1993).

These substances are co-extracted with the DNA product and can affect almost all components of the PCR reaction including the template DNA, the nucleotides, the amplification primers, $Mg2^{+}$ and the polymerase enzyme. While the effect of the presence of inhibitors is well known, the mechanisms of PCR inhibition are often unclear (Alaeddini, 2012). The majority of studies on the mechanism of PCR inhibition have been focused on its effects on the polymerase enzyme. The polymerase can be degraded by proteinases (Powell et al., 1994), denatured by phenol (Katcher and Schwartz, 2010) or detergents [Rossen et al., 1992) and inhibited by the blocking of the active site by the inhibitor (Akane et al., 1994).

Severe inhibition will lead to the loss of alleles from the STR profile, whereas slight to moderate inhibition can result in an underestimation of the DNA concentration or the loss of the larger STR loci (Kontanis and Reed, 2006). Such effects of PCR inhibitors can be mistakenly attributed to severe template degradation (Alaeddini et al., 2010). The use of real-time PCR can give a strong indication of the presence of inhibitors and is generally applied prior to the PCR process (Opel et al., 2010).

1.11.1 Overcoming PCR Inhibition

Once identified, the effects of the presence of PCR inhibitors can either be removed or reduced to enable successful STR analysis. A number of procedures have been developed to circumvent PCR inhibition. These procedures have been reviewed elsewhere (Alaeddini, 2012). Initially, the choice of the DNA extraction method will directly affect the level of PCR inhibitors present in the processed sample. Chelating agents such as EDTA and Chelex are known to inhibit the PCR reaction by competing for the polymerase enzyme binding site (Akane et al., 1994). Several methods have been proposed to overcome such inhibitions which include boiling, density gradient

centrifugation, filtration and magnetic capture hybridization (Alaeddini, 2012). In 1999 Bourke et al. developed a procedure that detaches inhibitors from DNA and releases them into solution by denaturing DNA with 0.4 mM NaOH. Passing through a Microcon-100, DNA is retained on the membrane while smaller sized inhibitors pass through (Bourke et al., 1999).

The selection of the polymerase enzyme itself has been suggested to improve the severity of inhibition (Kim et al., 2015). It was found that although the *Taq* polymerase is commonly used in forensic laboratories and it is the polymerase enzyme of choice for standard multiplex short tandem repeat (STR) kits, it is in fact among the most sensitive to inhibition (Al-Soud et al., 1998). Alternative polymerases such as the Ex Taq HS, FastStart Taq, and PicoMaxx HF have all been suggested which will improve the PCR reaction (Kim et al., 2015).

Furthermore, the addition of amplification facilitators has been found to improve the specificity of PCR through binding to, and therefore inactivating the inhibitors (Alaeddini, 2012). Another method and possibly the simplest is to reduce the effects of inhibition by diluting the DNA product thereby diluting the inhibitor. However, caution must be taken when diluting environmentally insulted samples as they may contain a low level of DNA concentrations and diluting the sample may result in failure of the PCR reaction.

1.11.2 Detection PCR Inhibition

The most common indicator used for the detection of PCR inhibitors is the shift in the cycle number in the Internal Positive Control (IPC) of common commercial quantification kits. In the presence of inhibitory activities on real-time amplification plots, reactions cross the detection threshold at later cycles and the exponential phase slopes decrease. Suppressed amplification efficiencies also have a negative effect on the linear phase and as a result, samples with partial inhibition reach lower plateau fluorescence values at the end of the reaction (Kontanis and Reed, 2006). Additionally, other technologies such gel electrophoresis, dot-blots, high-pressure liquid chromatography and calorimetric assays have all been used to detect the presence of inhibitors (Alaeddini, 2012).

Another method to detect both DNA degradation and inhibition simultaneously was developed by the introduction of two internal amplification controls (IACs) (Zahra et al., 2011). The two IAC fragments of 90 and 410 base pairs (bp) in length were designed so that they would co-amplify with DNA samples and were used to monitor amplification efficiency and detect PCR inhibitors. By monitoring the level of amplification of the 410 fragment, the effects of PCR inhibition and detection were successfully detected. Recently, newly developed commercial STR multiplex kits have introduced such internal amplification controls; one such kit is the Investigator® 24plex kits (Qiagen, Germany).

1.12 WORKING HYPOTHESIS:

Environmental Insults play a major role in the degradation of DNA, the sensitivity and correct identification of presumptive and confirmatory tests.

1.13 AIMS AND OBJECTIVES

The work in this thesis aims to provide an improved understanding of the impact of environmental insults on body fluids. In particular, the environment of the local area in the UAE will be assessed in relation to presumptive and confirmatory tests and also the persistence of DNA within body fluids exposed to the environment.

1.13.1 Objectives

1- To review the success rate of genetic information generated from forensic casework samples submitted to the DNA Unit for a three year period from 2012 to 2014.

2- To analyze the nature of partial STR profiles from the same three year period in terms of missing loci.

3- To identify specific sample types which contribute to the increased proportion of inconclusive STR results, either by elevated levels of inhibition or degradation.

4- To investigate the efficiency of eight extraction techniques in isolating DNA from three different body-fluid types (blood, semen and saliva).

5- To establish sensitivity limits for each body−fluid tested compared to published materials or manufacturers' claims
6- To conduct DNA extraction and quantification on certain body−fluid dilutions that was identified as "sensitivity limit" for each test.
7- To identify whether quantifiable DNA quantities are still available when sensitivity limits of the presumptive and confirmatory tests are reached.
8- To investigate the effects of environmental insults on the ability to identify body fluids commonly found in crime scenes (blood, semen and saliva), using four presumptive and four confirmatory tests.
9- To determine whether the type of material that the body-fluids are deposited on will have any effect on the outcome of these tests.
10- To evaluate how the outcome of these test results reflect on the ability to produce downstream genetic STR results.
11- To conduct two pilot studies as a platform to inform on the interval and duration of sampling.
12- To conduct a full scale experiment on the effects of environmental insults on DNA degradation, which include three types of body-fluids.
13- To compare the outcome of three quantification kits.
14- To carry out statistical analysis on the different DNA quantification values and how they relate to the different material types and to write up the PhD thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 General Materials

Microtest tube, screw-cap, 1.5 mL, DNA-free

Pipette tips, sterile, various sizes

Heating block

Microcentrifuge

Precision pipettes, to dispense various volumes

Timer

Microcon YM 100 units, sterile

MilliQ water

TE buffer

2.2 DNA Extraction

2.2.1 Sample Collection

All sample types (blood, semen and saliva) were collected from the researcher, a healthy male, aged 33 at time of collection. Blood samples were drawn by trained clinic nurse working for Ras Al Khaimah (RAK) Police (blood counts attached in Appendix 1). Seminal fluid was collected from the researcher after minimum 5 day abstinence. Saliva samples were collected from the researcher after abstinence from food and drink for at least one hour prior to sampling.

All experiments were conducted at the forensic DNA unit at the Police head quarters of Ras Al Khaimah. Samples were stored at -20 °C until time of experiment.

2.2.2 Sample Preparation

Volumes of 5 µl of each body-fluid type (blood, semen and saliva) were pipetted on a tip of a sterile cotton swab. For each sample type, eight such samples were prepared and left to air dry overnight at room temperature before extraction was carried out.

All elutes from the extraction methods used were made to a final volume of 50 µl and although samples were collected from the same source, the period of testing varied from time of collection until performing the extraction. Both the Chelex and the organic methods were tested simultaneously at the same day. Similarly, the QIAamp®

DNA Investigator Kit and the InnuPREP forensic extraction kit were also tested together at the same time. All automated machines (EZ1® system, AutoMate Express™ System, the Maxwell® 16 Forensic instrument and the InnuPure® C16 instrument) were all tested in the space of the same week and therefore can be directly compared.

All sample elutes were collected at a final volume of 50 µl, when the final elution volume was more than 50 µl, Microcon-100 tubes (Millipore, Ireland) were used to concentrate to a final volume of 50 µl.

2.2.3 Chelex-100 Method

Eight samples of each body-fluid type were extracted using the Chelex-100 (Sigma-Aldrich, USA) procedure as described by Walsh et al. in (1991). The following amendments were made. A volume of 200 µl of 10% Chelex-100 (Sigma-Aldrich, USA) was added to 1.5 ml tubes containing the whole swab of each body-fluid type and a volume of 20 µl (10 mg/ml) proteinase K (Sigma-Aldrich, USA) was added to the tubes containing the swabs. In addition a volume of 7 µl DTT (10 mM) (Sigma-Aldrich, USA) was added to all the semen samples only. The mixture was then placed in a water bath at 56 °C for 4 h followed by 8 min at 100 °C. The samples were then centrifuged for 3 min at 13000*g* and the supernatant was then transferred to a separate 1.5 tube ready for DNA quantification. The elute was concentrated to 50 µl using Microcon-100 tubes (Millipore, Ireland).

2.2.4 Phenol/Chlorophorm Method

Body-fluid samples were extracted using Phenol/Chlorophorm (Kochl et al., 2005). A volume of 0.5 ml of DNA extraction buffer containing (10 mM Tris, 100 mM NaCl, 10 mM EDTA and 2% SDS) was added to 1.5 ml tube containing a whole swab of 5 µl of body-fluid. A volume of 10 µl (10 mg/ml) proteinase K (Sigma-Aldrich, USA) were added. Finally A volume of 7 µl of (10 mM) DTT (Sigma-Aldrich, USA) was added to semen samples only. The mixture was then incubated at 56 °C for 1 h. The phenol/chlorophorm mixture (Millipore, Germany) was prepared following the manufacturer's instructions provided. A volume of 0.5 ml Phenol/Chlorophorm solution was added, vortexed and centrifuged for 5 min at 13000*g*. Without disturbing the white interface layer, the aqueous (upper) phase was transferred to a new 1.5 ml

tube. This process of adding Phenol/Chlorophorm solution was repeated 3 times. The DNA was concentrated using ethanol precipitation. A volume of 50 µl of 2 M Na-Acetate and 1.0 ml of absolute ethanol were added to the aqueous solution, mixed and centrifuged for 10 min at 13000*g*. The alcohol was removed and 1.0 ml of 70% alcohol was added, mixed and centrifuged for 5 min at 13000*g*. The tubes were then placed at 37 °C for 5 min and finally 50 µl of TE buffer were added.

2.2.5 QIAamp® DNA Investigator Kit

The QIAamp® DNA Investigator Kit protocol: (isolation of total DNA from body-fluid stains) was followed with no amendments to the provided manual. For each sample, the whole swab was placed in the provided 2 ml tube and 300 µl of Tissue Lysis Buffer (ATL) and 20 µl of proteinase K were added. Similarly, A volume of 20 µl (10 mM) DTT was also added for all semen samples). The mixture was then placed in a thermo-mixer at 56 °C for 1 h with frequent vortexing. A volume of 300 µl of Lysis Buffer (AL) was then added and the tube was incubated at 70 °C for 10 min. A volume of 150 µl absolute ethanol was added to the lysate and mixed thoroughly. After briefly centrifuging, the supernatant was transferred to the QIAamp® MinElute column placed in a 2 ml collection tube provided and centrifuged for 1 min at 6000*g*. A volume of 500 µl of diluted Wash Buffer 1 (AW1) was added and the column was placed in a centrifuge at 6000*g* for 1 min. The MinElute column was then transferred to another 2 ml tube and 700 µl of Wash Buffer 2 (AW2) added. The column was then centrifuged at 6000*g* for 1 min and the MinElute column was transferred to a new 2 ml tube and 700 µl of absolute ethanol was added followed by centrifugation at 6000*g* for 1 min. The column was transferred to a new 2 ml tube and centrifuged at 13000*g* for 3 min. Finally the MinElute column was transferred to a 1.5 ml tube and left to air for 10 min followed by addition of 50 µl distilled water and suspension at room temperature for 1 min and a final centrifugation at maximum speed for 1 min for collection of DNA elute.

2.2.6 InnuPREP® Forensic Extraction Kit

DNA extraction was carried out according to manufacturers' "Protocol 3" from the innuPREP® Forensic Kit manual provided. A volume of 400 µl of the provided lysis solution was added to 25 µl of proteinase K, for semen samples an additional 30 µl of

DTT was added. The contents were mixed and centrifuged at 10000*g* for 1 min and the supernatant transferred to a fresh 1.5 ml tube. A volume of 400 µl of provided binding solution was then added and the sample was then applied to the spin filter provided by the manufacturer and centrifuged for 2 min at 10000*g*. The filter was then transferred to a new 2 ml tube and 500 µl of the provided washing solution was added and then centrifuged for 1 min at 10000*g*. A volume of 750 µl of a second washing solution was then added and the tube centrifuged again for 1 min at 10000*g*. The filter was then placed in a new tube and a drying spin was performed for 2 min at maximum speed. Finally, 50 µl of elution buffer was added and aloud to stand still at room temperature for 1 min then centrifuged for 1 min at 6000*g*.

2.2.7 EZ1® System

For blood and saliva samples, protocol: “pre-treatment for stain on fabric” provided by the manufacturer and the procedure was carefully followed. The whole swab was placed in the provided 2 ml tube and 190 µl of diluted G2 buffer were added (dilution was prepared in distilled water in 1:1 ratio). 10 µl Proteinase K was then added and the tube incubated at 56 °C for 15 min followed by incubation at 95 °C for 5 min. The tubes were then placed in the EZ1® system and protocol "Tip dance" was selected with a final elution volume of 50 µl.

Since there was no specific protocol for the extraction of semen samples in the provided protocol manual, therefore for semen samples, protocol: (pre-treatment for epithelial cells mixed with sperm cells) provided by the manufacturer was followed from “Point number 10”.In each 1.5 ml tube containing whole swab of semen samples, 180 µl of G2 buffer, 10 µl Protienase K and 10 µl DTT (10 mM) were added and mixed thoroughly. The tube was then incubated at 56 °C and left overnight, followed by incubation at 95 °C for 5 min. the tubes were then placed in the EZ1 system and protocol "Tip dance" was selected with a final elution volume of 50 µl.

2.2.8 AutoMate Express™ System

The manufacturers’ procedures were followed as per their manual. The master mix for lysis was prepared by adding [number of samples (24) X (500 µl of PrepFiler Lysis buffer + 5 µl DTT]. 500 µl of the lysis buffer was then added to the LySep™ column

provided containing sample and placed in a thermal shaker for 40 min at 70 °C. The lysate was then transferred to the sample tube by centrifuging the LySep column for 2 min at 10000*g*. The lysate in the sample tube was then processed on the Automate Express™ Forensic DNA extraction instrument using the PrepFiler Express™ instrument protocol.

2.2.9 Maxwell® 16 Forensic instrument

The DNA IQ™ Casework Pro kit protocol manual provided by the manufacturer was followed (Section A: Samples on a solid support) with the following amendment. The Extraction mix was prepared [number of samples (24) X 386 µl of Casework Extraction Buffer + 10 µl Proteinase K (10 mg/ml) + 4 µl 1-Thioglycerol]. A volume of 400 µl of the Extraction Mix was then added directly to the DNA IQ™ Spin Basket provided containing sample and placed in a thermal shaker for 30 min at 56 °C. The lysate was then transferred to the sample tube by centrifuging the DNA IQ™ Spin Basket for 2 min at 16000*g*. A volume of 200 µl of the Lysis Buffer provided was then added to the tube containing the extract. The sample tube was then processed on the Maxwell® 16 Forensic instrument using the Maxwell® 16 LEV instrument protocol.

2.3 Sensitivity of Presumptive and Confirmatory tests

2.3.1 Serial Dilutions

An initial experiment was performed to estimate the limit of sensitivity for all products used. This study involved a large number of dilution sets which spanned a wide range of concentrations. The result from this experiment was used to establish the sets of dilutions to be prepared in triplicates.

All sample dilutions were diluted past the manufacturers' sensitivity claims. When no kit was available for the test, dilutions were prepared to the sensitivity reported in published papers; otherwise a wider range of dilutions was made. A set of stock dilutions for each body fluid was made and stored at -20 °C and used for all subsequent experiments.

2.3.1.1 Blood

Serial dilutions were prepared from stock liquid blood in EDTA in the following dilutions 1:200, 1:300, 1:400, 1:500, 1:700, 1:10000, 1:15000, 1:20000, 1:25000, 1:150000, 1:200000 and 1:250000 in deionised water to be used for all subsequent experiments.

2.3.1.2 Semen

Serial dilutions were prepared from stock seminal fluid in the following dilutions 1:2000, 1:3000, 1:4000 and 1:5000 in deionised water to be used for all subsequent experiments.

2.3.1.3 Saliva

Serial dilutions were prepared from stock saliva sample in the following dilutions 1:350, 1:400, 1:450, 1:500, 1:900, 1:1000 and 1:1100 in deionised water to be used for all subsequent experiments.

2.3.2 Presumptive Testing

2.3.2.1 Kastle-Meyer (Phenolphthalein) Test for blood

The test was carried out following manufacturer's procedures with no change to the protocol provided except that the test was preformed directly using a filter paper instead of taking a swab from the assumed substance. A volume of 100 µl of the following dilutions; 1:10000, 1:15000 and 1:20000 were pipetted onto a filter paper and one drop of ethanol followed by one drop of the phenolphthalein reagent were added using the provided dropper. Finally, a drop of hydrogen peroxide was added using the provided dropper. Positive results were noted as such if a bright pink colour formed within two minutes. The test was repeated in triplicates.

2.3.2.2 Hemastix® Test for blood

The test was carried out following the instructions provided by the manufacturer except that samples were pipetted directly on to the strip instead of the strip being dipped into the sample solution. In addition, cut-off time was extended from 60 s to 2 min. 100 µl of the following dilutions: 1:150000, 1:200000 and 1:250000 were pipetted

directly onto the test strip. Positive results were scored when any green colour change appeared within 2 min. The test was repeated in triplicates.

2.3.2.3 Phosphatesmo KM® Test for semen

The test was carried as manufacturers' recommendations with no amendments to the protocol provided except that the cut off time of reading the results was extended from "few seconds" as manufacturers' notes to two minutes. The test paper was removed from its container and was exposed by pulling apart the ends of the plastic tape, 100 µl of each dilution was then pipetted directly onto the centre of the test paper. Positive results were scored when a purple colour change appeared within 2 min. Since there was no sensitivity limit indication provided by the manufacturer and no value found in the literature; a wide range of dilution values was tested. Based on a preliminary study (results not shown) the dilutions used here were 1:3000, 1:4000 and 1:5000.

2.3.2.4 Phadebas® Test for saliva

A set of three dilutions was prepared as follow; 1:900, 1:1000 and 1:1100. Following the manufacturers' "directions for use". 200 µl of each dilution was pipetted into a 15 ml tube and 4 ml of distilled water was then added and the tubes were placed in a 37 °C water bath for 5 min. One Phadebas® tablet was then added to the tube, vortexed for 10 s and then left at 37 °C for 15 min. The reaction was then stopped by adding 1 ml of 0.5 M NaOH, vortexed immediately and then centrifuged at 1500*g* for 5 min. The change from clear to blue colour observed by the naked eye indicated the presence of amylase.

2.3.3 Confirmatory Testing

2.3.3.1 RSID™-Blood

The following dilutions were made from stock; 1:300, 1:400 and 1:500. Manufacturers' procedures were followed. A volume of 100 µl of each dilution was pipetted onto the tip of a sterile cotton swab; the whole swab was then broken off into a 1.5 ml tube and 250 µl of the provided extraction buffer added. The samples were then left at room temperature for 1.5 h with occasional agitation. A volume of 20 µl of the sample/extraction buffer mixture was added to 80 µl of the running buffer provided,

and the whole 100 µl mixture was pipetted onto the provided strip. The results were viewed after 10 min and positive results were indicated by the presence of two red lines on the strip.

2.3.3.2 Hexagon® OBTI Blood

The sensitivity of the Hexagon® OBTI kit was tested by preparing the following dilutions; 1:15000 and 1:20 000 and 1:25 000 and the manufacturers' provided procedures were followed. A volume of 100 µl of each dilution was pipetted onto the tip of a cotton swab, the whole swab was then broken into the provided sample collection tube containing the extraction buffer and left for 2 min and mixed with gentle agitation. Two drops of the mixture were then placed on the provided cassette using the provided dropper and results were viewed within 2 min. Positive results were indicated by the presence of two blue lines on the strip.

2.3.3.3 RSID™-Semen

The following dilutions were made from stock; 1:2000, 1:3000 and 1:4000. Manufacturers' procedures were followed. A volume of 100 µl of each dilution was pipetted onto the tip of a cotton swab. The whole swab was then broken off into a 1.5 ml tube and 250 µl of the provided extraction buffer added. The sample was then left at room temperature for 1.5 h with occasional agitation. A volume of 20 µl of the sample/extraction buffer mixture was added to 80 µl of the running buffer provided, and the whole 100 µl mixture was pipetted onto the provided strip. Strips were viewed after 10 min and positive results were indicated by the presence of two red lines on the strip.

2.3.2.4 RSID™-Saliva

The following dilutions were made from stock solution and they included 1:400, 1:450 and 1:500. Manufacturer's procedures were followed. A volume of 100 µl of each dilution was pipetted onto the tip of a cotton swab; the whole swab was then broken off into a 1.5 ml tube and 250 µl of the provided extraction buffer added. The sample was then left at room temperature for 1.5 h with occasional agitation. A volume of 20 µl of the sample/extraction buffer mixture was added to 80 µl of the running buffer provided, and the whole 100 µl mixture was pipetted onto the provided strip. Results

were viewed after 10 min and positive results were indicated by the presence of two red lines on the strip.

2.3.4 Determination of Minimum DNA Quantities

Selected dilutions where weak positive results for both presumptive and confirmatory tests were further analysed to examine the DNA quantities present. The same amounts of diluents (100 µl) were used for the extraction as for the sensitivity study. Hence the extracted DNA quantity could directly reflect the results from the presumptive and confirmatory tests. Table 2.1 below summarise the different body fluid dilutions used for DNA extraction and the corresponding amount of the body-fluid in micrograms.

Table 2.1: Table showing the different dilutions of body-fluids used to determine the DNA quantity using 100 µl of starting material (200 µl for phadabes).

Body-fluid	Dilution	Amount
Blood	1:400	0.25
	1:10 000	0.01
	1:15 000	0.007
	1:200 000	0.0005
Semen	1:2 000	0.05
	1:4 000	0.025
saliva	1:400	0.5
	1:900	0.22

2.3.5 DNA Extraction

All samples set for DNA extraction were pipetted on sterile cotton swabs as what was the standard procedure for evidence collection at the Police Department. All body fluid samples were extracted in triplicates.

2.3.5.1 Blood

A volume of 100 µl of the following Blood dilutions were pipetted in triplicates on to the tips of different cotton swabs, they included 1: 400 (0.25 µl), 1:10 000 (0.01 µl), 1:20 000 (0.005 µl) and 1:200 000 (0.0005 µl). The whole swab was then broken into a

1.5 ml tube. The samples were extracted using the Chelex method (Welsh et al., 1991). To each tube, 175 µl of 10% Chelex (Sigma-Aldrich, Germany) and 20 µl proteinase K (10 mg/mL) (Sigma-Aldrich, USA) were added. The mixture was then placed in a water bath at 56 °C for 2 h followed by 8 min at 100 °C. The samples were then centrifuged for 3 min at 13000*g* and the supernatant was then transferred to a separate 1.5 tube ready for DNA quantification.

2.3.5.2 Semen

A volume of 100 µl of the following semen dilutions were pipetted in triplicates on to the tips of different cotton swabs, they included 1:2000 (0.05 µl) and 1:4000 (0.025 µl). The whole swab was then broken into a 1.5 ml tube. The samples were extracted using the Chelex method (Welsh et al., 1991). To each tube 175 ul of 10% Chelex (Sigma-Aldrich, Germany) 20 µl proteinase K (10 mg/ml) (Sigma-Aldrich, USA) and 7 µl of DTT (10 mM) (Sigma-Aldrich, USA) were added. The mixture was then placed in a water bath at 56 °C for 4 h followed by 8 min at 100 °C. The samples were then centrifuged for 3 min at 13000*g* and the supernatant was then transferred to a separate 1.5 tube ready for DNA quantification.

2.3.5.3 Saliva

A volume of 100 µl of the following saliva dilutions were pipetted in triplicates on to the tips of different cotton swabs, they included 1:400 (0.25 µl) and 1:900 (0.11 µl). The whole swab was then broken into a 1.5 ml tube. The samples were extracted using the Chelex method (Welsh et al., 1991). To each tube 175 µl of 10% Chelex (Sigma-Aldrich, Germany) and 20 µl proteinase K (10mg/mL) (Sigma-Aldrich, USA) were added. The mixture was then placed in a water bath at 56 °C for 4 hours followed by 8 min at 100 °C. The samples were then centrifuged for 3 min at 13000*g* and the supernatant was then transferred to a fresh 1.5 tube ready for DNA quantification.

2.3.6 DNA Quantification

All DNA samples extracted from the different body fluids dilutions were quantified using the Quantifiler™ Human DNA Quantification kit on a an ABI 7500 real-time PCR machine (Applied Biosystems Foster City, USA). Amplification reactions and amounts were carried as recommended by the manufacturer. A volume of 2 µl of target DNA

was amplified with 23 µl of prepared master mix of 10.5 µl of Quantifiler™ human primer mix and 12.5 µl Quantifiler™ PCR reaction mix to give a final total volume of 25 µl reactions. Provided DNA standard was prepared at 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 23 µl of master mix was loaded separately into the wells. A volume of 2 µl of each DNA standard concentration was loaded into its corresponding well in duplicate. A volume of 2 µl of the extracted DNA samples were then loaded on the plate and the plate was sealed with an optical adhesive cover (Applied Biosystem). The plate was then placed into the ABI 7500, which was already prepared for running DNA quantification. The thermal cycler protocol was performed according to the manufacturer instructions (Applied Biosystem): Holding stage 1, 50 °C for 2 min and holding stage 2 at 95 °C for 10 min followed by a two-step cycle of 40 cycles; step 1 at 95 °C and step 2 at 60 °C. After completion of amplification, the DNA concentration for each sample was estimated in ng/µl.

2.4 Endurance of DNA, presumptive and confirmatory tests subject to environmental insults

2.4.1 Experimental Setup

The setup of this experiment was designed to endure outdoor environment for 51 days in which sampling took place every other day for the initial 15 days and every third day thereafter. Prior experience was gained through the conduction of two pilot studies from which the sample sizes and the sampling rate has been adjusted. All eight presumptive and confirmatory tests mentioned in Section 2.2.2 and Section 2.2.3 in addition to DNA samples were tested to investigate their respective endurance to environmental insults.

A white roof tile (50 X 50 cm) was used to secure three types of materials; glass (microscope slides), cloth (100% pure cotton) and metal (a large stainless steel kitchen knife). Each material type was placed in triplicate, one for each body-fluid type. Grids

of 1 cm ^{2} were drawn on all material types with a marker pen in which 5 µl of each body-fluid type was pipette. Each row contained six grids; three grid contents were used for triplicate DNA analysis, the rest of the grid contents were used for the presumptive and confirmatory testing. Some grids were left as a negative control which was sampled every third sampling day (Figure 2.1).

All parts of this set up were cleaned thoroughly before being assembled. The roof tile, kitchen knives and glass slides were first cleaned with 70 % bleach solution followed by spraying with TriGENE (Medichem International, UK), they were all then wiped clean with deionised water before being fixed to the tile. The cotton cloth was soaked in 70 % bleach for 2 h then soaked for 2 h in deionised water followed by ironing.

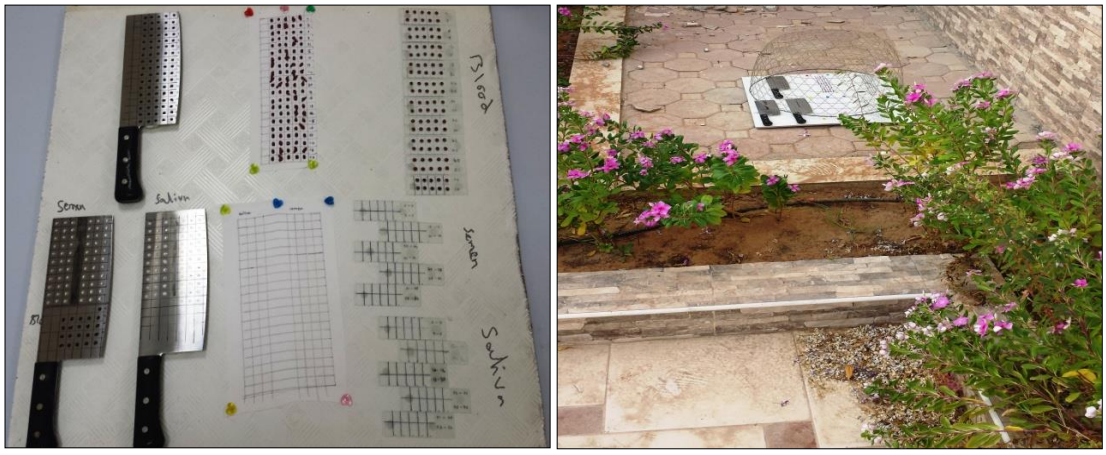


Figure 2.1 A pictures showing the set up of the experiment. Blood, semen and saliva samples were deposited on cloth, metal and glass materials and placed outdoors exposed to environmental insults for the duration of the experiment.

The roof tile was then taken outdoors in an open and uncovered area exposed to environmental insults for the duration of the experiment. A dome-like metallic grid (used for catching crabs in the region) was placed on top of the roof tile to stop scavengers from entering the setup without compromising the exposure of the experiment to the environment.

The whole inside of the 1 cm^2 grid was either swabbed with a wet cotton swab moistened with deionised water (in the case of glass and metal) or cut out with a

sterile disposable scalpel and placed immediately in a sterile 1.5 ml tube and stored at -20 °C until further work was conducted.

2.4.2 Accumulated Degree-Days (ADD)

Accumulated degree-days (ADD) as defined in (Chapter 1) was calculated from the hourly readings using data-logger placed at the experiment site. ADD was calculated using the following formula:

ADD = (Maximum + Minimum Temperature)/2

2.4.3 DNA Quantification

2.4.3.1 Quantifiler™ Human

All samples extracted were quantified using the Quantifiler™ Human DNA Quantification kit on a 7500 real-time PCR machine (Applied Biosystems) as described in (Section 2.2.6).

2.4.3.1 Quantifiler™ Trio

A total of 445 samples extracted were also quantified using the Quantifiler™ Trio DNA Quantification kit (Applied Biosystems Foster City, USA) on a an ABI 7500 real-time PCR machine. Amplification reactions and amounts were carried as recommended by the manufacturer. A volume of 2 µl of target DNA was amplified with 18 µl of prepared master mix of 9 µl of Quantifiler™ Trio primer mix and 9 µl Quantifiler™ PCR reaction mix to give a final total volume of 20 µl reactions. Provided DNA standard was prepared at 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 18 µl of master mix was loaded separately into the wells. A volume of 2 µl of each DNA standard concentration was loaded into its corresponding well in duplicate. A volume of 2 µl of the extracted DNA samples were then loaded on the plate and the plate was sealed with an optical adhesive cover (Applied Biosystem). The plate was then placed into the ABI 7500, which was already prepared for running DNA quantification. The thermal cycler

protocol was performed according to the manufacturer instructions (Applied Biosystem): Holding stage 1, 50 °C for 2 min and holding stage 2 at 95 °C for 10 min followed by a two-step cycle of 40 cycles; step 1 at 95 °C and step 2 at 60 °C. After completion of amplification, the DNA concentration for each sample was estimated in ng/µl.

2.4.4 STR Analysis

A total of 47 samples (comprising all body-fluid and material types tested) from ADD 0, 338.5, 724.5, 1250.25, 1926.25 and 2201.5 were selected to examine the effect of environmental insults on DNA profiles generated using the AmpF/STR® Identifiler® Plus kit (Applied Biosystems).

STR amplification was carried out following the manufacturer's recommendations in a 25 µl reaction containing 10 µl Identifiler® Plus Reaction Mix, 5 µl Identifiler Plus® Primer Mix, extracted DNA samples from blood to a final amount of 1 ng and the total volume of the reaction mix was made up to 25 µl with TE buffer. PCR was carried out in a GeneAmp® 9700 (Applied Biosystems). Amplification reactions were carried as recommended by the manufacturer, with the following cycling conditions: 95°C for 11 min, 28 cycles at 94°C for 20 sec and 59°C for 2 min. Data were then collected using ABI 310 Prism® Genetic Analyser (Applied Biosystems).

2.4.5 Capillary Electrophoresis

The amplified multiplex PCR products were assessed using an ABI 310 Prism® Genetic Analyser (Applied Biosystems). DNA fragment analysis was carried out using a 47 cm long capillary using POP™- 4 polymer (Applied Biosystems). Electrophoresis running buffer was used at 1X concentration. The GS STR POP4 (1 ml) G5v2. md5 run module with dye set DS-33 (Dye set G5): 6-FAM (blue), VIC (green), NED (yellow), PET (red) and LIZ (orange) was used with the following parameters: run temperature 60 °C, syringe pump time 150 s, pre-run voltage 15 kV, pre-run time 120 s, injection time 5 s, injection voltage 15 kV, run voltage 15 kV and run time 28 min.

A volume of 24 µl of Hi- Di™ formamide, 0.5 µl GeneScan™ LIZ-500 internal size standard (Applied Biosystems) and 1.5 µl of PCR product was used. The samples were

mixed, briefly centrifuged and then incubated at 95 °C for 5 min followed by 5 min at 4 °C.

2.4.6 Analysis of DNA Profiles

The data obtained from capillary electrophoresis (CE) were analysed using GeneMapper™ ID version 3.2 (ABI 310, Applied Biosystems, UK).The parameters for the analysis of the multiplex amplicons were kept constant for each run and were described in Table 2.2.

Table 2.2 Table contained the parameters for the analysis of PCR fragments.

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

2.5 Statistical Analysis

R-Studio Version 3.0.1 was used for all statistical analysis carried out in this thesis. Data are expressed, where possible, as graphical representations or electropherograms and as mean ± standard deviation (SD) supported using ANOVA or Tukey's (HSD). Data are also supported by "n" and "p" values where appropriate.

CHAPTER 3

DNA DATA ANALYSIS

3.1 Overview:

In January 2008 the Ras Al Khaimah (RAK) Police "DNA Genotyping Unit" was established as part of the Forensic Laboratory. This was done in order to add to the overall arsenal in the fight against crime, with the goal to improve the crime detection rate in the Emirate. Since then, DNA testing has become especially important in criminal cases involving sexual assault, kinship testing and in the identification of human remains.

The number of submitted cases to the Forensic DNA Unit at RAK police has been increasing steadily over the past 8 years. Initially, most casework received by the laboratory involved samples relating to illegal sexual crimes and reference samples taken from both victims and suspects, mainly due to the limited understanding of the crime solving potential of DNA analysis by the investigating officers. Upon reception, all forensic evidence enters an examination laboratory in which the items are inspected for relevant biological materials. Presumptive and confirmatory tests are also carried out at this stage. Following the identification of biological material, the sample is then taken to the next processing laboratory in which extraction is performed. The Chelex-100 method is the technique of choice but other extraction and purification methods and platforms are available for use. Next the sample is quantified in a pre-PCR room. Real-time PCR is performed to estimate the quantity and quality of the DNA present if any. Several quantification kits are used frequently at this stage; the Quantifiler® Human, Duo and Trio (Applied Biosystems) are the main methods employed. Amplification of the DNA present in the evidence samples is carried out by one of several STR multiplex kits; typically the Identifiler® Plus kit (Applied Biosystems) is used for the PCR, however, depending on the case, sample type and quantification results, other multiplex systems such as the Mini Filer and Y-Filer can also be used. Finally, fragmentation analysis is carried out in capillary electrophoresis and genetic STR profiles are then assessed and reported on.

3.2 Aims and objectives:

The aim of this study was to investigate the success of DNA profiling at the Forensic DNA Unit in RAK police measured by the success rate of producing full STR profile from submitted casework samples. This was done in order to evaluate factors influencingthese success rates as related to the sample types processed. These aims can be achieved through the following objectives:

1- A review of the rate of genetic information generated from forensic casework samples submitted to the DNA Unit for a three year period from 2012 to 2014.
2- To analyze the nature of partial STR profiles from the same three year period in terms of missing loci.
3- Identification of specific sample types that contribute to the increased proportion of inconclusive STR results, either by elevated levels of inhibition or degradation.

The findings from this chapter directly influenced the design of further studies conducted to evaluate and seek to improve the overall success rate of scene of crime samples submitted to the Forensic DNA Unit in RAK Police Forensic Laboratory.

3.4 Results:

The workflow of RAK Police DNA unit is shown in the chart flow in Figure 3.1.

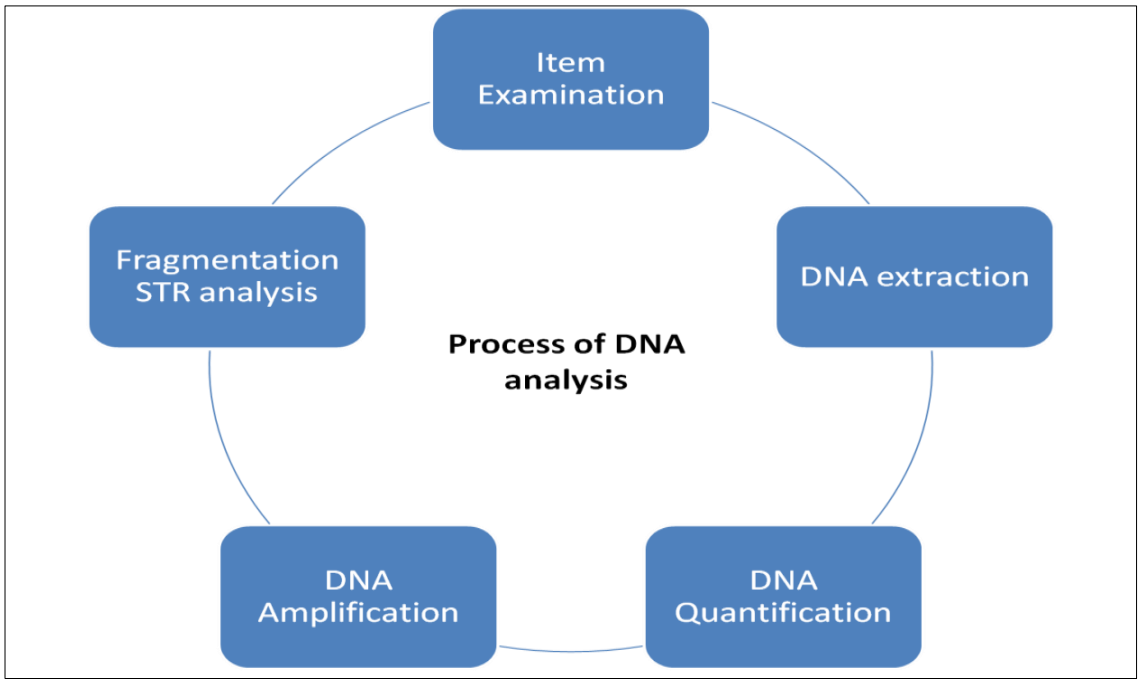


Figure 3.1 A flow-chart representing the main stages involved in the processing of biological evidence to produce a DNA profile. They include item reception and examination, DNA extraction, DNA quantification and amplification, DNA fragmentation and STR analysis.

3.3.1 Overview of case work submitted

As can be seen from Figure 3.2, the number of submitted cases to the Forensic DNA Unit has continued to increase since the year 2009. After the initial surge from 2009 to 2011 (56 to 152 cases), the number of case submission was almost unchanged from the year 2011 until 2013. However, the following year (2014) saw a surge in the number of cases submitted, reaching almost double the number from the previous year (from 171 cases in 2013 to 325 cases in 2014).

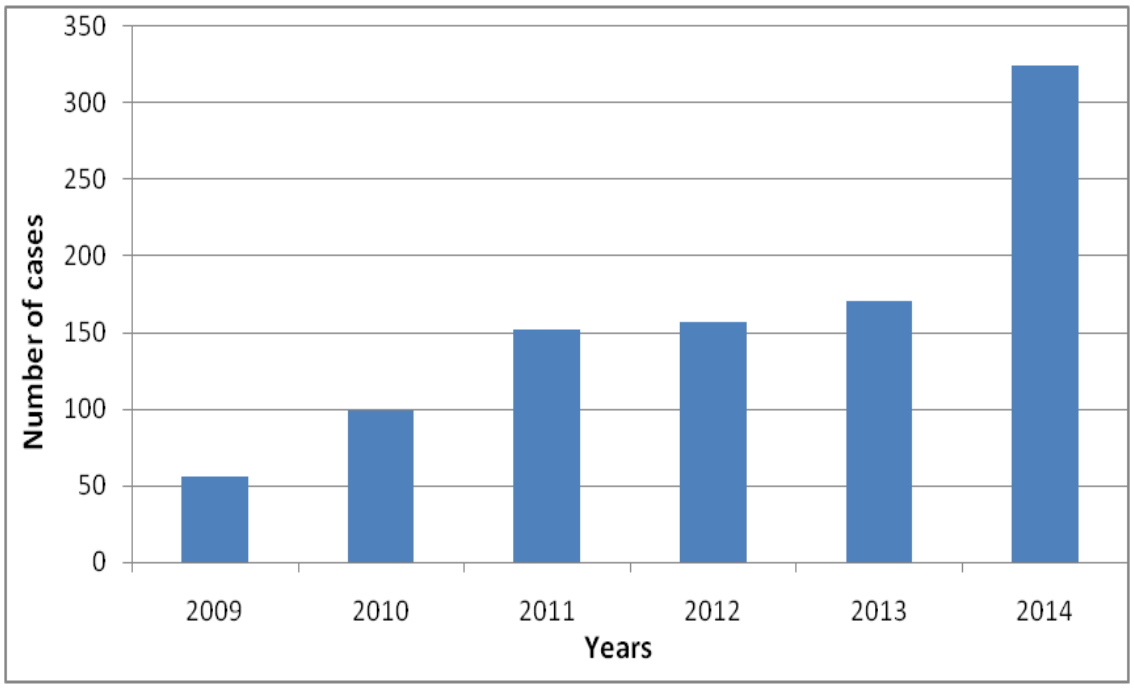


Figure 3.2 A Bar-chart showing the increase in the number of cases received by the forensic DNA unit from the year 2012 until 2014. Note the large increase in 2014.

3.2.2 Analysis of success rates

Over 96% of samples received by the unit fell in the body-fluid category. They included either blood, semen or touch (saliva) samples. Therefore all analysis work was only limited to this type of samples shown in (Table 3.1).

Table 3.1 Table showing the percentage of each body-fluid type is calculated from the total number of samples received each year, also the percentage was calculated for the sum of the three years

	2012	2013	2014	Combined %
Blood	24.2%	21.50%	18.3%	21.0%
Semen	18.1%	16.6%	14.9%	16.3%
Touch (Saliva)	53.8%	59.9%	65.4%	59.3%
Nails	1.9%	1.1%	1.1%	1.3%
Hair	1.5%	0.7%	0.0%	0.7%
Urin	0.4%	0.0%	0.3%	0.2%
Bone	0.0%	0..4%	0.0%	0.1%

STR profiles generated from identified body-fluids (blood, semen and saliva) and encountered in the last three years were analyzed to calculate the success rate of each body-fluid type. The profiles were classified as full profiles including mixtures (FP), partial profiles which also include mixtures (PP) and no profile (NP).

The results show that blood samples were the most robust of the body-fluid types. Full profiles were obtained in 85.7% of blood samples submitted for DNA analysis in 2012. Semen samples have also showed a high percentage of full profile yield at 80.9 %, although this percentage was as low as 55.8 % in 2014. On the other hand, touch (saliva) samples maintained a relatively consistent proportion of full profiles in the 3 years of examination with percentages ranging between 44% in 2012 and 48.4% in 2013. Inhibition was seen in all body fluid types in every year they were analyzed, except for semen samples in 2012. Moreover, inhibition as indicated by the Quantifiler® kits' Internal Positive Control (IPC) was remarkably higher in blood samples with around 8% of all blood samples profiled in that period exhibiting inhibition. In comparison, only around 3.4% of semen and touch (saliva) samples showed any form of inhibition in the same period (Table 3.2).

Table 3.2 Table showing the percentage success rate of STR profiles produced from DNA analysis of different body-fluid types for the years from 2012 until 2014.

	YEAR	FP	PP	NP	INHIBITION
Blood	2012	85.70%	6.4%	0.0%	7.9%
	2013	75.40%	9.8%	6.6%	8.2%
	2014	81.30%	6.3%	4.7%	7.8%
Semen	2012	78.7%	4.3%	17.0%	0.0%
	2013	80.9%	8.5%	4.3%	6.4%
	2014	55.8%	17.3%	23.1%	3.9%
Touch (Saliva)	2012	44.0%	32.1%	21.6%	2.2%
	2013	48.4%	29.6%	17.6%	3.8%
	2014	45.7%	36.5%	13.7%	4.1%

FP= full profile, PP= partial profile, NP= no profile

3.2.3 Evaluation of STR profiles

After reviewing case work results for blood, semen and saliva samples from 2012 until 2014, the STR electropherograms generated using the identifiler kit were assessed to identify the characteristics of partial profiles produced. In the period tested, there were a total of 242 partial profiles which varied in the number of missing loci and alleles. Partial profiles were only called as such when both alleles in a given loci were missing. Examples of inhibited and degraded samples are shown in Figures 3.3 and 3.4, respectively.

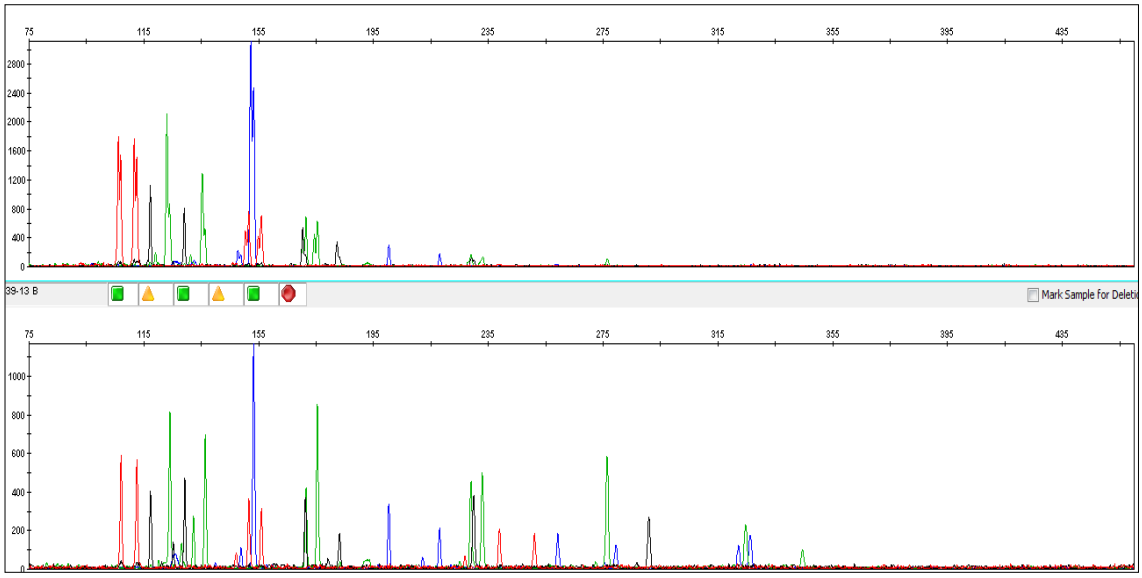


Figure 3.3: An electropherogram showing two STR profile for the same sample when inhibited (top); the larger alleles are missing but a characteristic split peak at the smaller size loci indicates the presence of inhibition. 1:10 dilution of the same sample restores some genetic information (bottom).

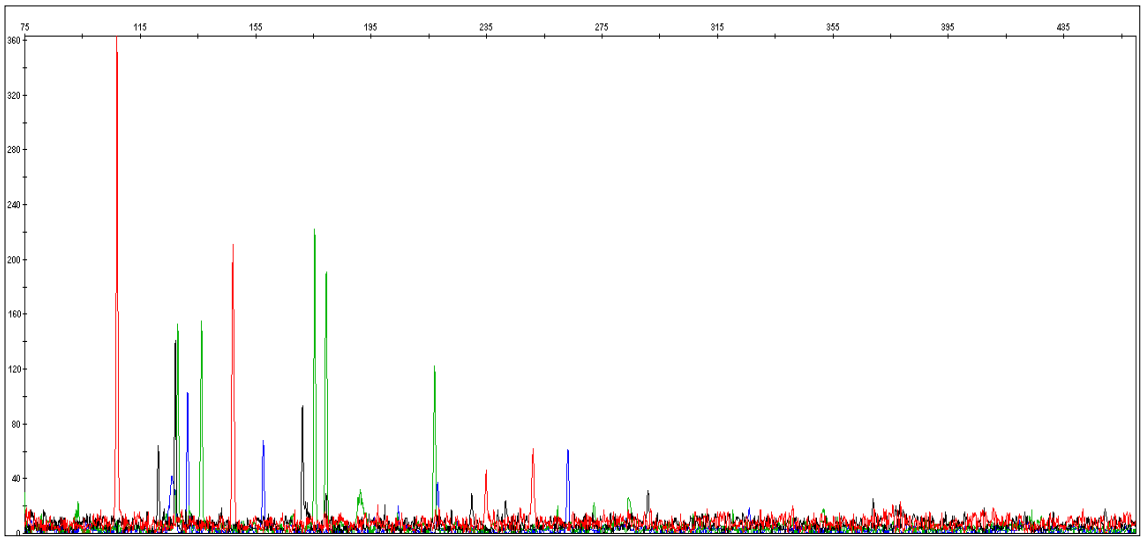


Figure 3.4: An electropherogram showing a degraded STR profile. Characteristically, smaller sized loci are amplified and are present, loci gradually dropout as the size of the loci increase.

The data in Figure 3.5 demonstrate a general pattern of increased loss of allele calling with the increase in the PCR product size of the different loci. However the pattern does not follow a straight line and it appears that some loci are more prone to allele drop outs than others. The D18S51 has the third largest product size in the Identifiler kit yet showed the highest rate of "no-calls", with the locus almost always missing when partial profiles are encountered (98.4% of the time). Whereas the D3S1358 locus which has the third smallest product size, was the locus with least missing alleles of the partial profiles analyzed (12% of the time). Interestingly, all the loci labelled with the green (VIC) dye showed unexpected better results in comparison to their product size. Similarly, with the exception of the vWA locus, all loci labelled with the yellow (black) (NED) dye performed worse than expected for their respective product size range.

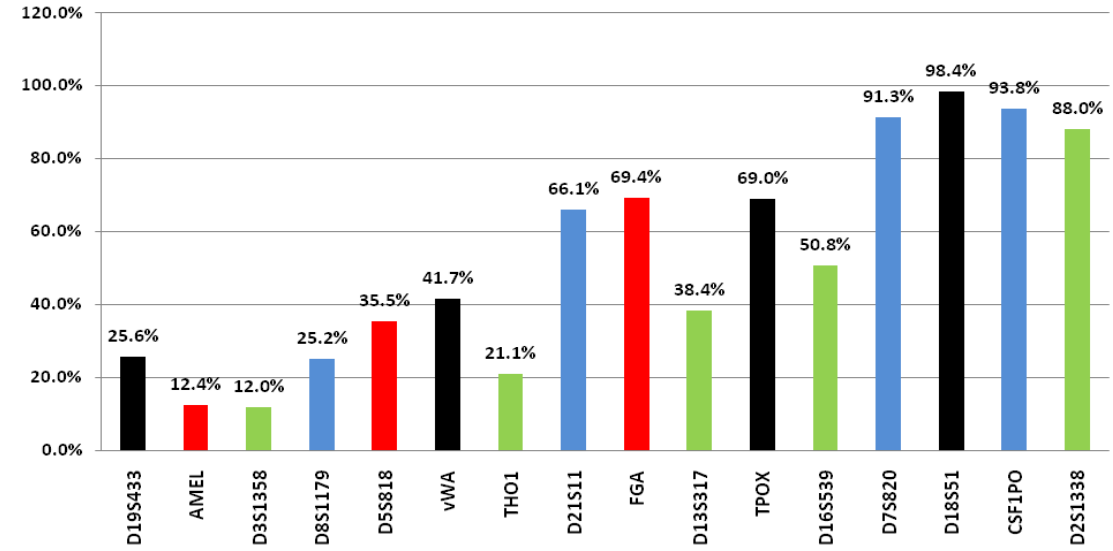


Figure 3.5: Bar charts of the percentage of missing loci calculated from the number of partial profiles from 2012 until 2014. Loci are arranged in order of PCR product sizes in the Identifiler plus kit. Black bars represent the NED dye label, red is PET, green is VIC and blue is the 6-FAM day label.

2.4 Discussion

The DNA Forensic Unit at RAK police is a small through-put laboratory serving a population of fewer than half a million people (DED, 2012). However, the number of submitted cases has risen continuously since the establishment of the DNA unit. The establishment of the Crime Scene Unit in 2012 in RAK has contributed greatly to the increase in sample types and numbers, as well as the increased diversity in the types of cases being dealt with by the DNA unit. This increase in variety and number of samples being processed has led to the increased proportion of inconclusive STR profiles generated within the laboratory. The inclusion of samples from volume crime cases along with other major crimes has increased the number of mixture profiles and partial profiles seen when genetic analysis is carried. Furthermore, the unusual increase in case submission rate between the years 2013 and 2014 can be directly attributed to the establishment of the Scene of Crime unit in August 2012. Intelligence-led crime scene processing has been shown to increase the effectiveness of forensic laboratories (Ribaux et al., 2010). Bond and Hammond conducted a study in 2008 in which they showed that the most significant predictor of successful DNA typing was found to be

Crime Scene Examiner accreditation with offense type and the DNA sample condition (Bond and Hammond, 2008). In addition to the increasing presence of the crime scene unit, another reason for the recent increase in case work received is that the forensic DNA unit has demonstrated its efficiency in the detection of crime to investigating officers. Historically, this has shown to prompt the increased reliance on DNA analysis as a first line of investigation rather than as a last resort avenue of inquiry (Barrows and Tarling, 2004).

The results shown in Table 3.1 demonstrate that DNA analysis has been highly successful in producing informative genetic information from the sample types examined, especially from blood and semen samples. Where degradation and inhibition are thought to give rise to most of the partial and no profile results observed in blood and semen samples, DNA collection and extraction are thought to be the main factor for the majority of failures in the saliva (touch) sample types (Franke et al., 2008). PCR inhibition was a major factor affecting the outcome of STR results. While all body fluid types exhibited some degree of inhibition, blood samples were the most affected and resulted in around 8% of all samples being partially or completely inhibited. The increased level of observed inhibition in blood samples is thought to be a consequence of the extraction method. Samples extracted using the Chelex-100 method has long been identified to be unable to remove inhibitors of the PCR reaction, especially with blood samples (Higuchi, 1988; Walsh et al., 1991; Fridez and Coquoz, 1996). In addition, as seen in Figure 3.3, simple serial dilutions can restore the inhibited profile into a full profile. Information regarding STR success rates from other state laboratories is scarce. However, in a 2012 study, 15 samples of degraded bone were analyzed using STR profiling and the success rates were predictably lower than those seen in this study (Harder et al., 2012). In addition to the lower STR success rates, the relative ratio between analyzed loci was also different to the ones observed here. In another study conducted by Wilson-Wilde and co-workers, the success rates for selected casework samples was calculated (Wilson-Wilde et al., 2013) and the results were slightly higher for blood samples and comparable to our touch (saliva) samples, however their study did not include any semen samples. According to the Association of Chief Police Officers (ACPO), the success rate for body-fluid samples

from the United Kingdom was stated at 87% for blood samples, 90% for semen and 40% for saliva samples which is again reasonably comparable to the results shown here (APCO, Good practice manual, 2005 2nd Ed.).

STR analysis of all partial profiles produced in the duration of this study have shown a direct correlation between the PCR product sizes of the loci and the rate of which these loci have dropped out in the identifiler kit STR profiles. Few loci did not conform to this direct relationship (the larger the product size of a locus, the more this locus will be missing in a partial profile), particularly with the (VIC) dye label, where it was shown that markers labelled with this dye were more likely to contain called alleles regardless of their size in relation to other dyes. It is not clear whether this observed phenomenon is a localized issue specific to our genetic analyzer or whether the matter is more widely observed in the forensic community. Nevertheless, there have been some suggestions that the Chelex extraction chemistry can influence the intensity of certain dye labels (personal communication). Furthermore, size ranges were calculated from the identifiler kit allelic ladder (Butler, 2007) without any non-template addition such as mobility modifiers, which may differ from the observed size relative to an internal size standard (Butler, 2006). Therefore, the PCR Product sizes for some markers may appear different in size to what is seen on an electropherogram. Interestingly, the three largest VIC dye labelled loci all have mobility modifiers added to adjust their apparent size during electrophoresis (Butler, 2006). Finally, the genetic structure of the local population may have contributed to the non conformity of some of these markers. Alleles with shorter repeat units may be more prominent in a population for a certain locus, making the STR marker appear shorter in size than for the population with higher allele frequency of larger repeat units for the same locus.

Reviewing genetic case work data from the past three years has highlighted two main areas of concern which may require further investigations. The first is the issue of PCR inhibition and how it is related to our extraction methodology and the issue of DNA degradation, particularly in local climates and how these environmental insults affect DNA recovery. Second, presumptive and confirmatory tests are also an issue of interest that may have a direct impact on increasing the proportion of successful STR profiling in our unit. Both semen and saliva samples have resulted in relatively large

percentages of “no-profile” calls, with the largest being semen samples in 2014 with almost one quarter of all semen samples which tested positive for at least one presumptive or confirmatory test still produced no genetic information.

In conclusion, the results of this Chapter highlights the percentages of success rates at one of the regions’ most recently developed Forensic DNA Units and identifies particular loci in which drop-out rates are unusually higher than expected in relation to their size. This section of the study also points to the curiously high rate of unsuccessful profiling of semen samples especially in the year 2014.

CHAPTER 4

EVALUATION OF EIGHT DNA EXTRACTION METHODS

4.1 Introduction

The search for more efficient extraction protocols has been ongoing since the development of DNA-based genotyping (Thompson et al., 2012). The more routinely used extraction methodologies rely on either organic substances, chelating agents or silica-based capture. However, in more recent years the use of automated bench-top systems which typically utilise the use of magnetic particles to purify DNA has also gained popularity in the forensic community (Liu et al., 2012).

Forensic casework samples submitted for DNA analysis can undergo a series of processes to generate STR profiles. In many cases, forensic laboratories have to deal with samples containing degraded or low quantity (touch) DNA, often producing inconclusive PCR amplification results (Oorchot and Jones, 1997; Fondevila et al., 2007). Aside from the DNA quantity, the presence of PCR inhibitors such as humic acid from soil and heme in blood, can inactivate or hinder the process of amplification presenting forensic laboratories with another challenge to obtain STR profiles (Phillips et al., 2012). In fact, with PCR inhibition being the most common cause of PCR failure when adequate amounts of DNA are present (Alaeddini, 2012), the ability to extract the maximum amount of DNA coupled with no or minimal inhibiting substances is a key step in what has become "the gold standard in personal identification" that is forensic DNA profiling (Caputo et al., 2013).

The Chelex-100® extraction method has been the technique of choice at the Forensic DNA Unit in Ras Al Khaimah police force since its establishment in January 2008. In this chapter, three different body fluid types (blood, semen and saliva) were extracted using a total of eight extraction techniques. The phenol/chlorophorm (Sambrook et al, 1989) and Chelex-100 (Welsh et al, 1991) methods represent traditional liquid-based extractions (organic and inorganic), whereas the QIAamp® DNA Investigator Kit (Qiagen, Germany) and InnuPREP® forensic extraction kit (Analytikjina, Germany) represent silica-based extraction methods. Four automated magnetic-based extraction systems were also compared; these were the EZ1® system using the investigator card (Qiagen), the AutoMate Express™ System using the PrepFiler™ Express kit (Applied Biosystems), the Maxwell® 16 Forensic instrument with the DNA IQ™ chemistry

(Promega, USA) and the InnuPure® C16 using the innuPREP forensic DNA kit-IPC16 (Analytikjina, Germany).

4.2 Objective

To investigate the efficiency of eight extraction techniques in isolating DNA from three different body-fluid types (blood, semen and saliva). The techniques were assessed on the DNA yield quantity and quality measured by the Quantifiler® Human kit (Applied Biosystems) and the amplification of its' Internal Positive Control (IPC). The results of this study directly impacted on the methodology used for the rest of the research presented in the following Chapters as well as an impact on the extraction methodology to be employed in our DNA unit in Ras Al Khaimah.

4.3 Method

As described in Chapter 2

4.4 Results

All sample types were able to produce DNA quantities in the range recommended by the manufacturer of the STR kit used in our laboratory (Identifiler Plus kit, Applied Biosystems, USA), except for blood samples when extracted using the organic method and saliva samples when the InnuPREP® Forensic Extraction Kit and QIAamp® DNA Investigator Kit were used. The results were particularly uncharacteristic of the organic method and the test was therefore repeated 2 additional times resulting in comparable results. The bar chart in Figure 4.1 below combines the quantification results for the extraction methods tested for all body fluid types.

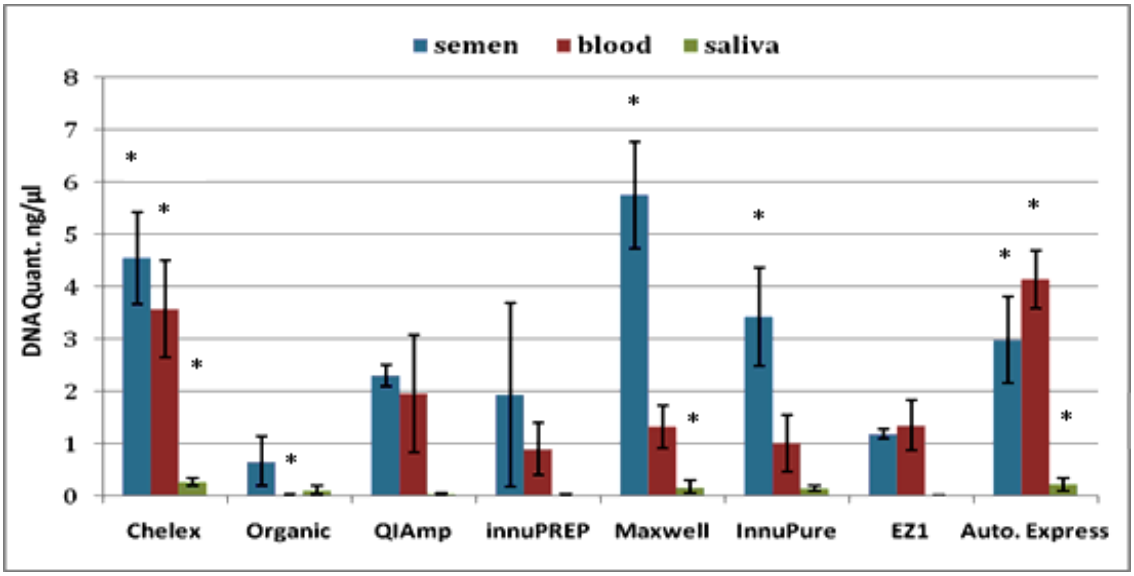


Figure 4.1 Bar chart comparing the mean yield of DNA from 8 repeats of 5 µl blood, semen and saliva samples, using 8 different extraction methods with 50 µl elution volume. Data are mean ± SD, n=8. *= $p<0.05$.

Figure 4.1 shows that each different body-fluid type was best extracted using a different extraction method. Semen samples gave the highest DNA yield when extracted using the Maxwell® 16 Forensic instrument with 5.75 ng/µl of DNA, whereas the AutoMate Express™ System resulted in the highest DNA yield from blood samples (4.13 ng/µl). Expectedly, saliva samples produced the least DNA amounts, with the Chelex-100 method yielding the highest DNA quantity from saliva samples (0.26 ng/µl). Semen samples produced the highest DNA yield across all methods tested compared to the other body-fluids. However, both the EZ1® and the AutoMate Express™ systems yielded more DNA from blood samples.

4.4.1 Blood

Figure 4.2 shows that for blood samples, the AutoMate Express™ System yielded the highest DNA quantity extracted in comparison to all other methods, with an even distribution and a mean of 4.1 ng/µl. Followed closely by the Chelex-100 method with a mean of 3.6 ng/µl, a positively skewed distribution and a wider range of results (SD=± 0.94 ng/µl). The organic (Phenol/chlorophorm) method was shown to yield around 137 times less DNA than the Chelex-100 method with a mean DNA quantity of 0.025 ng/µl. In terms of the silica-based methods; the QIAamp® DNA Investigator Kit yielded more

than twice the DNA from blood samples than its counterpart the InnuPREP® Kit with a mean DNA yield of 2.0 ng/µl and 0.7 ng/µl respectively. With the exception of one outlier at 2.1 ng/µl, the InnuPREP® Kit gave a very narrow distribution of DNA yield. The QIAamp® DNA Investigator Kit showed a wide data distribution resulting in the largest standard deviation across all methods tested (SD=± 1.13 ng/µl). With the exception of the AutoMate Express™ System, all other automated bench top systems performed similarly when extracting from blood samples. The mean values for the EZ1® System, the InnuPure® C16 instrument and Maxwell® 16 Forensic instrument were 1.3 ng/µl, 1.01 ng/µl and 1.32 ng/µl respectively.

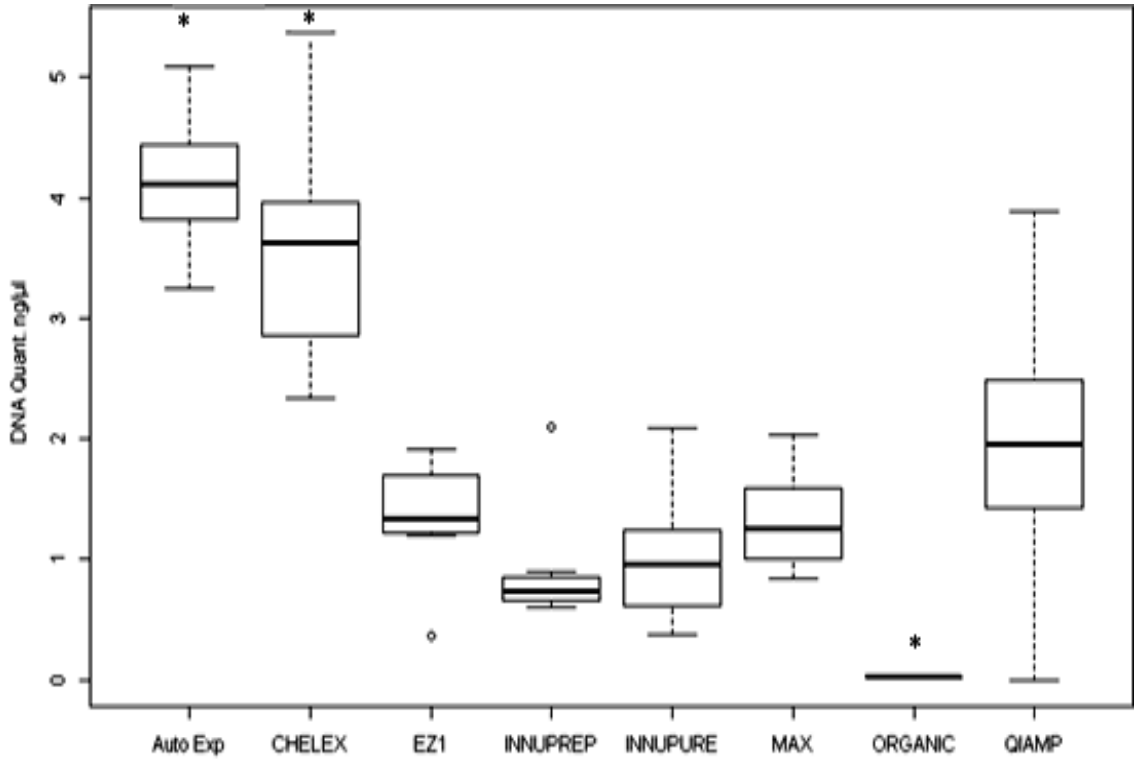


Figure 4.2 A Box plot of 5 µl of blood samples extracted using 6 different extraction methods. Data are mean ± SD, n=8. *= $p<0.05$ compared to other methods.

However, when ANOVA was carried out for all extraction methods for blood samples a P-value of 2.0e-16 was calculated, indicating a significant difference in DNA yield when the different extraction methods were used. Tukey's Honest Significant difference (HSD) test was carried out to identify pair-wise differences between all extraction methods tested with 95% confidence level, the full table of results and a graphical

representation are shown in (Appendix 2). The Tukey's (HSD) analysis showed that the majority of the significant difference in the DNA yield was due to the Chelex-100 method and the AutoMate Express™ system. Significant differences were also seen with the Organic method when compare to the Maxwell® 16 Forensic instrument, EZ1® system and the QIAamp® DNA Investigator Kit methods (Table 4.1). Where the significant differences (p= <0.005) obtained were due to an increased yield from the Chelex-100 method and the AutoMate Express™, the significant difference seen with the organic method was sustained due to a very low DNA yield.

Table 4.1 Table showing pairwise analysis using the Tukey's (HSD) statistics for all extraction techniques when blood samples were used. All pairs in this table were identified to yield significantly different amounts to each other.

Extraction Type Pairs	p-value
EZ1-Auto Exp	0.000
INNUPREP-Auto Exp	0.000
INNUPURE-Auto Exp	0.000
MAX-Auto Exp	0.000
ORGANIC-Auto Exp	0.000
QIAMP-Auto Exp	0.000
EZ1-CHELEX	0.000
INNUPREP-CHELEX	0.000
INNUPURE-CHELEX	0.000
MAX-CHELEX	0.000
ORGANIC-CHELEX	0.000
QIAMP-CHELEX	0.000
ORGANIC-EZ1	0.003
ORGANIC-MAX	0.005
QIAMP-ORGANIC	0.000

4.4.2 Saliva

The results of box plots in Figure 4.3 show that the Chelex extraction method gave the highest mean extracted DNA (0.26 ng/µl) and although the AutoMate Express™ System showed an outlier at 0.49 ng/µl (the highest DNA yield from a saliva sample) the mean value (0.22 ng/µl) came second to that obtained with Chelex. The organic extraction

showed improved performance with saliva in comparison to blood samples, with a DNA quantification mean of 0.11 ng/µl. Compared to the relatively even distribution of the Chelex data, the quantification results for the organic method was positively skewed with a relatively large SD of 0.084 ng/µl.

With the exception of the automated EZ1® System, silica-based extractions were the least effective in extracting DNA. The QIAamp® DNA Investigator Kit yielded over twice the quantity of DNA than the InnuPREP® Kit (median= 0.04 ng/µl and 0.02 ng/µl respectively). When comparing automated systems, the AutoMate Express™ System gave the highest DNA yield in contrast to the EZ1® System which was the least yielding extraction method tested (mean= 0.22 ng/µl and 0.002 ng/µl, respectively). Again, the InnuPure® C16 instrument and Maxwell® 16 Forensic instrument were similar in the amount of extracted DNA and the distribution of the yields.

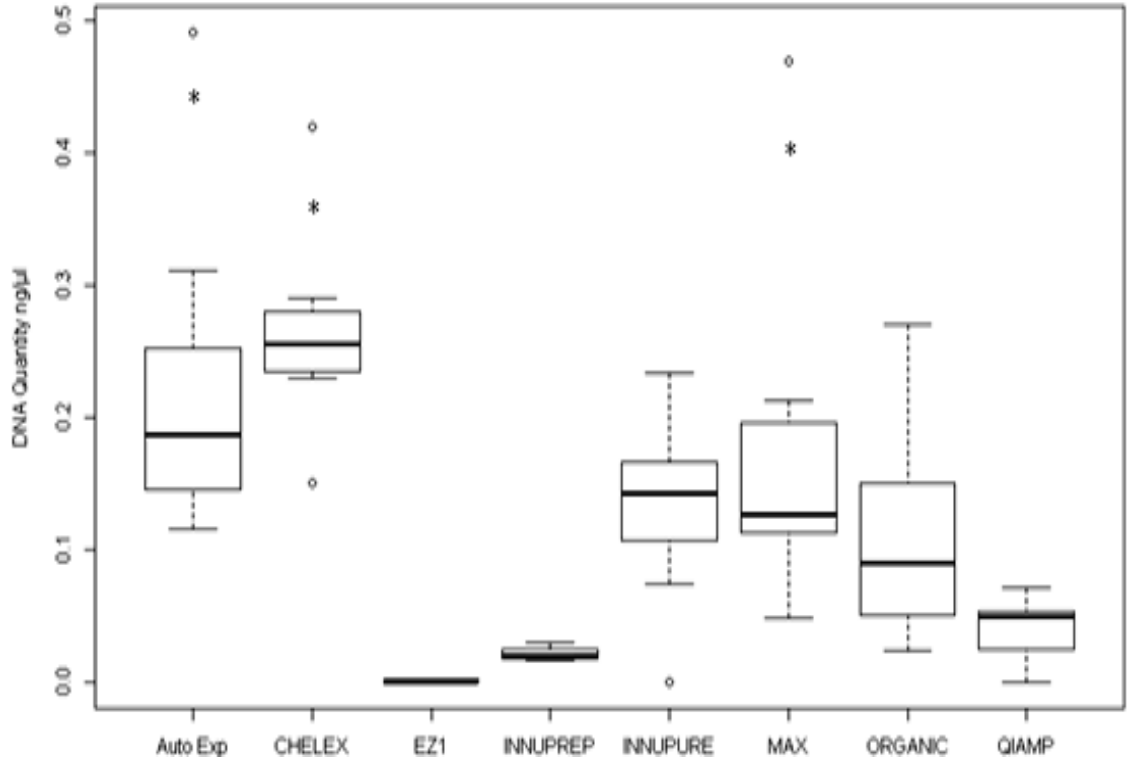


Figure 4.3 A Box plot of 5 µl of saliva samples extracted using 8 different extraction methods. Data are mean ± SD, n=8. *= p<0.05 compared to other methods.

ANOVA calculation indicated a significant difference in the DNA yield when different extraction methods were used for saliva samples (P-value= 2.82e-10). The Tukey's

(HSD) analysis was carried out to identify extraction techniques which contributed to the significant difference observed within the ANOVA calculation (Appendix 3). Again, the Chelex-100 method had a major effect on the significant difference observed when the ANOVA calculation was carried. Notably, there was no significant difference between the Chelex-100 method and all the automated techniques tested except for when compared to the EZ1® system. Significant differences were also seen with the AutoMate Express™ when compared with the InnuPREP® and the QIAamp® methods. In addition, the Maxwell® also showed significant differences when compared to the EZ1® and InnuPREP® methods. The number of pair-wise significant differences for saliva samples were much less than that observed for both blood and semen samples (see Table 4.2).

Table 4.2 Table for statistical data analysis showing pairwise analysis using the Tukey's (HSD) statistics for all extraction techniques when saliva samples were used. All pairs in this table were identified to yield significantly different amounts to each other.

Extraction Type Pairs	p-value
INNUPREP-Auto Exp	0.000
QIAMP-Auto Exp	0.001
EZ1-CHELEX	0.000
INNUPREP-CHELEX	0.000
QIAMP-CHELEX	0.000
MAX-EZ1	0.001

4.4.3 Semen

Figure 4.4 shows the DNA quantities extracted using the different methods investigated in this study. The results show the widest data distribution between the sample types tested. The extracted DNA quantity from semen samples ranged between 5.75 ng/µl for the Maxwell® 16 Forensic instrument and 0.66 ng/µl for the organic extraction method. The Chelex method produced large DNA quantities with a mean yield of 4.54 ng/µl, second only to the Maxwell® and seven times more than that produced by the organic method. However, the organic extraction was more

consistent in terms of yield (SD= 0.87 ng/µl and 1.15 ng/µl respectively). In terms of silica-based techniques, both the QIAamp® DNA Investigator Kit and the InnuPREP® Kit showed comparable averages of DNA yields, but while the latter showed the widest range of data distribution among methods tested (SD =1.76 ng/µl) In contrast, the QIAamp® DNA Investigator Kit was the most consistent (SD= 0.2 ng/µl). Direct comparison between all automated systems tested show a broad range of DNA extraction capabilities from semen samples. The Maxwell® was the highest yielding automated system while the EZ1® System gave the lowest average quantity of extracted DNA 1.19 ng/µl. However, both the AutoMate Express™ and InnuPure® systems gave similar quantities (3.0 ng/µl and 3.42 ng/µl respectively) and similar data distributions.

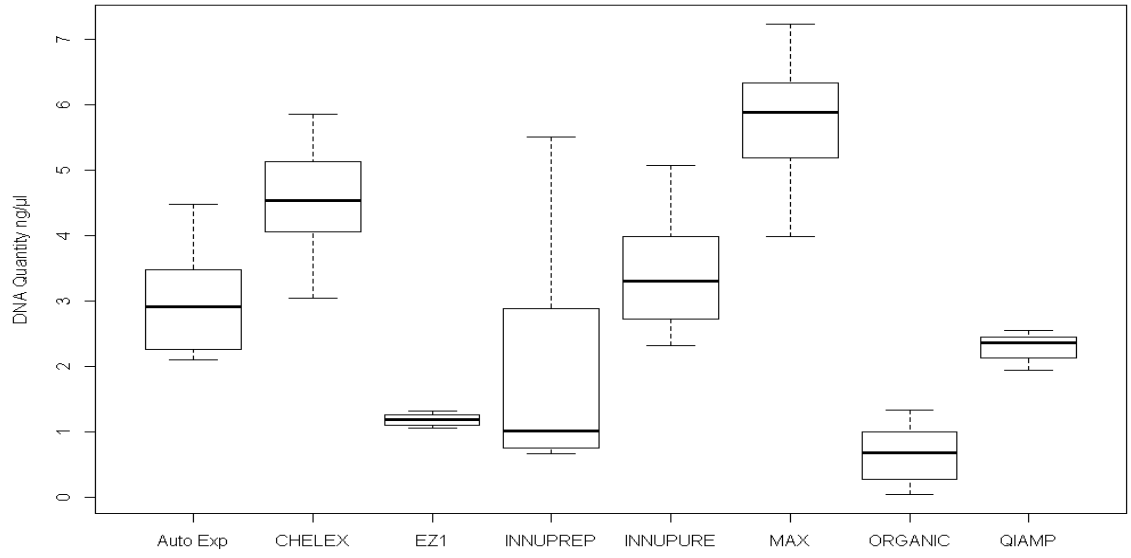


Figure 4.4 A Box plot of 5 µl of semen samples extracted using 8 different extraction methods. Data are mean ± SD, n=8.

Calculating ANOVA for the DNA yield from semen, the Null hypothesis that there is no significant difference in the DNA yield when different extraction methods were used was rejected (P-value= 5.98e-16).

The Tukey's (HSD) was carried out to investigate pair-wise differences between the different extraction methods (Appendix 3). The results showed that for semen samples, the significant difference highlighted by the ANOVA calculation was due to

several extraction techniques. As opposed to blood and saliva samples, when semen samples were extracted, most of the extraction methods used showed pair-wise significant differences. When blood and saliva samples were tested, only three methods contributed to the overall significant difference (Table 4.3).

Table 4.3 A table showing pair-wise analysis using the Tukey's (HSD) statistics for all extraction techniques when semen samples were used. All pairs in this table were identified to yield significantly different amounts to each other.

Extraction Type Pairs	p-value
EZ1-Auto Exp	0.006
MAX-Auto Exp	0.000
ORGANIC-Auto Exp	0.000
EZ1-CHELEX	0.000
INNUPREP-CHELEX	0.000
ORGANIC-CHELEX	0.000
QIAMP-CHELEX	0.000
INNUPURE-EZ1	0.000
MAX-EZ1	0.000
MAX-INNUPREP	0.000
MAX-INNUPURE	0.000
ORGANIC-INNUPURE	0.000
ORGANIC-MAX	0.000
QIAMP-MAX	0.000

The bar charts in Figure 4.5 show that almost all extraction methods tested in this experiment yielded good quality DNA with an IPC value of around the 27 Ct mark. The exception was the high value of 39.0 Ct obtained when blood samples were extracted using the Chelex method and to some extent with the AutoMate Express™ with values near 29.0 Ct for both blood and saliva samples. In both cases, these extraction methods were the highest yielding methods used.

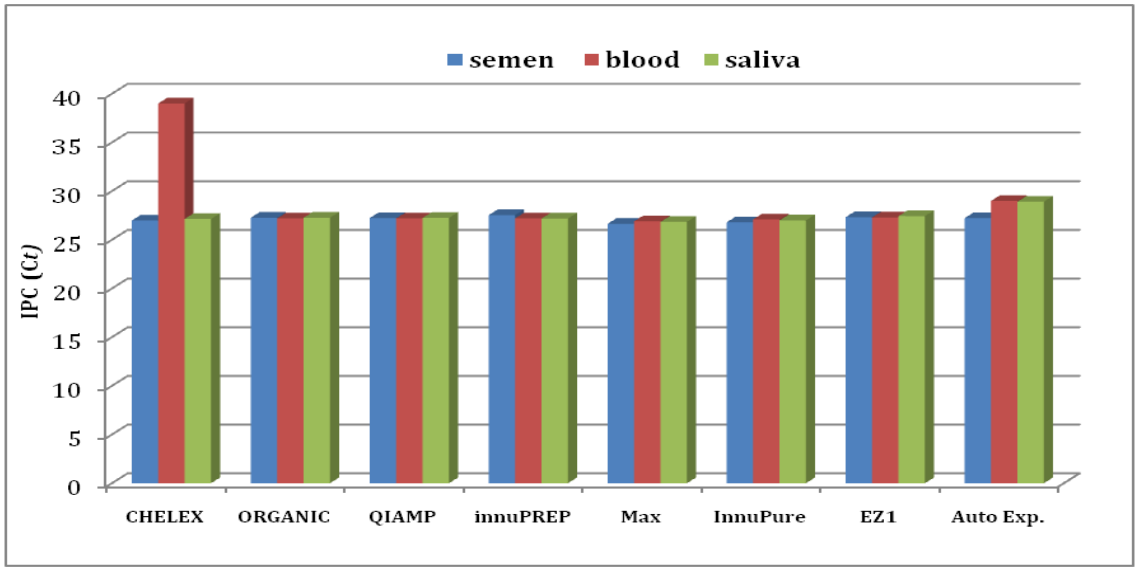


Figure 4.5 Bar charts comparing the mean value of the internal positive control (IPC) cycle time (C*t*) for samples extracted using 8 different extraction methods with 5 µl of blood, semen and saliva as starting material and 50 µl elution volume.

In addition to the analysis of results displayed in Figure 4.5, subjective comparison of additional factors such as DNA yield, consistency, quality, simplicity and price per sample cost. Table 4.4 indicates that the AutoMate Express™ System has an advantage over the other methods tested, particulary in terms of DNA yield and time efficinecy and simplicity; however, set up costs are considerably high.

Table 4.4: Table showing a subjective comparison between the 8 methods tested and rated according to their observed performance in this experiment.

Extraction method	DNA Yield	Quality	Consistency	Time/simplicity	Cost	Total
Chelex®-100	5	3	2	3	5	18
Organic	1	5	5	1	4	16
Investigator	3	5	3	3	3	17
InnuPREP®	2	5	1	3	3	14
Maxwell®	4	5	3	4	2	18
InnuPure®	3	5	3	4	2	17
Auto Exp.™	5	4	3	5	2	19
EZ1®	2	5	5	4	1	17

1= V. Poor 2= Poor 3= Good 4= V. Good 5= Excellent

4.5 Discussion

The results in Section 4.3 showed that the Chelex extraction method was more effective in extracting DNA when compared to the organic extraction. It is evident that the organic extraction did not perform to its expected potential. The method is a well-established technique in the forensic community and known for its large quantity yield (Kochl et al, 2005). This unusual result prompted the repeat of the organic test alongside the Chelex method two additional times in different laboratories; nevertheless, the results were comparable to the ones shown here. A possible way to improve on the yield for the organic method is to increase the lysis incubation time from four hours to overnight incubation. However, the large number of factors influencing the effectiveness of the procedure, coupled with the use of hazardous chemicals renders the phenol/chlorophorm extraction an undesirable technique for extraction in forensic laboratories and has prompted the search for alternative extraction methods in the forensic community (Carpi et al. 2011). In addition, a plethora of studies have shown that the organic extraction did not give the highest or most consistent DNA yield when compared to other extraction methods and kits (Cler et al., 2006; Davoren et al. 2007; Guo et al., 2009; Cawthorn et al., 2011 and Babaei et al., 2011).

Unlike the organic method, the Chelex extraction procedure relies on the chelating properties of the resin which poses minimal hazard risk to the analyst. In fact, the Chelex method showed higher yields from low DNA starting materials such as saliva samples when compared to all techniques tested in this study. Disadvantages of the Chelex method have been discussed in previous studies (Fridez and Coquoz, 1996). DNA extracted from blood samples using the Chelex method showed an unusually high value for IPC C*t* compared to all other methods indicating the presence of PCR inhibitors, arguably the only weakness of the technique. Otherwise, the values for IPCs were within accepted range as specified by the real time kit manufacturers. The most likely source of this inhibition is porphyrin compounds (heme) (Higuchi, 1988). It was suggested by Walsh et al in 1991 that the increased release of these compounds is due to the presence of Proteinase K. Therefore it might be advisable to reduce the amount of this enzyme when samples known to contain large amounts of blood are processed.

The addition of an extra purification step such as the use of the MinElute® PCR Purification kit (Qiagen, Germany) might also be a suitable solution, although this undermines the advantages offered by Chelex. It is important to note that the exact procedure may vary from one laboratory to another when wet-based extractions such as the phenol/chlorophorm and the Chelex methods are carried out, which might affect the performance of the techniques and might explain the discrepancy found in the literature (Fridez and Coquoz, 1996; Sweet et al., 1996). In this current study Chelex was found to be a much more reliable technique in extracting DNA from body-fluids than the organic method. This is in concordance with a study conducted by Jung et al. in 1991 which concluded that Chelex presents three clear advantages over the phenol/chlorophorm technique: Firstly, Chelex increases the magnitude of DNA amplification by a factor of six. Secondly, it avoids the use of toxic organic solvents and thirdly, it involves less time and work. Moreover, the sensitivity of new generation STR multiplex kits are such that usually minimal amounts of diluted DNA are required for optimal PCR amplification, since dilutions to the DNA sample will also dilute the PCR inhibitor. This has reduced the effects of the types of inhibitions based on polymerase binding site competition (Lee et al., 2014).

In terms of silica-based extractions tested in this experiment, the QIAamp® DNA Investigator Kit was shown to yield almost twice as much DNA as the InnuPREP® kit for all sample types tested and the IPC values for both kits were comparable; both showing a good level of purity in extracting DNA from body fluid samples. The only area where the InnuPREP® kit performed better than the QIAamp® DNA Investigator Kit was its consistency; the standard deviation value for the InnuPREP® kit was lower for all body fluid types except for semen, however the QIAamp® DNA Investigator Kit is well known for its high DNA yield and reproducibility (Cler et al., 2006; Guo et al., 2009 and Bogas et al., 2011). A close look at the results show that when the DNA quantities were high for both kits such as the case with semen samples, the InnuPREP® kit showed a higher standard deviation value than the QIAamp® DNA Investigator Kit indicating that the higher consistency levels for the InnuPREP kit were maintained due to lower DNA yields. Both commercial silica-based extraction kits used here do not disclose the components of their extraction chemistry or the composition of their silica

columns. Nevertheless, the bases for such extraction methods generally rely on the same basic steps (Tan and Yiap, 2009). Although the cell lysing components may differ slightly, it is more likely that the fundamental factor for the different efficiency of these kits is the composition of the silica-based component (Poeckh et al., 2008). Several studies were conducted to understand the driving forces governing the interaction between DNA and silica to maximize yield and elution (Melzak et al., 1996; Balladur et al., 1997 and Saeki et al., 2010). For instance, in 2008 Yu et al. concluded that DNA extraction conditions such as pH and loading capacity are the main factors influencing the outcome of DNA binding to silica particles. Possible inhibition can also occur from carry over salts contained in elution buffers (Yu et al., 2008), an issue that was not encountered in this experiment.

Direct comparison between the four different automated systems tested in this study did not show a clear advantage for a particular method. While semen samples produced the highest yield of DNA when extracted using the Maxwell® 16 Forensic instrument, DNA from blood and saliva samples was better extracted using the AutoMate Express™ system. On the whole, all automated systems were able to produce good quality and consistently sufficient DNA amounts for downstream STR analysis from all body-fluid types (except EZ1® system with saliva samples). Most automated systems relay on the use of magnetic particles consisting of one or more magnetic cores [generally magnetite (Fe_3O_4) or maghemite (gamma Fe_2O_3)] with a coating matrix of polymers, silica or hydroxyapatite with terminal functionalized groups which enables the capture of DNA molecules (Carpi et al., 2011). The effectiveness of the coating matrix type used and the chaotropic agent facilitating the binding of DNA is difficult to assess as most manufacturers fail to give detailed information on these aspects (Witt et al., 2012). However, the use of spin baskets in the lysis step seems to give an advantage in the overall DNA yield in the two systems which utilise such technology (the AutoMate Express™ and the Maxwell® 16 Forensic instrument). The advantage of the use of such columns was also observed by Davis et al. (2011) when comparing three automated extraction systems. While the EZ1® Advanced system lacked the use of spin baskets, recent releases of the QIAmp® kit do incorporate such columns. According to the manufacturer, the LySep® columns

included in the PrepFiler® Express kit increase the surface area for more DNA binding and easy access for the wash and elution buffers to reach maximum number of attached DNA molecules (Applied Biosystem publication, 2008).

The wide use of the EZ1® system over the last decade has been generally focused on the extraction of DNA from reference samples containing large quantities of DNA (Rockenbauer et al., 2009; Phillips et al., 2012) or as a means of purifying and concentrating samples containing large quantities of inhibitors (Anslinger et al., 2005). Either way, the results obtained from this experiment indicated that the EZ1® system is more suitable for DNA extraction from samples suspected of containing inhibitors when the DNA amount is not limiting, rather than challenging crime scene samples with limited DNA levels, a suggestion that is supported by earlier studies (Kishore et al., 2006). On the other hand, the better DNA yield of the AutoMate™ Express system found in this study was In-line line with two recent papers. The first compared the system with the QIAamp® Investigator kit on the QIAsymphony® system (Qiagen, Germany) (Stangegaard, 2013). The second paper compared the AutoMate Express to the manual QIAamp® DNA Investigator Kit (Qiagen), DNA IQ System Kit (Promega, USA) and the Chelex 100 extraction technique. Both studies found the AutoMate™ Express system to have advantages over the QIAamp® DNA Investigator Kit (Bogas et al., 2011).

In terms of DNA yield, of all the extraction methodologies examined in this study, the Chelex method and the AutoMate™ Express along with the Maxwell® 16 Forensic instrument all showed superior ability to extract DNA from the different body fluid types tested when compared to the other techniques. However, recent studies have demonstrated the susceptibility of the DNA IQ™ chemistry to a range of upstream products used in sample collection (adhesive tapes), presumptive testing (Hemastix®) or fingerprint enhancement reagents, due to the impact of competition for binding to the magnetic beads caused by these substances (Laurin et al., 2015). Other studies have also demonstrated reduced DNA yields with the PrepFiler™ Express kit when sample material on cotton swabs were used (Witt et al., 2012). This was observed to be due to the inability of the magnet to efficiently collect the PrepFiler™ magnetic beads. On the other hand, the InnuPure® C16 system has demonstrated good flexibility

and efficiency in extracting DNA from body-fluids, a finding reviewed by Tan and Yiab in 2009.

Modifications and improvements of existing extraction methods and systems are constantly investigated and implemented (Sweet et al., 1996; Nagy et al., 2005; Yu et al., 2008 and Caputo et al., 2013). Ultimately, the quest for the ultimate DNA extraction methodology can only be an inter-laboratory investigative matter.

In conclusion, the investigations carried out in this Chapter critically demonstrated that for a low through-put laboratory, the manual Chelex-100 extraction method is a sufficient DNA extraction technique which may require additional purification steps when dealing with forensic samples containing suspect inhibitors.

CHAPTER 5

SENSITIVITY OF PRESUMPTIVE AND CONFIRMATORY TESTS

5.1 Overview:

The general principle of presumptive testing is based on relatively simple chemical reactions, which result in a colour change to indicate the presence of a particular body fluid in a stain. These tests are well documented and some have been well established and used for over a century (Takayamah, 1912). On the other hand, although some forms of confirmatory testing have been available for a long time, such as microscopic examination of semen, the use of confirmatory tests as such has gained popularity with the advent of immunocromatographic kits in the last two decades. Both confirmatory and presumptive tests save time and money by prioritizing the samples sent for DNA analysis (Johnston et al., 2008).

Crime scenes often contain substances that can appear as body fluids (Virkler and Lednev, 2009); presumptive testing at the scene of crime may limit the number of samples taken to the forensic laboratory. While further presumptive and confirmatory tests in the laboratory may exclude the material as being a body fluid or of human origin and remove the need for further analysis such as DNA profiling.

Identifying biological materials can prove to be a challenging task particularly when body-fluids are present in minute amounts. Furthermore, although the number of readily available body−fluid identification kits has increased substantially in recent years (An, 2012), the number of independent studies focused on characterizing these tests is limited. When available, these studies show a wide range of discrepancies in the reported sensitivity of these presumptive and confirmatory tests, which can ultimately lead to misguided conclusions in crime investigations where evidence containing vital information can be disregarded as invaluable due to negative presumptive or confirmatory results.

The aim of this section of the project was to investigate the sensitivity of a group of presumptive and confirmatory tests commonly used in the region and to establish a link between the sensitivity results and the ability to obtain further downstream genetic information.

5.2 Aims and Objectives:

To establish and evaluate the sensitivity limit of detection of four presumptive and four confirmatory tests in their ability to detect three different body−fluid types: blood, semen and saliva. This chapter also aims to identify the DNA quantity present at the point of the sensitivity limit for each body−fluid type tested. Experiments were designed to:

1- To establish sensitivity limits for each body−fluid tested compared to published materials or manufacturers' claims
2- To conduct DNA extraction and quantification on certain body−fluid dilutions that were identified as "sensitivity limit" for each test.
3- To identify whether quantifiable DNA quantities are still available when sensitivity limits of the presumptive and confirmatory tests are reached.

5.3 Methods: As described in Chapter 2 of this study

5.4 Results:

To test the sensitivities of each of the four presumptive and four confirmatory tests, sets of dilutions were prepared according to the manufacturers' claims of sensitivity. When not available, literature was searched for data to give an indication of the dilution levels to be prepared. Since the experiment was set up to identify relative sensitivities between the presumptive and confirmatory tests rather than absolute sensitivities, all body−fluids were taken from one male to limit the variation between samples.

5.4.1 Presumptive Testing

5.4.1.1 Kastle−Meyer (Phenolphthalein) Test for Blood

The Kastle−Meyer (phenolphthalein) test for blood is well established and commonly used in the forensic community. The test involves chemical reactions that result in a colour change from clear to purple upon contact with blood. Although some colour changes began to develop beyond the 2 minutes cut−off time for the reaction, the maximum sensitivity of the Kastle−Meyer test was noted

at 1:10000 where all three triplicates gave a positive colour change within 2 minutes. Both the 1:15000 and 1:20000 dilutions started to develop a colour change after 2 minutes of the reaction and the results were considered as negative (Table 5.1).

Table 5.1: A table showing the results of the sensitivity of the Kastle–Meyer test for blood when 100 µl of dilutions (1:10000, 1:15000 and 1:20000) were tested in triplicates.

Dilution	1st repeat	2nd repeat	3rd repeat
1:10000	+++	+++	++
1:15000	–	–	–
1:20000	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

The picture shown in Figure 5.1 was taken after a few minutes following the 2 min cut–off point and therefore most of the negative samples show a faint pink which was considered as a negative result.

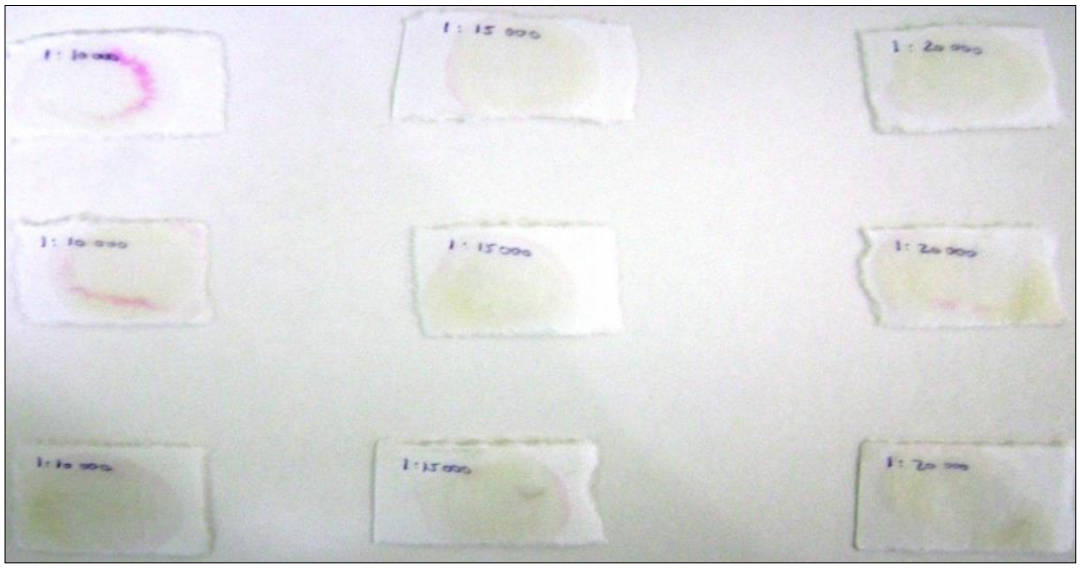


Figure 5.1 Original photograph showing three sets of dilutions (1:10000, 1:15000 and 1:20000) of blood when the Kastle–Meyer test (phenolphthalein) was carried out.

5.4.1.2 Hemastix® Test for blood

In addition to its easy application, the Hemastix® presumptive test for blood was the most sensitive presumptive test examined. The Hemastix® strip gave positive results as indicated by a change in colour from yellow to green within 2 minutes. Dilutions of up to 1:200000 were detected by this test as shown in Figure 5.2.

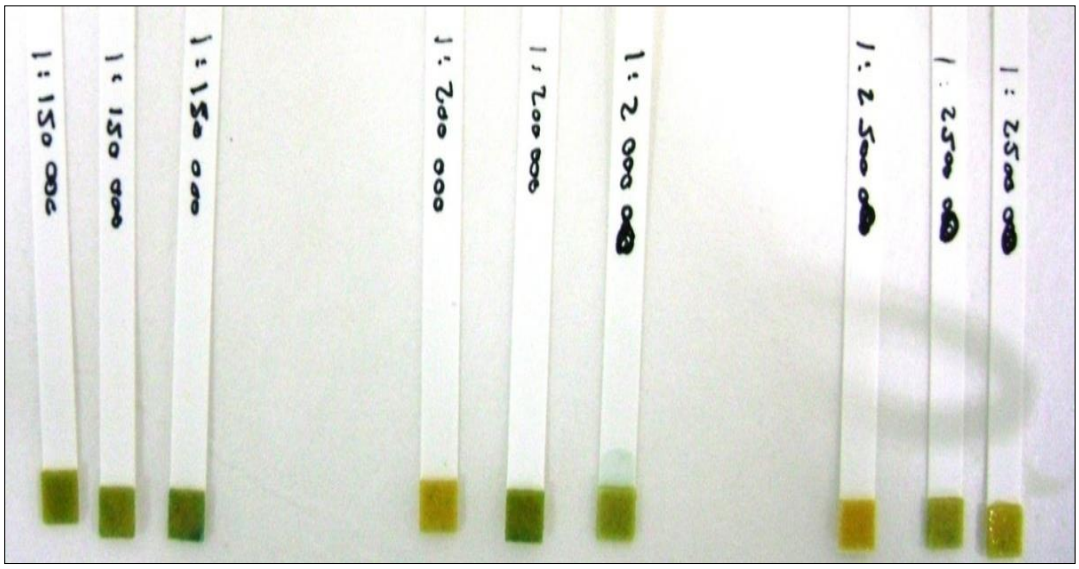


Figure 5.2 Original photograph showing Hemastix® strips when dilutions of 1:150000, 1:200000 and 1:250000 of blood were added. One strip at dilution 1:200000 gave a very weak positive result, whereas at 1: 250000 one strips gave a very weak positive result within the 2-minute window.

Beyond the 1:200000 dilutions, the Hemastix® test became unreliable as the colour change was only observed in one stick, another stick was also inconclusive and showed late colour development. The sensitivity limit for the Hemastix® test was noted at 1:200000 dilution of the blood sample. Table 5.2.

Table 5.2: Table showing the results for the Hemastix® strips test when a triplicate set of 100 µl dilutions of blood were tested (1:150000, 1:200000 and 1:250000). Consistent positive results were still observed at 1:200000 dilutions.

Dilution	1st repeat	2nd repeat	3rd repeat
1:150000	+++	+++	+++
1:200000	+++	++	+
1:250000	+	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.1.3 Phosphatesmo KM® Test for semen

The Phosphatesmo KM® test is a common acid phosphates test that changes colour to purple upon contact with the enzyme. Dilutions were set up as mentioned in section (2.2.3.1) and results were collected within 2 minutes of application. One of the triplicate 1:5000 dilution samples gave a very weak positive result just after the 2 minutes window and results were recorded as slightly positive, Figure 5.3.

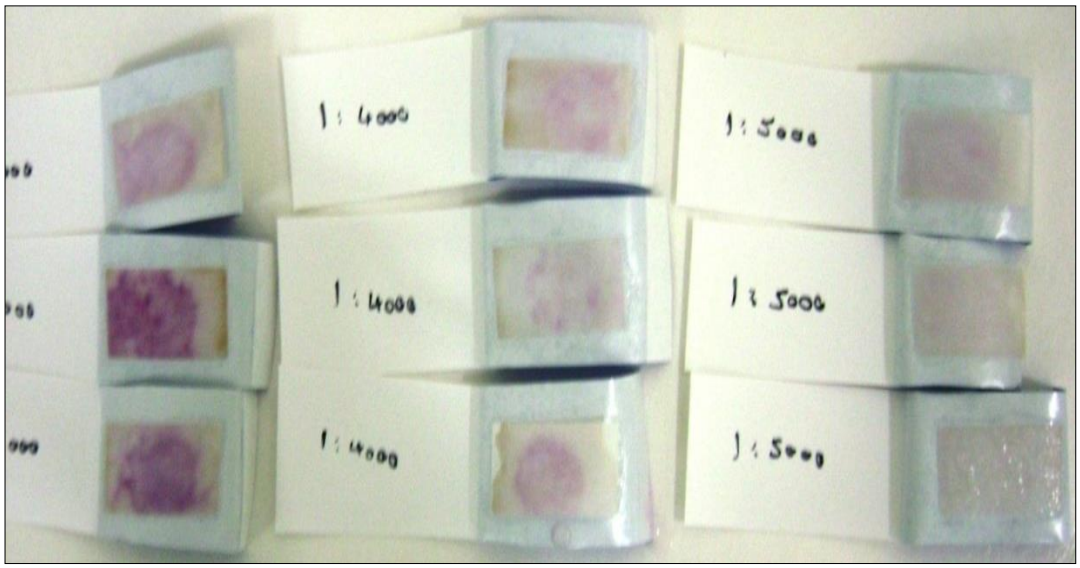


Figure 5.3 Original photograph showing three triplicate sets of the Phosphatesmo KM® with semen dilutions of 1:3000, 1:4000 and 1:5000. Only one sample at 1:5000 dilution gave a very weak positive result.

There was no prior predicted sensitivity limit for this test as none was found in the literature or the materials provided by the manufacturer (Table 5.3). The limit of

detection for the Phosphatesmo KM® (Macherey–Nagel, Germany) test for semen was noted at 1:4000 dilutions, after which the test paper did not change colour and gave negative results.

Table 5.3: Table showing results for Phosphatesmo KM® test when 100 µl of each dilutions of semen were tested in triplicate (1:3000, 1:4000 and 1:5000). Consistent positive results were still observed at dilution 1:4000.

Dilution	1st repeat	2nd repeat	3rd repeat
1:3000	+++	+++	+++
1:4000	++	+++	++
1:5000	+	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.1.4 Phadebas® Test for saliva

The Phadebas® saliva presumptive test was assessed for its sensitivity to identify the product of amylase activity from a triplicate set of three dilutions of saliva: 1:900, 1:1000 and 1:1100. The blue colour change indicative of amylase presence was observed by the naked eye with comparison to the negative control. Results are shown in Figure 5.4.

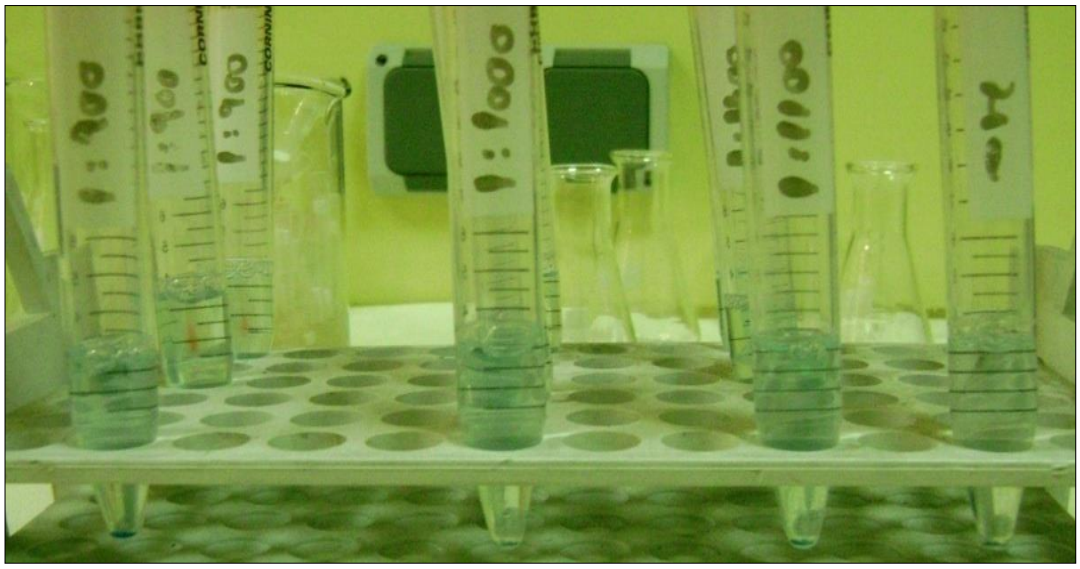


Figure 5.4 Original photograph showing test tubes containing tablets of the Phadebas® saliva presumptive test with saliva samples diluted to 1:900, 1:1000 and 1:1100.

Clear colour changes were observed with dilutions up to 1:900. The results were estimated with the naked eye relying on the judgment of the examiner. A very weak positive result was estimated for one sample of the 1:1000 dilutions, Table 5.4.

Table 5.4: Table showing the sensitivity results for the Phadebas® test for Saliva. 200 µl of each dilution of saliva was tested in triplicate (1:900, 1:1000 and 1:1100). Consistent positive results were observed at dilution 1:900.

Dilution	1st repeat	2nd repeat	3rd repeat
1:900	+++	+++	+++
1:1000	+	–	–
1:1100	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

Clear colour changes were observed with dilutions up to 1:900. The results were estimated with the naked eye relying on the judgment of the examiner. A very weak positive result was estimated for one sample of the 1:1000 dilutions.

5.4.2 Confirmatory Testing

5.4.2.1 RSID™–Blood

A set of blood dilutions were prepared as described in (Section 2.2.1.1) to test the sensitivity of the RSID™–Blood confirmatory kit. The results are shown in Figure 5.5 below.

Figure 5.5 RSID™–Blood test strips with different sets of dilutions. Two samples showed weak positive results at dilution 1:400.

The test showed positive results for dilutions of up to 1:400 after which all subsequent dilutions were negative. As seen from Figure 5.5, the detection of positive sample lines were difficult to identify in higher dilutions. Even though one sample from the 1:400 dilution was negative, still the sensitivity limit for the RSID™–blood kit was noted at 1:400.

Table 5.5: Table showing sensitivity of the RSID™–Blood. 100 µl of each dilution of blood was tested in triplicate (1:300, 1:400 and 1:500). Negative results were obtained with dilutions higher than 1:400.

Dilution	1st repeat	2nd repeat	3rd repeat
1:300	+++	++	++
1:400	+	+	–
1:500	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.2.2 Hexagon® OBTI for blood

Out of the three dilution sets prepared, positive results were only observed with the 1:15000 dilutions. One out of the three repetitions of this dilution gave a weak positive result (Figure 5.6). Higher dilutions of blood tested negative with the Hexagon® OBTI kit.

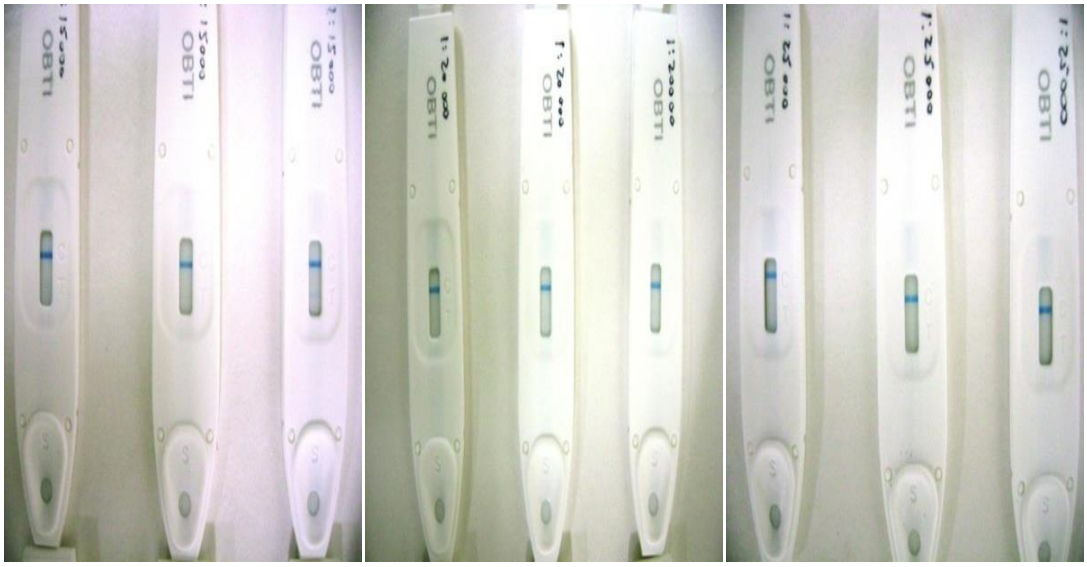


Figure 5.6 Original photographs showing the Hexagon® OBTI test for Blood when blood dilutions of 1:15000 (left), 1:20000 (centre) and 1:25000 (right) were tested. Only weak positives were obtained with the 1:15000 dilution, all further dilutions of blood gave negative results.

The Hexagon® OBTI proved to be a much more sensitive confirmatory test than the RSID™–blood. It was shown in this experiment to be over 35 times more sensitive (Table 5.6).

Table 5.6: Table showing the sensitivity of the Hexagon® OBTI test for Blood. 100 µl of each dilution of blood was tested in triplicate (1:15000, 1:20000 and 1:25000). Only the 1:15000 dilutions gave positive results.

Dilution	1st repeat	2nd repeat	3rd repeat
1:15000	++	+	+
1:20000	–	–	–
1:25000	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.2.3 RSID™–Semen

The manufacturers of RSID™–Semen test state that semenogelin in as little as 1 µl of human seminal fluid can be detected. Results of the present study showed that the manufacturers' claims were met (and exceeded) at 0.05 µl equivalent to a 1:2000 dilution (Figure 5.7).

Figure 5.7 RSID™–Semen test strips with three sets of dilutions (1:2000, 1:3000 and 1:4000). Two samples showed weak positive results at dilution 1:2000 and all further dilutions gave negative results.

Table 5.7 shows that the sensitivity limit of detection for the kit was 1:2000, with two out of the three triplicate samples giving a weak positive red line on the strip. Further dilutions of semen stock solution gave negative results.

Table 5.7: Table showing the sensitivity of the RSID™–Semen kit. An aliquot of 100 µl of each dilution of semen was tested in triplicate (1:2000, 1:3000 and 1:4000). Only the 1:2000 dilutions gave positive results.

Dilution	1st repeat	2nd repeat	3rd repeat
1:2000	++	+	–
1:3000	–	–	–
1:4000	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.2.4 RSID™–Saliva

The RSID™–Saliva confirmatory test for the presence of α amylase in human saliva was set up to investigate the sensitivity of the kit as described in (Section 2.2.2.4) Weak but clear positive results were observed at dilutions of up to 1:400 whereas

at dilution 1:450 the test gave a very weak result that is difficult to call with a barely visible positive line (Figure 5.8).

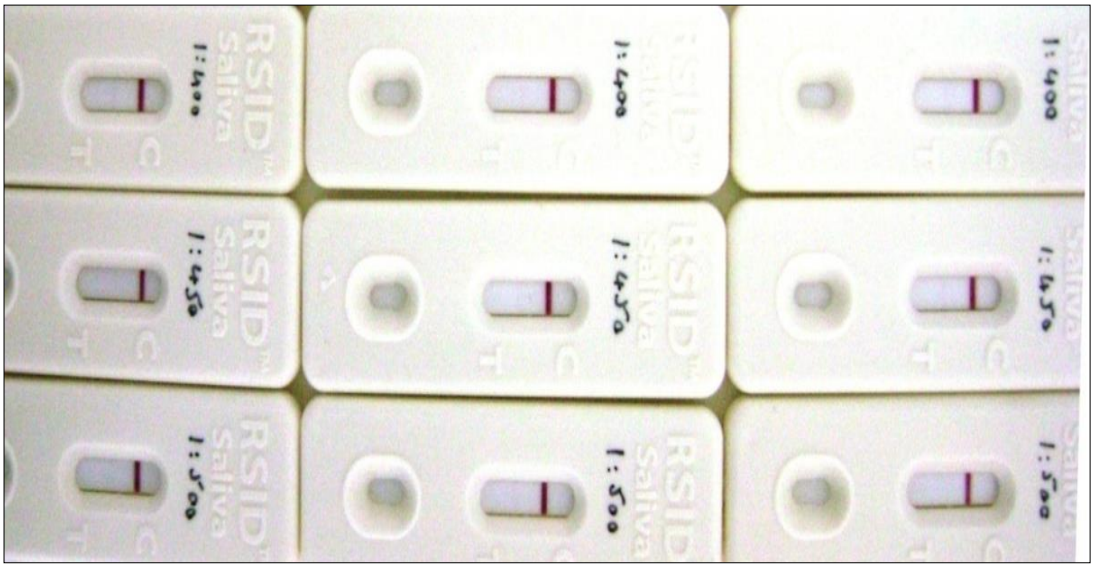


Figure 5.8 Original photograph showing the RSID™–Saliva test strips with three sets of dilutions (1:400, 1:450 and 1:500). All samples showed weak positive results at dilution 1:400 whereas one strip at dilution 1:450 gave a very weak positive result.

Since only one strip at dilution 1:450 gave a very weak positive result, the sensitivity of this kit was identified at 1:400 dilutions (Table 5.8).

Table 5.8: Table showing the sensitivity of the RSID™–Saliva kit. 100 µl of each dilution of saliva was tested in triplicate (1:3500, 1:400, 1:450 and 1:500). Clear positive results were observed with 1:400 dilutions.

Dilution	1st repeat	2nd repeat	3rd repeat
1:400	++	++	++
1:450	+	–	–
1:500	–	–	–

(+++) Strong positive; (++) Weak positive; (+) Very weak positive and (–) Negative.

5.4.3 Results Summary for Presumptive and Confirmatory Tests

The present findings showed that the most sensitive presumptive test was the Hemastix® test for blood which was able to detect blood samples diluted up to 1:200000 (equivalent to 0.0005 µl of blood). In contrast, the least sensitive

presumptive test was the Phadebas® test for saliva where it was only able to detect saliva up to 1:900 dilutions or the equivalent of 0.22 µl of saliva. Similarly, the most sensitive confirmatory test was the Hexagon® OBTI test detecting up to 1:15000 dilution of blood equivalent to 0.0067 µl of blood, and the least sensitive confirmatory test was the RSID™–test for saliva by detecting a 1:400 dilution of saliva equivalent to 0.2 µl of saliva. In addition, the Hexagon® OBTI confirmatory test for blood was shown to be more sensitive in detecting human blood than the presumptive Kastle–Meyer (phenolphthalein) test. All other presumptive tests examined were more sensitive in detecting their relevant body-fluid than their respective confirmatory tests (Table 5.9).

Table 5.9: Table showing a comparative table showing the sensitivity of all tests (presumptive and confirmatory) carried out in this study showing the number of positive samples at any given dilution.

Presumptive tests				
Dilution	Reagent			
	Hemastix®	Kastle-Meyer	Phosphotesmo KM	Phadebas®
1:900	3	3	3	3
1:1000	3	3	3	1
1:4000	3	3	3	0
1:5000	3	3	1	0
1:10000	3	3	0	0
1:15000	3	0	0	0
1:200000	3	0	0	0
1:250000	1	0	0	0

Confirmatory tests				
Dilution	Reagent			
	OBTI Hexagon	RSID™-blood	RSID™-semen	RSID™-saliva
1:400	3	2	3	3
1:500	3	0	3	0
1:2000	3	0	2	0
1:3000	3	0	0	0
1:15000	3	0	0	0

When available, claims of the manufacturer for their products' sensitivities were found to be sound and reliable as none of the results showed the product to be less sensitive than claimed in the kit. On the other hand, a wide range of discrepancies were observed when the results of the current study were compared to results found in the literature. Table 5.10 shows a summary of the current results compared to the values found in the literature for all presumptive and confirmatory methods tested in this study.

Table 5.10: Table showing a comparison between presumed sensitivity found in the literature (highest and lowest) compared to the manufacturers' claims and the results found in this experiment in terms of dilutions and Microliter.

Body fluid	Test	Presumed (high)	Presumed (Low)	Results (µl)
Blood	*K-M*	1:100 000 (Tobe et al.2007)	1:10 000 (Johnston et al. 2008)	1:10000 (0.01)
	Hemastix®	1:1000 000 (Webb et al. 2006)	1:100 000 (Tobe et al. 2007)	1:200000 (0.0005)
	Hexagon OBTI	1:1000 (Johnston et al. 2008)	1:100 000 (Hochmeister et al. 1999)	1:1500 (0.0067)
	RSID™- Blood	0.05 µl (Schoweer et al. 2008)	0.25 µl (Turrina et al. 2008)	1:400 (0.25)
Semen	*Phosphatesmo KM*	Unknown	Unknown	1:4000 (0.025)
	RSID™-Semen	1:100 000 (Pan & Cheung, 2007)	1:512 (Boward & Wilson, 2013)	1:2000 (0.05)
Saliva	*Phadebas®*	1:200 (Mayer & Adkins, 2008)	1:100 (Pang & Cheung, 2008)	1:900 (0.22)
	RSID™- Saliva	1:500 (Casey and Price 2009)	1:10 000 (Pang & Cheung, 2008)	1:400 (0.2)

5.4.4 DNA Quantification

Dilutions which were determined to be the sensitivity limits to examine presumptive and confirmatory tests were selected for DNA extraction followed by DNA quantification as described in (Sections 2.2.5 and 2.2.6). Table 5.11

summarizes the quantity of DNA extracted from body-fluid dilutions that gave the sensitivity limit of detection for the presumptive and confirmatory methods tested.

Table 5.11: Table showing the mean DNA amount in (ng/µl) extracted in triplicate to a final volume of 50 µl from 100 µl of the threshold dilutions for both presumptive and confirmatory methods tested.

Body Fluid	Test	Type	Dilution	Quant. (ng/µl)	SD
Blood					
	Kastle-Meyer	Presumptive	1:10 000	0.001	N/A
	Hemastix®	Presumptive	1:200 000	N/A	N/A
	Hexagon OBTI	Confirmatory	1:15 000	0.002	N/A
	RSID™- Blood	Confirmatory	1:400	0.01	0.001
Semen					
	Phosphatesmo KM	Presumptive	1:4000	0.01	0.011
	RSID™-Semen	Confirmatory	1:2000	0.003	0.001
Saliva					
	Phadebas®	Presumptive	1:900	0.004	0.002
	RSID™- Saliva	Confirmatory	1:400	0.014	0.02

DNA quantification was carried out for all triplicate dilution samples and a mean of the DNA value was taken. Both quantification results from the presumptive and confirmatory tests were around 100−fold below the quantity recommended by our STR kit providers (1 ng/µl).

5.5 Discussion:

5.5.1 Presumptive and Confirmatory Testing

In this series of experiments four presumptive tests namely; Kastle−Meyer, Hemastix®, Phosphatesmo KM® and Phadabas®, were tested in order to determine their sensitivity limit in identifying their corresponding body-fluids. In addition, four confirmatory tests were also tested for their sensitivity limit of detection, these

were RSID™−Semen, RSID™−Blood, RSID™−Saliva and Hexagon® OBTI for blood. The results were then compared to reported sensitivities provided either by the manufacturer or published materials.

This study showed that presumptive tests were generally more sensitive in detecting body-fluids than confirmatory tests. The greater sensitivity of presumptive tests is thought to be a product of their nature. They have been developed to detect substrates that are abundant in their respective body-fluids, whereas confirmatory tests target more specific antibodies that are generally present in body-fluids at lower quantities. Table 5.12 shows a comparison of the different body-fluid components tested for in this experiment.

Table 5.12 Table showing a comparison between the quantities of presumptive and confirmatory active components of the tests used in the experiments.

Body fluid	Presumptive component	Confirmatory component
Blood	Haemoglobin= $1.3x10^{+15}$/ml *(Laux, 2011)*	Glycophorin A = 5 900,000/Cell *(Chasis & Mohandas, 1992)*
Semen	Acid phosphatase= 480 K units/L *(Laux, 2011)*	Semenogelin= 19mg/ml *(Sato et al., 2004)*
Saliva	α amylase = 85 U⁄µL *(Backes et al., 2015)*	α amylase = 85 U⁄µL *(Backes et al., 2015)*

5.5.1.1 Blood

The Hemastix® test for blood was by far the most sensitive test and the easiest to use. According to the literature, the predicted sensitivity of the strip was stated as 1:100000 (Webb et al., 2006). It was clearly shown here that the sensitivity of the Hemastix® strips is at least twice that previously reported. The sensitivity obtained here was comparable to the sensitivity stated by Tobe et al. in 2007. Although easy to use, the nature of the Kastle−Meyer (KM) test required more handling of liquid chemicals in a multi-step process that may render it less efficient. A wide discrepancy is reported regarding the sensitivity of presumptive testing for blood which is most probably due to variability in the application of the methods used. In

a previous study, Tobe et al., in 2007 found that the sensitivity of the Kastle−Meyer depended partly on the reaction time it is allowed (Tobe et al., 2007).

The sensitivity of the KM test from previous publications ranged from 1:10000 for blood−soaked cloth (Cox, 1991) to 1: 100000 (Webb et al., 2006) and even 1:10000000 for haemoglobin solutions (Hunt et al., 1960). The current findings were more comparable with the results published by Cox in 1991 in which the test was applied in a similar fashion.

The sensitivity limit of detection of the Hexagon® OBTI confirmatory test was measured in this study at 1:15000 which is 15 times more than the 1:1000 limit stated by Johnston et al. in 2008. Again, there is a wide discrepancy in the reported limit of sensitivity for this test. For example, Hochmeister et al. (1999) found the lower limit of detection for Hexagon® OBTI to be 1:100000, however, the volume of the buffer solution present in the test tube was reduced in that experiment to increase the concentration of the blood samples. Nevertheless, others have stated the value at 1:1000 (Hermon et al., 2003). The likely cause of this wide sensitivity range is due to differences in applications, including incubation time and type of materials used for body-fluid deposition.

The RSID™−Blood kit was by far the least sensitive of all the confirmatory and presumptive tests carried out in this experiment. Even though the findings of this study agree with the manufacturers reported sensitivity, the results obtained showed the RSID™−Blood kit to be less sensitive than previously reported (Schoweer et al., 2007) but it is in line with the results reported by Turrina et al. (2008). The long incubation time for this kit combined with the low sensitivity of the product makes the RSID™−Blood kit a less desirable confirmatory test for critical and urgent casework samples.

Sensitivity values for presumptive and confirmatory tests show substantial discrepancies in the literature and variables. These include sample donors − which may have different ranges of active components – and moreover will have a major role in the detection level of the method. The issue of the wide range discrepancies regarding sensitivity values in the literature was addressed by Tobe et al. (2007), in which they explained it in terms of differences in reagent concentrations, methods

of preparation of samples, reagents and results, and also differences in the type of material containing the samples. Other studies further add that many of the discrepancies observed are probably due to the application methods of the test. For example, test reagents being added directly to a dilute body−fluid solution rather than on a material containing the dilute body−fluid (Grodsky et al., 1951). It was observed from the results in this section that expressing results in terms of dilutions may have a misleading effect as starting materials may differ from one experiment to another. This is evident in the results of sensitivity limits for the RSID™−blood in which Turrina et al., (2008) proposed the sensitivity at 1:250. Although the results from this current series of experiments were determined at 1:400, both experimental results ultimately state the sensitivity limit at 0.25µl of blood, which is a sounder base for comparisons. The wide range of reported sensitivity may raise doubt on the reliability of these tests. More significantly, this study highlighted the need for a standardised method of application and communication for presumptive and confirmatory testing in order for results of different studies to be compared soundly. In 2011, Laux suggested the necessity for the development of diluted biological standards for the quality assurance of presumptive testing reagents, thus ensuring the sensitivity of the test reagents.

5.5.1.2 Semen

The test for the presence of acid phosphatase (AP) is the most widely used presumptive test for semen (An et al., 2012). In this experiment Phosphatesmo KM® papers were tested for their sensitivity in detecting different dilutions of seminal fluid. Both Khaldi et al. (2004) and Evers et al. (2009) stopped at describing the test as sensitive, but no quantitative sensitivity limit was found for this test in the literature. According to the results from the present study, the sensitivity limit of detection for the Phosphatesmo KM® papers was established at 1:4000 dilution equivalent to 0.025 µl of semen.

The RSID™−Semen kit which detects the seminal vesicle specific antigen (semenogelin) in human seminal fluid gave positive results at dilutions of up to 1:2000 (equivalent to 0.05 µl of semen). This is the closest comparison between a presumptive and a confirmatory test examined in this study. This reflects the fact

that semenogelin is present in high concentrations in semen. Still the RSID™−Semen kit was shown to be considerably less sensitive than stated by Pang and Cheung (2007) but more sensitive than that stated by Boward and Wilson (2013) (1:100000 and 1:512 respectively). The seminal vesicles typically contribute around 50−80% of the seminal volume and although it undergoes rapid proteolytic digestion by prostate−specific antigen (PSA), it is still present in larger quantities than other secretions from the prostate gland (including acid phosphatase), which represents approximately 20–30% of the total volume of the seminal fluid (Duncan and Thompson, 2007). The results of this current study question the need to perform enzymatic presumptive tests in laboratory conditions on forensic samples when confirmatory tests are available.

5.5.1.3 Saliva

The presence of saliva is normally indicated by the positive identification of α−amylase present in saliva (Vilkler and Lednev, 2009). Phadebas® tablets, which are a commonly used presumptive test for saliva, were tested for their sensitivity to saliva dilutions. The result showed that the Phadebas® test is sensitive to dilutions of up to 1:900 of human saliva or 0.22 µl of saliva. Although quite sensitive, the test was laborious and required large amounts of evidential material 200 µl of stock saliva, which is not often available in forensic cases. Therefore, the Phadebas® tablet test for saliva is not recommended for forensic use and a possible alternative could be the paper form of the test that is less laborious and can be exposed to larger surface areas. Mayer and Adkins (2008) demonstrated that the Phadebas® test will continue to give positive results up to 1:200 dilution of saliva using the paper form of the test but failed to state the initial volume of starting material. Hedman et al. (2007) again stated the sensitivity of the test at 1:200 with 50 µl of starting material. This is consistent with the findings of this experiment and highlights the importance of stating the volume of the starting material. On the other hand, Pang and Cheung (2008) started with 100 µl of saliva (half the volume of human saliva dilutions used in this experiment) but noted the sensitivity of the Phadebas® test at 1:100, nine times less than the results obtained in the present experiment.

The RSID™–Saliva confirmatory test showed half the sensitivity produced by the Phadebas® presumptive test with a sensitivity limit of 1:400 dilutions of saliva. However, a value 100 µl of the starting material was used initially which is half the amount that was used as a starting material for the Phadebas® test. Therefore the final sensitivity of both products are believed to be similar. Furthermore, the results obtained from this experiment were very close to the sensitivity discussed earlier by Casey and Price (2009), and 25 times less sensitive than stated by Pang and Cheung (2008), even when the same volume of starting material was used. This is possibly due to the different methodology used by different researchers. In addition, the wide range of amylase enzyme levels present in human saliva can differ widely from one individual to another, and even from the same individual at different sampling times (Auvdel, 1986). This highlights the need for a unified method for the application of sensitivity testing. Comparisons of presumptive and confirmatory test are better evaluated by relative sensitivities. To avoid such variability, samples for this experiment were taken from the same person to maintain comparable relative results.

5.5.2 DNA Quantification

Positive preliminary presumptive and confirmatory tests usually precede down−stream analysis such as DNA extraction, quantification and STR analysis. Often these positive results will result in no STR profiles due to inhibition, degradation or low quantities of DNA molecules (Alaeddini, 2012). DNA extraction and quantification were carried out for the lowest concentration that was able to give a positive result for each of the body−fluid types used (sensitivity limit), in order to establish a relationship between the limit of detection for each presumptive and confirmatory test and its corresponding DNA quantity as measured by real−time PCR.

Dilutions relating to confirmatory sensitivity limits contained more DNA than those relating to presumptive ones and therefore may be more likely to yield some useful genetic information. The content of DNA per µl quantified from the sensitivity limit of all presumptive and confirmatory test were shown to be considerably less than the recommended concentration for STR analysis using Identifiler Plus kit (0.1

ng/µl). This finding is essential in understanding that both confirmatory and presumptive testing can give positive results but fail to give profilable DNA that can identify the depositor of a body-fluid. Although this is true for all body-fluids, the RSID™ kits showed greater chance of obtaining more genetic information than all other tests due to their low sensitivity, making them more reliable in predicting the presence of STR profiles in downstream analysis.

In conclusion, the results have clearly demonstrated that presumptive rests were more sensitive in identifying their respective body-fluids than confirmatory tests. In addition, positive confirmatory test results were more reliable in producing amplifiable DNA for downstream analysis.

CHAPTER 6

ENDURANCE OF PRESUMPTIVE AND CONFIRMATORY TESTS SUBJECT TO ENVIRONMENTAL INSULTS

6.1 Overview:

The identification of body fluids in crime scenes can be a key factor in solving crimes. Body−fluid evidence associated with a crime can provide essential information that may help solve a case, collaborate witness testimony, define a scene of crime, link a suspect and scene, or simply point the investigation in a new direction (Tobe et al., 2007). In many cases body−fluids are positively identified using presumptive and confirmatory tests, but later fail to give STR profiles. Conversely, samples that produce negative presumptive and confirmatory test results may result in full or partial STR profiles. Environmental insults such as ultra violet radiation (UV), heat and humidity may play an important role in the outcome of these tests. However, at the time of the conduction of this study, there was no published work to our knowledge on this subject matter in the peer−reviewed literature. Furthermore, it is of great interest to relate the outcome these presumptive and confirmatory tests to the state of subsequently produced STR profiles.

6.2 Aims and Objectives:

This chapter was designed to investigate how environmental insults may affect the outcome of presumptive and confirmatory tests and whether this outcome is further affected by the type of material the body fluids are deposited on. The objectives of the current study were:

1- To investigate the effects of environmental insults on the ability to identify body fluids commonly found in crime scenes (blood, semen and saliva), using four presumptive and four confirmatory tests.

2- To determine whether the type of material that these body fluids were deposited on had any effect on the outcome of these tests.

3- To evaluate how the outcomes of these test results were related to the ability to produce downstream genetic STR results.

6.3 Method: As described in Chapter 2 of this study

6.4 Results:

6.4.1: Presumptive tests

A set of three different body fluid types were deposited on different materials and collected periodically for a total period of 51 days (ADD2201.5). Presumptive and confirmatory tests were then carried out on these body fluids to investigate the effects of environmental insults on the identification capabilities of these tests (Section 2.3.1). Table 6.1 summarizes the outcome results of the different presumptive tests studied.

Table 6.1 Table showing the time course results (in triplicates) of four presumptive tests for blood, semen and saliva deposited on glass, metal and cloth in relation to ADD and time in days.

		MATERIAL TYPE			**MATERIAL TYPE**			**MATERIAL TYPE**			**MATERIAL TYPE**		
		G	M	C	G	M	C	G	M	C	G	M	C
DAY	ADD	HEMASTIX			PHENOLPHATHALIN			KM			PHADABAS		
1	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3	85.25	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++
5	164	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++
7	250.5	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	+	++
9	338.5	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
11	428	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
13	515.25	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
15	599	+++	+++	+++	+++	+++	+++	++	++	++	–	–	–
18	724.5	+++	+++	+++	+++	+++	+++	++	++	++	–	–	–
21	852.75	+++	+++	+++	+++	+++	+++	++	++	++	–	–	–
24	986.5	++	+++	++	+++	+++	+++	++	+	+	–	–	–
27	1118.5	++	++	++	++	++	+++	+	+	+	–	–	–
30	1250.25	+	++	++	+	++	+++	–	–	–	–	–	–
33	1381	+	++	+	+	++	+	–	–	–	–	–	–
36	1523.25	+/–	++	+	+/–	+/–	+	–	–	–	–	–	–
39	1660.5	+/–	++	+	+/–	+/–	+/–	–	–	–	–	–	–
42	1793.25	–	++	+	–	–	+/–	–	–	–	–	–	–
45	1926.25	–	++	+	–	–	–	–	–	–	–	–	–
48	2062. 5	–	+	+/–	–	–	–	–	–	–	–	–	–
51	2201.5	–	+/–	–	–	–	–	–	–	–	–	–	–

*+++ = strong positive, ++ = positive, + = weak positive, +/– = very weak positive, – =negative. **G**=glass, **M**=metal, **C**= cloth*

The Phadebas® presumptive test for saliva was only able to produce “strong positive” results on the first day of sampling, followed by “positive” and “weak positive” outcomes until ADD 250.5 (Day 7). After the one-week mark, all saliva samples tested with Phadebas® were “negative”. Saliva samples on glass were the first to give “negative” results at ADD 250.5. All other material types gave “negative” results on the following sampling day (ADD 338.5).

In contrast to the saliva test, the semen Phosphatesmo KM® presumptive test for the presence of acid phosphatase continued to show “strong positive” results for all material types until ADD 515.25 (day 13). For the following period of 8 days (ADD 852.75), the intensity of the colour change was observed as only “positive”, which progressively kept decreasing to “weak positive” and “very weak positive” until ADD 1118.5 (day 27). All subsequent samples deposited on all the three different materials gave “negative” results.

The presumptive tests for blood were more effective than their counterparts for semen and saliva. All blood samples tested “strongly positive” with the phenolphathalein (Kastle–Meyer) test up to ADD 986.5 (day 24), after which the intensity of the colour change began to weaken and results gradually changed from “positive” to “weak positive” and “very weak positive” until ADD 1660.5 (day 39). Negative results were not observed until ADD 1793.25 (day 42) for blood on glass and blood on metal. Negative results for blood on cloth were observed the following sampling day at ADD 1926.25 (day 45). All subsequent readings were “negative” for this method. On the other hand, the Hemastix® presumptive test for blood proved to be the most affected test by the type of material body fluids were deposited on. Where the results of this test gave “very weak positives” from ADD 1523.25 (day 36) and “negative” results on ADD 1793.25 (day 42) for blood deposited on glass, it continued to show “weak positive” and “very weak positives” for blood deposited on cloth until ADD 2062.5 (day 48). Interestingly, blood samples placed on metal did not produce any negative results over the duration of the experiment when using the Hemastix® presumptive test.

6.4.2: Confirmatory tests

Table 6.2 summarizes the outcome results of the different confirmatory tests studied.

Table 6.2 Table showing time course results of four confirmatory tests for blood, semen and saliva deposited on glass, metal and cloth materials in relation to ADD and time in days.

		MATERIAL TYPE			MATERIAL TYPE			MATERIAL TYPE			MATERIAL TYPE		
		G	M	C	G	M	C	G	M	C	G	M	C
DAY	ADD	OBTI			RSID−Blood			RSID−Semen			RSID−Saliva		
1	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3	85.25	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
5	164	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
7	250.5	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
9	338.5	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
11	428	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
13	515.25	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
15	599	+++	+++	+++	+++	+++	++	+++	+++	+++	–	–	–
18	724.5	+++	+++	++	+++	+++	+	+++	+++	+++	–	–	–
21	852.75	++	++	+	++	++	+	+++	+++	+++	–	–	–
24	986.5	+	+	+	++	++	+	+++	+++	+++	–	–	–
27	1118.5	+	+	+	+	+	–	+++	+++	+++	–	–	–
30	1250.25	+	+	+	–	–	–	+++	+++	+++	–	–	–
33	1381	–	–	–	–	–	–	+++	+++	+++	–	–	–
36	1523.25	–	–	–	–	–	–	++	++	++	–	–	–
39	1660. 5	–	–	–	–	–	–	+	+	++	–	–	–
42	1793.25	–	–	–	–	–	–	+	+	++	–	–	–
45	1926.25	–	–	–	–	–	–	+	–	++	–	–	–
48	2062. 5	–	–	–	–	–	–	–	–	+	–	–	–
51	2201.5	–	–	–	–	–	–	–	–	–	–	–	–

***+++** = strong positive, **++** = positive, **+** = weak positive, **+/−** = very weak positive, **−** =negative.*
***G**=glass, **M**=metal, **C**= cloth*

When saliva samples were tested using the RSID™-Saliva kit, the results were very similar to those obtained using presumptive tests. Initially the results were "strongly positive" which rapidly turned "negative" by ADD 338.5 (day 9) for all material types; this corresponded to the ADD where the presumptive Phadebas® test for saliva also

failed. On the other hand, semen samples tested with the RSID™-Semen showed increased resilience to environmental insults than the Phosphatesmo KM® presumptive test for semen. The RSID™-Semen kit was able to produce "positive" and "weak positive" results for all material types up to ADD 1793.25 (day 42). Semen samples deposited on metal were the first to produce "negative" results on ADD 1926.25 (day 45), followed by semen deposited on glass on ADD 2062.5 (day 48). Semen samples deposited on cloth continued to give weak positive results except for the last day of the experiment at ADD 2201.5 (day 51). This was in contrast to the results obtained for blood samples when used with confirmatory tests. Both the Hexagon® OBTI kit and the RSID™ Blood were evidently more susceptible to environmentally insulted samples than their corresponding presumptive tests. The Hexagon® OBTI kit began to show “weak positive” results in the period between ADD 986.5 (day 24) and ADD 1250.25 (day 30), followed by negative results for the rest of the experiment for all material types. The RSID™-Blood kit was even more vulnerable to environmentally insulted body-fluids and produced “weak positive” for both blood on glass and metal and “negative” results for blood samples on cloth at ADD 1118.5 (day 27). All blood samples tested thereafter were negative.

6.4.3: Material types

The effects of the different material types on the outcome of the presumptive and confirmatory results were also investigated. For comparison, results are presented graphically as contingency plots for each material type in Figures 6.1, 6.2 and 6.3 for glass, metal and cloth respectively.The scores in these plots were calculated by multiplying the positive results by two to enable better presentation and statistical analysis of the results. Three positive signs from Tables 6.1 and 6.2 above equal a score of “6”, two positive signs equal “4” and one positive sign equal “2”. Weak positive signs were assigned “1” and negative results are indicated in the plot with zero.

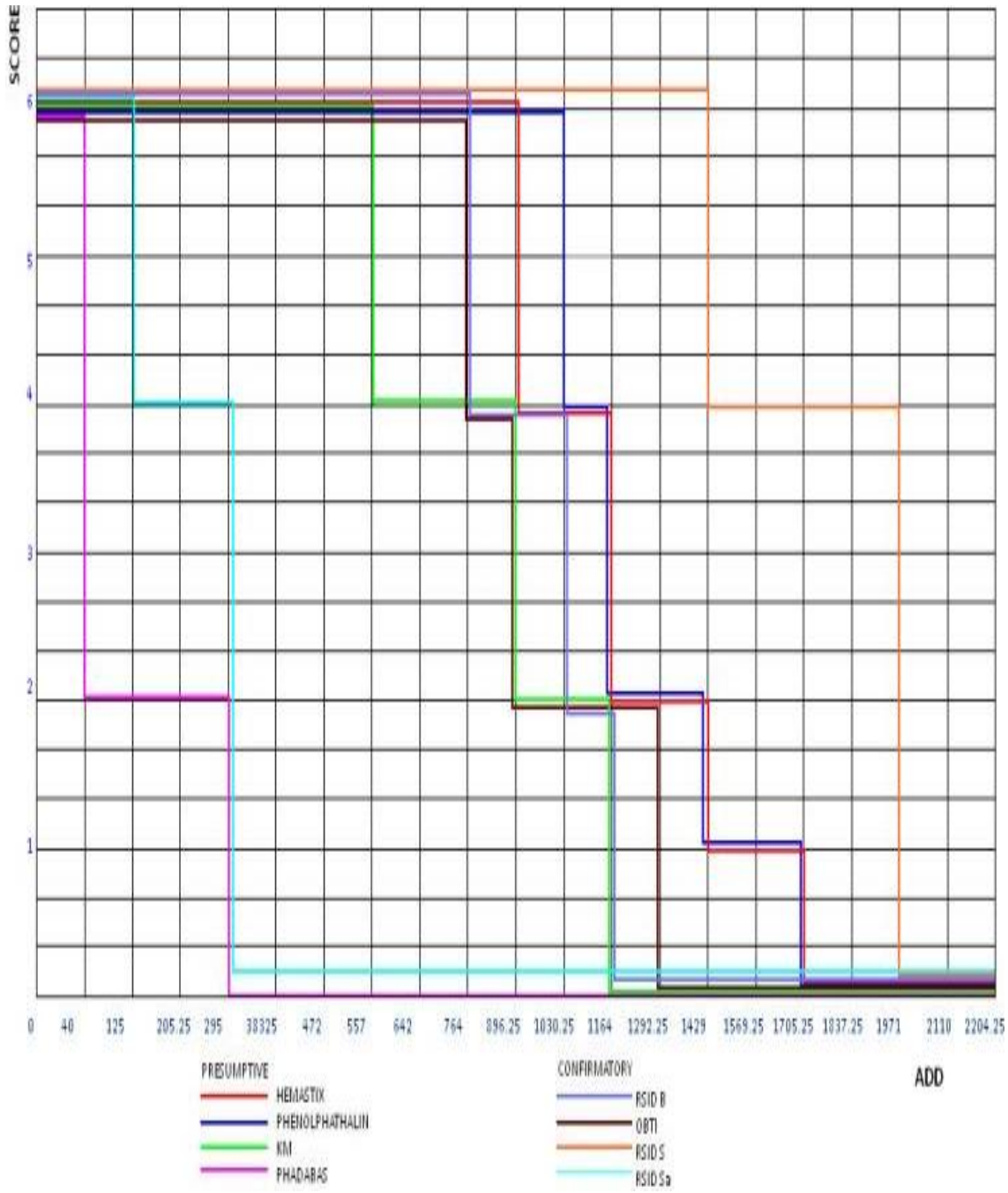


Figure 6.1 A contingency plot showing both presumptive and confirmatory test scores against ADD, indicating the effects of environmental insults on body fluids when placed on glass material.

With the exception of saliva samples, all other body-fluid types (blood and semen) produced strong positive reactions for a period ranging from ADD 724.5 to 986.5 (day 18 to 24) after which the intensity of the reaction colour decreased gradually. Interestingly, both presumptive tests for blood behaved in a similar manner when blood samples were placed on glass. Both tests showed identical patterns of

degradation, yielding negative results beyond ADD 1660.5 (day 39). The only difference between the two presumptive test types was that the phenolphathalin test continued to give "strong positive" results for an additional 133.75 ADD (3 days) than the Hemastix® test. The intensity of colour change of the presumptive test for semen (Phosphatesmo KM®) dropped as early as ADD 515.25 (day 13) whereas the confirmatory test RSID™–semen kit did not show any signs of colour intensity change until ADD 1381 (day 33). In addition, while the presumptive test for semen gave negative results after ADD 1118.5 (day 27), the confirmatory test continued until beyond ADD 1926.25 (day 45) to yield negative results. The difference between the results for semen exhibited the largest variation between a presumptive and a confirmatory test for any body-fluid type on glass. On the other hand both the presumptive and confirmatory tests for saliva showed more consistency, producing negative results at ADD 338.5 (day 9). However, the confirmatory RSID™–saliva kit showed more stability, characterised by the gradual decrease in the colour change intensity compared to its' counterpart the presumptive Phadebas® test.

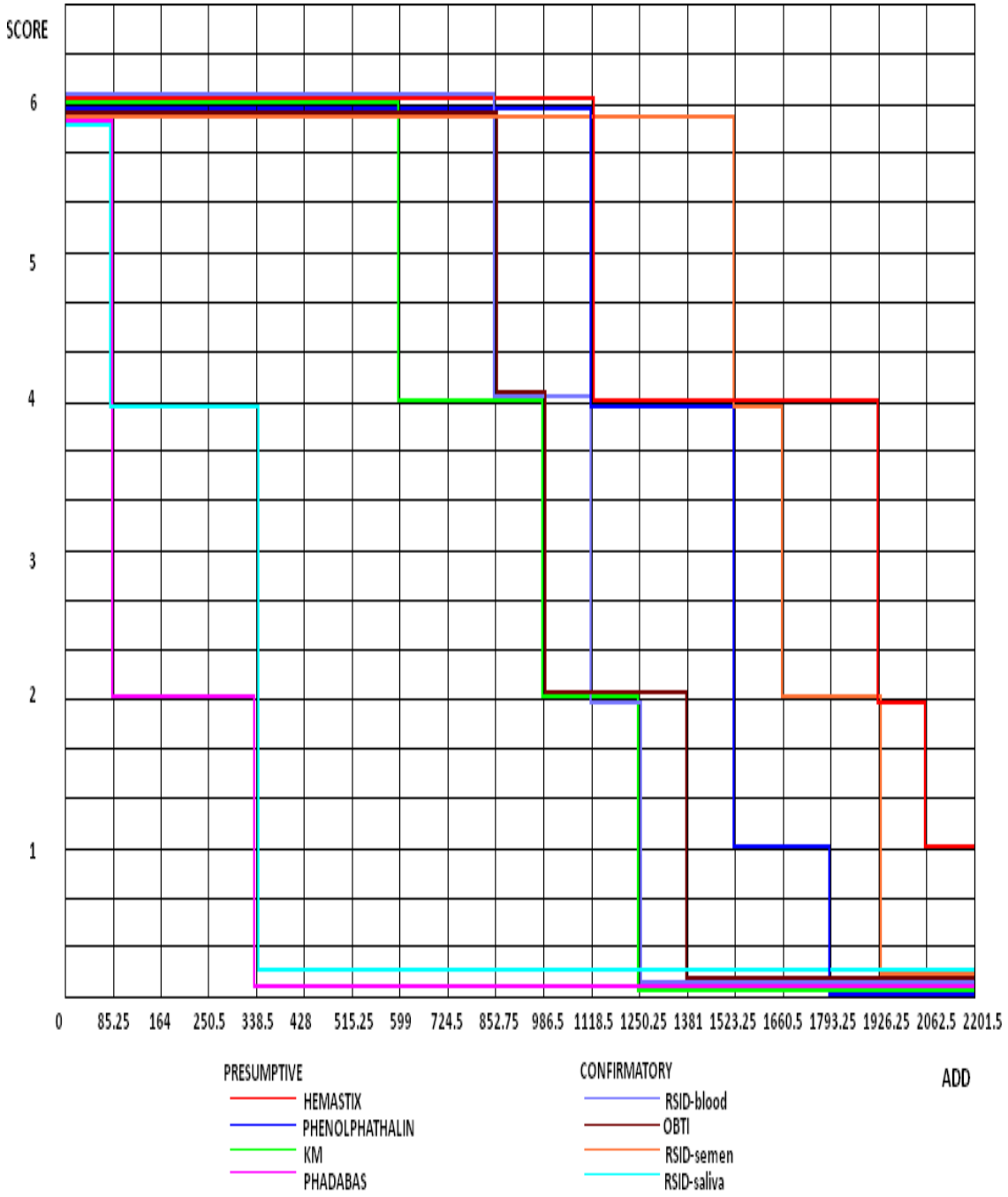


Figure 6.2: A contingency plot showing both presumptive and confirmatory test scores against ADD, indicating the effects of environmental insults on body fluids when placed on metal material.

Saliva samples on metal reacted in a very similar manner to when placed on glass (Figure 6.2). Both presumptive and confirmatory tests for saliva failed to develop positive results after ADD 250.5 (day 7). Again the confirmatory test RSID™–saliva was more gradual in reaching negative results than the Phadebas® presumptive test.

Similarly, the effects of environmental insults on semen samples placed on metal followed the same pattern as that for glass, for both presumptive and confirmatory tests. The Phosphatesmo KM® showed identical results as it continued to show colour change up to ADD 1118.5 (day 27), whereas the RSID™–semen kit failed to give positive results beyond ADD 1793.25 (day 42), 133 ADD (3 days) less compared to when semen was placed on glass. Noticeably, blood samples were less affected by environmental insults when placed on metal than on glass. The phenolphathalin test continued to show positive reactions up to ADD 1793.25 (day 42), where it failed beyond ADD 1660.5 (day 39) on glass. More significantly, the Hemastix® test failed to show any negative results and continued to give weak positive results for the duration of the experiment ADD 2201.5 (day 51), in contrast to blood samples on glass tested with Hemastix® where negative results began to develop after ADD 1660.5 (day 39).

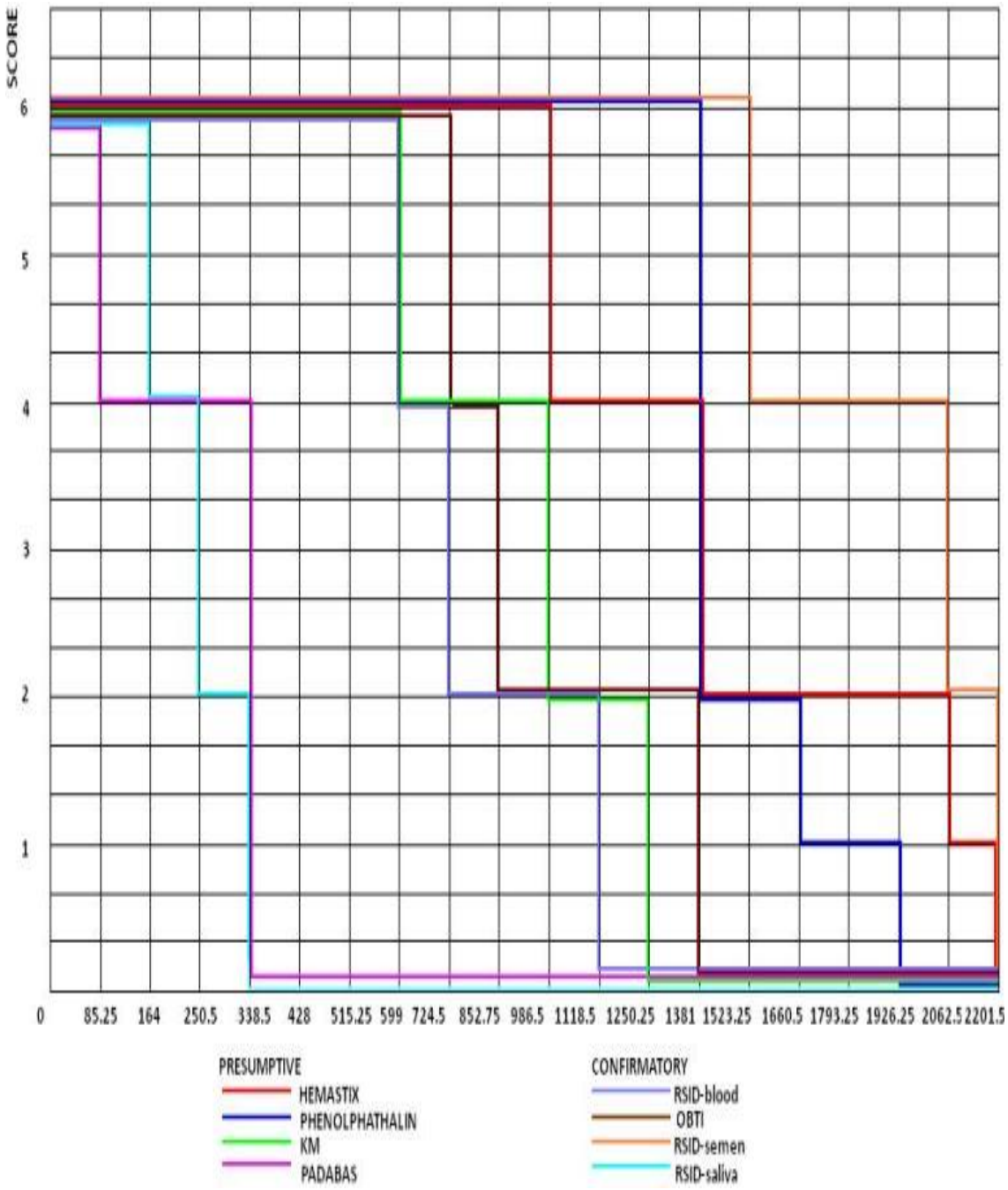


Figure 6.3 A contingency plot showing both presumptive and confirmatory test scores against ADD indicating the effects of environmental insults on body fluids when placed on cloth material.

When body fluid samples were placed on cloth material, saliva samples were again the first to produce negative results for both presumptive and confirmatory tests. Reminiscent of saliva samples on glass and metal, both tests failed to give any positive results beyond ADD 250.5 (day 7) and again the confirmatory test RSID™–saliva was more gradual in reaching that point. In the case of semen samples, the gap difference

between the presumptive Phosphatesmo KM® test and the confirmatory RSID™–Semen (Figure 6.3) seem to have widened. The intensity of the colour change of the Phosphatesmo KM® test was quicker in fading on cloth material and quicker to give negative results than with both metal and glass. On the contrary, the RSID™–Semen continued to show weak positive results until ADD 2062.5 (day 48) and only gave negative results on the last day of the experiment on ADD 2201.5 (day 51). Interestingly, the colour intensity of the RSID™–Semen began to fade from strong positives on the exact same ADD (1381) regardless of the material it was placed on. However, negative results varied from ADD 2062.5 for glass, ADD 1926.25 for metal and ADD 2201.5 for cloth.

6.4.4: Statistical analysis

The Chi-square test of association was carried out to further investigate whether the material type on which the body fluids were placed had any effect on the outcome of the individual presumptive and confirmatory tests. From Table 6.3 below it can be seen that when comparisons of individual presumptive or confirmatory tests were made, there were no significant difference on the outcome of the results between the different material types.

Table 6.3: Table showing acomparison between the scores of positive and negative results for each presumptive and confirmatory test when used on body-fluids placed on different materials. *P*- value is calculated for each test individually.

TEST TYPE	GLASS		METAL		CLOTH		*P* VALUE
	POSITVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	
HEMASTIX	16	4	20	0	19	1	**0.059**
PHENOL.	16	4	16	4	17	3	**0.859**
KM	12	8	12	8	12	8	**1**
PHADABAS	3	17	4	16	4	16	**0.859**
RSID B	13	7	13	7	13	7	**1**
OBTI	12	8	12	8	11	9	**0.934**
RSID S	18	2	17	3	19	1	**0.574**
RSID Sa	4	16	4	16	4	16	**1**

With the exception of blood samples, Table 6.3 shows that there was little or no difference in the number of positive and negative results obtained when body-fluids were placed on different materials. In fact, both the RSID™–Blood and RSID™–Saliva in

addition to the Phosphatesmo KM® all resulted in a *p*-value of 1 with identical performance on different materials. The Phenolphathalin test, Phadebas®, Hexagon OBTI and RSID™-Semen all gave a very large *p*-value. The exception to the similarity between the results on different materials was the Hemastix® test for blood, where the *p* value was only slightly higher than the 0.05 confidence level at 0.059.

To evaluate whether there was any preference in the results between presumptive and confirmatory tests types in relation to body-fluids types and the type of materials on which they are deposited on, positive results for both test types were assessed and *P*-values of the difference was calculated using the Chi-square test of homogeneity (Table 6.4).

Table 6.4 Table showing data of the comparative effects of the use of presumptive tests against confirmatory tests on the outcome of the results for the different body fluids and material used. The *P*-value figure indicates the significance (*= $p < 0.05$) in the difference between the two test types.

PRESUMPTIVE AND CONFIRMATORY TESTS	GLASS	METAL	CLOTH
BLOOD**	0.084	0.004*	0.002*
SEMEN	0.028	0.077	0.008
SALIVA	0.677	1	1

**Results from 2 presumptive and 2 confirmatory tests were added together for the calculation of the Chi-square test of homogeneity for blood samples.

For blood samples deposited on glass, that the results show that there was no significant difference whether presumptive or confirmatory tests were used (*p*-value of 0.084). However, there was a significant difference when blood samples that were placed on both metal, *p*-value= 0.004 and cloth *p*-value= 0.002. Semen samples did not show any significant difference on glass or metal materials when presumptive or confirmatory kits were used. However, when semen was placed on cloth the *p*-value was 0.008 indicating a significant difference between the two test types. The type of test or the material did not affect saliva samples. In fact, both presumptive and confirmatory tests gave identical results for saliva samples on metal and cloth materials. Table 6.4 shows a bar-chart demonstration of the test type/material

relationship on the different body fluids. Positive results from each test type were added to give a score out of 20 (the total number of readings).

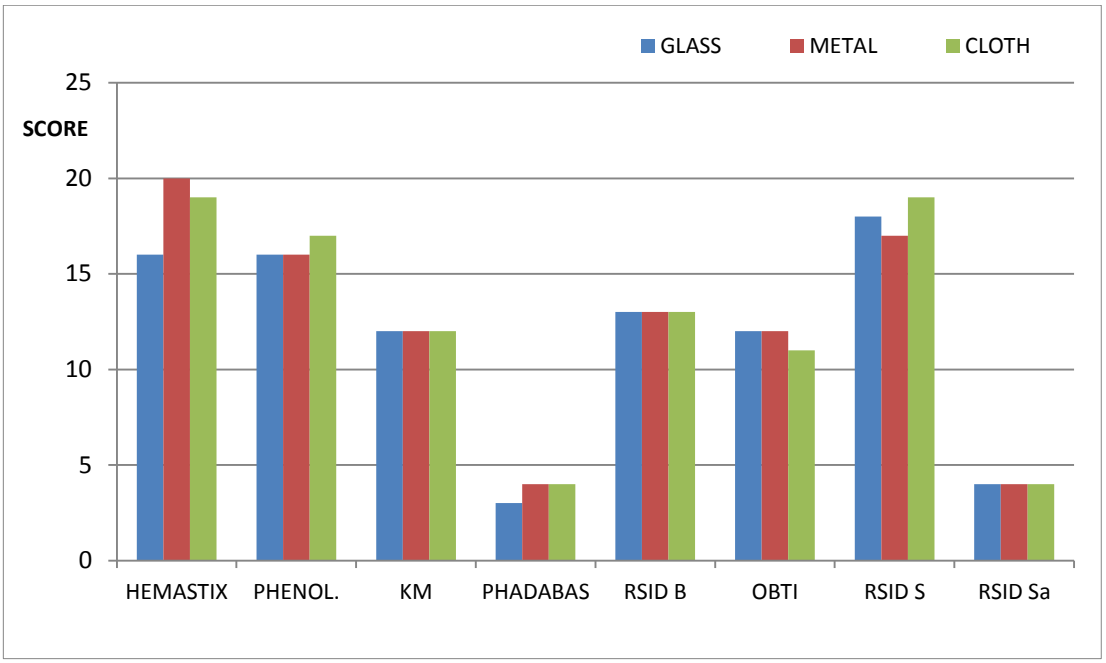


Figure 6.4 Bar chart showing the different test types and how their score compares when different materials were used. PHENOL= phenolphathalin, KM= Phosphatesmo KM®, B=Blood, S=Semen and Sa=Saliva. $P > 0.05$ for all material types tested.

6.4.5 STR analysis

Genetics STR analysis was carried out on a selected number of the environmentally insulted body-fluid samples. This was carried out to assess the state of the DNA present at the points where presumptive and confirmatory tests fail to identify their respective body fluids. For the purpose of comparison, full STR profile (29 alleles) of the researcher generated with the Identifiler Plus kit (Applied Biosystems, USA) is also included in Figure 6.5. In order to compare the performance of the presumptive and confirmatory test, only the 6-FAM™ dye “blue” line of the profile is illustrated.

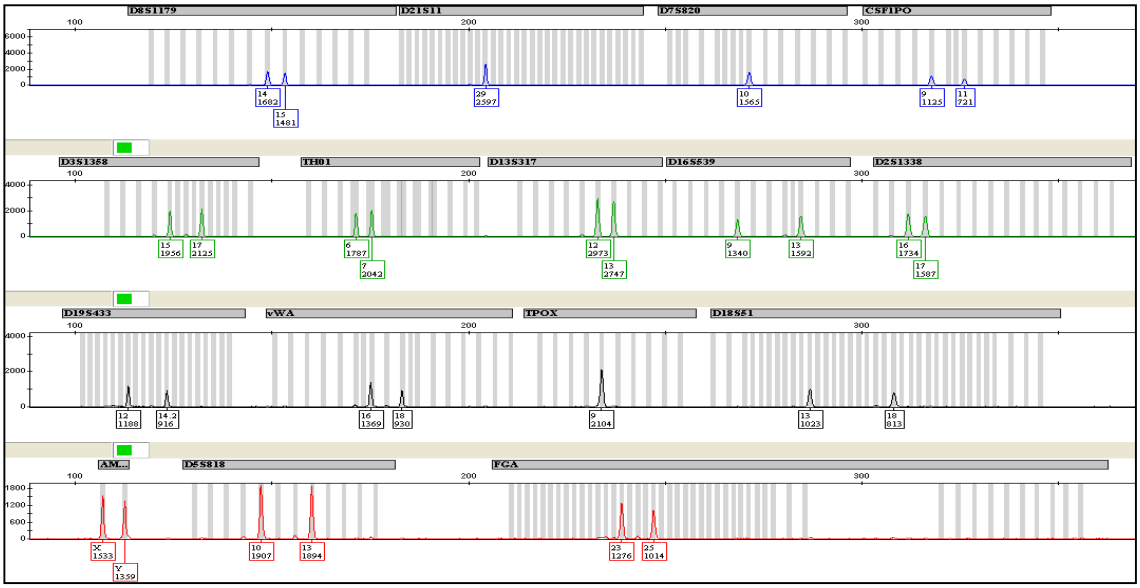


Figure 6.5: Original electropherogram illustrating a full STR profile of the biological material generated using the Identifiler® Plus kit (Applied Biosystems, USA).

Figure 6.6 below shows that blood samples continued to produce full profiles when deposited on cloth even when both presumptive and confirmatory tests failed to give positive results. At Day 30 (1250.25 ADD) all tests for blood gave positive results except for the RSID™–Blood test, while at Day 45 (1926.25 ADD) and Day 51 (2201.5 ADD) only the Hemastix® test was still producing positive results.

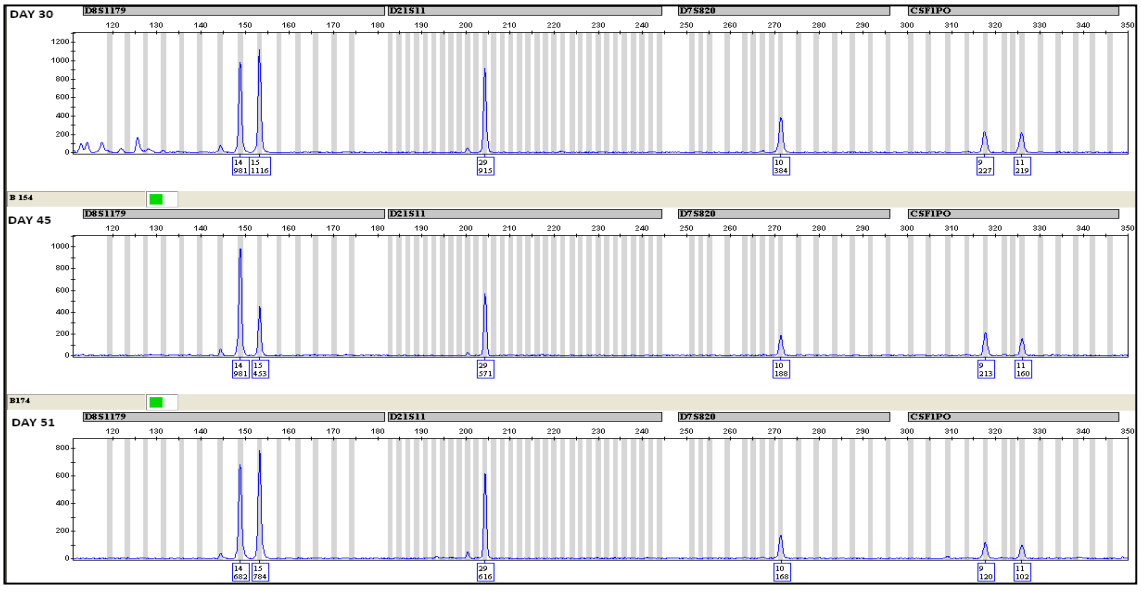


Figure 6.6: Original electropherogram showing STR profile of blood samples on cloth material on Day 30 (both presumptive and confirmatory positive), Day 45 and Day 51.

The nature of the STR profiles was completely different when blood samples were placed on metal. Barely any genetic information was obtained from the selected samples with negative presumptive and confirmatory test results. Figure 6.7 shows that the genetic information for blood samples on metal is a true reflection of the presumptive and confirmatory results obtained earlier. Both presumptive and confirmatory tests failed to give full profiles with the Identifiler Plus kit. Blood samples on metal from day 51 produced a weak STR partial profile containing 5 alleles; one of these alleles was identified as a drop-in contaminant. Both presumptive and confirmatory test results were reflective of the state of the DNA present. As a whole, there was no informative DNA profiles produced when negative presumptive and confirmatory results were obtained.

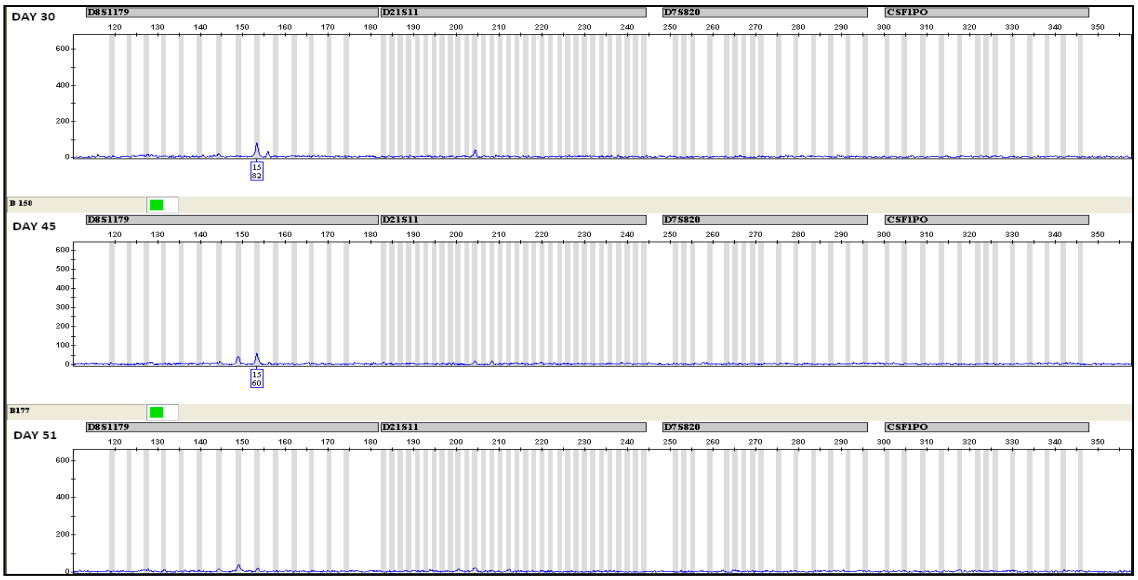


Figure 6.7: Original electropherogram showing STR profile of blood samples on metal material on Day 30, Day 45 and Day 51.

Blood samples deposited on glass gave a different outcome to that of blood samples placed on either cloth or metal (Figure 6.8). Interestingly, confirmatory tests produced more genetic information - demonstrated by nearly complete profiles (27 alleles at day 30) - at their degradation detection limit. Whereas presumptive tests gave rise to weaker partial profiles with limited informative genetic identification powers at the point at which they gave negative results (17 and 14 alleles at day 45 and 51 respectively).

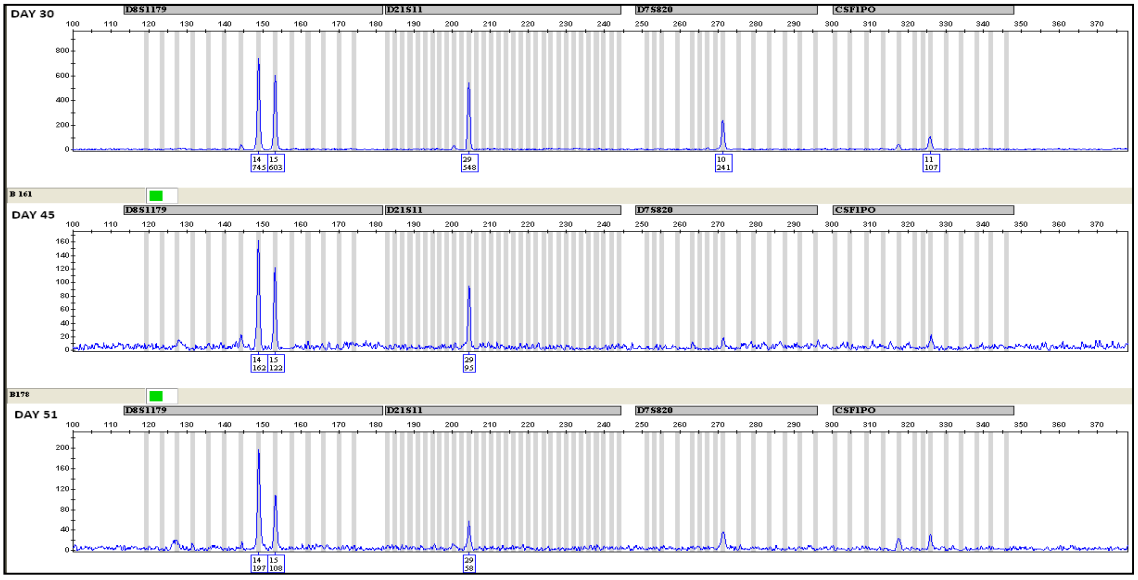


Figure 6.8: Original electropherogram showing STR profile of blood samples on glass material on Day 30, Day 45 and Day 51.

The presumptive test for semen (Phosphatesmo KM®) tested negative for semen samples on day 30 for all material types, whereas the confirmatory RSID™-Semen kit continued until day 51 for cloth, Day 48 for glass and Day 45 for metal to give the same negative results. Figure 6.9 shows that on day 30, semen samples on cloth material generated partial STR profiles (14 alleles). The partial profile was progressively weaker at day 45 (7 alleles) and no profiles were generated for semen samples on cloth material on day 51. Importantly, an initial negative result with the presumptive Phosphatesmo KM® test could still result on some genetic information. On the other hand, although the RSID™-Semen kit continued to show positive reactions, no or very little genetic information was recovered from days 45 and 51.

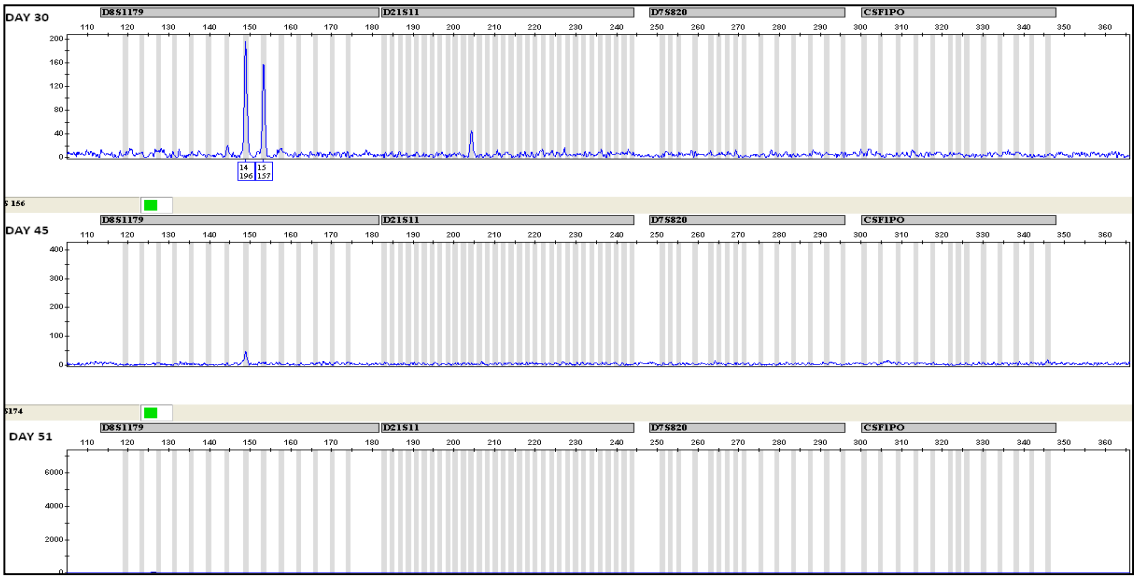


Figure 6.9: Original electropherogram showing STR profile of semen samples on cloth material on Day 30, Day 45 and Day 51

It is clear from Figure 6.10 that for metal, only on day 30 that some genetic information was present (10 alleles), but no profiles were obtained at day 45 and day 51 for semen samples on metal.

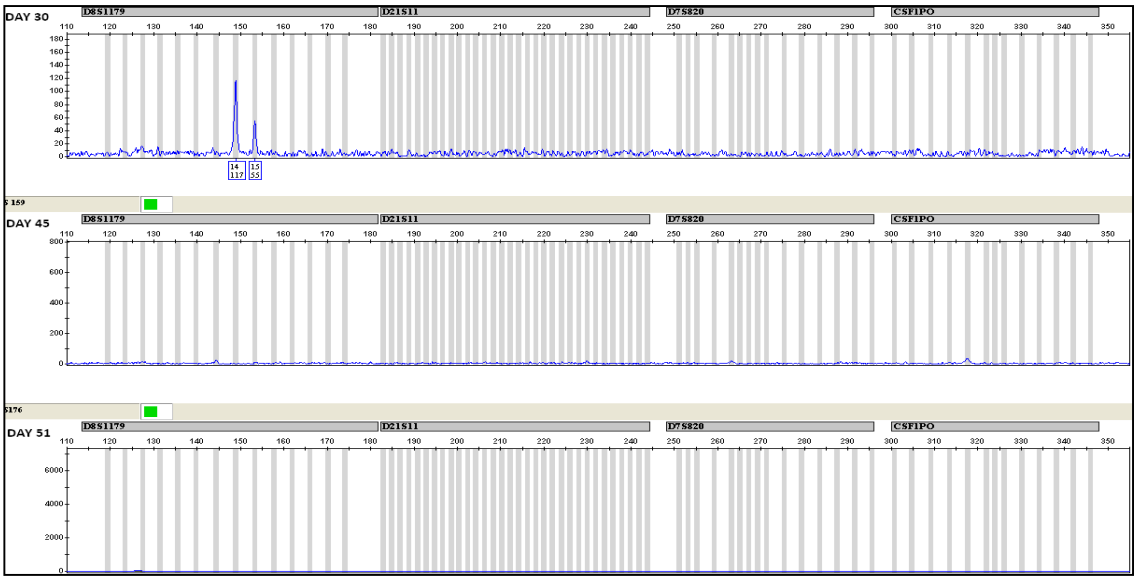


Figure 6.10: Original electropherogram showing STR profile of semen samples on metal material on Day 30, Day 45 and Day 51.

Semen samples on glass were able to generate a partial profile on day 30 (19 alleles) even when presumptive testing was negative for the presence of semen. On day 45

only weak partial profiles were produced (3 alleles) and similar results were obtained for semen samples on glass material on day 51 (7 alleles) (Figure 6.11).

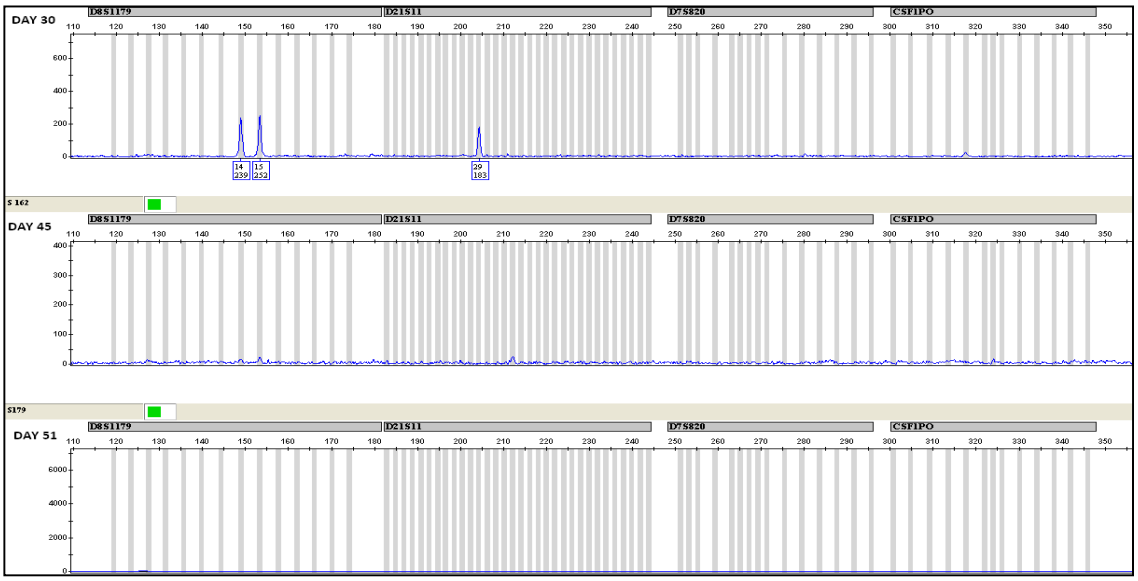


Figure 6.11: Original electropherogram showing STR profile of semen samples on glass material on Day 30, Day 45 and Day 51.

Both presumptive and confirmatory tests for saliva failed to give positive results from day 9 of the experiment except for saliva samples deposited on glass material, where presumptive tests gave negative results earlier on day 7. The data in Table 6.12 shows that no genetic information was obtained from saliva samples on any material on day 9 except for glass where a partial profile was generated (22 alleles). Although, the presumptive test for saliva was the first to fail to give positive results on glass at day 7, some genetic information was still available at that point. Saliva samples on glass material showed some informative genetic profiles at day 9. However, the generated profile demonstrated the presence of contamination of a foreign unidentified source.

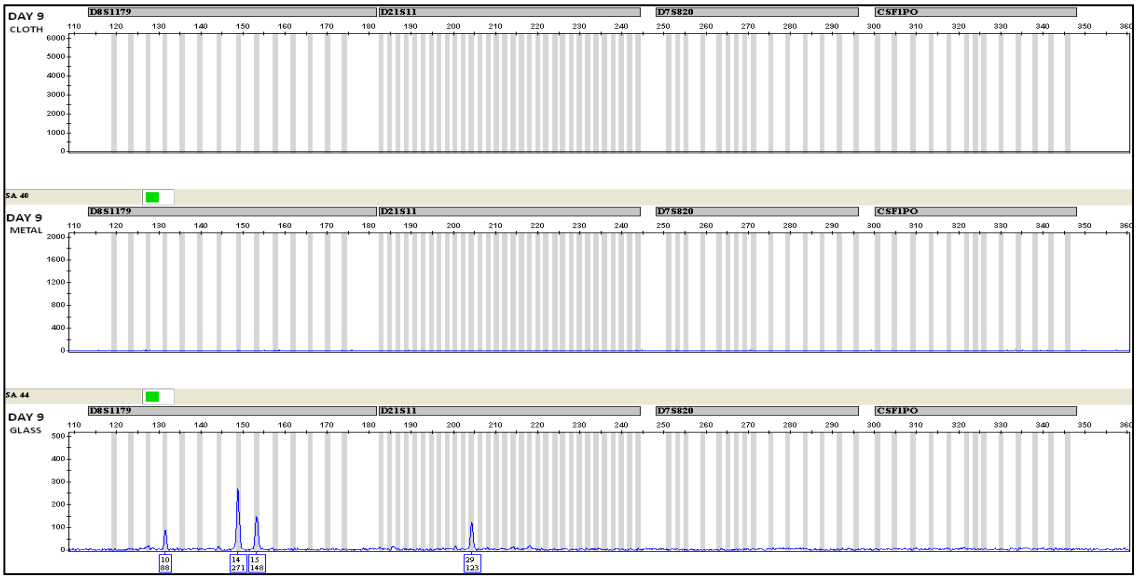


Figure 6.12: Original electropherogram showing STR profile of saliva samples at day 9 deposited on cloth, metal and glass materials (from top to bottom).

Table 6.5 summarises the relationship between the presumptive and confirmatory tests and the outcome of the STR profiles. The results show that only negative presumptive tests for semen were only able to yield weak partial STR profiles, while samples from the negative confirmatory tests did not yield any STR profiles at all. Saliva samples tested negative at day 9 and STR profiles for the same day were all negative and no profiles were generated for that day for either presumptive or confirmatory tests. The exception was when saliva was placed on glass. Strong partial STR profiles were still being generated, even though both presumptive and confirmatory test results were negative. However, these strong partial profiles contained strong contamination from an unknown source. Blood samples were the most variable body fluid depending on the type of material it was placed on. With cloth, blood samples were capable of producing full STR profiles for the duration of the experiment regardless of the outcome of the presumptive and confirmatory tests. While on metal material, it failed to give any informative genetic information with either test types. Blood samples on glass were able to produce close to full STR profiles with negative presumptive tests, in addition to some informative genetic information when confirmatory tests gave negative results.

Table 6.5: Table summarizing the relationship between the presumptive and confirmatory tests and the outcome of the STR profiles*.

	Blood on cloth		*Blood on metal*		*Blood on glass*	
Test type	Day	No. alleles	Day	No. alleles	Day	No. alleles
Hemastix	51	F.P.	∞	5 -mix	42	14
Phenol.	45	F.P.	42	5	42	14
OBTI	33	F.P.	33	6	33	27
RSID	27	F.P.	30	6	30	27

	Semen on cloth		*Semen on metal*		*Semen on glass*	
Test type	Day	No. alleles	Day	No. alleles	Day	No. alleles
KM	30	14	30	10	30	19
RSID	51	0	45	2	48	3

	Saliva on cloth		*Saliva on metal*		*Saliva on glass*	
Test type	Day	No. alleles	Day	No. alleles	Day	No. alleles
Phadabas	9	0	9	1	7	25
RSID	9	0	9	1	9	22 - mix

*For days where STR profiles were not analysed, number of alleles was stated from the nearest day profiled

6.5 Discussion

Body-fluid identification plays an important role in crime scene investigation, prompting the wide range production of presumptive and confirmatory screening tests (Virkler and Lednev; 2009). Factors determining the effectiveness of such tests do not only include the sensitivity and specificity of the reagents and active components of the kits, but also extend to their effects on downstream genetic analysis (Bittencourt et al., 2009). For the more widely used tests, such factors have been studied at length and are reported in the literature (An et al., 2012; Vennemann et al., 2014). However, body-fluids associated with crime scenes are often present in less favourable conditions. Long exposure to environmental insults such as high temperatures and humidity in addition to wind and UV radiation may all affect the outcome of these presumptive and confirmatory tests adversely. Although some studies have attempted to address the effects of environmental insults on DNA recovery and analysis, the

effects of environmental insults on body-fluid identification using presumptive and confirmatory tests have not previously been addressed in a comprehensive manner.

This series of experiment were designed to study the effects of local environmental insults on the most commonly encountered body-fluids in crime scenes; blood, semen and saliva (Virkler and Lednev; 2009). In addition, the effects of the type of material onto which the body fluid deposited were also investigated. A woven textile 100% cotton material (cloth), microscope glass slides (glass) and a large kitchen knife (metal) were used to study the significance of the role material types play on the identification of the different body-fluids. Furthermore, the relationship between both outcomes of presumptive and confirmatory tests and the genetic STR content of the samples in question were compared. The experiment was carried out on the hottest season of the year (June-August) in the Arabian Gulf region, in the UAE. The summer season of the Emirate of Ras Al Khaimah (RAK) spans the months between June and October in which shaded temperatures usually soar to over 50 °C and humidity reaches 100% (DED, 2012).

The results from Section 6.3 show that the ability of presumptive tests to withstand environmental insults varied between the different body-fluid types. The point after which the presumptive tests developed the first negative results will be referred to as the “degradation limit”. Blood presumptive tests showed high level of resistance to environmental insults compare to the other body-fluids examined in this experiment, most likely because of the abundance of the target substance haemoglobin in blood in comparison to the other target substances in their respective body fluids (Chapter 5).

The Hemastix® test for blood was the last to reach its degradation limit out of all presumptive kits tested. In fact, the degradation limit for Hemastix® was not determined for blood samples on metal material, as the test continued to produce positive results for the duration of the experiment (ADD 2201.5), whereas it reached its degradation limit with glass on ADD 2064.5 and even earlier on cloth at ADD 1660.5. The Hemastix® test has established itself as one of the most sensitive presumptive tests for blood since its introduction in forensic science (Tobe et al., 2007). However, the test is well known to produce false positive results with a large number of substances, including metal (Shaler and Saferstein,2002). Therefore, it is likely that the

continuity of the positive results in this experiment for blood samples on metal with Hemastix® were false positive results, a consequence of its low level of specificity. Interestingly, Loy and Dixon (1998) suggested the addition of EDTA to overcome much of the false positive results of the Hemastix® test. This modified method was later validated by Veall and Matheson (2014) and incorporated their findings successfully on degraded samples from archaeological specimens. Furthermore, the specificity of the Hemastix® test is improved when the colour change of the strip is noted rather than the colour change on the sample (Tobe et al., 2007), which was the method employed in this current experiment. The phenolphthalein test was more susceptible to environmental insults and reached its degradation limit on ADD 1660.5. The results of the present study have indicated that the Hemastix® test is better suited to identify blood samples subjected to environmental insults than the Phenolphthalein method. However, when assumed blood is encountered on metal material, caution should be taken with the interpretation of the results, additional and more specific confirmatory tests would also be recommended, however the sensitivity will be compromised.

As discussed in Chapter 5, both presumptive tests for blood used in this series of experiments rely on the peroxidase-like nature of Haemoglobin (Hb). Hb works as a catalyst in the oxidation of the reduced form of the substrate (e.g. phenolphthalein and 3,3′, 5,5′-tetramethylbenzidine), in the presence of an oxidizing agent such as hydrogen peroxide (Tobe et al., 2007). Since ageing of blood samples alone (at room temperatures) does not have any effects on the outcome of both the Hemastix® and Phenolphthalein tests (Webb et al., 2006), the results suggest that the limiting factor for these presumptive tests is a combination of their affinity to the level of degradation of the heme molecules exposed to environmental insults and the chemical composition of the buffers which enables dried haemoglobin to be dissolved into the screening system. The nature of haemoglobin degradation is beyond the scope of this study. However, biophysical alterations of bloodstain analysis have been conducted for many years in the context of forensic science and age determination of bloodstains (Wu et al., 2009; Strasser et al., 2007). Although still unclear, it is thought that the structural alterations of the Hb molecule to hemichrome will alter the peroxidase-like activity of haemoglobin (Bremmer et al., 2012). Therefore, it is important to note that

the degradation rate of Haemoglobin from Oxy-Hb to met-Hb and then hemichrome is a process highly dependent on the actions of temperature and humidity (Bremmer et al., 2011). Moreover, with average daily temperatures reaching over 45 °C for a period of over six weeks, dehydrated blood samples become insoluble in water (the soluble medium for both the Phenolphthalein and the Hemastix® tests), possibly culminating in negative results even when Haemoglobin is present, as has been earlier suggested by Hochmeister et al., (1999). In the study conducted by Dorrill and Whitehead (1979), the solubility of haemoglobin was substantially improved with the substitution of water with a chemical protein extractant such as ammonia allowing for the identification of bloodstains as old as four years that were not previously identifiable, with the unmodified method. The advantage of the Hemastix® test, as seen in the results of this current study, can be attributed to the chemical composition of the strips; according to the Hemastix® patent application filed by Miles Laboratories Inc. (US Patent Application 777,002. 1977 Mar 14). The reagent strip contains many other chemicals whose functions are to stabilise the reactive ingredients as well as to enhance the colour development of the oxidized tetramethylbenzidine (Poon et al., 2009), which may also improve the solubility of haemoglobin.

Confirmatory tests for blood are based on immunological methods; the Hexagon® OBTI utilises anti-human haemoglobin (Hb) antibodies to provide a means of detection for the presence of human (primate) Hb (Johnston et al., 2008) and the RSID™-Blood uses two anti-glycophorin A (red blood cell membrane specific protein) monoclonal antibodies (Schweers et al., 2008). Both of these kits are used in lateral flow strip test format to detect human blood.

The Hexagon® OBTI test was able to produce positive results for an additional 131.75 ADD than the RSID™-Blood kit and extended to 263.75 ADD for blood samples deposited on cloth. The extended “limit of degradation” for the Hexagon® OBTI test can be attributed to the large number of haemoglobin molecules in comparison to the glycophorin A protein present in blood. The current results of this study are in agreement with the findings of Turrina and collogues (2008), although the environmental conditions and periods of exposure were not stated in their study, they conclude that the Hexagon® OBTI test was more efficient in identifying blood samples

from aged and degraded stains than the RSID™-Blood kit (Turrina et al., 2008). In a study conducted by Taborelli and co-workers (2011) on the effects of decomposition of skin cells, they found that glycophorin A was detected using immunohistochemical staining up to 15 days in room temperature. On the other hand, Hochmeister and co-workers demonstrated in 1999 that the Hexagon OBTI test continued to show positive results for the duration of one month in various environmental conditions, including exposure to ambient outdoor conditions during the summer months (Hochmeister et al., 1999). However, the average temperature was much lower than that experienced in the current study. Furthermore, Misencik and Dale (2007) tested the Seratec-Hem Direct kit (a descendant of the Hexagon OBTI test) and demonstrated that haemoglobin was not detected in blood samples exposed to the environment on different materials after four weeks. Again, the environmental conditions were not specified. However it seems from all the literature available that the Hexagon OBTI test failed to produce positive results after a one month period. It was argued by Hochmeister et al., (1999) that the lack of solubility of haemoglobin in the extraction medium of the kits rather than the level of degradation is the main cause of the negative results observed. Furthermore, they demonstrated that with the addition of 5% ammonia solution, positive results can be produced from 15 year-old blood stains stored in room temperature that previously tested negative. It is not clear whether the local environmental insults of the current experiment affect the haemoglobin structure directly to the extent of epitopal misrecognition, or whether these effects work on the binding forces of heme (and blood generally) on the material type they are deposited on, which renders the process of haemoglobin solubility and extraction more challenging.

In contrast to blood samples, the presumptive test for semen was more susceptible to environmental insults than the confirmatory test. The Phosphatesmo KM® reached its degradation limit at ADD 1118.5 whereas the RSID™–semen kit did not reach the same point until ADD 2062.5. The low degradation limit of the Phosphatesmo KM® in comparison of that of the RSID™–semen kit can be explained in terms of the enzymatic activity of the acid Phosphatase protein. Environmental insults such as temperature and humidity can alter the integrity of the peptide structure of the enzyme leading to

the loss of its biological activity (Voet and Voet, 1992) and hence, the manifestation of negative results with the Phosphatesmo KM®. In fact, it was previously demonstrated that of all the proteins with enzymatic activity present in semen, the acid phosphatase enzymatic activity exhibits the highest level of inactivation when subjected to such environmental insults (Jimenez-Verdejo et al., 1994). In addition, although acid phosphatase is present in large quantities in seminal fluid the rapid and natural degradation of the acid phosphatase enzyme is well studied and established *in vivo* and postcoital samples (Keil et al., 1996; Khaldi et al., 2004).

On the other hand, Semenogelin is the major component in human seminal plasma, accounting for about 40% of the total seminal proteins (Sato et al., 2004). The immunochromatographic nature of its detection (rather than enzymatic) means that even with considerable amount of structural change, the denatured peptides are still recognisable by the kits' antibodies. In addition, the relatively small size of the semenogelin I, with a molecular weight of less than 30 kDa, compared to other proteins present in the seminal fluid (Dunacan and Thompson et al., 2007) may play a role in the prolonged degradation limit of the RSID™–Semen kit found in this study. It is plausible that the effects of high temperatures requires longer periods of exposure to denature the small structural peptides in the semenogelin I protein. This is in agreement with the findings of Sato et al. (2004) in which they concluded that the antigen Semenogelin is identifiable even after exposure to temperatures as high as 150 °C for a relatively short period of only 1 hour.

Both the presumptive and confirmatory tests for saliva reached their degradation limit on the same sampling day at ADD 250.5. Although both tests make use of the α-amylase enzyme, the RSID™–Saliva test is designed to detect the presence of human salivary α–amylase by means of antigen-antibody recognition, whereas the Phadebas® test utilises the enzymatic activity of the α–amylase. Amylase is the most abundant and resistant enzyme in saliva (Willott, 1974). Early studies showed that 100% of the enzyme activity is retained after 7.5 months period at room temperatures, which drops to 10% of the original activity after 28 months (Nelson and Kirk, 1963). The exact amino acid sequence of the target epitope of the RSID™–Saliva kit is not disclosed and is not found in the literature. However the similarity in the degradation limits between

the Phadebas® test and the RSID™–Saliva suggests a similar size and structure for the target region on the α–amylase. The α–amylase enzyme possesses extra substrate binding regions common in starch-degrading enzymes and is critically important for their function (Cockburn et al., 2015). Situated on the catalytic domain, these surface binding sites (SBSs) enhance the activity of the enzyme and mutations at these sites can eliminate the ability of binding starch. The results from this current study proposes that It is possible that the RSID™–Saliva kit targets these branch point binding sites which are more susceptible to environmental insults and can explain the similarity in degradation limits between the presumptive and confirmatory tests for saliva.

Interestingly, studies have found that the enzymatic activity of the α–amylase persists at room temperature for extended periods reaching 119 days for saliva stains on cotton swabs (Auvdel 1986). In a recent study, forensic samples as old as 26 years stored at room temperature were tested for both the presence of the α–amylase enzyme (using the RSID™–saliva) and its enzymatic activity using the BNP-Amylase test (Sclavo-Dasit, Milano, Italy). Both tests showed almost identical results and were able to identify amylase in the saliva samples (Carboni et al., 2014). Again, the findings suggest that both the target for the RSID™–saliva kit and the (SBSs) responsible for the enzymatic activity of the protein are closely linked.

The change in the material type did not seem to have any significant effect on the outcome of the individual tests. However, the results showed that the Hemastix® test was the most affected test by the change of the material type (*p*-value of 0.059). It seems that the low *p*-value is a result of the increased level of false positives for this test compared to others (Virkler and Lednev, 2009). Although the effects of environmental insults on presumptive and confirmatory tests have not been previously studied, a few studies looked into the interactions of blood stains on different materials focusing on cell morphology and adhesion forces. One previous study demonstrated that the adhesion forces of the red blood cells differ between blood stains on mica and glass, reaching its maximum on day 27 when samples were placed in an uncontrolled outdoor environment (Wu et al., 2009). In comparison, Stresser et al. (2007) emphasised the loss of elasticity of RBC in time. A feature they proposed for the age determination of the bloodstains (Stresser et al., 2007). Currently, there is

insufficient data regarding the physical interactions between body fluids and deposit materials and how these materials affect the molecular structure of the body-fluids. This current study demonstrated that when body-fluids are subjected to environmental insults, a common presumptive or confirmatory test will function similarly regardless of the type of material the body-fluids are found on. However, the decision to pursue either presumptive or a confirmatory tests will play a more significant role in the success of identifying such samples.

Only blood samples deposited on metal and cloth, in addition to semen samples on cloth showed a significant difference in the results between presumptive and confirmatory testing. The differences seen in the blood and the semen samples on cloth material are likely to be a limitation of the extraction buffer of the RSID kits in extracting dried body-fluid stains, rather than an aspect of material property. However, it was shown previously that materials made of natural cotton can retain the contents of blood samples longer than many other material types (Verdon et al., 2013). On the other hand, the significant level of difference between presumptive and confirmatory tests for blood samples on metal materials is largely caused by what is hypothesised to be false positives results. Unfortunately, in spite of its imperative value in forensic biology and genetics, the subject area of body-fluid identification and their physical and environmental interactions has not been comprehensively studied.

Following positive presumptive and confirmatory tests, DNA analysis is normally carried out to relate the identified body-fluid to a specific person. Presumptive tests are expected to be less specific but more sensitive. Therefore, they continue to give positive results even when DNA is not expected to be present (Allard and Rankin, 2010). This is unlike confirmatory tests that are generally less sensitive and therefore have more chance of containing DNA material. In fact, the confirmatory RSID™ kit producers state that their kits have been adjusted so that a positive RSID kit result correlates with the likelihood of obtaining a genetic STR profile (Old et al., 2009; Schweers et al., 2008). It is important to point out that all the studies present in the literature only refer to DNA quantities in terms of body-fluid sensitivity; while the relationship between the outcome of a presumptive or confirmatory test and the DNA quantities that could be extracted following exposure to environmental insults have

not been investigated previously. In this study, with the exception of blood on cloth and blood on glass, informative DNA profiles were not always obtained from positive presumptive and confirmatory tests. Interestingly, body-fluid samples deposited on glass seem to produce better DNA profiles than on other materials. The findings of this study demonstrate that environmental insults have a greater impact on DNA degradation than on presumptive and confirmatory tests. The effects of environmental insults on DNA will be investigated further in the following chapter.

In conclusion, the findings of this study have clearly demonstrated that environmental insults have a greater impact on DNA degradation than on presumptive and confirmatory tests. Confirmatory tests were generally more susceptible to environmental insults than their counter-part presumptive tests.

CHAPTER 7

EFFECTS OF ENVIRONMENTAL INSULTS ON DNA DEGRADATION

7.1 Overview

The effects of environmental insults on DNA recovery from forensic evidence has been previously studied with respect to individual specific environmental factors which include; temperature (Barbaro and Cormaci, 2008), humidity (Lund and Dissing, 2004), soil (Shahzad et al, 2009) and ultra-violate radiation (McNally et al., 1989). Experimental work to investigate a single or a few of these environmental factors have generally been conducted in controlled environments in which one or more of these factors can be monitored in laboratory conditions, which does not accurately reflect a genuine real-life casework scenario.

The results from casework examination (Chapter 3) have suggested that DNA degradation may be an important factor limiting the percentage of successful DNA genotyping by limiting the number of full STR profiles encountered in everyday analysis.

For this chapter, two pilot studies were conducted as precursors for a wider experiment carried out in the period between March 2012 and August 2014 with the aim to shed light on how local environmental insults can affect DNA degradation from body fluids samples when found on different commonly found materials.

7.2 Aims and Objectives

The aims of this sections were to shed light on the effects of local environmental insults on the degradation of DNA isolated from three different body fluids (blood, semen and saliva). It also aimed to investigate whether the material types these body fluids were incubated on would have any effect on the outcome of the DNA analysis. Objectives of this chapter were:

1- To conduct two pilot studies as a platform to inform on the interval and duration of sampling.

2- To conduct a full scale experiment on the effects of environmental insults on DNA degradation, which include three types of body-fluids.

3- To compare the outcome of three quantification kits.

4- To carry out statistical analysis on the different DNA quantification values and how they relate to the different material types.

7.3 Results

For this series of experiments (two pilots and one full experiment), body-fluids were positioned on different materials and placed in outdoor environment for a duration ranging from six weeks for Pilot 1 and ten weeks for the full experiment. The samples were then collected at regular intervals and stored at -20 °C. DNA extraction was then carried out and followed by three different types of quantification. The resultant quantities were then used to generate STR profiles to identify the effects of environmental insults of the body-fluid samples.

7.3.1 Effects of environmental insults on DNA from blood samples in spring time (Pilot study 1)

7.3.1.1 Sample Collection

One sample was collected every 3rd day from each material type. Although care was taken in dispensing the blood samples within the area of the grid, a few days into the experiment some blood samples on metal and glass started to flake due to complete dehydration. In a row containing such samples, the most complete grid was taken for DNA extraction and quantification (Figure 7.1).

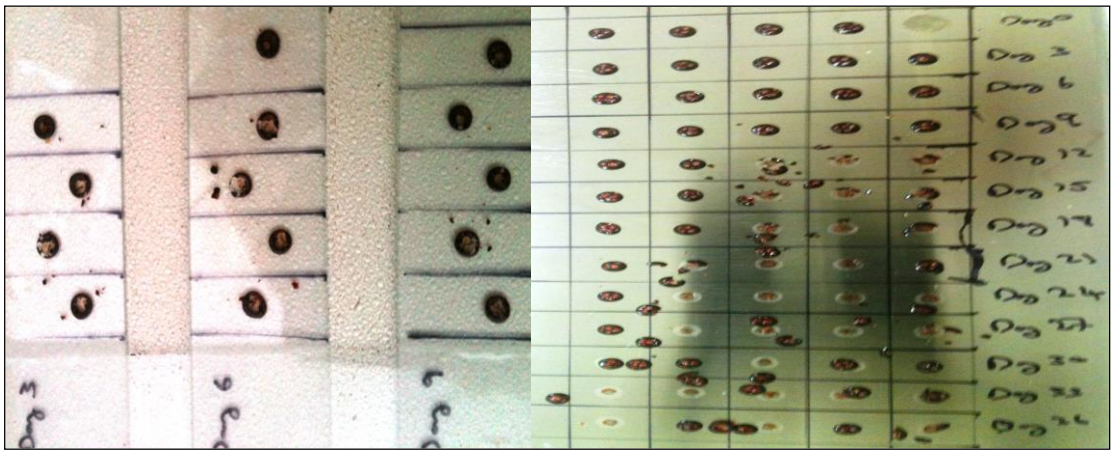


Figure 7.1: A photograph showing blood samples on glass (left) and metal (right) with the blood spots flaking and displaced after few days of environmental exposure.

7.3.1.2 DNA Quantification

In total, 42 samples in addition to negative controls, were extracted in the 48 days period of the project. Using the Chelex-100 extraction method followed by concentration using Microcon-YM 100 filters to 50 µl, all samples were quantified using the Quantifiler® Human kit. The amount of DNA present was compared for blood samples deposited on metal, glass and cotton in relation to ADD. Table 7.1 shows the time course and recovery of DNA quantity of blood samples on different materials against ADD measured in a period of 48 days between 31st of March until 19th May 2012.

Table 7.1: Table showing the values of DNA extracted from blood samples on different materials after being subjected to environmental insults. Accumulated degree-days (ADD) were calculated.

No. DAY	Neg. control	DNA Quantity ng/µl			TEMP °C
31MAR-19MAY		GLASS	METAL	CLOTH	ADD
0	0	1.255	0.997	2.482	0
3		1.263	1.223	1.397	78
6		0.769	0.651	1.102	167.5
9	0	0.756	0.596	0.221	255
13		0.754	0.852	0.441	368.5
17		0.309	0.312	0.163	475
21	0	0.388	0.209	0.263	582
25		0.411	0.181	0.24	693.5
29		0.335	0.229	0.441	809
33	0	0.261	0.132	0.264	943
37		0.23	0.218	0.171	1080
41		0.196	0.136	0.177	1215.5
45	0	0.213	0.147	0.27	1357
48		0.144	0.054	0.252	1425.5

The results showed that blood samples deposited on cotton materials gave the highest DNA yield, starting with 2.482 ng/µl of DNA at day 0 and reaching 0.252 ng/µl by the end of the experiment on day 48. On the other hand, blood samples on glass had a starting DNA content of 1.255 ng/µl which reached 0.144 ng/µl by the end of the experiment. Blood samples deposited on metal showed the highest rate of DNA depletion as DNA amounts fell from 0.997 ng/µl to 0.054 ng/µl in 48 days. However, these were results from only one sample per time-point readings. Triplicate sample readings were conducted in (Section 7.3.3).

Interestingly, the extracted DNA concentrations from the different materials were more comparable to each other starting from day 3 at ADD 78 rather than at ADD 0. The rate of DNA degradation (from day 3 until day 48) showed that degradation was fastest for blood on metal, with over 95% of the original DNA quantity depleted during the length of the experiment and a rate of around 2.1% decrease in DNA quantity per day compared to the quantity present at Day 3. Glass came second with 88.6% degradation, equivalent to the rate of 1.97% per day. Blood samples deposited on cloth seemed to be the most resistant to DNA degradation. Only 82% of the starting DNA amount was depleted by the last day of the experiment, with a rate of 1.82% per day. The line-graph in Figure 7.2 shows that most of DNA degradation occurs around the initial 500 ADD (just over two weeks). The following 29 days show a steady increase in ADD but the rate of DNA degradation slows.

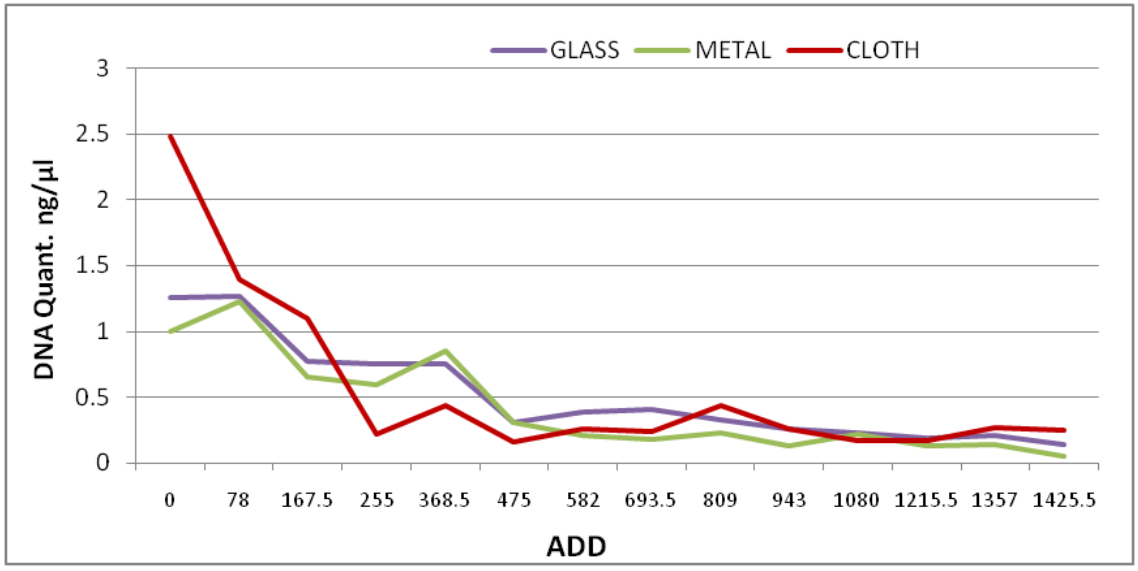


Figure 7.2 Time course line-graph showing a schematic representation of the DNA depletion pattern in relation to ADD. Data are the mean of 3 samples for each point.

To assess the level of variation between the three types of materials, analysis of variance was conducted and a probability value of p= 0.747 was calculated, indicating that there was no significant difference between the different groups and that the type of material on which blood samples were deposited on did not affect the outcome of the DNA degradation. In agreement with the ANOVA calculation, the box-

plot representation in Figure 7.3 shows that most variation comes from within the group rather than between groups.

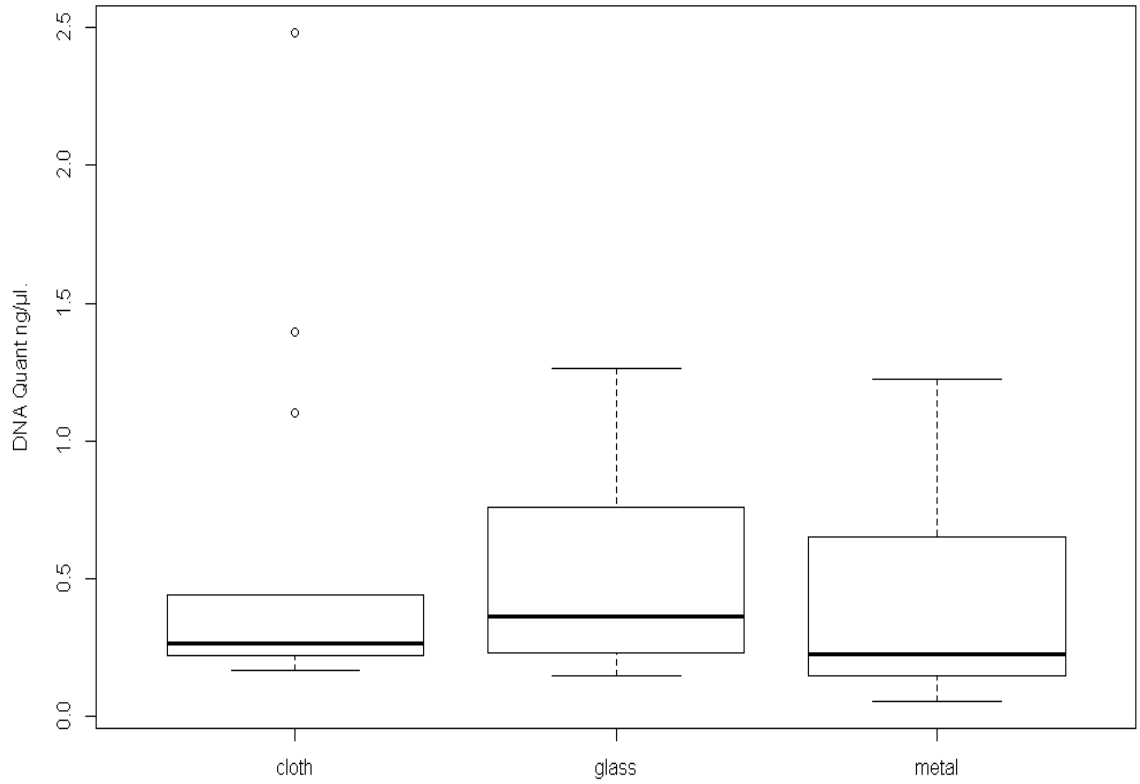


Figure 7.3 Box-plot representation of the DNA quantities extracted from blood on the different materials tested in this experiment. n= 3, $p < 0.05$ for cloth material, glass and metal.

7.3.1.3 STR Analysis

To assess the effects of environmental insults on STR profiles, a selection of samples representing the duration of the experiment from day 0, 17, 33 and 48 were chosen for STR analysis. These included samples from all three material types. Based on the quantification results, 1ng of DNA from each tested sample was used when available; otherwise 10 µl of the extraction product was used for the multiplex reaction, as per manufacturer's recommendation.

STR profiles generated from blood samples deposited on cloth showed a progressive decrease only in the quantities of the DNA product. Although full profiles were still observed at ADD 1425.5 (day 48), the combined allele peak height of the profile was

calculated to be 41,916 at ADD 0 for blood samples on cloth which decreased to 21,045 at ADD 1425.5 (Figure 7.4).

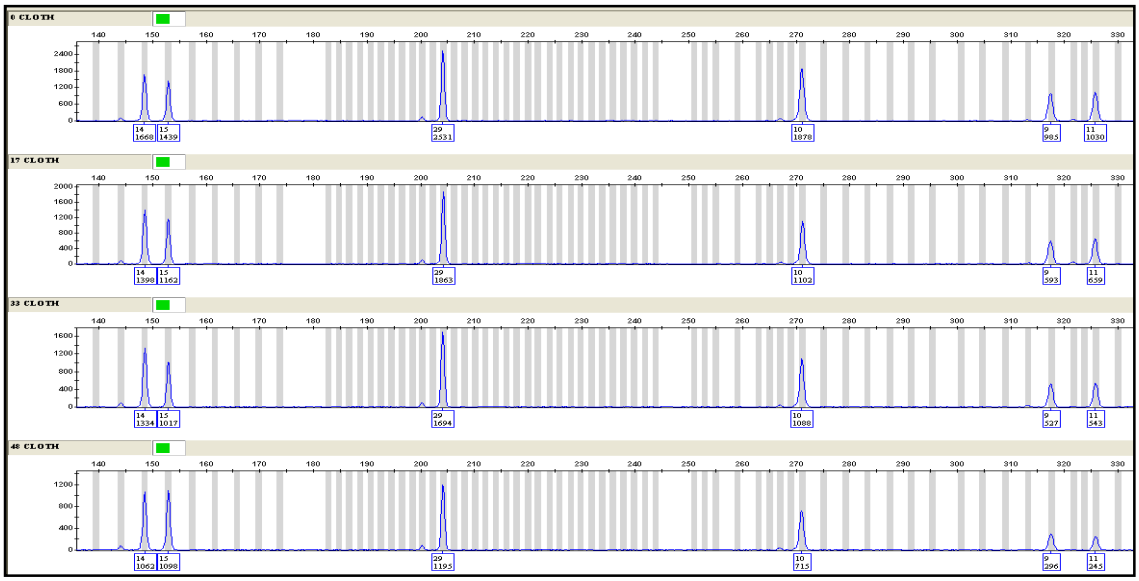


Figure 7.4 Original electropherogram (6-FAM) showing the gradual degradation of DNA profiles of blood samples on cloth from days 0, 17, 33 and 48 corresponding to accumulated degree days of 0, 475, 943 and 1425.5 respectively. Representative plot, n=3.

Similarly, blood samples deposited on glass material gave full profiles up to 1425.5 ADD (day 48). In addition to the slight peak imbalance at the FGA locus, the combined allele peak height of the profile showed a much lower value from that obtained from blood samples on cloth. The combined allele peak height was calculated to be 21,301 RFUs at 0 ADD and 14,576 RFUs at 1425.5 ADD (Figure 7.5).

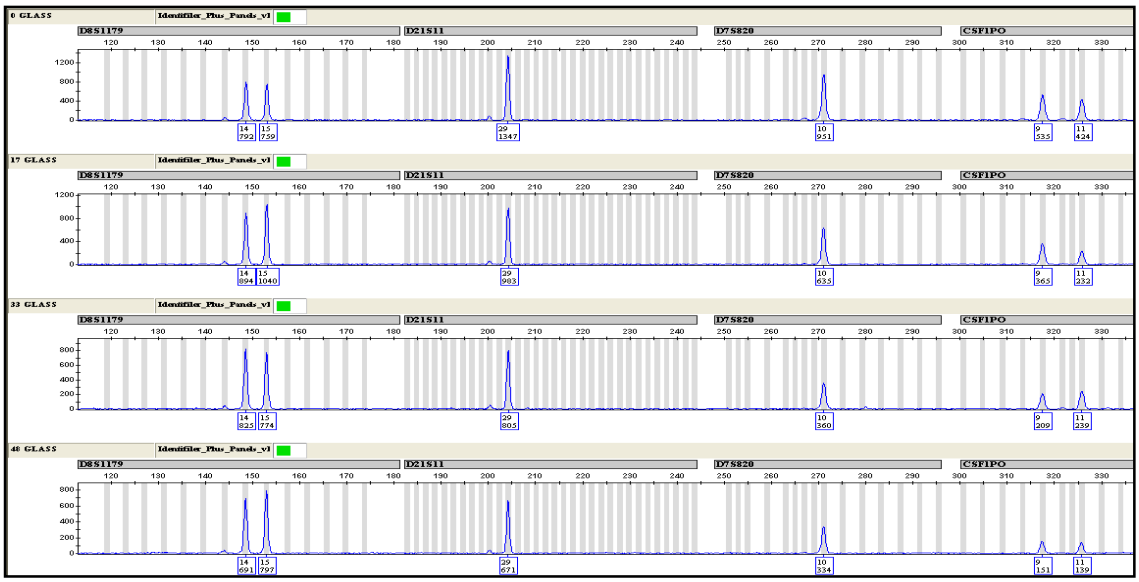


Figure 7.5 Original electropherogram showing the gradual degradation of DNA profiles of blood samples on glass from days 0, 17, 33 and 48 corresponding to accumulated degree days of 0, 475, 943 and 1425.5 respectively, n=3.

Profiles of blood samples deposited on metal were the most affected by the increase in ADD. As can be seen from Figure 7.6, the STR profile at ADD 1425.5 exhibited complete drop-out of loci CSF and FGA and one allele drop-out at loci vWA and D18. The difference in the combined allele peak height was also most affected when blood samples were deposited on metal compared to cloth and glass. The value of the combined allele peak height at ADD 0 was 43,694 RFUs which dropped drastically to 4,358 RFUs at ADD 1425.5.

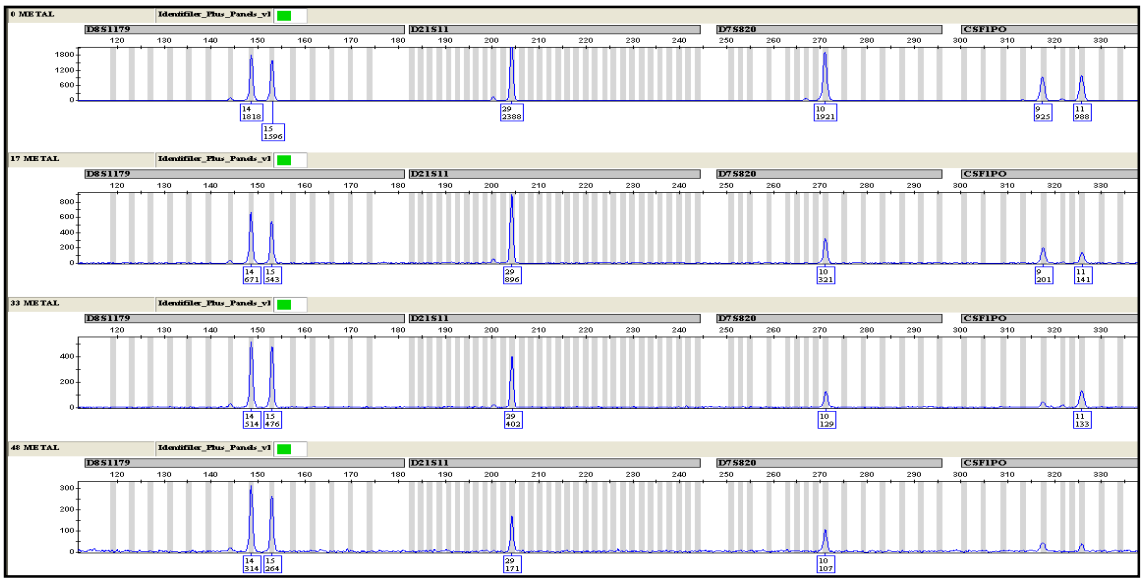


Figure 7.6 Original electropherogram showing the gradual degradation of DNA profiles of blood samples on metal from days 0, 17, 33 and 48 corresponding to accumulated degree days of 0, 475, 943 and 1425.5 respectively, n=3.

7.3.2 Effects of environmental insults on DNA from three different body-fluid types (Pilot study 2)

7.3.2.1 Sample collection

The experiments conducted in (Pilot 1) were repeated with the addition of two more body-fluids to total three different body-fluids (blood, semen and saliva). The experiment was also carried out in the summer season instead of spring. Again, no repeats of measurements were taken and only one sample for each body-fluid on a specific material was taken for further analysis.

Figure 7.7 A photograph showing the set up of (Pilot 2) experiment. Blood, semen and saliva samples were deposited on cloth, metal and glass materials and placed outdoors exposed to environmental insults for the duration of the experiment.

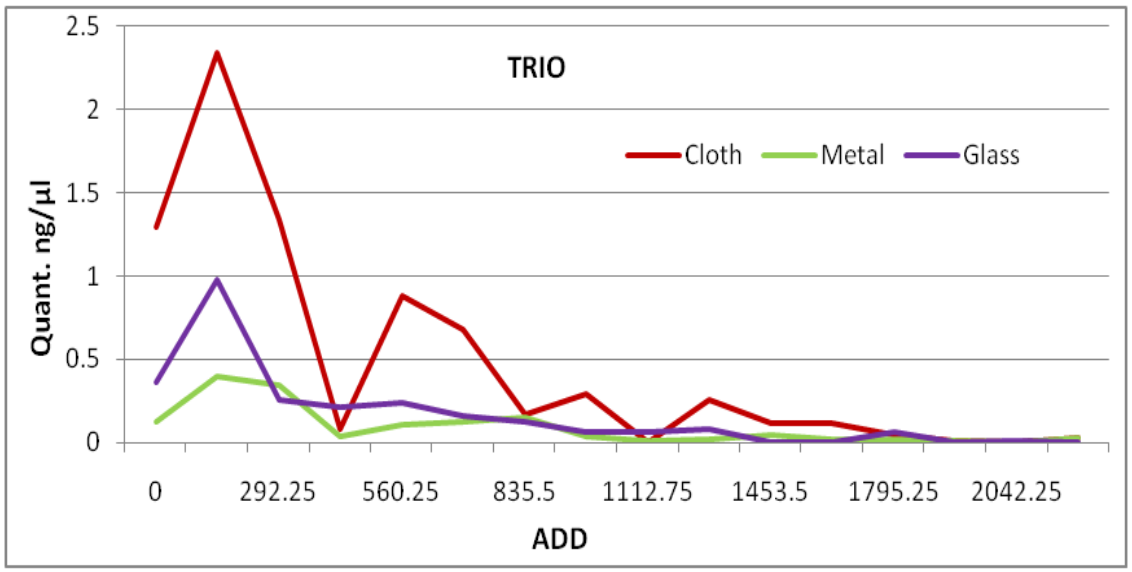
Sampling was carried out every 3rd day with a total of 48 samples taken for blood, 54 for semen and 27 for saliva.

7.3.2.2 DNA Quantification

DNA quantification was carried out using two different quantification kits; the Quantifiler® Human and the Quantifiler® Trio (Applied Biosystems) with the objective to identify the more reliable and suitable kit for our purpose. Figures 7.8, 7.9 and 7.10 show that the majority of DNA degradation occurs during the initial 300 ADD. This holds true for all body-fluid types in all materials tested, except for saliva samples where the rate of degradation is highest in the initial 150 ADD. The same pattern was observed for blood samples in (Pilot 1) at a similar ADD; the majority of DNA extracted from blood samples carried out in that experiment degraded in the initial 300 ADD, around Day 17.

Interestingly, DNA quantity, as measured by both the Quantifiler® Human and Trio, continued to increase for the first 150 ADD before beginning to decrease, this characteristic was exclusively observed for blood samples on different materials and was not seen in other body-fluid types. Additionally, all body-fluid types on cloth

contained larger DNA quantities initially compared to the other material types. However, this increase in DNA quantities rapidly ceased and fell to normal levels within few days of sampling.



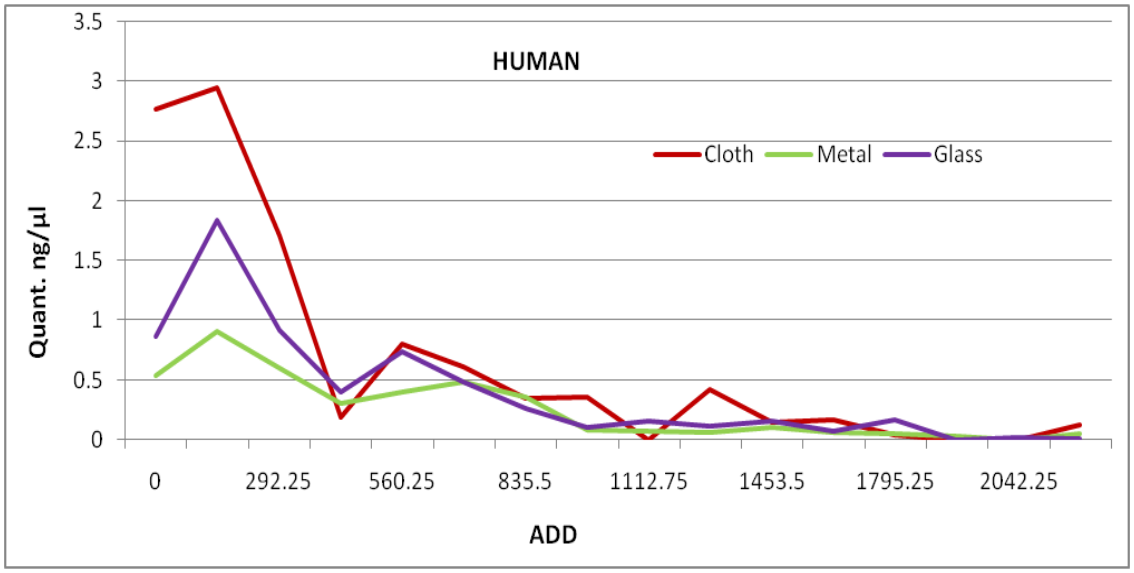


Figure 7.8 Time course graphs showing the rate of DNA degradation against ADD from 5µl of blood when deposited on cloth, metal and glass using two quantification kits (Quantifiler® Trio and Quantifiler® Human), n=3, data are plotted as mean values.

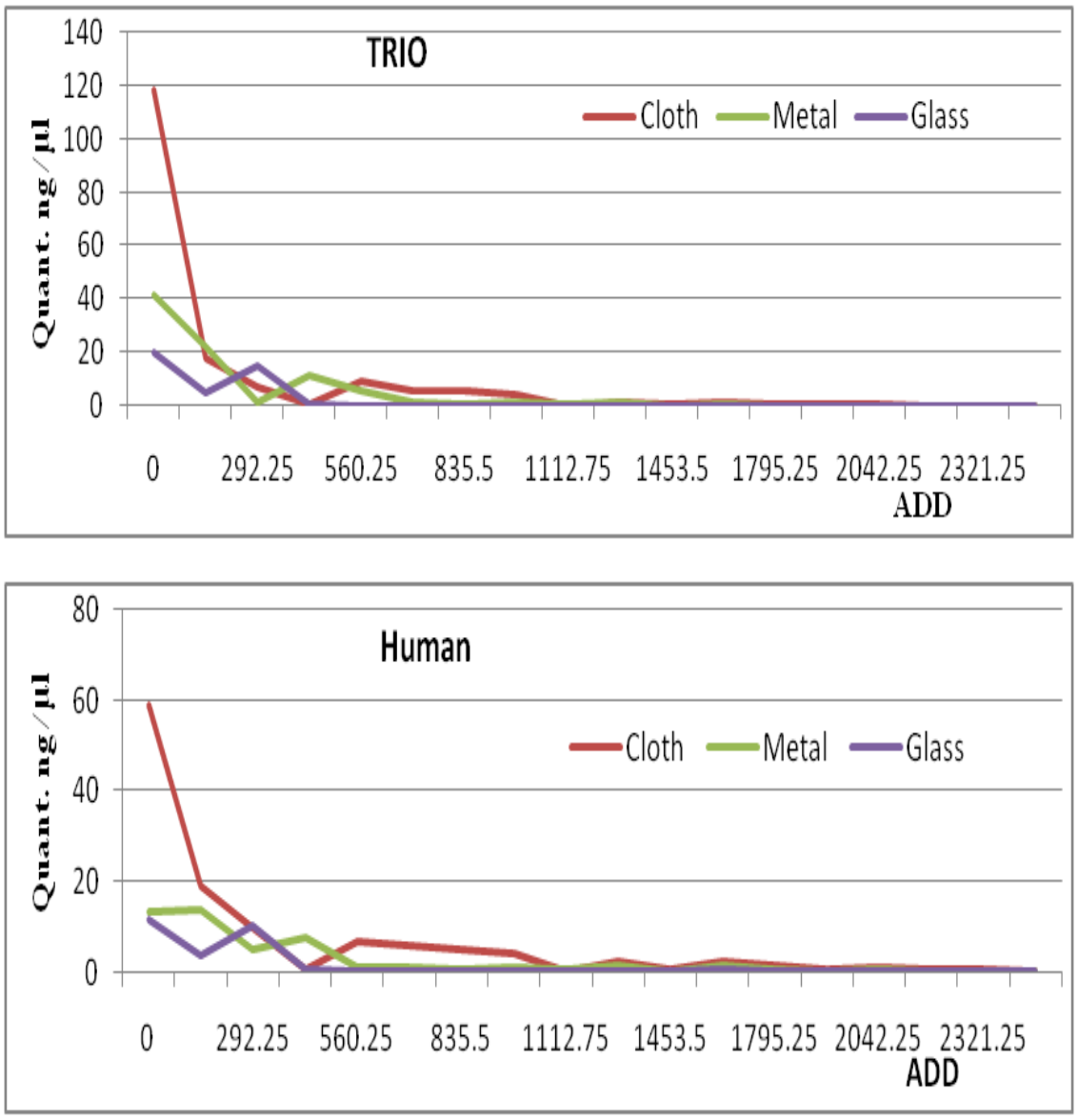


Figure 7.9 Time course graphs showing the rate of DNA degradation against ADD from 5µl of semen when deposited on cloth, metal and glass using two quantification kits (Quantifiler® Trio and Quantifiler® Human) , n=3. Data are plotted as mean values.

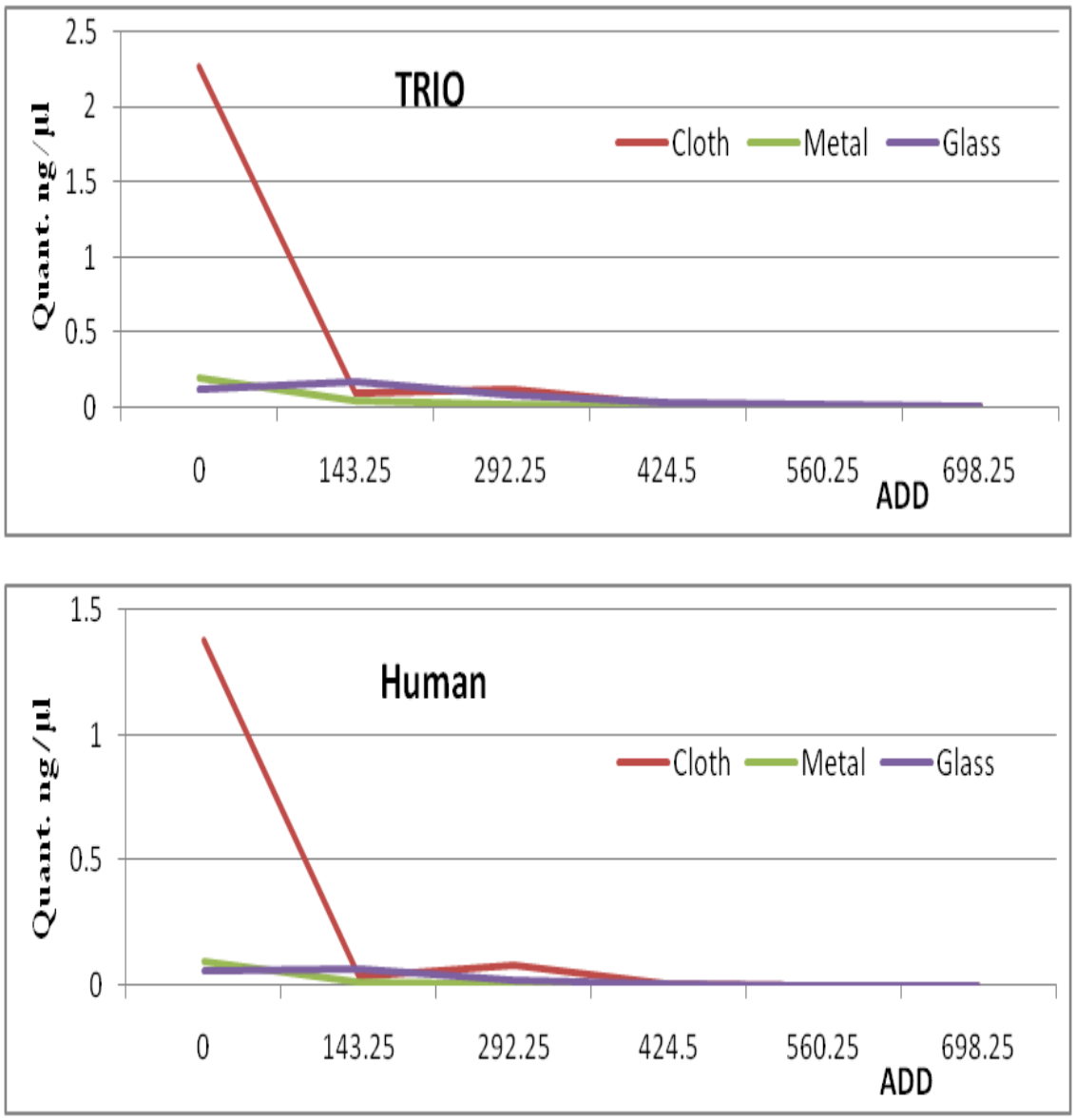


Figure 7.10 Time course graphs showing the rate of DNA degradation against ADD from 5µl of saliva when deposited on cloth, metal and glass using two quantification kits (Quantifiler® Trio and Quantifiler® Human) , n=3, data are plotted as mean values.

When comparing blood samples on different materials, ANOVA test showed that there was no significant difference between the two quantification kits tested, except for when metal was used (p=0.0284). Otherwise there was no significant difference between blood samples on cloth and glass (p=0.518 and p=0.0979 respectively). A box-plot representation of the DNA quantities extracted is shown in (Figure 7.11).

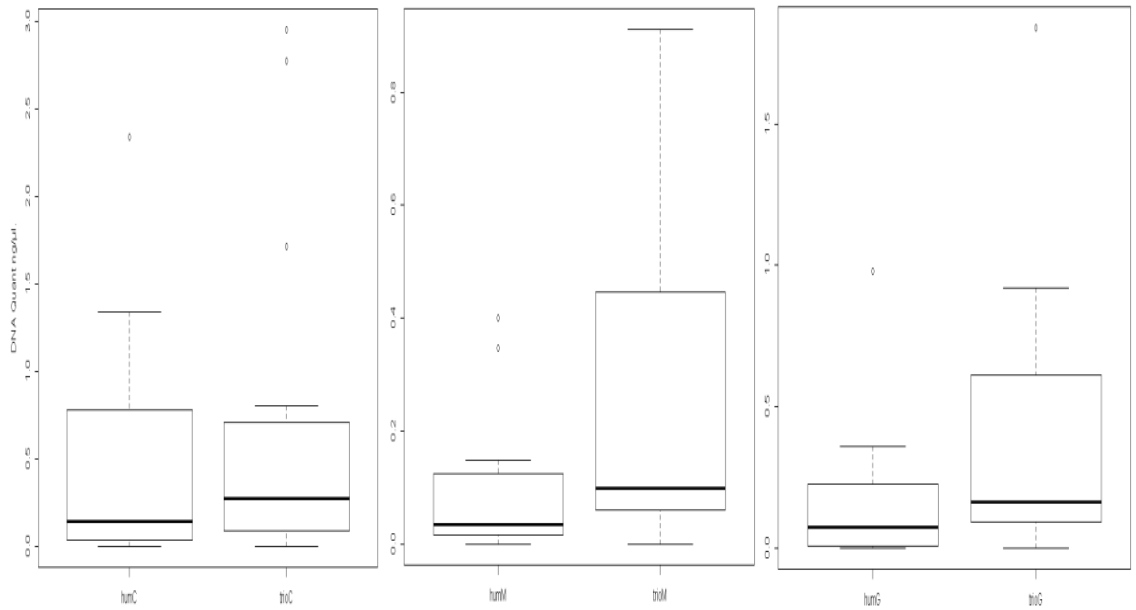


Figure 7.11 Box-plot representations of the difference between quantifying using Quantifiler® Human and Quantifiler® Trio for blood samples on three different materials; cloth (right), metal (centre) and glass (left), n=3.

In addition, ANOVA analysis has shown that there was no significant difference in DNA quantities extracted from degraded semen samples from different materials when using the different quantification kits. However, the box-plot representations of the DNA quantities for the different materials show a large number of positive outliers which may have influenced the outcome of the ANOVA results (Figure 7.12).

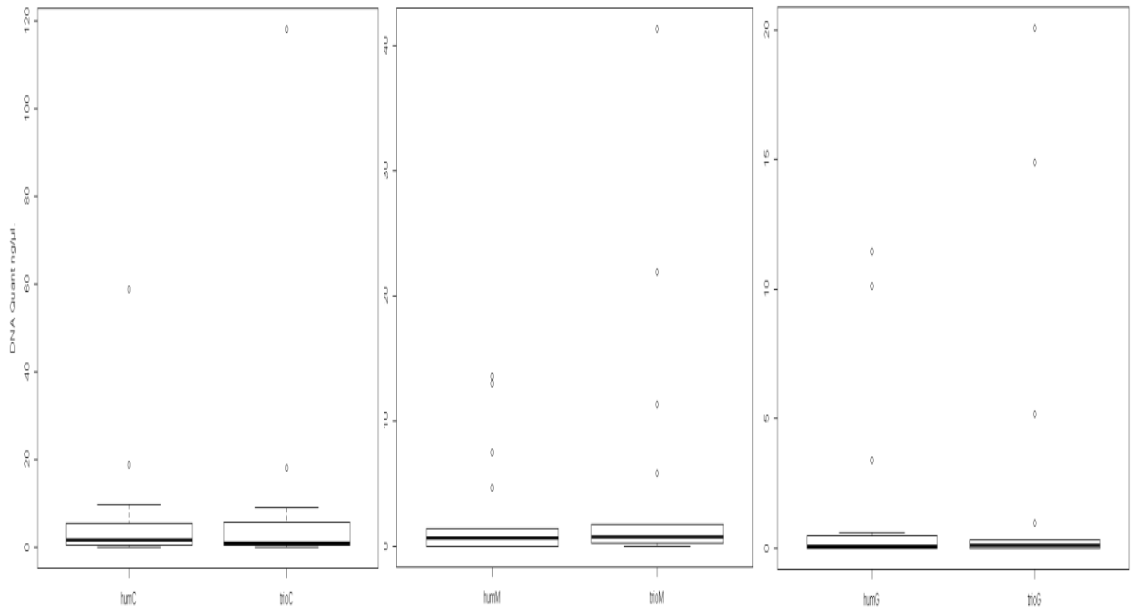


Figure 7.12 Box-plot representations of the difference between quantifying using Quantifiler® Human and Quantifiler® Trio for semen samples on three different materials; cloth (right), metal (centre) and glass (left), n=3.

Similar to the semen quantification results, the degraded saliva samples did not show any significant difference between the two quantification kits on any of the different materials when ANOVA was calculated. However, in contrast to semen samples, variation in DNA quantities between the two kits may have occurred in the lower end of the quantification kits' detection levels (as opposed to the higher end of the quantification kits' detection levels seen with semen and identified by the large number of positive outlier data points seen in Figure 7.10. The Box-plots in Figure 7.13 show that for all material types, the data were tightly distributed except for when saliva samples were deposited on glass and measured with the Quantifiler® Trio.

To establish if there was a significant difference between the readings of the two quantification kits, the first five semen samples (largest DNA quantities) were compared between the two different kits. Calculation of ANOVA still showed no significant difference between samples with exceedingly large amounts of DNA. The same was carried out for the last five saliva readings (smallest DNA quantities). Again ANOVA showed that there was no significant difference (results not shown).

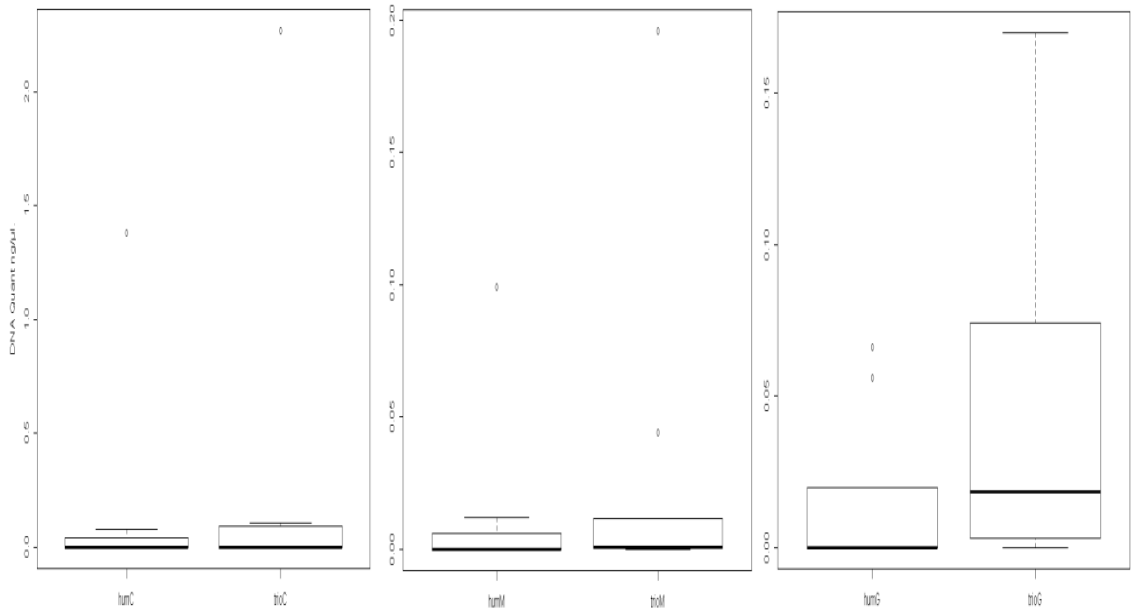


Figure 7.13: Box-plot showing of the difference between quantifying using Quantifiler® Human and Quantifiler® Trio for saliva samples on three different materials; cloth (right), metal (centre) and glass (left). n=3, $p > 0.05$ for different materials.

To investigate the effect of material types on the quantity of DNA measured, ANOVA was again calculated for each body-fluid type with all three different materials. No significant difference was found between the different materials for any particular body-fluid and this was true for both quantification kits tested.

The Degradation Index (DI) function of the Quantifiler® Trio did seem to increase in value as ADD increased. However the trend was not distinct with blood samples. According to the values obtained for the DI, severe degradation has occurred to both semen and saliva with DI values reaching nearly 1000 for semen and 150 for saliva, whereas the maximum DI value registered for any blood sample did not exceed 35. (Figures 7.14, 7.15 and 7.16).

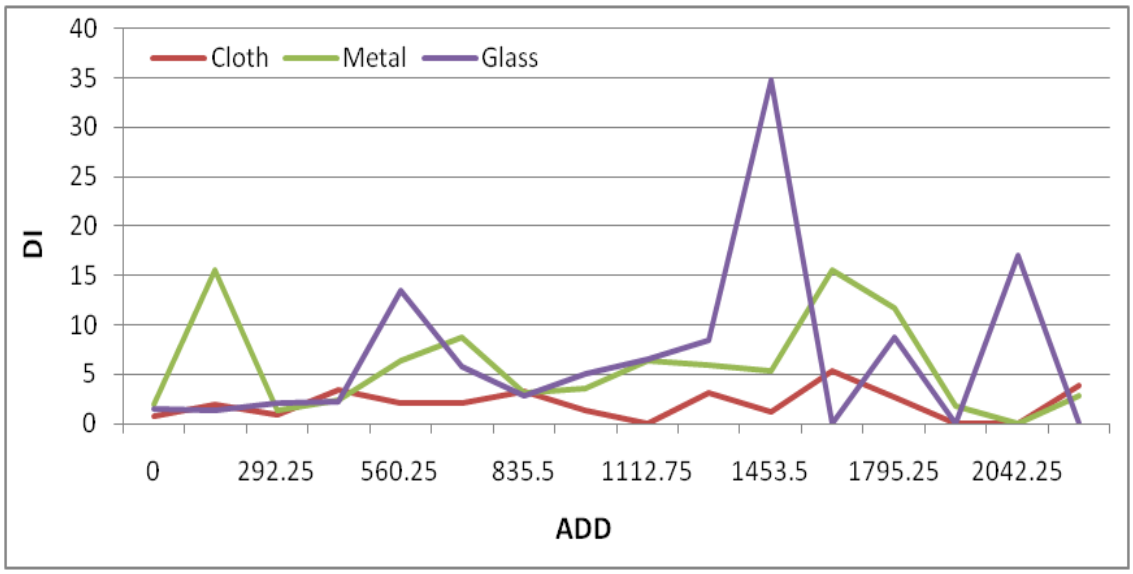


Figure 7.14 Time course graphs showing the change in DI in relation to ADD for blood samples on different materials. n=3. Data are plotted as mean values.

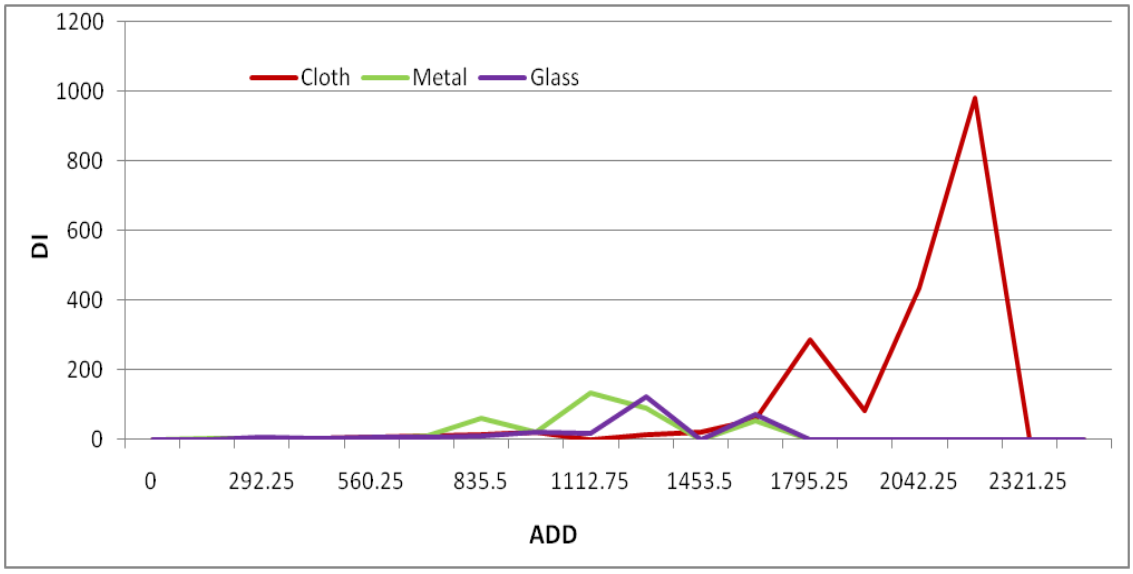


Figure 7.15 Time course graph showing the change in DI in relation to ADD for semen samples on different materials.

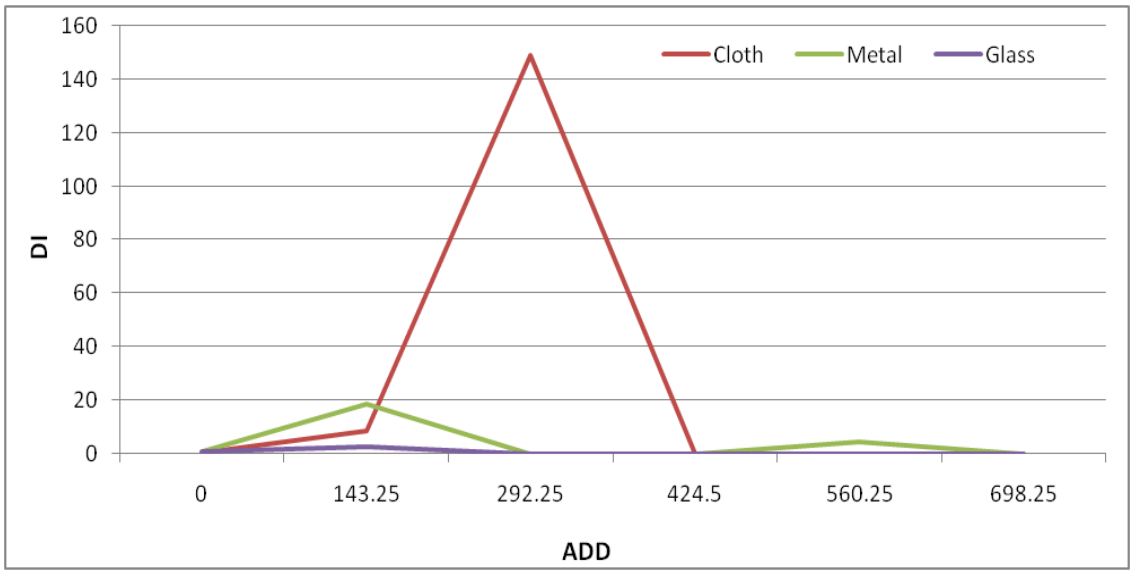


Figure 7.16 Time course graph showing the change in DI in relation to ADD for saliva samples on different materials. n=3. Data are plotted as mean values.

The IPC value was consistently higher for all body-fluid types and in all materials when the Quantifiler® Human kit was used. As expected, the Cycle time (Ct) seems to fluctuate depending on the type of sample and material being quantified. However, with the Quantifiler® Trio the Ct value remains almost unchanged throughout the experiment with a constant C*t* value of just over 26 cycles. The highest values for the IPC were recorded with blood samples using the Quantifiler® Human kit with average C*t* reaching up to 29.5 cycles for blood on metal.

7.3.2.3 STR analysis for semen samples

Since semen samples displayed the highest DNA quantity values and also the highest DI values, STR analysis was only carried out for semen samples for this pilot study. A sample from every material type at three different periods (start, middle and end of the experiment) was taken for STR analysis. For direct comparisons, DNA volumes were calculated twice with a target value of 1ng, once according to the Quantifiler® Trio results, the other according to the Quantifiler® Human. Surprisingly, semen samples have shown little resistance to degradation. Electropherogram results (Figures 7.18 to Figure 7.26 below).

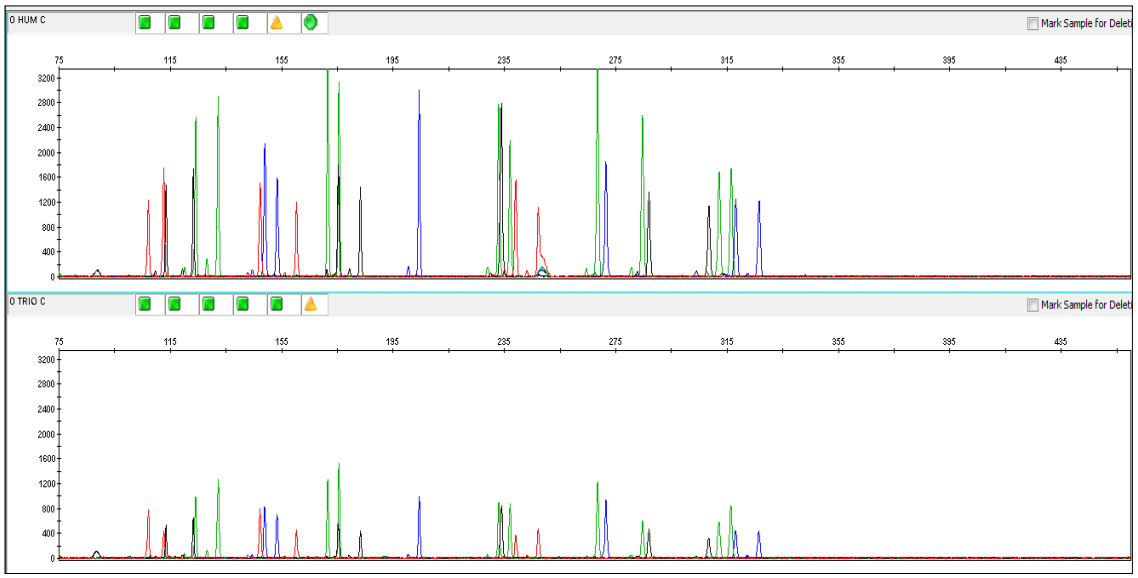


Figure 7.18 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on cloth at DAY 0.

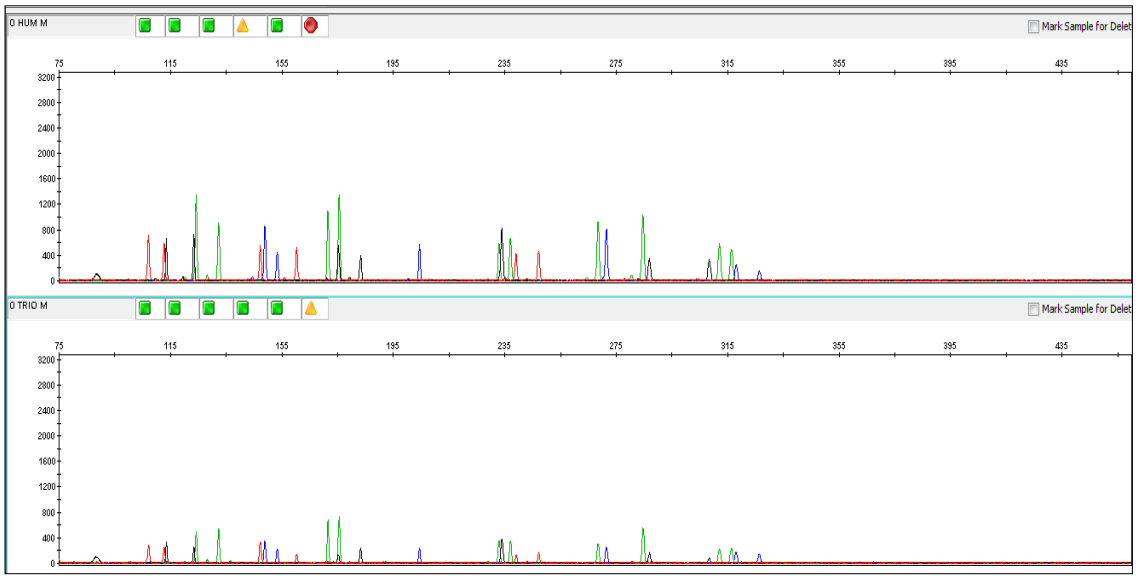


Figure 7.19 Two electropherograms comparing STR results from Quantifiler® human (top) and Quantifiler® Trio (bottom) for semen samples on metal at DAY 0.

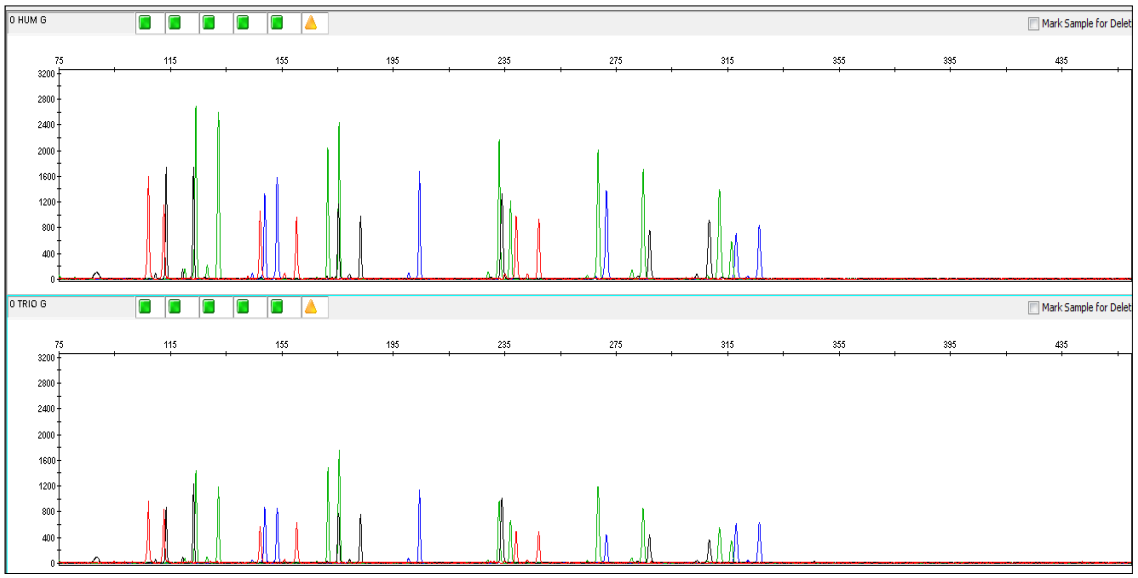


Figure 7.20 Two electropherograms comparing STR results from Quantifiler® human (top) and Quantifiler® Trio (bottom) for semen samples on glass at DAY 0.

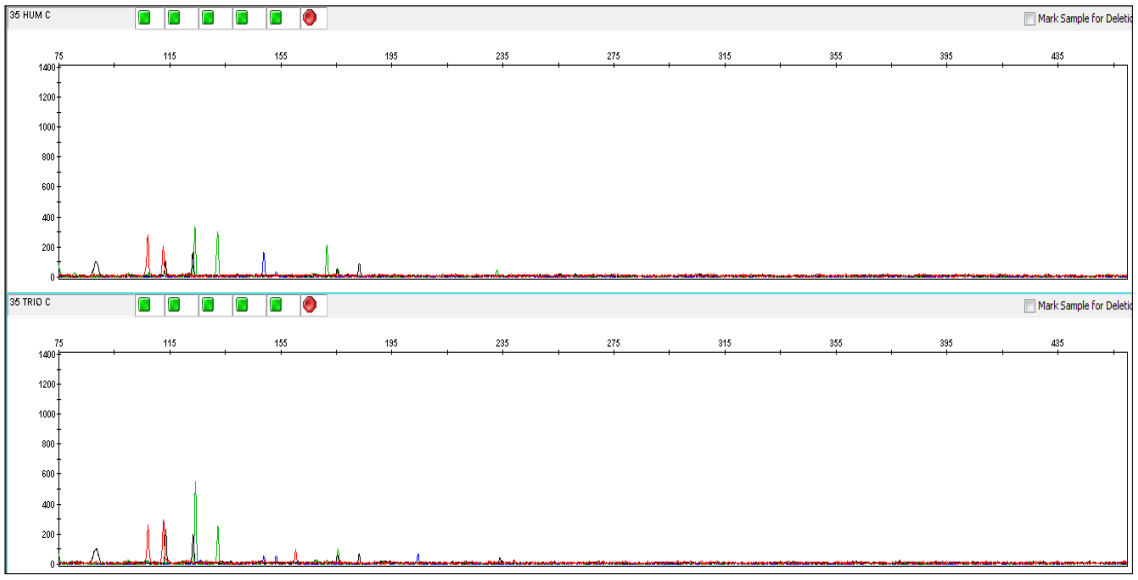


Figure 7.21 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on cloth at DAY 35.

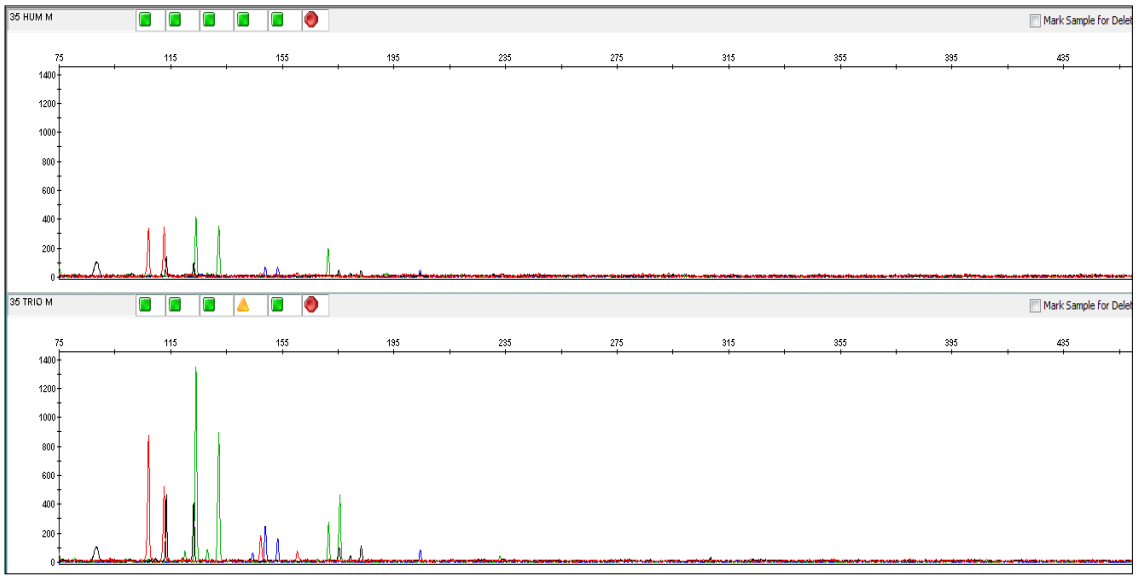


Figure 7.22 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on metal at DAY 35.

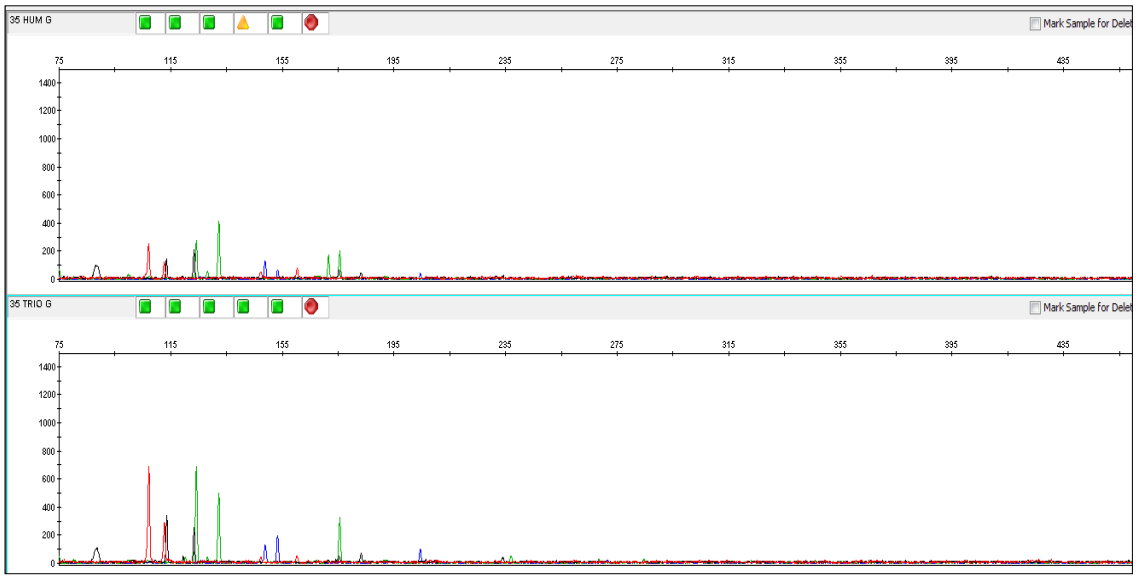


Figure 7.23 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on glass at DAY 35.

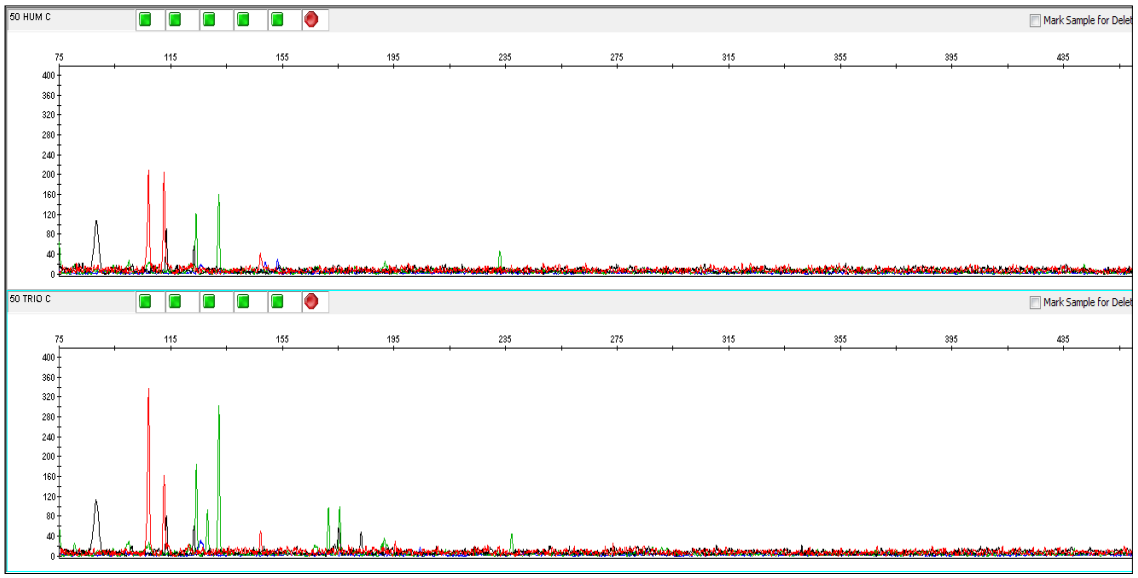


Figure 7.24 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on cloth at DAY 50.

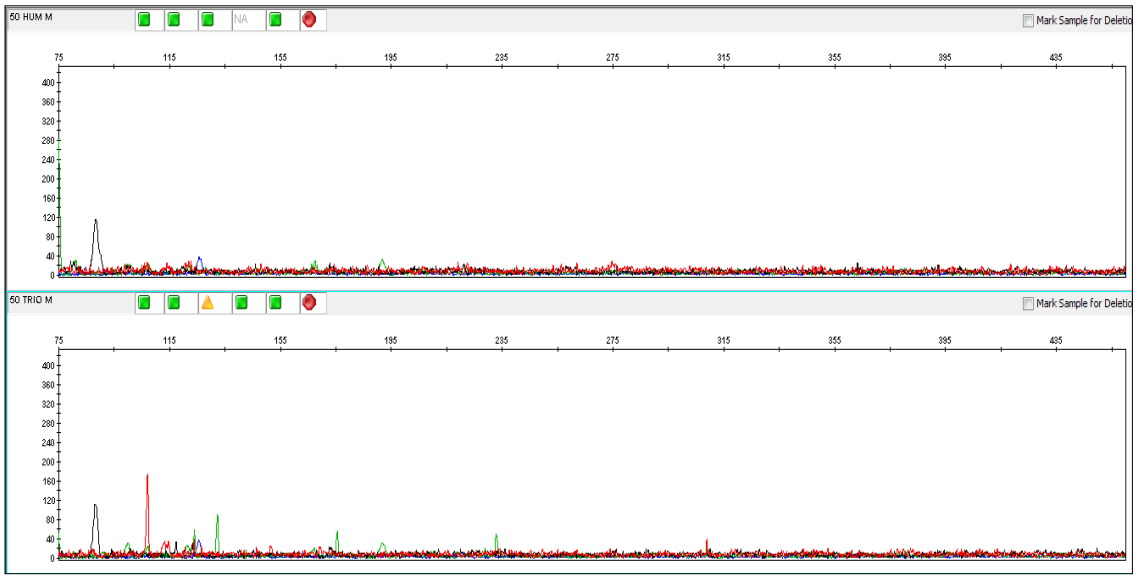


Figure 7.25 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on metal at DAY 50.

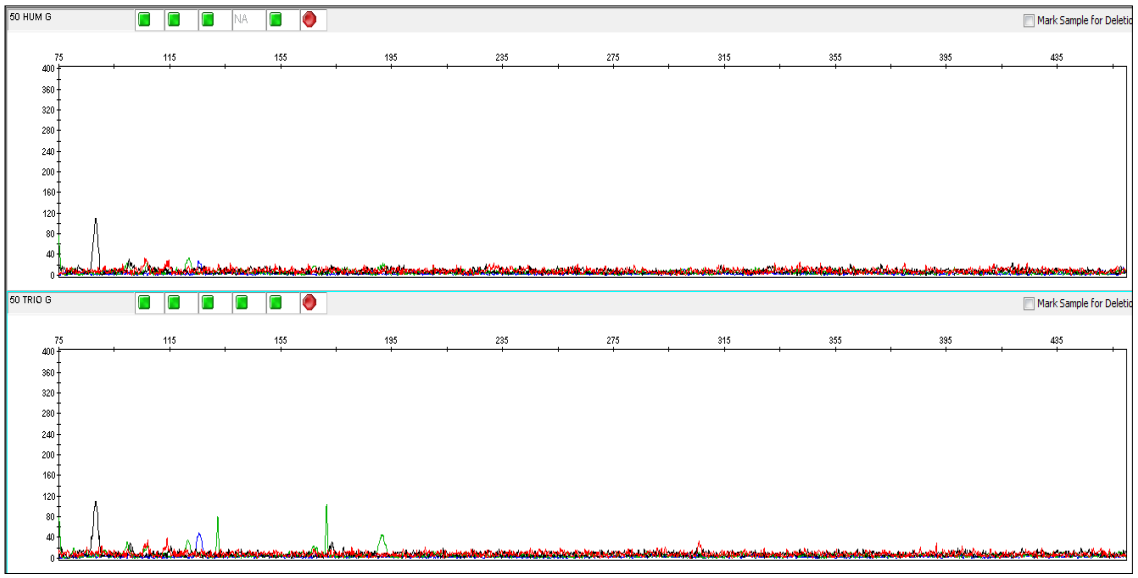


Figure 7.26 Two electropherograms comparing STR results from Quantifiler® Human (top) and Quantifiler® Trio (bottom) for semen samples on glass at DAY 50.

Both results from thee quantification kits produced full STR results for semen samples on all materials at DAY 0. However, the Quantifiler® Human produced profiles with almost double the peak heights of those produced with the Quantifiler® Trio. In addition, both quantification kits did not show any signs of inhibition and the DI of the Quantifiler® Trio was below 1 for both cloth and glass and 1.7 for metal. At DAY 35, all STR amplification products from both quantification kits resulted in partial profiles, with all amplicon sizes of over 200bp dropping out. Quantification results from both quantification kits were comparable and therefore allele peak heights from both methods were similar.

The DI given by the Quantifiler® Trio indicated severe degradation with values of over 50 for both cloth and metal and over 70 for glass material. The Quantifiler® Human was only able to produce weak partial profiles at DAY 50; with product sizes of under 150bp for cloth samples and failed to produce any genetic information for both metal and glass materials. On the other hand, the Quantifiler® Trio produced peaks heights at just under 200bp from cloth material and also produced weak partial profiles of few peaks from metal and glass materials. The DI for cloth material was given at 978.9 whereas both metal and glass material did not register any value for the degradation index.

In general, at higher DNA quantities the Quantifiler® Human produced better STR profiles with larger peak heights than the Quantifiler® Trio. However, the Quantifiler® Trio seemed to be more sensitive in detecting low quantity degraded DNA samples.

7.3.3 Effects of local environmental insults on DNA extracted from three different body-fluids

7.3.3.1 Sample collection

Based on results observed in pilot study 1 and pilot study 2, samples were collected in triplicates from each body-fluid type every other day for the first 15 days and every 3rd day thereafter. Therefore, results from this "full" experiment are expected to be more reliable and coherent. The same issue of blood samples flaking as mentioned earlier in this study was also seen here.

7.3.3.2 DNA quantification

Following DNA extraction using the Chelex-100 method, DNA quantification was carried out using the Quantifiler® Trio (Applied Biosystems) which contains an internal control for measurements of the extent of DNA degradation present in a sample (Degradation Index). The resulting effect of environmental insults on DNA degradation are shown in Figure 7.25 for the three body-fluid types tested on three different materials. To normalise the data, the natural log of the quantification results was taken. Complete triplicate quantification results along with standard deviations and degradation index are shown in Appendix 5.

DNA extracted from blood samples produced an unexpected pattern of DNA degradation when on cloth material. While blood samples on both metal and glass material mimicked the characteristic initial increase in DNA quantity in the first 150 ADD seen in (Pilot 2).The results showed that DNA quantity from blood samples on cloth continued to increase for the majority of the duration of the project (Figure 7.27).

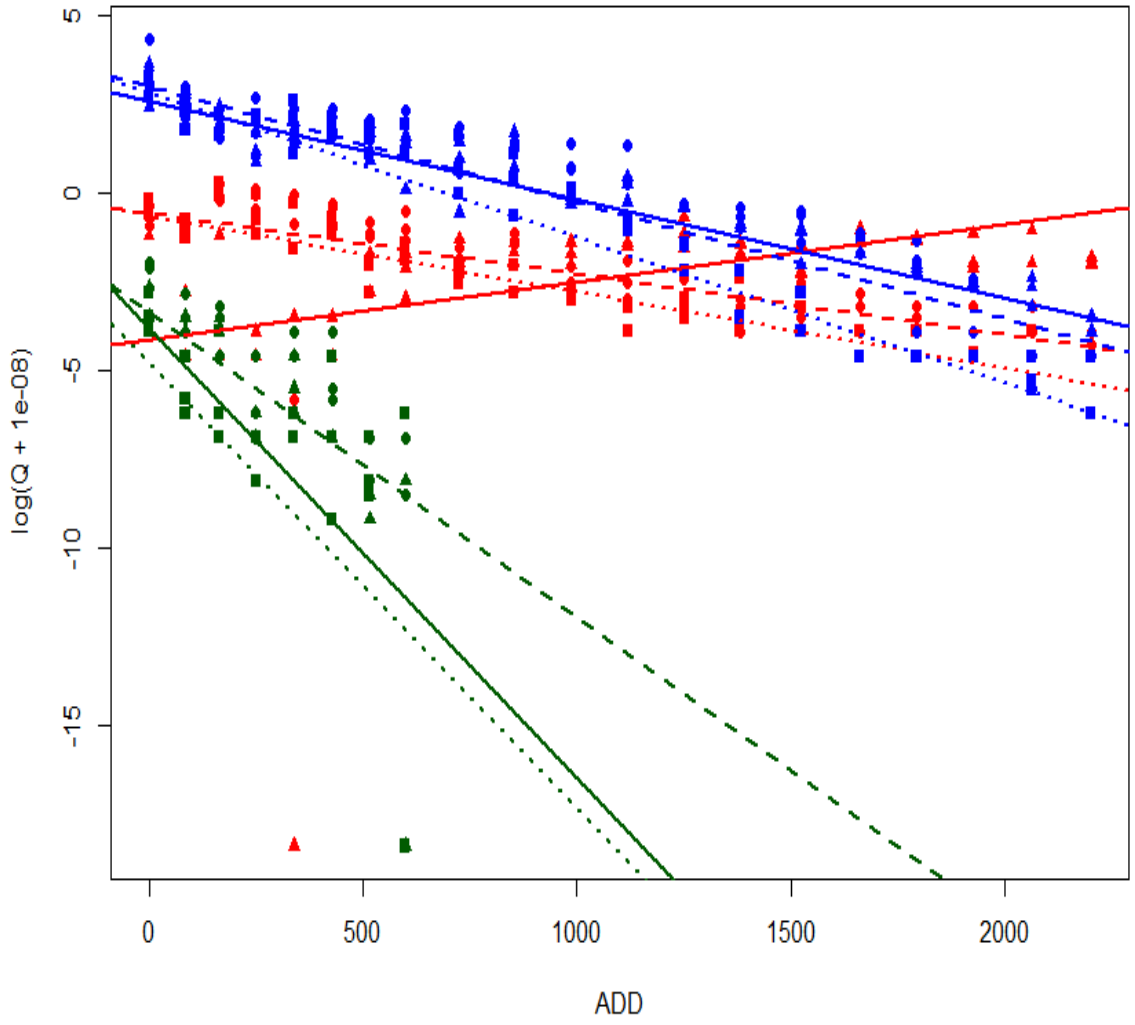


Figure 7.27 Scatter plots showing regression lines for the DNA quantity against the increase in ADD for blood (red), semen (blue) and saliva (green) samples when placed on cloth (solid-line, triangles), glass (broken-line, circles) and metal (dotted-line, squares). n=3.

Interestingly, for blood samples on cloth, the DNA quantity from ADD 0 were very similar to that obtained 50 days later at ADD 2201.5. However, after the initial increase in DNA quantity, both blood samples on metal and glass continued their gradual decrease until the end of the experiment.

DNA quantities extracted from semen samples continued a gradual decrease, with the majority of DNA degraded in the initial 300 ADD, a trend similar to that seen in (Pilot 2). Saliva samples were the quickest to degrade, with the majority of the DNA quantity degraded within the first 250 ADD and almost all DNA degraded by 599 ADD.

The Degradation Index results given by the Quantifiler® Trio kit was also analyzed and presented in Figure 7.28 below. Again, the data were normalised by taking the natural log of the data.

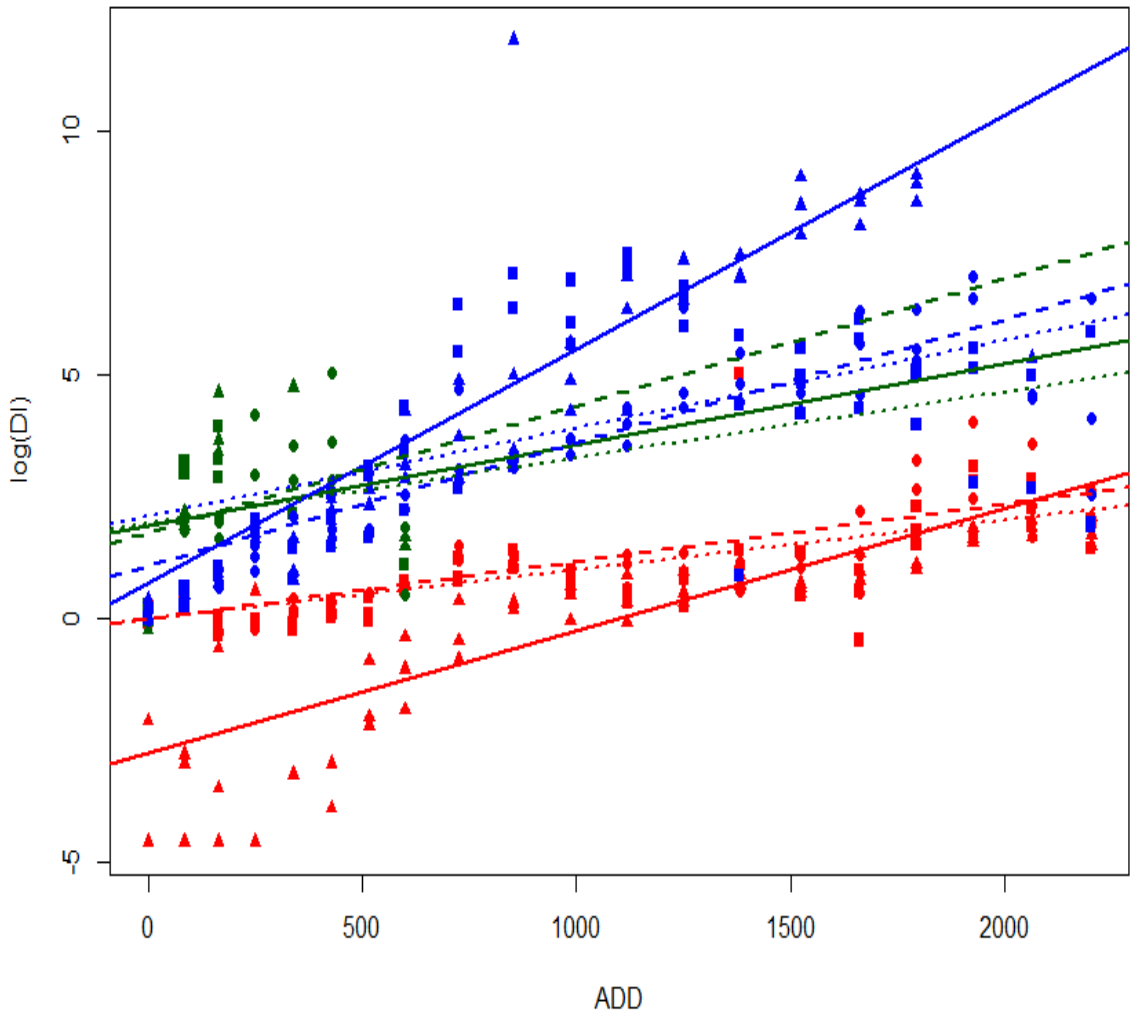


Figure 7.28 Scatter plots showing regression lines for the Degradation Index (DI) against the increase in ADD for blood (red), semen (blue) and saliva (green) samples when placed on cloth (solid-line, triangles), glass (broken-line, circles) and metal (dotted-line, squares). n=3.

As expected, the results for the value of the DI for all body-fluid types on the different materials continued to increase with the increase in ADD, with the presence of outlier residuals from the regression lines. With the exception of saliva, body-fluids on cloth showed the highest level of DNA degradation represented by an increased rate in the regression slope exceeding all other materials. No presence of inhibition was detected

by the Quantifiler® kit as given by the IPC Ct values of all the samples, with the average IPC Ct value not exceeding 27.0 cycles for the entire runs. According to the manufacturers' recommendations (Quantifiler® Trio DNA Quantification kit, User manual, 2014), when the presence of inhibition is not expected, the degradation index interpretation should be interpreted as follow:

Table 7.2 Table summarising the interpretation of the degradation index in relation to the quality of the DNA present when the IPC C*t* flag in the Quantifiler® kit is not triggered.

Degradation Index	Quality Index interpretation
<1	DNA is not degraded or inhibited
1 to 10	DNA is slightly to moderately degraded. PCR inhibition is possible but not enough to suppress IPC amplification
> 10 or blank	DNA is significantly degraded. PCR inhibition is also possible but not enough to suppress IPC amplification

The DI values for blood samples on both cloth and metal did not exceed 7 units for the duration of the experiment, indicating slight to moderate degradation. Whereas it reached beyond 10 units when on glass, which suggests that significant DNA degradation has occurred. Although the DI for semen samples on glass was much higher than it was with blood samples, reaching up to 902 towards the end of the experiment, the DI was much higher for cloth and metal material where the DI reached over 47000 unit and 1400 unit respectively.

7.3.3.3 STR analysis

In order to relate the function of DI to the quality of genetic information produced, STR analysis was carried out for a total of 46 samples (one sample from each body-fluid type on three different materials from six different ADD periods for blood and semen but only 3 ADD periods for saliva see Figures 7.27 to Figure 7.42). The STR master mix was prepared according to manufacturers' recommendations and when available 1 ng/µl of target DNA was applied, otherwise the maximum volume of 10 µl was added to the master mix.

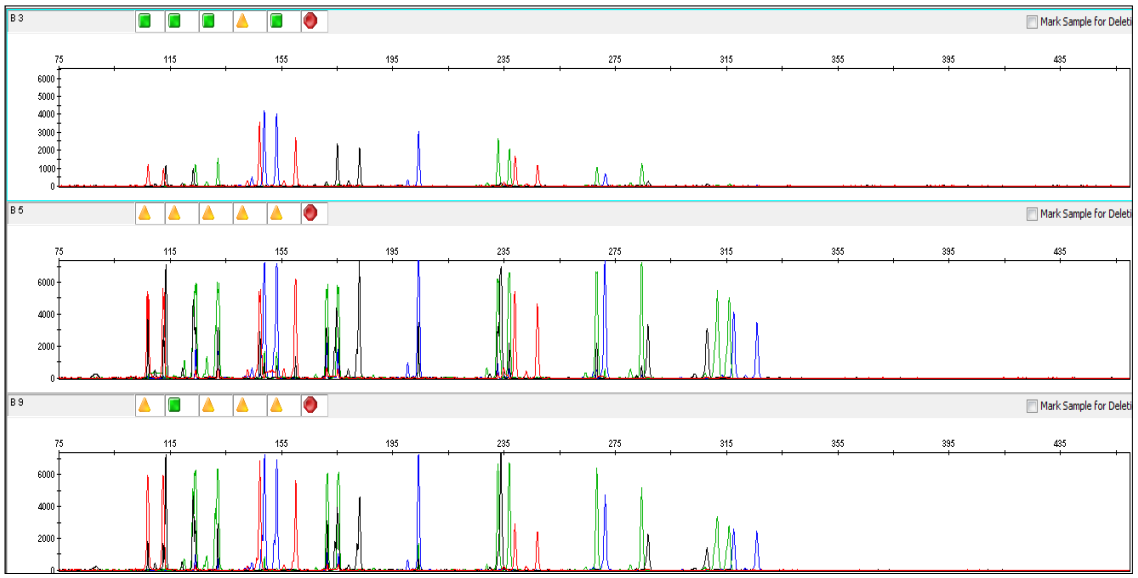


Figure 7.29 Three electropherograms of blood samples at 0 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

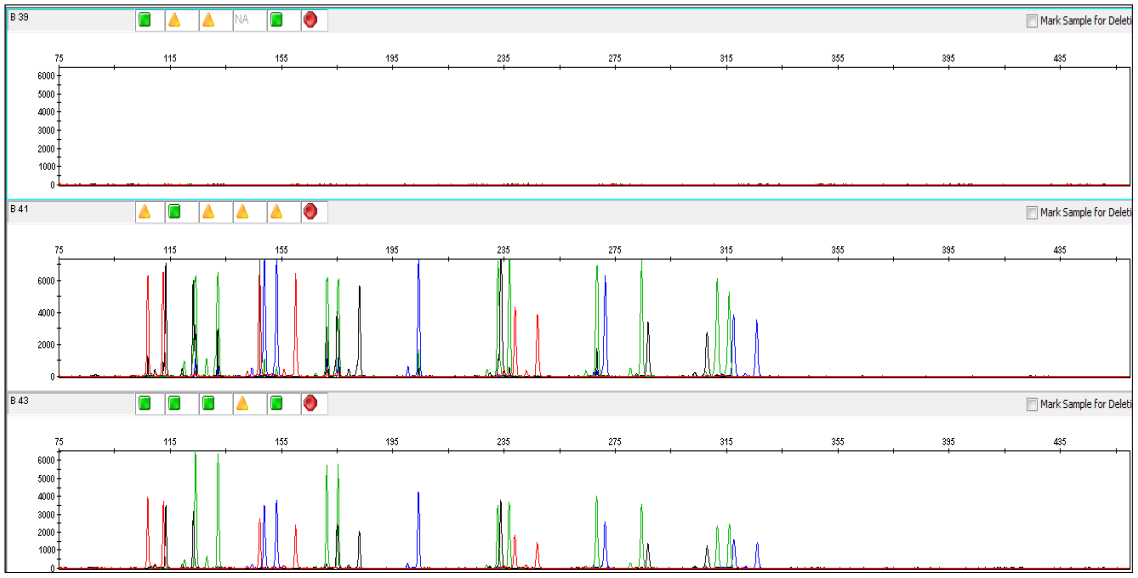


Figure 7.30 Three electropherograms of blood samples at 338.5 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

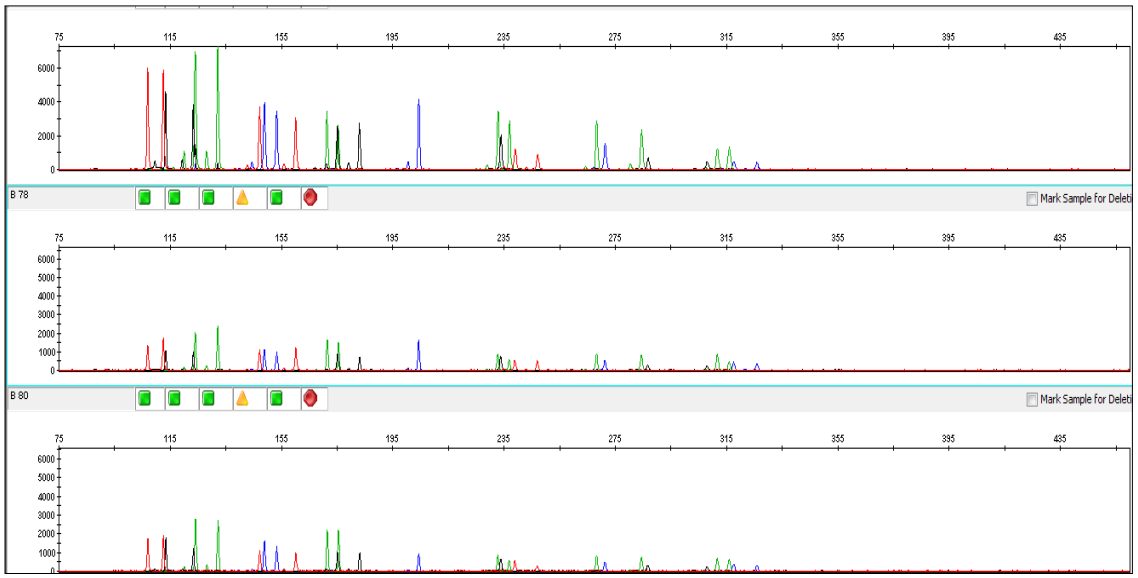


Figure 7.31 Three electropherograms of blood samples at 724.5 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

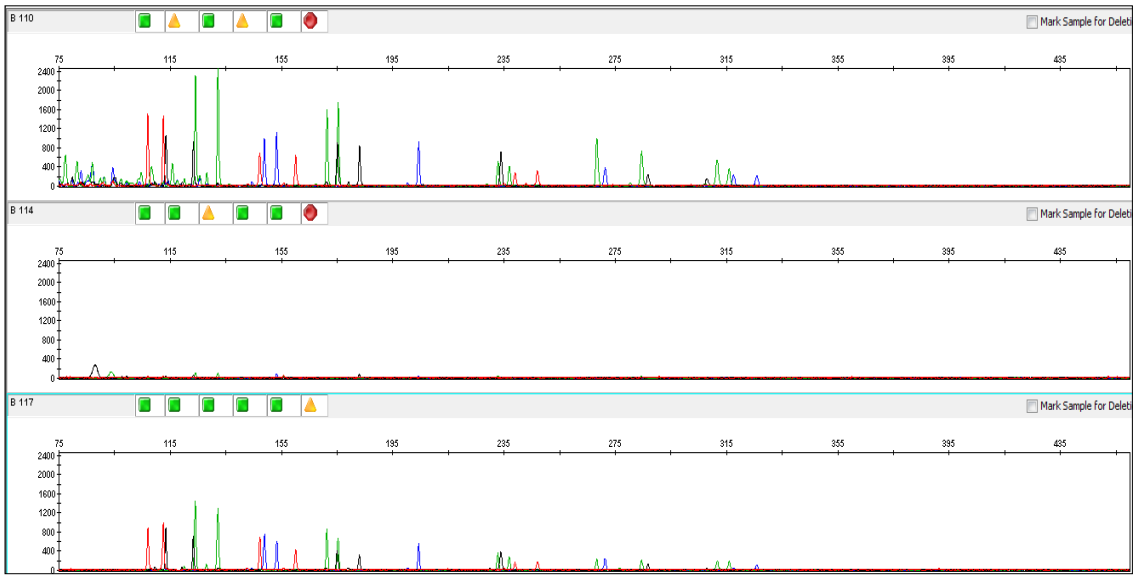


Figure 7.32 Three electropherograms of blood samples at 1250.25 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

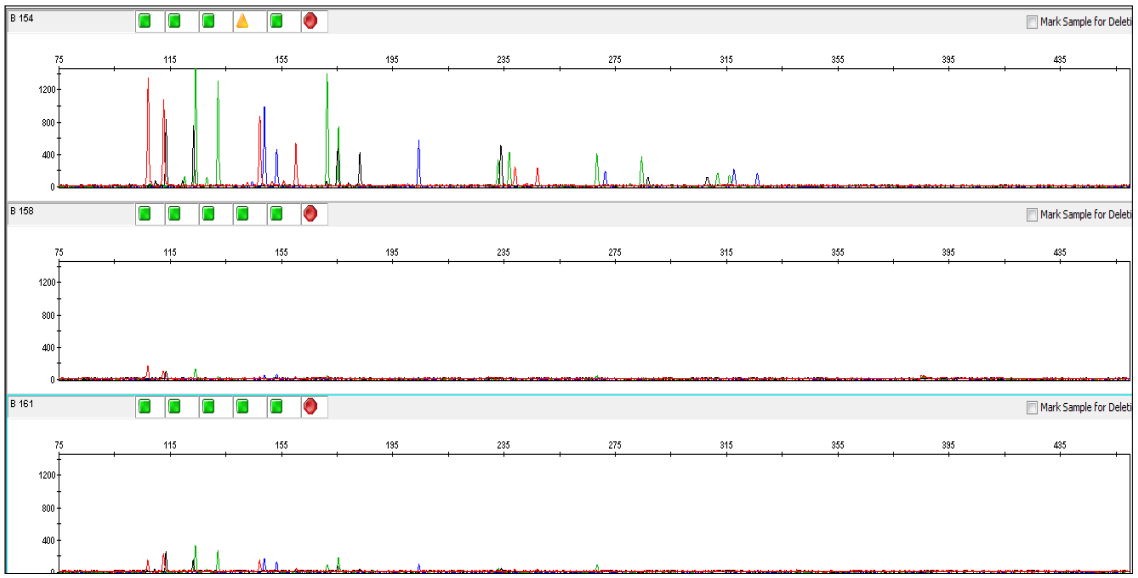


Figure 7.33 Three electropherograms of blood samples at 1926.25 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

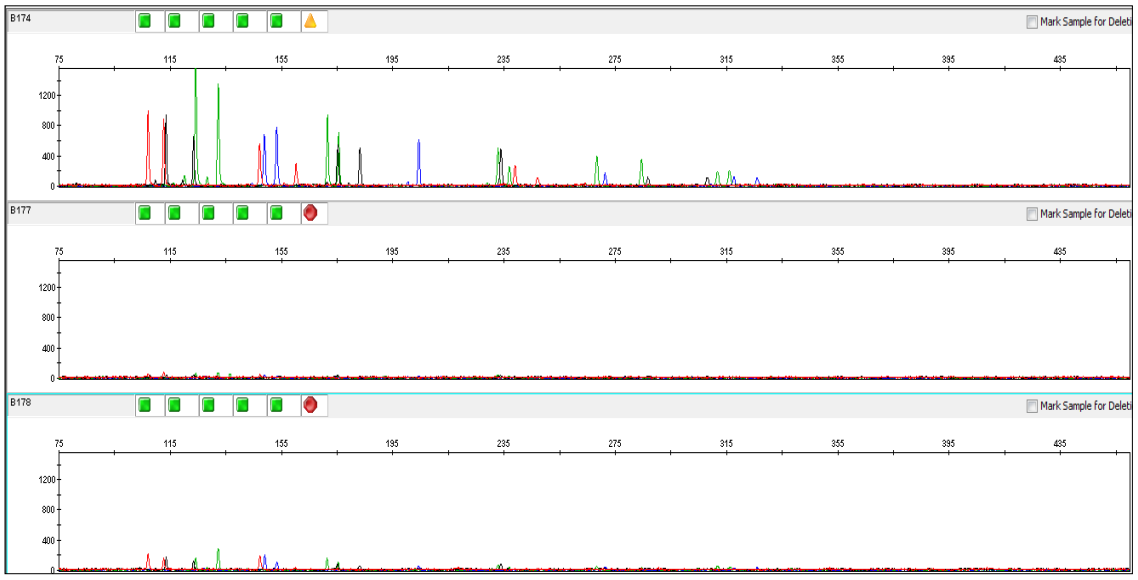


Figure 7.34 Three electropherograms of blood samples at 2201.5 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

When the recommended amounts of DNA were introduced to the STR master mix and genetic analysis was carried out, blood samples behaved in an uncharacteristic manner. Samples from 0 ADD on cloth failed to produce a full profile, with only few of the alleles with larger size amplicons dropped out, the degradation index was 0.04. Both the other blood samples on metal and glass produced full profiles as expected at 0 ADD, although the degradation index was higher than that registered for cloth

material with 1.04 and 1.29 for blood on metal and glass respectively. In the following analysis period (338.5 ADD), the cloth sample failed to generate any STR result even though DI did not exceed 0.04. Again, both metal and glass materials produced good quality STR results with DI of 0.83 and 1.24, respectively.

The following ADD period (724.5 ADD) gave a contrasting result, with cloth samples producing a full profile and producing the largest peak heights compared to the other material types. Both metal and glass samples gave full profiles of very low RFU heights. DI values were 0.82, 2.59 and 3.74 for cloth, metal and glass materials respectively. By ADD 1250.25, blood samples on metal produced a very weak partial profile (DI=1.99) while the performance of blood samples on glass was better with only few alleles with larger amplicon sizes dropped from the STR profile (DI=2.56). Blood samples on cloth at the same ADD still managed to produce a full STR profile even though the DI of 1.87 was comparable to that of metal.

Furthermore, blood samples on cloth were still able to produce full STR profiles for the duration of the experiment, even with DI value of 5.94 at ADD 2201.5, at the same time as no or very weak profiles were generated when blood samples were placed on metal (DI= 8.51) or glass (DI= 9.16).

Although statistically there was no significant difference between the natural log of the quantities of the DNA extracted from blood samples on the different materials (p= 0.266), the natural log of the values for DI indicated a significant difference in the degradation levels when different materials were used (p=0.00242) (Figure 7.35).

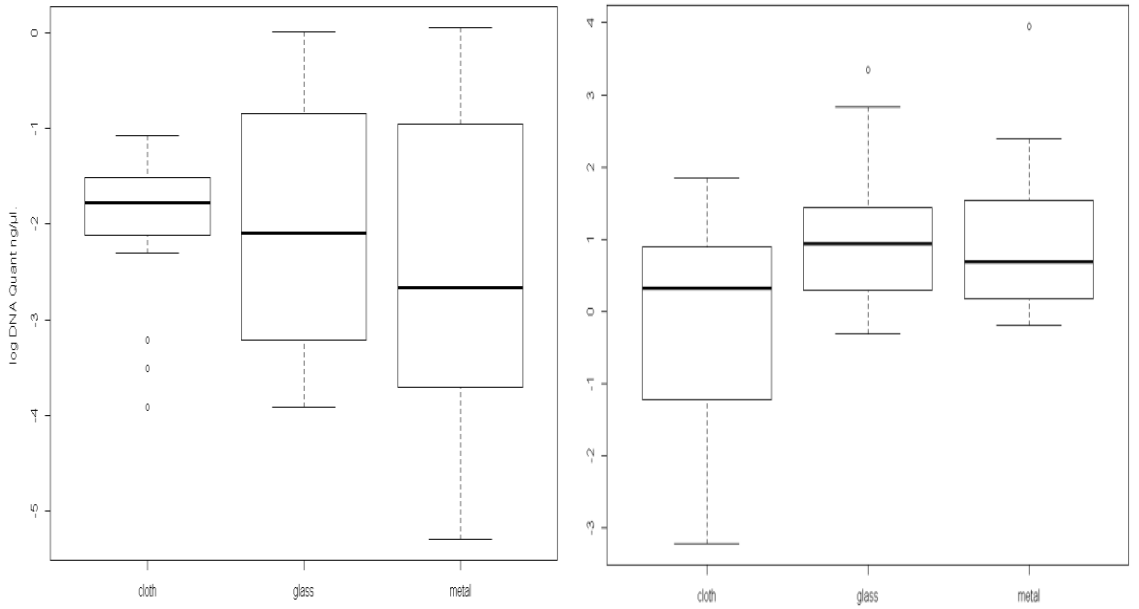


Figure 7.35 Box-plot representation of the variation in the log DNA quantities when blood samples were placed on different materials (left) compared to the variation in the log DI values for the same materials (right), n=3.

Both metal and glass materials gave similar DI medians, while blood samples on cloth showed a significantly lower DI and a relatively higher DNA quantity medians. This is in concordance with STR profiles where blood on cloth continued to produce good genetic information for the duration of the experiment.

The STR results for semen samples on different materials at different ADD periods are shown in Figures 7.36 to 7.41 below.

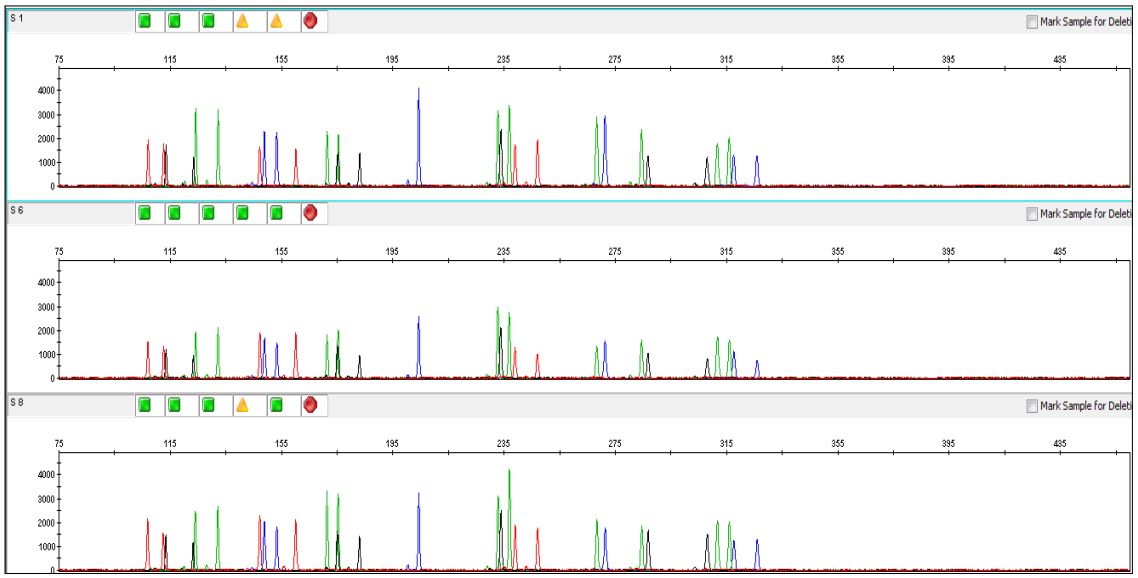


Figure 7.36 Three electropherograms of semen samples at 0 ADD on cloth (top), metal (middle) and glass (bottom) materials.

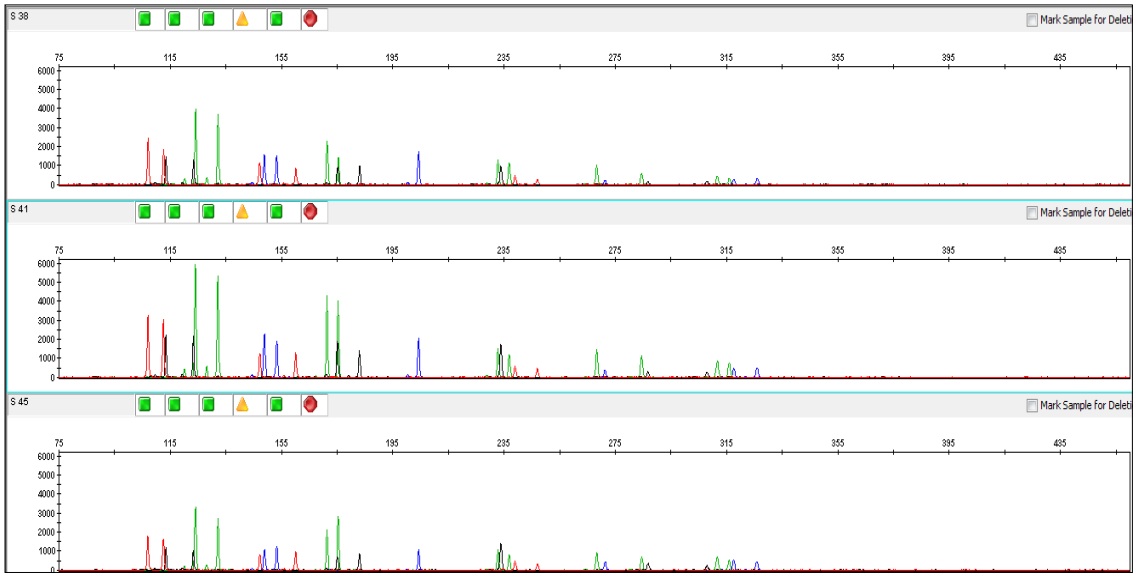


Figure 7.37 Three electropherograms of semen samples at 338.5 ADD on cloth (top), metal (middle) and glass (bottom) materials.

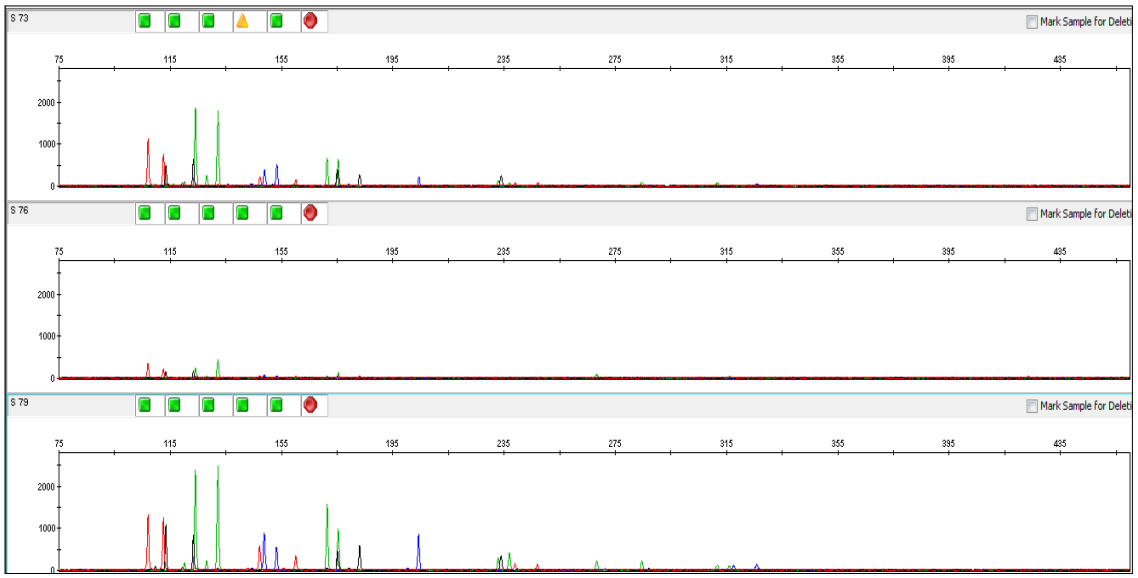


Figure 7.38 Three electropherograms of semen samples at 724.5 ADD on cloth (top), metal (middle) and glass (bottom) materials.

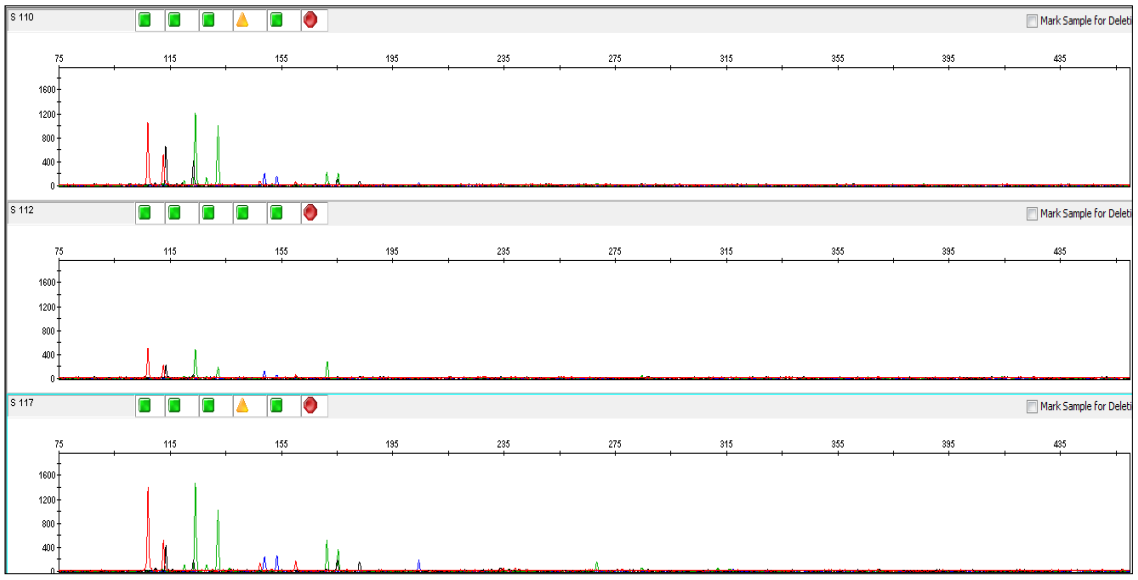


Figure 7.39 Three electropherograms of semen samples at 1250.25 ADD on cloth (top), metal (middle) and glass (bottom) materials.

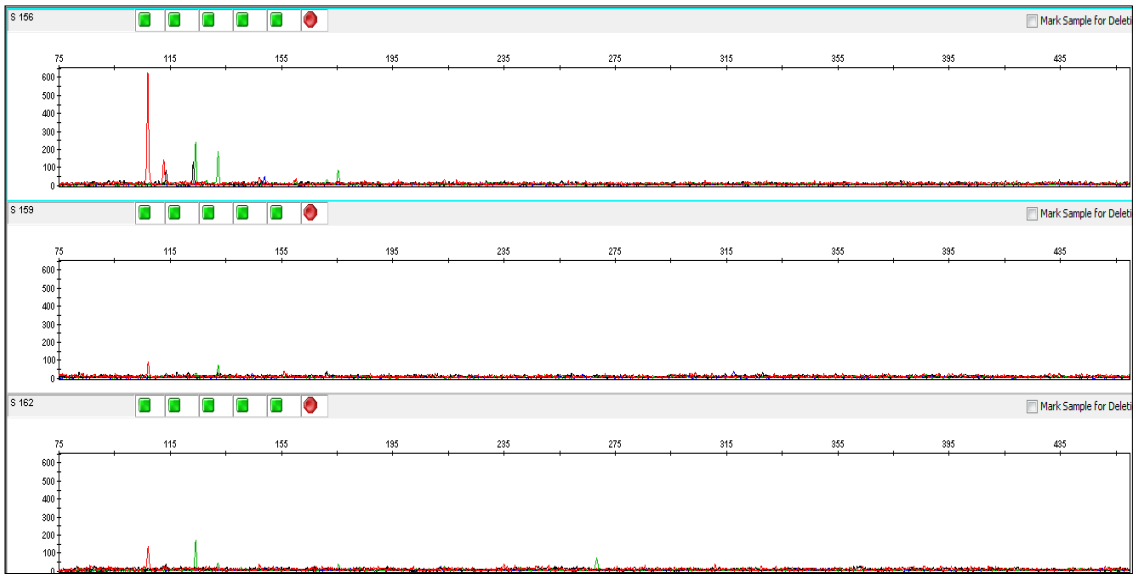


Figure 7.40 Three electropherograms of semen samples at 1926.25 ADD on cloth (top), metal (middle) and glass (bottom) materials.

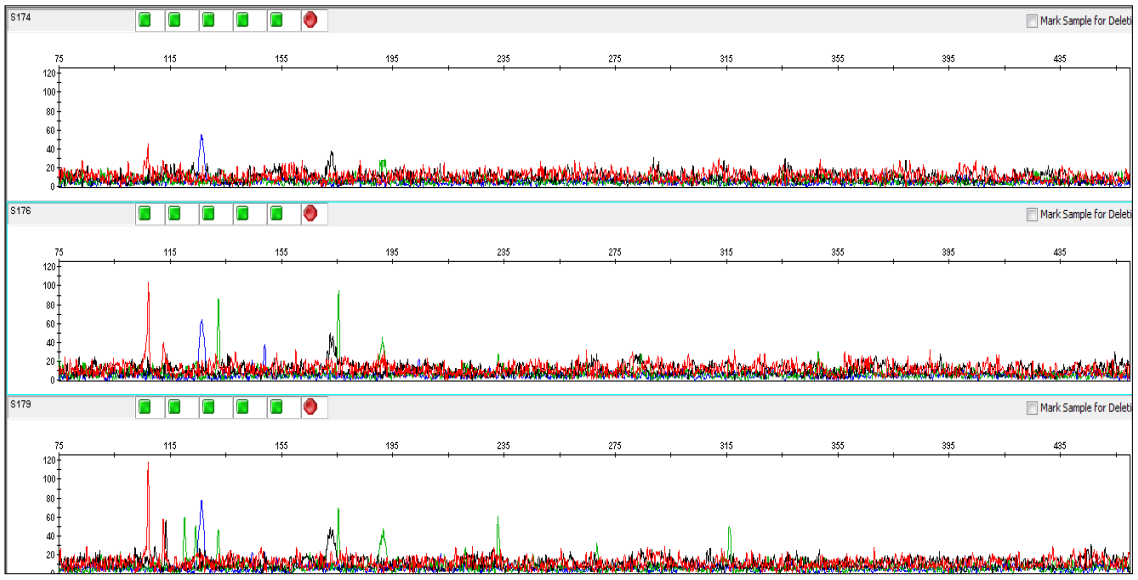


Figure 7.41 Three electropherograms of semen samples at 2201.5 ADD on cloth (top), metal (middle) and glass (bottom) materials.

The STR degradation pattern for semen samples on different materials behaved in a similar manner up to ADD 338.5. According to the results collected from the six different ADD periods, all semen samples produced full STR profiles, which decreased in peak heights at similar rates until drop-outs started to occur at ADD 724.5.

At ADD 338.5 the degradation index for all samples was comparable between the different material types, ranging from 4.83 for cloth to 8.47 for metal. At ADD 724.5,

semen samples on metal materials seem to be the most affected by environmental insults as many of the STR peaks have dropped out, giving a weak partial profile. This was reflected by the large DI value of 236.1. Semen placed on both cloth and glass had some of the larger amplicons drop-out, but the profiles still maintained strong genetic information. However, the DI for both cloth and glass materials was considered large with values of 40.5 and 21, respectively.

Partial STR profiles were obtained for all material types at ADD 1250.25 with DI values reaching 1527 for semen samples on cloth and 747.1 and 102.9 for semen samples on metal and glass, respectively. The level of genetic information obtained was negligible from ADD 1926.25 onwards with very few peak calls for all material types, and DI values failing to register for semen on cloth, while they decreased from previous values for metal and glass materials to reach 357.4 and 61.1 on the final ADD readings.

One way ANOVA calculation showed no significant differences between material types in both quantity of DNA extracted (p=0.392), nor the DI given for these samples from the different materials (p=0.133), Figure 7.42.

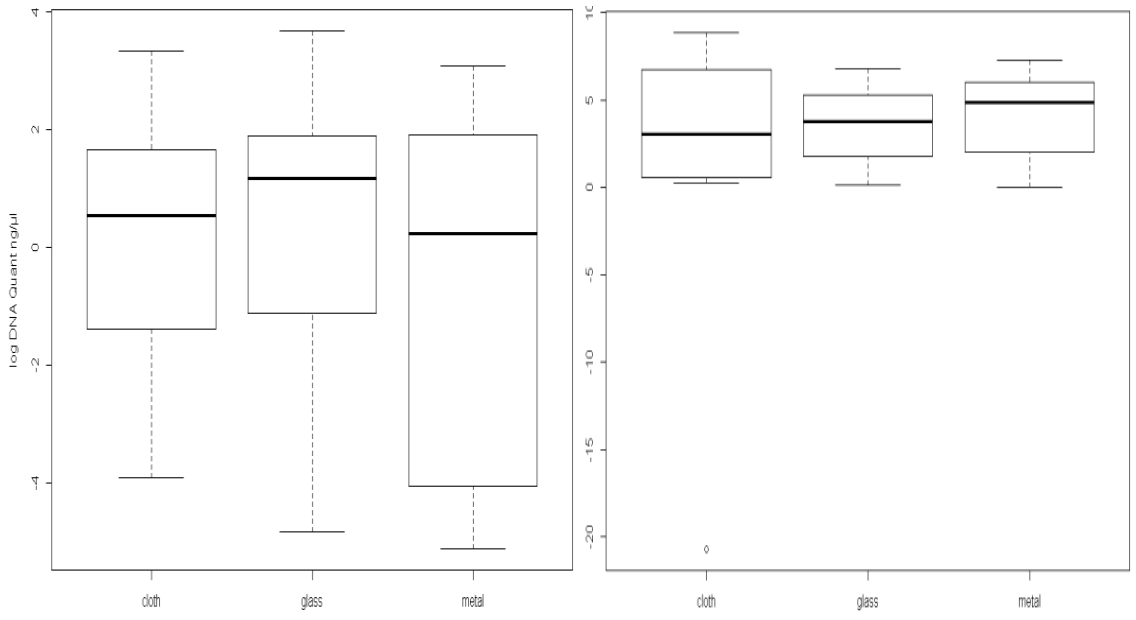


Figure 7.42 Box-plot representation of the variation in the log DNA quantities when semen samples were placed on different materials (left) compared to the variation in the log DI values for the same materials (right). n=3, *p*>0.05.

With lower starting DNA materials, the saliva samples were expected to produce the weakest STR profiles. Therefore the duration of sampling was much shorter than that for blood and semen and only 3 periods of ADD were analyzed (up to 338.5 ADD). Figures 7.43 to 7.45.

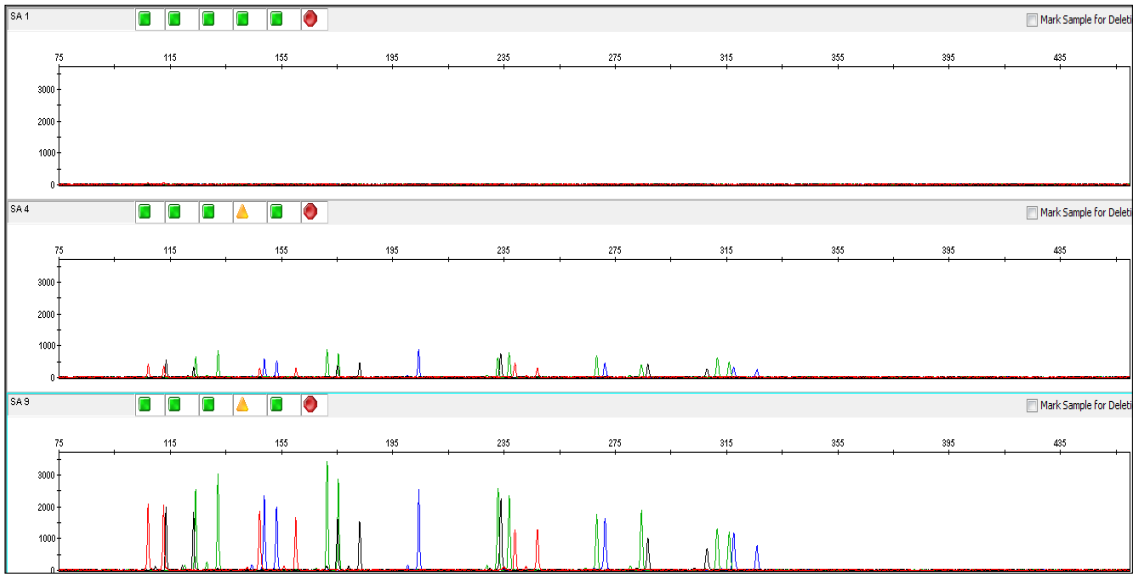


Figure 7.43 Three electropherograms of saliva samples at 0 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3

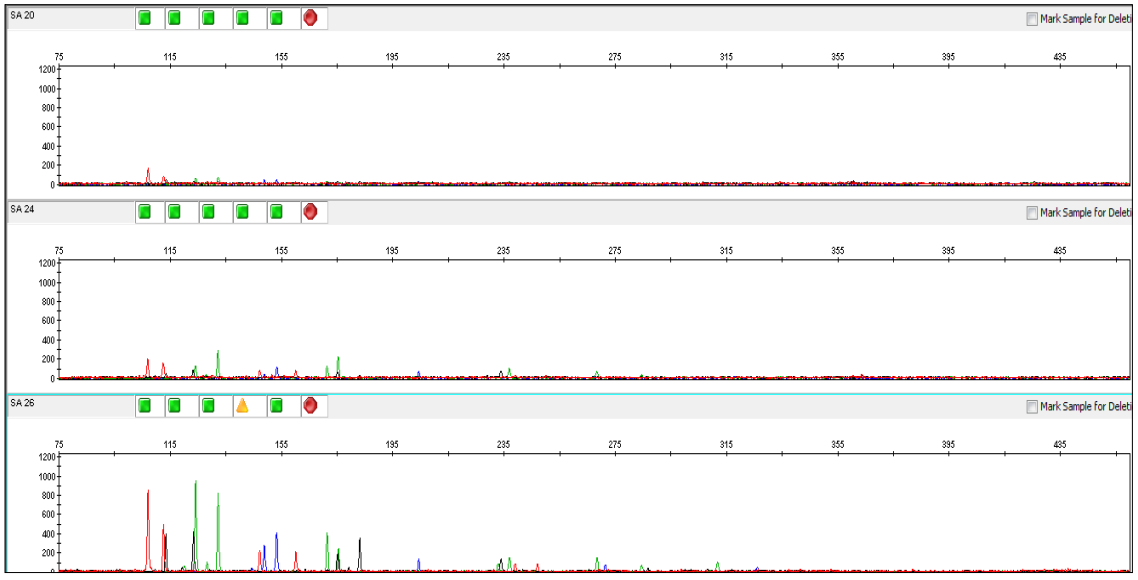


Figure 7.44 Three electropherograms of saliva samples at 164 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

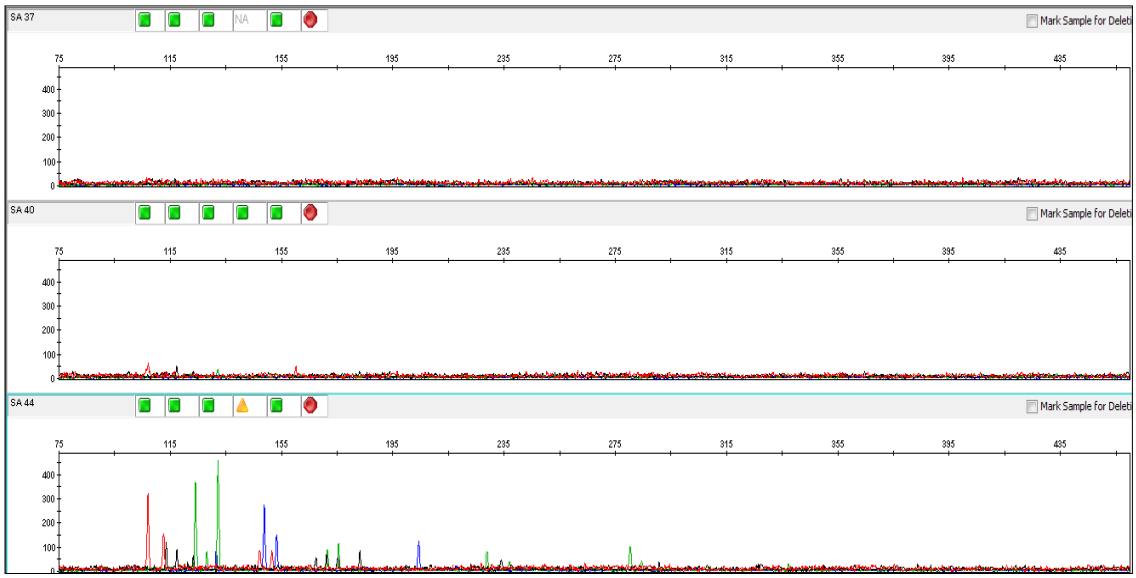


Figure 7.45 Three electropherograms of saliva samples at 338.5 ADD on cloth (top), metal (middle) and glass (bottom) materials. n=3.

Saliva samples on cloth material failed to produce any STR results at ADD 0, yet the DI was only 1.2. On the other hand, a full STR profile with high RFU peaks was generated from saliva samples on glass with DI of 1.18 whereas when placed on metal, the saliva samples still managed to produce full profile but with lower peak heights and DI of 1.06. The trend appeared to continue at ADD 164. A weak partial profile was obtained for saliva on cloth material but with a much larger DI of 101.2 than that on ADD 0. Saliva on metal produced a weak partial profile with DI of 18 and saliva on glass produced a strong partial profile with only 3 alleles missing from the profile and DI of 7.13. At the final ADD period for saliva samples (338.5 ADD) no profile was obtained for cloth samples with a large DI of 113.6 calculated by the Quantifiler® Trio kit and a quantification value of 0.01 ng/µl. Similarly, no profile was obtained for saliva samples when placed on metal at ADD 338.5, which produced a value of 8.43 for the DI and a quantification value of 0.001 ng/µl. However, saliva samples on glass produced a weak partial STR profile which contained some contamination of an unknown source. The degradation index was given at 35 and the quantification value was estimated at 0.02 ng/µl.

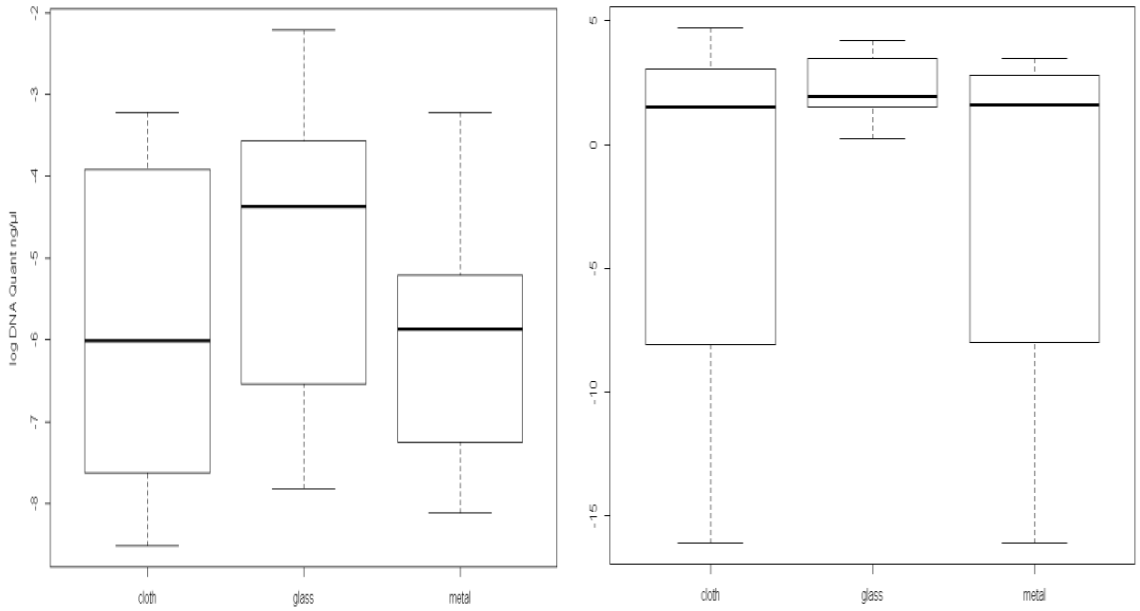


Figure 7.46 Box-plot representation of the variation in the log DNA quantities when saliva samples were placed on different materials (left) compared to the variation in the log DI values for the same materials (right). n=3, p>0.05.

Although there was a wide data distribution, Figure 7.46 shows that the mean values for both the log value of the DNA quantities and the log value for the degradation index were comparable. ANOVA calculation showed that there was no significant difference between the DNA quantities extracted from saliva samples when placed on three different material types (p=0.441). Similarly, the degradation index was also not significantly different between the three material types (p=0.327).

Table 7.3 Table showing the effects of environmental insults on different body-fluids on different materials. The number of alleles generated from an STR profile, the Degradation Index (DI) and the Combined Peak Heights (CPH) are displayed along the Added Degree Days (ADD). A full profile contains 29 alleles.

Body fluid	No. alleles			DI			C.P.H			ADD
	cloth	metal	glass	cloth	metal	glass	cloth	metal	glass	
blood	24	29	29	0.12	0.89	1.26	37,258	167,475	148,191	0
	0	29	29	0.04	0.79	1.17	0	170,862	94,880	338.5
	29	29	29	0.62	3.4	3.54	85,859	28,295	31,546	724.5
	29	1	29	2.57	2.5	3.82	25,348	105	14,134	1250.25
	29	4	10	5.12	5.62	11.7	16,983	494	1,989	1926.25
	29	0	11	5.5	13.8	13.3	14,250	0	1,880	2201.5
semen	29	29	29	1.46	1.01	1.14	61,652	46,514	61,943	0
	29	29	29	2.18	4.19	8.03	34,846	54,134	32,350	338.5
	17	7	26	40.5	236.1	21	10,468	1,636	18,369	724.5
	11	7	16	1527	747.1	102.9	5,711	1,995	7,368	1250.25
	5	0	2	N/A	167	701.3	1,326	0	310	1926.25
	0	1	1	N/A	357.4	61.1	0	104	118	2201.5
saliva	0	29	29	1.2	1.06	1.18	0	14,943	52,835	0
	1	8	20	101.2	18	7.13	167	1,365	6,953	338.5
	0	0	10	113.6	8.43	35	0	0	2,180	724.5

7.3.4 Comparison of 3 quantification kits

DNA quantification for all environmentally insulted samples from the full experiment (Section 7.3.3) was carried out two additional times with two different quantification methods to investigate the accuracy and reproducibility of the three different methods. Results from the Quantifiler® Human (Applied Biosystems) and the Quantus™ Fluorometer (Promega, USA) were compared to the results previously obtained by the Quantifiler® Trio (Section 7.3.3.2).

Based on the same principles of real time amplification, the Quantifiler® Human kit has been in use for years in the forensic community, while the Quantus™ Fluorometer detects the intensity of fluorescent dyes bound to double stranded DNA. The results from the comparison of the three quantification methods are shown in Figure 7.47.

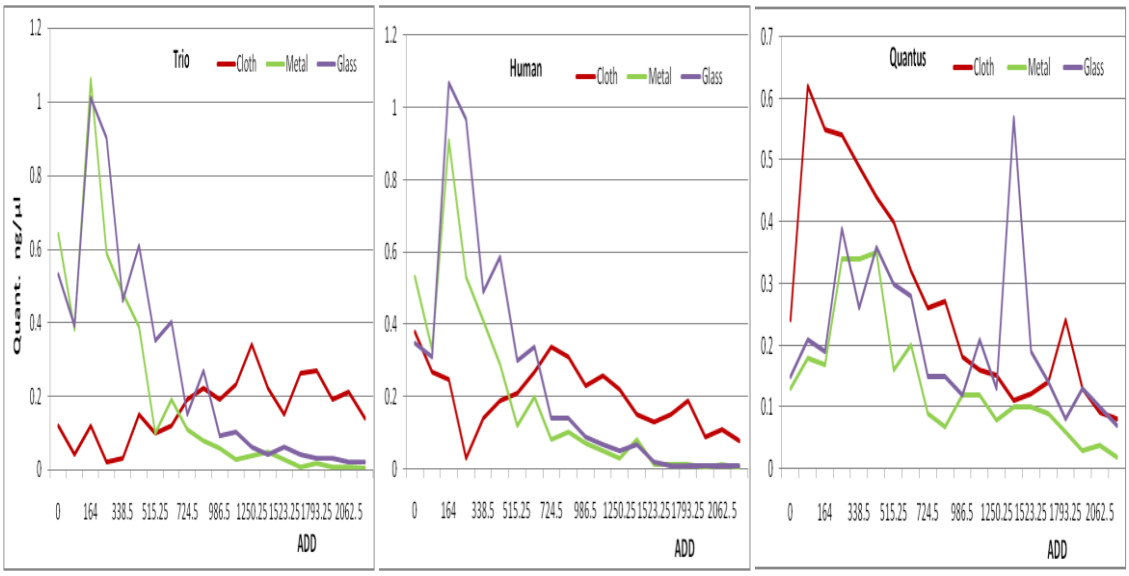


Figure 7.47 Figure showing 3 line-graphs for the mean DNA quantification results extracted from blood samples on different materials, using 3 different quantification methods. Quantifiler® Trio (left), Quantifiler® Human (centre) and Quantus™ (right). n=3.

The mean quantification results from both the Quantifiler® Trio and the Quantifiler® Human were very comparable for blood samples on all material types except for when on cloth. Where the mean quantification value continued to increase with the Trio until around ADD 1250, the Quantifiler® Human showed an initial dip in DNA quantities at around ADD 250 followed by a period of DNA quantity increase until ADD 725 where it subsequently decreased.

Different results for blood samples on cloth were obtained with the Quantus™ method. Unlike both Quantifiler® kits, the mean quantification value obtained by the Quantus™ was initially highest for cloth samples which then continued to decrease at almost a steady rate for the duration of the experiment. Blood samples on metal and glass and to some extent cloth (most apparent with Quantifiler® Human) all had a characteristic dip in DNA quantities in the initial 100 ADD before reaching the peak followed by a gradual decrease until the end of the experiment.

Although the data show no statistically significant difference between the quantification values of the different methods for the different materials when blood was quantified, it is worth noting that ANOVA showed that the difference between the

methods was most significant when cloth material was compared alone (p>0.05), Figure 7.48.

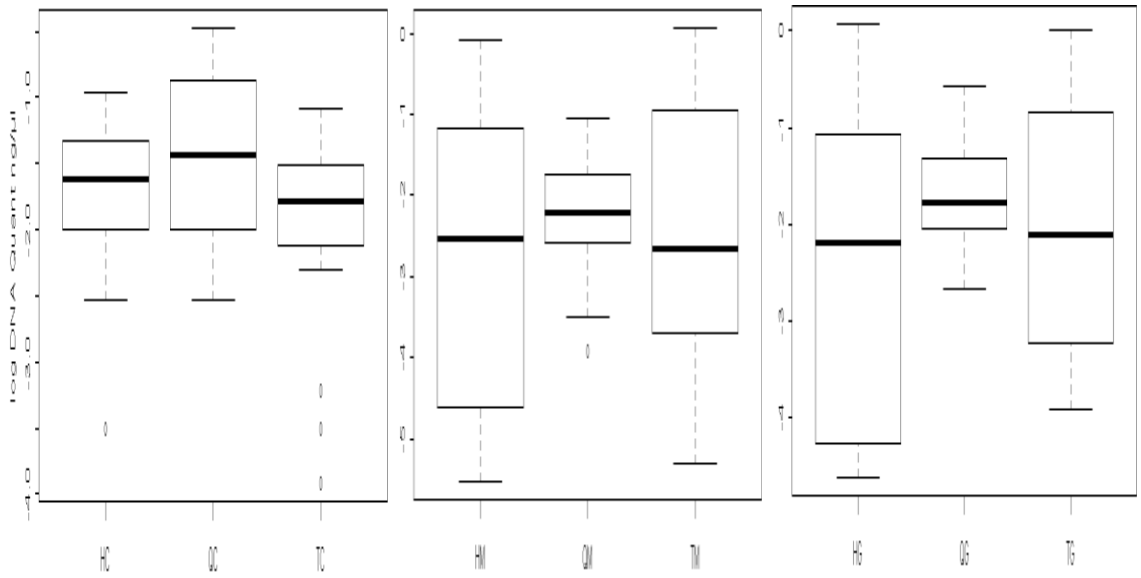


Figure 7.48 Box-plot of the log of DNA quantities extracted from blood samples as given by the different quantification kits. H=Quantifiler® Human, Q=Quantus™ and T= Quantifiler® Trio. Different materials were plotted separately with C=cloth (right), M=metal (centre) and G=glass (left). n=3, p> 0.05.

The mean DNA quantification results for semen samples on different materials, as given by the Quantifiler® Trio, show that the majority of the DNA degradation occurs within the first 350 ADD, then the quantity of DNA continued to decrease in a slower steady rate until the end of the experiment. On the other hand, both the Quantifiler® Human and the Quantus™ Fluorometer quantification results reached a much lower scale than that given by the Quantifiler® Trio. Reminiscent of the quantification results for the blood samples, both the Quantifiler® Human and the Quantus™ fluorometer displayed the same characteristic dip in the value of the quantified DNA amounts before rising again followed by a final steady decrease. However, the initial dip in DNA quantity came later at around ADD 300 for all material types, although it was more pronounced on metal (Figure 7.49).

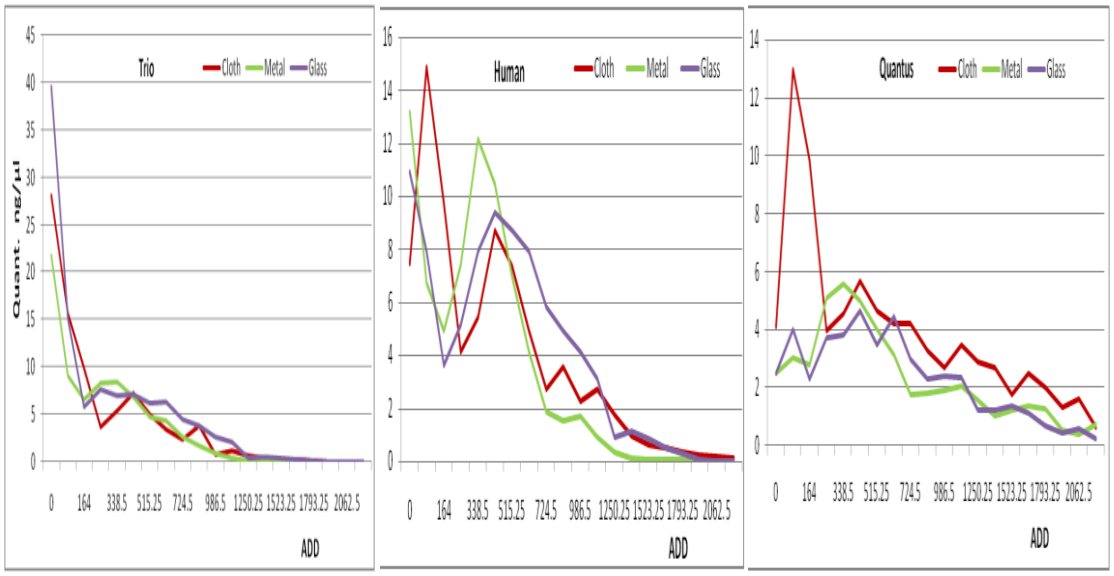


Figure 7.49 Figure showing 3 line-graphs for the mean DNA quantification results extracted from semen samples on different materials, using 3 different quantification methods. Quantifiler® Trio (left), Quantifiler® Human (centre) and Quantus™ (right). n=3.

According to ANOVA calculation, there was no significant difference between the three quantification methods when semen samples were used. However, the data distribution of the log quantities indicated that the Quantus™ Fluorometer was not as sensitive in measuring DNA amounts as both the Quantifiler® kits when the DNA quantity was too large or too small (Figure 7.50).

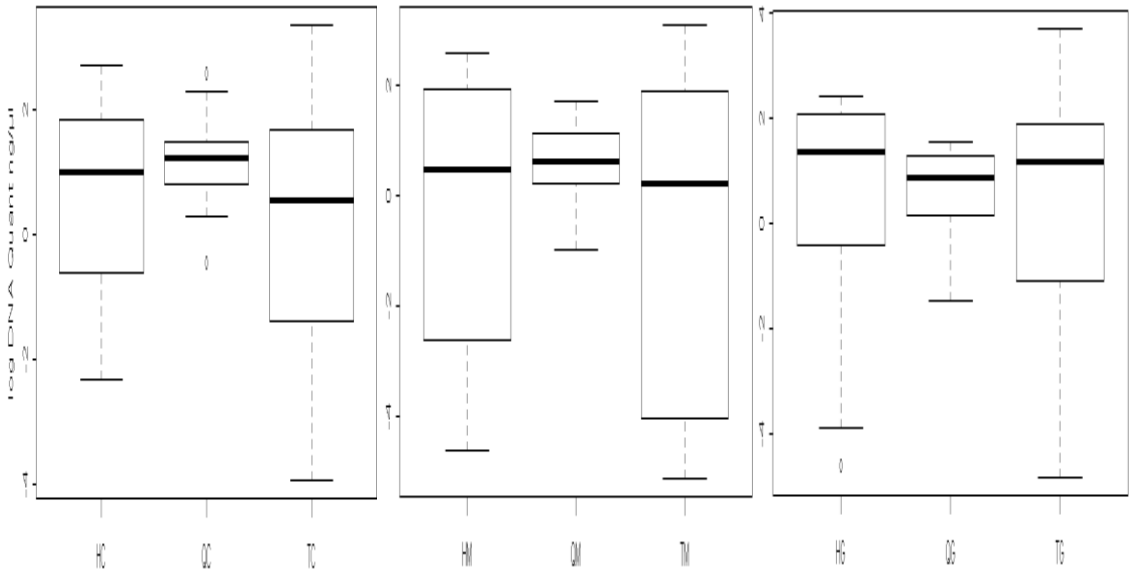


Figure 7.50 Box-plot of the log of DNA quantities extracted from semen samples as given by the different quantification kits. H=Quantifiler® Human, Q=Quantus™ and T= Quantifiler® Trio. Different materials were plotted separately with C=cloth (right), M=metal (centre) and G=glass (left). n=3, $p > 0.05$.

Both quantification results obtained from Quantifiler® Trio and Human were comparable to each other. Initially saliva on glass gave a much higher mean DNA quantity than on cloth or metal but came down to comparable values around ADD 85. However, the mean DNA quantity was much lower in saliva samples than the other body-fluids tested and therefore slight variations were more pronounced.

Interestingly, values obtained from the Quantus™ were unlike those obtained from the Quantifiler® kits; both saliva samples on metal and glass produced mean DNA values that dipped in quantity in the initial 164 ADD and continued to rise from then on with a relatively large fluctuation in the mean DNA quantity. The mean DNA quantity extracted from saliva samples on cloth did not exhibit such fluctuation and continued to decrease in value until the end of the experiment (Figure 7.51).

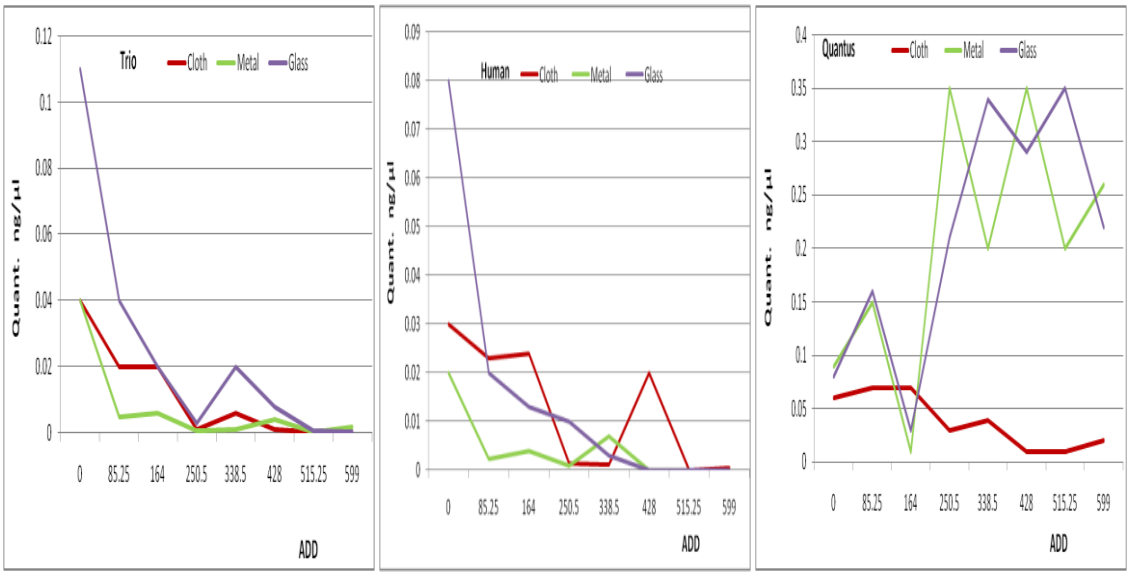


Figure 7.51 Figure showingthree line-graphs showing the mean DNA quantification results extracted from saliva samples on different materials, using 3 different quantification methods. Quantifiler® Trio (left), Quantifiler® Human (centre) and Quantus™ (right). n=3, $p < 0.05$.

More variation was seen between the different quantification methods with saliva samples than all other body-fluid types Figure 7.52). Statistical analysis using ANOVA showed that large variation was present between cloth materials but a p value of 0.095 indicated that the difference was not significant. With metal however, ANOVA calculation indicated a strong correlation between the quantity of the DNA present and the type of quantification method that was used (P= 0.001). Again, although there was a significant difference between the measurements of DNA amounts when quantified with the different quantification kits, a p-value of 0.00578 calculated by ANOVA suggests that the difference is “fairly” insignificant. Again, the data distribution points for the Quantus™ Fluorometer was the least spread, indicating consistency. While the Quantifiler® human showed the widest data distribution towards the lower end of the results.

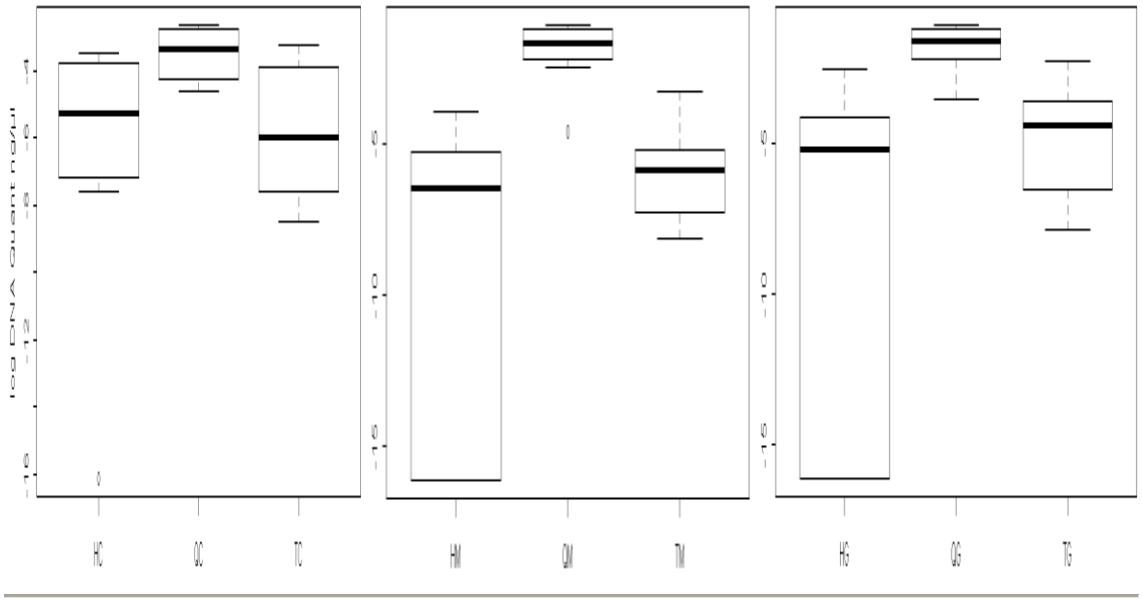


Figure 7.52 Box-plot of the log of DNA quantities extracted from saliva samples as given by the different quantification kits. H=Quantifiler® Human, Q=Quantus™ and T= Quantifiler® Trio. Different materials were plotted separately with C=cloth (right), M=metal (centre) and G=glass (left).

The sum of means of the Standard Deviation (SD) between the triplicate readings of each sample was taken as an indication of the consistency of each method. However, other factors such as the efficiency of the extraction between samples will also have influenced the SD values (Table 7.4). n=3, $p < 0.05$.

Table 7.4 Table showing the SD between the triplicate readings for each sample compared between the different quantification methods. The red figures indicate the largest mean SD between the methods for each material type.

Material	Blood			Semen			Saliva		
	Human	Trio	Quantus	Human	Trio	Quantus	Human	Trio	Quantus
Cloth	0.09	0.07	0.1	1.03	1.6	0.67	0.01	0.01	0.02
Metal	0.07	0.08	0.07	1	1.42	0.63	0.01	0.01	0.08
Glass	0.1	0.1	0.13	1.5	3.18	0.82	0.18	0.01	0.1

Noticeably, the Quantifiler® Human quantification method showed the least SD in comparison to the other methods tested, the only exception was with saliva samples on glass. In contrast, the Quantifiler® Trio kit was consistently the most inconsistent of all the methods when large quantities of DNA were present such as with semen

samples. However, the method was the most consistent when DNA quantities were lowest such as with saliva samples. The Quantus™ Fluorometer showed good consistency with semen samples, though the method was not able to measure accurately when large amounts of DNA were present.

Finally, out of the three quantification methods tested, both Quantifiler® kits contained an internal control to detect and estimate the presence of inhibitors in the DNA extract, whereas the Quantus™ Fluorometer does not contain such controls. However, the Quantifiler® Trio did not show any sign of inhibitors in any of the samples quantified using this system for the whole experiment. This was indicated by the Internal Positive Control Ct (IPC C*t*) flag in the Quantifiler® kit is not being triggered. On the other hand, for the same samples, the Quantifiler® Human did show increased IPC *Ct* values especially for blood samples on cloth and glass (Table 7.5).

Table 7.5 A table comparing the mean IPC Ct values calculated from the total number of samples for both the Quantifiler® kits for all body-fluid types on different materials.

	IPC Ct Value								
Body-fluid	**Blood**			**Semen**			**Saliva**		
Material	Cloth	Metal	Glass	Cloth	Metal	Glass	Cloth	Metal	Glass
Human	29.29	27.42	28.455	27.84	27.485	27.76	27.3125	26.825	26.95
Trio	25.7	26.1	26.6	26.5	26.1	26.3	26.8	26.3	26.5

7.4 Discussion

Limited number of studies have addressed the effects of environmental insults on DNA from body-fluid stains. This was probably due to the fact that there is a large variation in climates between different geographical locations which renders the standardization of such effects practically impossible (Larkin et al., 2010).

Generally, the focus has been directed towards studies of DNA degradation from decayed tissue and organs either for the purpose of postmortem interval investigation (William et al., 2015) or ancient DNA analysis (Hofreiter et al., 2001). With the intention to conduct a full experiment on the effects of environmental insults on DNA

degradation, two pilot studies were carried out to give an indication of the behavior of DNA in such climates as those seen in the region.

Both pilot studies carried out with singlet samples, but while (Pilot 1) contained only blood samples, while (Pilot 2) was conducted on blood in addition to semen and saliva samples. The results from the pilot studies suggested the need for a longer experimental duration with triplicate readings to conduct reliable statistical analysis. It also demonstrated the requirement for more frequent sampling in the initial stages of the experiment where DNA degradation rates are highest.

For all samples the donor was kept constant to eliminate variation in DNA quantities from different individuals, especially since variation in DNA degradation rate is thought to be affected more by light, temperature and UV light than between individual DNA samples (Bender et al., 2004). The results have shown that for all body-fluid types, the majority of DNA degradation occurs in the initial period of exposure to environmental insults which were measured in ADD. In this series of 5 experiments ADD was chosen since it accounts for measurements of both temperature and time combined. ADD is the cumulative total of the average daily temperatures and it is used in forensic entomology and anthropology to estimate the postmortem interval (Larkin et al., 2010).

Factors effecting the degradation of DNA molecules were reviewed by Alaeddini et al. in 2010. These factors are divided into two types and they included enzymatic and non-enzymatic degradation, both of which are influenced to different degrees by environmental insults (Alaeddini et al., 2010). Three sub-groups of endonuclease are known to affect the degradation of DNA molecules in cell death (Walker et al., 1995) and two of which are cation dependent (Mg^{+2} and Ca^{+2}). The effect of these endonucleases is thought to be limited in the present experiment to semen and saliva samples due to the presence of EDTA in the blood collection tube, which acts as chelating agent sequestering metal ions. lysosomal proteases are cation independent and are mainly involved in the removal of histone proteins facilitating the DNA molecule for further cleavage by other endonucleases, but are not involved directly in DNA degradation (Alaeddini et al., 2010). It is thought that desiccation and high temperatures will inactivate these enzymes in the early stages of the experiment (up

to 500 ADD) giving way to non-enzymatic processes such as oxidation, hydrolytic reactions and solar radiation (Hofreiter et al., 2001) which is responsible for the observed increase and then the slow and relentless DNA degradation that follows.

In this study, both blood and semen samples showed increased rates of DNA degradation within the initial 500 ADD. However, for saliva samples these effects were seen earlier between ADD 250 – 300, indicating that the cell type of the body-fluid might have a role in the rate of DNA degradation.

Semen samples exhibited the highest level of DNA degradation upon exposure to environmental insults as illustrated by the extreme value of DI estimation from the Quantifiler® kit and also by the STR genetic profiling results. This result was interesting since the majority of studies conducted on the longevity of DNA in semen samples reported excellent resilience for tens of years. However, all these studies were conducted from samples stored in room temperatures (Hara et al., 2007; Nakanishi et al., 2014; Hara et al., 2015). Conversely, environmental insults such as high temperatures, UV light and humidity were expected to increase the vulnerability of the DNA molecules and increase the degradation rate of the semen samples (Bender et al., 2004). Nevertheless, the increased rate of DNA degradation in semen samples suggests that factors other than those mentioned might play a more important role in the drastic degradation of DNA seen especially with semen samples in the initial period of the experiment. Endogenous nucleases feature amongst the first agents to initiate the process of DNA fragmentation in the post-mortem period (Hofreiter et al., 2001), while digestion of chromatin proteins by lysosomal proteases would in turn facilitate the process of random digestion of DNA by endonucleases. The organization of sperm chromatin has long been known to be unique among all the cell types and it was suggested that spermatozoa have a more active chromatin structure (Ward and Ward, 2004). In addition to the active chromatin structure of the spermatozoa, it was suggested that sperm cells contain a mechanism by which they can digest their own DNA when exposed to stressful environments such as high temperatures. This nuclease activity is part of a mechanism that the spermatozoon uses when it encounters a stressful environment to prevent fertilisation and to avoid the transmission of potentially damaged DNA to the embryo (Ward and Ward, 2004). This

may explain the unexpected fast rate of DNA degradation in semen samples in the initial period of the experiment.

In addition to endogenous nucleases activity, the rupture and release of the nutrient-rich contents of the sperm cell brought about by the cell shrinking, will encourage the growth of environmental microorganisms, therefore resulting in further aggressive degradation of the DNA molecule (Antheunisse, 1972). Storage of semen samples in freezing conditions before conduction of the experiment was also suggested to have major effects on sperm viability and DNA fragmentation (Lopez-Fernandez et al., 2008).

The ability to extract and amplify intact DNA from bloodstains stored for long periods of time at ambient condition is well studied and documented (Kobilinsky et al., 1992, Kline et al., 2002, Dissing et al., 2010). The most conclusive study on the effect of environmental factors on bloodstains was carried out in a controlled environment in 1989 by McNally and co-workers. Their study investigated the effects of ultraviolet light, heat, humidity, and soil contamination. Surprisingly, all the above studies concluded that environmental factors do not affect the quantity and quality of DNA until extreme condition of 100% humidity (Dissing et al., 2010) or temperatures of more than 150 °C (Barbaro et al., 2008). The results from the experiment in the present study were contradictory to those earlier findings and the assumption is they are more in line with the frequent observation in forensic laboratories: that poorly stored body-fluid stain samples or samples exposed to environmental insults will contain degraded DNA molecules (Fondevila et al., 2008).

Although there was a lack of statistical relevance between material types and DNA quantity obtained from the different body-fluid types, the current results demonstrated a relationship between the rate of DNA degradation as measured by DI and the material type on which the body-fluids were placed, particularly with blood samples. Blood samples on cloth showed a significantly lower rate (p <0.05) of DNA degradation than blood on metal or glass. Again, the literature has given insufficient attention to the effects of different materials on DNA degradation, however, in two previous experiments the surface adhesive-force of red blood cells (RBC) was studied and was found to be time and environment-dependent (Strasser et al., 2007; Wu et al.,

2009), These forces govern the shape and therefore the rupture and release of haemoglobin (Hb) to be readily available to environmental insults. Hb molecules in the dried state undergo oxidative changes and release reactive Fe(II) cations, which in turn undergo a series of reactions which generates the highly reactive hydroxyl radical (OH•) (Gutteridge, 1981; Puppo and Halliwell, 1988). The presence of Hb in the vicinity of DNA in dried bloodstains creates the opportunity for OH•-induced oxidative damage to the deoxyribose sugar and the DNA nucleobases (Marrone and Ballantyne, 2009). The study conducted by Wu and co-workers also showed that the adhesive properties of the RBC membrane is directly influenced by the material type in which blood is deposited (Wu et al., 2009). However only two material types were tested in that experiment (glass and mica) and more investigation involving a wider variety of material types would need to be conducted for more comprehensive comparisons. This study hypothesises that the fiber network of the cotton material may have helped to contain the shape of red blood cells which ultimately may have influenced the decrease in degradation rate seen with blood samples on cloth.

This absorbing property of cotton may also be responsible for the high level of inhibition seen with blood samples on cloth material in the first stages of the experiment. The most likely source of this inhibition is porphyrin (heme) compounds (Higuchi, 1988). It was suggested by Akane and co-workers (1994) that inhibitory contaminants are little co-purified with DNA from freshly prepared blood stains and they maintained that such contamination may be derived from degenerated haemoglobin (Hb). They later indicated that the contaminant was likely to be the product of protenase K digestion of some haem-blood protein complex (Akane et al., 1994). The following schematic chemical equation (Marrone and Ballantyne, 2009), can be used here to explain the continuous increase in the rate of inhibition in the initial period of the experiment as demonstrated by first; the drop in the allelic number and then the complete nullification of the STR profile of blood samples on cloth:

$$Hb + O_2 \rightarrow \textit{oxy}\ HbO_2 \rightarrow \textit{met}\ Hb - O_2 \rightarrow \text{hemichrome}$$

Figure 7.51: Equation showing raw Hb can easily be oxidised upon exposure to environmental insults to *met*-Hb which can no longer bind oxygen, over time *met*-Hb is converted to hemichrome which is a denatured form of the protein.

Two possible reasons for the cessation of inhibition from blood samples after 500 ADD. Firstly, the observation that after long periods of exposure to sunlight and high temperature, bloodstains become insoluble in water (Dorrill and Whitehead in 1979) and therefore, only the available cell contents contained within the cotton fabrics are released in the extraction step leaving the majority of the haem compounds attached to the cloth material. The second reason for the lack of PCR inhibition is the fact that the heme-blood protein complex responsible for the inhibition is completely degraded either by the complete digestion of the polypeptides in the protein complex or the complete denaturation of the haem complex into hemichrome (Bremmer et al., 2011). However, after 500 ADD and consequently the effects of inhibition is not seen from that point onwards. Furthermore, the effects of DNA degradation were evident in the downstream genetic analysis, generated from samples that contained optimal DNA quantities (more than 1 ng), but still failed to produce full STR profiles. Interestingly, the DI was almost always lower in cloth material compared to metal and glass, except with saliva samples where it showed the highest DI values between the different materials. Semen samples on cloth and particularly metal showed a higher DI than on glass. This is most likely because of the increased temperature of the metal surface that in turn increases the rate of DNA degradation by non-enzymatic damage.

Saliva samples were collected as “whole saliva” for the purpose of this experiment. Unlike blood and semen, saliva is known to be a host for several bacteria and bacterial products, viruses, fungi and food debris (Kaufman and Lamster, 2002). In addition, constituents of whole saliva include several non-salivary gland components such as nasal secretions, serum and blood derivatives from oral wounds (Pandeshwar and Das, 2014). It was hypothesised in this study that the fabric network of the cotton fibers may have formed a suitable niche for microbial growth in the saliva samples, hence DNA degradation in saliva was most observed in cloth material.

When different quantification methods were used to estimate the quantity and quality of the DNA produced from the current experiment, statistical analysis showed that there was no significant difference between the methods, except when the estimated DNA quantities were small as seen with the saliva samples, or when the sample was inhibited. This is especially particular in the case with blood samples on cloth. Inhibition was detected only by the Quantifiler® Human which is the reason for the significant difference between the methods when blood samples were deposited on cloth. In recent years the STR multiplex amplification kits have been improved in terms of sensitivity and robustness to PCR inhibitors (Tvedebrink et al., 2012). However, older quantification methods such as the Quantifiler® Human no longer match the sensitivity, precision, and accuracy of the STR amplification methods they now precede (Nicklas et al., 2012). On the other hand, the advent of the new Quantifiler® Trio was optimised for the use with the latest generation of the multiplex systems (Vernarecci et al., 2015), and explain the discrepancies between the Quantifiler® Human and the Quantifiler® Trio when dealing with inhibited samples when used with the Identifiler® Kit. Conversely, the Quantus™ system does not contain a mechanism for the detection of inhibition and therefore, the estimation of the DNA quantity was much higher with this system. Even though the mean SD of the three quantification kits showed the Quantus™ to be more consistent in its reading, the data distribution of the system indicates that this consistency is due to a narrow window of reading. Values for both very large and very low DNA quantities were not accurately read by the Quantus™ giving rise to a constant range of reading. All methods were able to detect a dip in DNA quantities in the initial 100 ADD which cannot be thoroughly investigated in the scope of this experiment. However, the physical pressures of environmental insults may have a role to play in the manifestation of this observation. Recent studies using atomic force microscopy (Strasser et al., 2007; Wu et al., 2009) has observed morphological, stiffness and adhesive force changes of sample surfaces upon environmental pressures. This correlates to our findings that initially extensive lysis of the cell-contained body-fluid stains had to be carried out to extract membrane enclosed DNA molecules, but soon after environmental insults began to take effect, the cell contents are release and are more readily available for DNA extraction. To conclude, the results of this chapter have clearly demonstrated that, different environmental insults such as

temperature and humidity will lead to rapid DNA degradation. However, exposure of biological samples to surfaces such as cloth, metal and glass does not have significant effects on the rate of this degradation. The results also showed that DNA present on different body-fluid samples tended to degrade faster than others as demonstrated by the DI results for blood samples.

CHAPTER 8

GENERAL DISCUSSION & CONCLUSION

8.1 General Discussion:

Forensic science has embraced the use of DNA molecular biology tools for diagnostic purposes more than any other scientific field (Budowle and Daal, 2009). The Forensic DNA Unit at RAK Police Forensic laboratory is one of the most recent Forensic DNA units to be established in the region. The analysis of case work data presented in Chapter 3 of this study shown that body-fluid samples processed generally produced high success rates with STR profiling. However, compared to the few limited data published in the literature regarding STR success rates (APCO, Good practice manual, 2005 2nd Ed; Wilson-Wilde et al., 2013), both the performance of semen and blood samples was under par. This presented an opportunity to investigate how to improve the success rate of samples, particularly with regard to evidence samples containing semen. The success rate for semen samples calculated from three years of casework samples was 71%. Given the large DNA content of semen samples due to the usually large number of sperm cells present (Duncan and Thompson, 2007) and the protective nature of the spermatozoa cell wall, a higher success rate had been anticipated.

One of the key factors affecting DNA success rates is the DNA sample condition (Bond and Hammond, 2008). Moreover, high daily temperatures and humidity levels are usually experienced in the region and the effects of environmental insults on DNA degradation and inhibition are documented and reviewed in the literature (Cadet et al., 2015; Baptista et al., 2015; Kim et al, 2015). In addition, the efficiency and effectiveness of laboratory procedures such as DNA extraction and quantification will play an important role in the overall success of the genetic profiling outcome. Furthermore, there are a number of laboratory procedures and chemistries that may introduce variability in the DNA signal (Rowan et al., 2015).

A large number of DNA extraction methodologies are available for forensic use. These include chelating agents and organic compounds. Commercial extraction kits based on silica and magnetic particles such as the QIAamp® and the InnuPREP® kits are also available. Many of which have been incorporated into robotic bench-top systems such as the EZ1®, the Maxwell®, the AutoMate Express™ and the InnuPure® systems. Initially, this study compared and evaluated the above-mentioned techniques before conducting further research on sample degradations due to environmental insults.

When subjective comparisons based on the current study (see Chapter 4) were conducted, the Automate Express™ system gave the highest total score considering the aspects mentioned in Table 4.4 (see Chapter 4). The greatest disadvantage of this system was the cost attached to processing each sample. In terms of kit alone, each sample extracted using this method will cost around $10 US dollars (not accounting for the price of the system, operation and maintenance). In return, extracted samples are expected to give excellent DNA quality and yield with minimum effort and time consumption. The Chelex-100 method is a much cheaper alternative that shows a similar DNA yield; however, the quality (especially with blood samples) and time are compromised. In a recent study conducted by Ip et al. (2015) in which they compared five different extraction techniques including the Chelex-100 method, their results showed that the Chelex-100 method yielded the least DNA of all the techniques and they concluded that the method must be confined to processing reference samples. As discussed earlier in (Section 4.4 in Chapter 4), along with other research found in the literature, this current study have found that -on the contrary- Chelex-100 yielded large DNA quantities sufficient for casework processing (Jung et al. 1991; Idris and Goodwin, 2015). Different procedural protocols may explain the discrepancies found in the literature. However, the current study employed the used of 10% Chelex rather than 5% Chelex. In addition, incubation time and Proteinase K volumes were also increased from the protocol described by Walsh et al. in 1991, which was followed by Ip et al. (2015). The Chelex-100 procedure is currently the method of choice in the Forensic DNA Unit at RAK Police and therefore the personal experience of the researcher may have a positive influence on the DNA yield when testing this method.

A comparison between a set of common presumptive and confirmatory tests was also conducted to establish the lower limit of sensitivity which detects their respective body-fluids. Generally, presumptive tests were more sensitive to their respective active components, most likely due to their relative abundance. However, the Hexagon® OBTI was shown to be more sensitive than the presumptive Kastle–Meyer test and although the OBTI test is considered to be a confirmatory test (Hermon et al., 2003), the test is known for its cross reactivity with higher mammals such as chimpanzees and gorillas (Hochmeister et al., 1999), and therefore some may consider

it to be a presumptive test. The sensitivity of these screening tests was found to be largely dependent on the nature of the active component of the body-fluid and its availability to act as a substance for either a chemical reaction in the case of presumptive tests or an antigen for confirmatory tests.

When subjected to local environmental insults, presumptive and confirmatory tests, in addition to the DNA quantities, showed great variability between the body-fluid types tested. However, statistical analysis showed that there was no significant difference ($p < 0.05$) in DNA quantities between the different material types, and the same was true for all screening tests. However, differences in DNA quantities from blood samples on different materials can be seen towards the end of the experiment as the ADD increases. These finding are particularly important when dealing with cases of various types of evidence materials, where the order of sample processing can be assigned depending on expected abundance of DNA content and recovery from the different material types present. The results discussed in Chapter 6 of this study indicated that material types (cotton cloth, metal and glass) do not influence the process of DNA profiling. However, factors such as the development of inhibitors (e.g. rust on metal) and prolonged exposure to environmental insults can produce differences between these materials. Analysis of variance for the last five ADD readings for blood showed a significant difference between the different materials (p=1.24e-14). Blood samples seem to retain their DNA content better on cloth material than on metal or glass. Table 8.1 shows that except for blood on cloth, DNA seemed to be more susceptible to environmental insults than the targets of presumptive and confirmatory tests, as indicated by the number of alleles present at the degradation limit of the screening test. This is in contrast to the general notion that proteins, enzymes and antigens present in blood are more susceptible to degradation than DNA molecules (Schiro, 2014). Interestingly, body-fluids on glass material seem to maintain a good level of genetic information even with negative screening tests results.

Table 8.1 A table comparing the effects of environmental insults on the detection limit of presumptive and confirmatory tests and the DNA quantity present along with the number of alleles present at the same ADD. The total Full Profile (FP) alleles are 29.

Screening test	Quant ng/µL			No. alleles			ADD
	C	M	G	C	M	G	
Hemastix®	0.14	0.01	0.02	FP	5	14	2201.5
Kastle–Meyer	0.26	0.01	0.04	FP	5	14	1660.5
Hexagon® OBTI	0.34	0.04	0.06	FP	6	27	1250.25
RSID™-Blood	0.23	0.03	0.1	FP	6	27	1118.3
Phosatesmo KM®	1.22	4.2	2.14	14	10	19	1118.3
RSID™-Semen	0.07	0.01	0.04	0	2	3	1926.25
Phadabas®	0.001	0.001	0.003	0	1	25	250.5
RSID™-Saliva	0.001	0.001	0.003	0	1	25	250.5

C=cloth, M=metal and G=glass

Generally, DNA quantification is carried out on forensic samples in order to adjust the concentration of template DNA used for STR analysis (Nielsen et al., 2008). Recent advances in the field of DNA quantification have produced kits with capabilities to estimate the degree of DNA degradation and inhibition (Opel et al., 2010; Nicklas et al., 2012).In this current study, three quantification techniques were assessed to identify the most suitable method which can infer the effects of environmental insults on DNA degradation and inhibition. The findings reviled that at adequate DNA amounts, tested quantification methods showed no significant difference in their estimates ($p > 0.05$). However, features such as the introduction of the Degradation Index (DI) were found to be useful and reliable. On the other hand, at low DNA concentrations, significant differences ($p < 0.05$) between the quantification methods were more apparent. The Quantus™ was identified as less sensitive and lacks additional informative features such as an Internal Positive Control (IPC) and therefore is a more suitable method for reference samples or high throughput database centres. Although repeated samples (n=3) were conducted to carry out this study, time and resources constrains have limited experimental work to the summer of only one year. Annual fluctuations in summer temperatures may account for significant differences which may lead to different outcomes in DNA degradation patterns.

8.2 Conclusion:

This study was conducted with the main aim to better the understanding of the impact of local environmental factors on forensic genetics processes, including sample screening and DNA profiling. Initial review of data suggested good success rate for body-fluids analysis with opportunities to improve current genetic profiling performance. However, lower genetic profiling success rates from body-fluid samples identified in the current study, in comparison to internationally published data, may be due to factors such as localised regional effects of environmental insults. Hot and humid climates will affect sample differently than cold and humid climates. It is the belief that inter- and intra-laboratory reference standards testing, carried out between laboratories in the region or laboratories of similar climates, will allow for better understanding and diagnosis of local laboratories.

The search for the optimum extraction technique can only be an inter-laboratory investigation. Along with factors investigated in this study, the method of choice will have to perform across a wide range of forensic samples, be easily adapted in the laboratory setting and will have to comply with the skills and capability of the laboratory personnel, a factor that is usually over-looked. It is also important to mention the critical role Crime Scene officers play in the collection of samples containing body-fluids. The most efficient extraction methodology cannot extract sufficient DNA quantities if the samples do not contain enough DNA molecules initially. The results of this study have found that the Chelex-100 was the most suited for our requirements, even though other techniques proved to be more dexterous.

The reliability and robustness of presumptive and confirmatory tests were studied and later compared to the DNA quantity and quality when subjected to environmental insults. The findings showed that caution must be taken when dealing with degraded body-fluid samples. With very few exceptions, body-fluids screening tests have demonstrated robustness towards environmental insults which surpass DNA degradation, so in practice positive results with presumptive and confirmatory tests may well be accompanied by an absence of amplifiable DNA.

Finally, recent advances in DNA quantity estimation techniques have increased the choice available for DNA quantification. This study has demonstrated here that when dealing with forensic samples, the accompanying quantification technique must have the ability to respond to a wide range of DNA quantities especially at very high (>20 ng/µL) and very low (< 0.1 ng/µL) concentrations. Moreover, additional features of the more advanced technology were found to be reliable, but they require equally advanced downstream analysis tools to compliment the results. The findings also suggested that the use of the less specific Fluorometric techniques for the measurement of DNA, are better suited for high through-put reference sample profiling rather than challenging forensic case-work samples.

In conclusion, the results have clearly demonstrated that local environmental insults have a direct effect on forensic samples containing body-fluids. The decline in both the DNA quantity and quality present, coupled to the decrease in the integrity and reliability of presumptive and confirmatory tests was found to be directly associated with temperature and time. The degree of such degradation was found to be associated to the type of body-fluid being tested. However, the type of material these body-fluids were deposited on had no or insignificant impact.

9.1 Scope for Future Studies:

a) With additional time and resources, further work could have been conducted to genetically analysis additional quantification results as estimated by both the Quantifiler® Human and the Quantus™ Fluorometer. Comparison of STR profiles from all three quantification methods tested would shed light on the nature of DNA degradation as a result of environmental insults.

b) The effects of environmental insults on presumptive and confirmatory tests in addition to DNA quantities were only collected and analyzed from readings of one year only and the repeat of this experiment for a minimum of 3 years would give a larger room for comparisons and will give more accurate statistical powers. In addition, the effects of seasonal fluctuation are also of great interest and might play an important role in the role of the rate of DNA degradation.

c) Comparative studies assessing DNA degradation and the use of mini-STRs and single nucleotide polymorphisms (SNPs) could be conducted to indicate a general prediction of genotyping methodology to be used. In addition, a number of new "next generation" STR kits have been developed such as the Global® Filer STR kit (Applied Biosystems) which require smaller amounts of input DNA in the reaction mix. The implementation of such new technology can be studied in the light of this experiment.

d) Investigation of the effects of environmental insults could have been completed with the execution of a parallel experiment carried out in indoor laboratory conditions. The results from such experiment would reveal direct comparisons between samples subjected to environmental insults and samples that are shielded from such factors.

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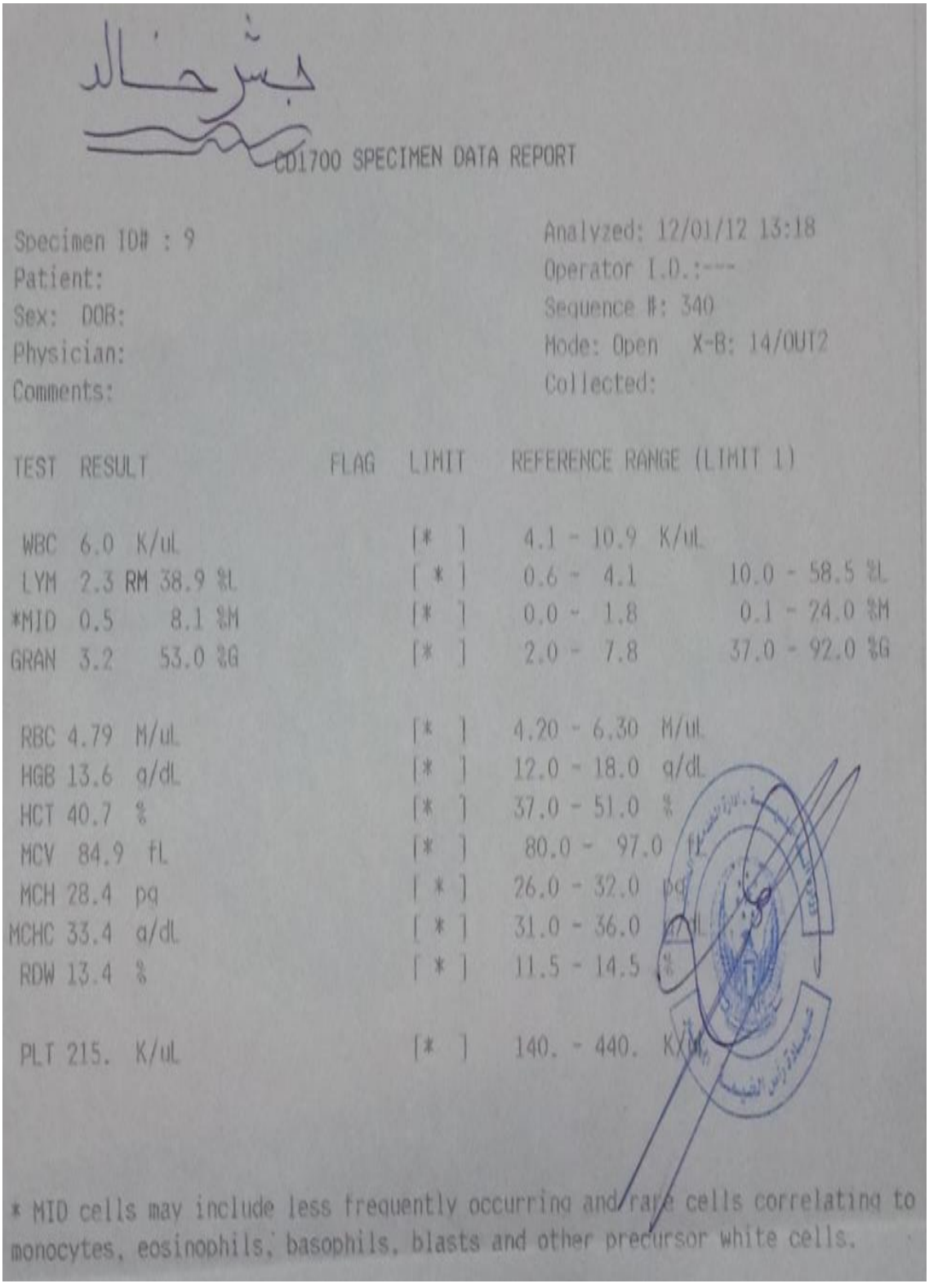
APPENDIX

Appendix 2. Examiner's specimen data report.



بشر خالد

CD1700 SPECIMEN DATA REPORT

Specimen ID# : 9
Patient:
Sex: DOB:
Physician:
Comments:

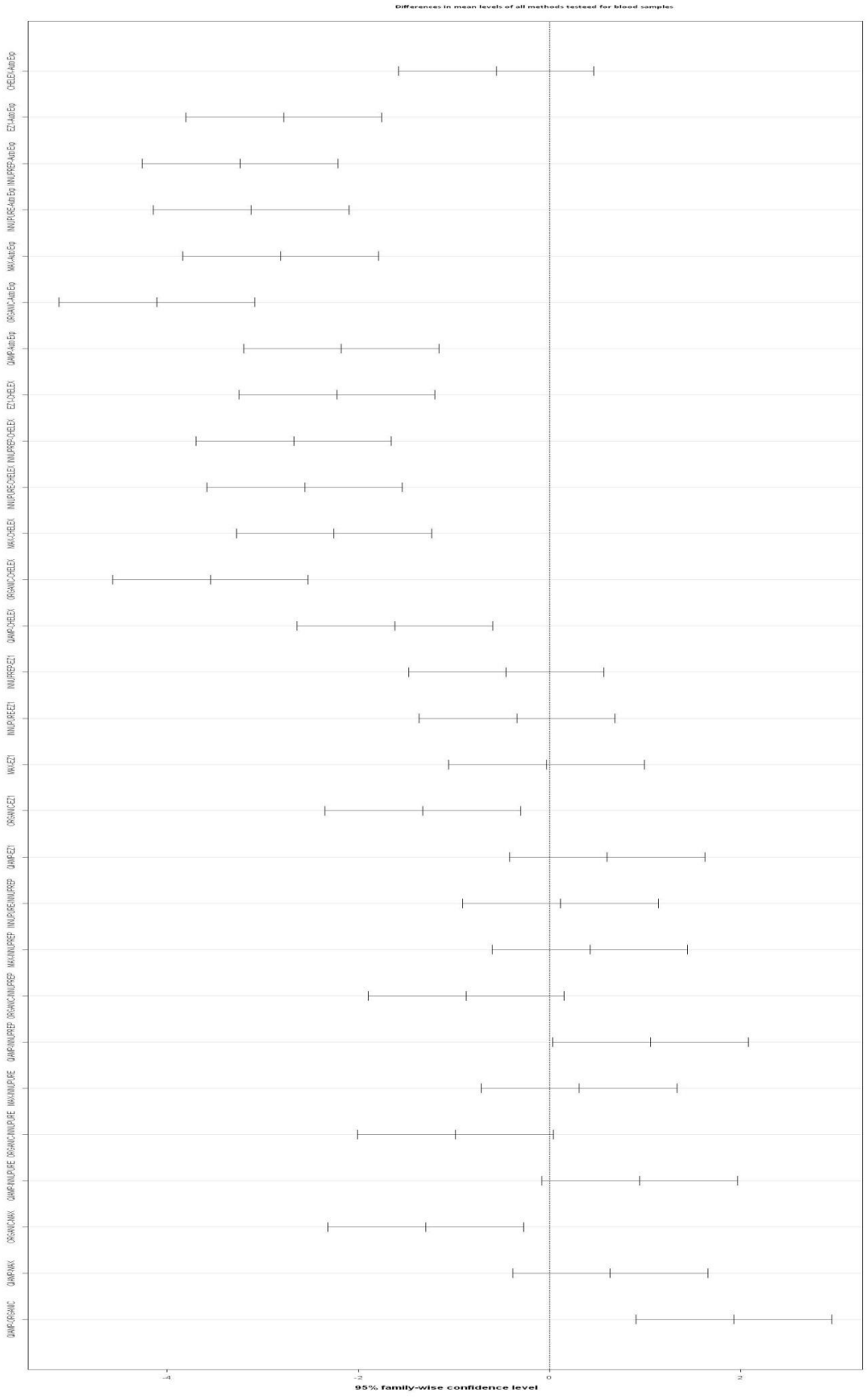
Analyzed: 12/01/12 13:18
Operator I.D.:---
Sequence #: 340
Mode: Open X-B: 14/OUT2
Collected:

TEST	RESULT	FLAG	LIMIT	REFERENCE RANGE (LIMIT 1)	
WBC	6.0 K/uL		[*]	4.1 - 10.9 K/uL	
LYM	2.3 RM 38.9 %L		[*]	0.6 - 4.1	10.0 - 58.5 %L
*MID	0.5 8.1 %M		[*]	0.0 - 1.8	0.1 - 24.0 %M
GRAN	3.2 53.0 %G		[*]	2.0 - 7.8	37.0 - 92.0 %G
RBC	4.79 M/uL		[*]	4.20 - 6.30 M/uL	
HGB	13.6 g/dL		[*]	12.0 - 18.0 g/dL	
HCT	40.7 %		[*]	37.0 - 51.0 %	
MCV	84.9 fL		[*]	80.0 - 97.0 fL	
MCH	28.4 pg		[*]	26.0 - 32.0 pg	
MCHC	33.4 g/dL		[*]	31.0 - 36.0 g/dL	
RDW	13.4 %		[*]	11.5 - 14.5 %	
PLT	215. K/uL		[*]	140. - 440. K/uL	

* MID cells may include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts and other precursor white cells.

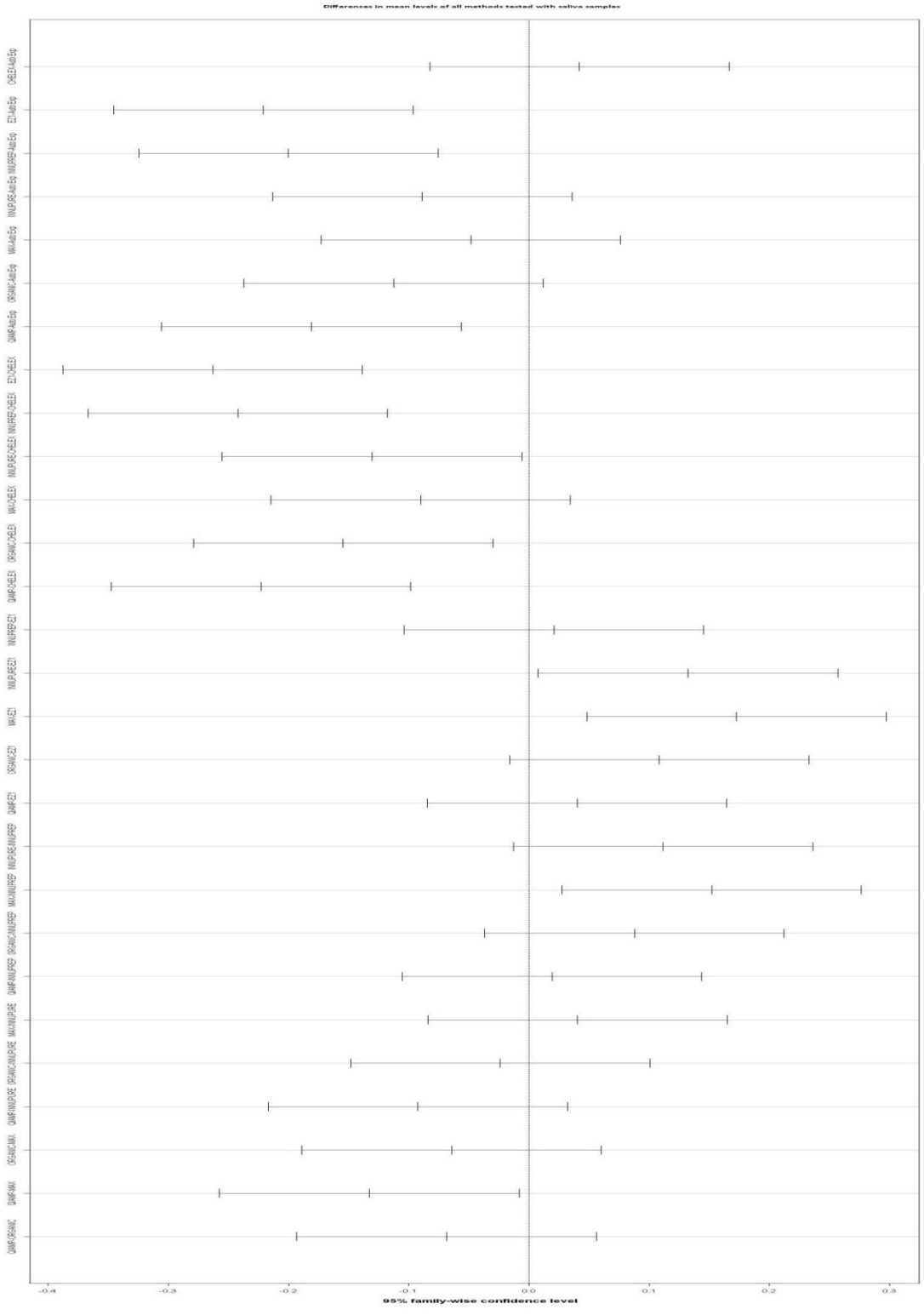
Appendix 2. A table of the pair-wise differences of mean values when the DNA yield from blood samples was compared using 8 different extraction techniques.

Extraction Type Pairs	p-value
CHELEX-Auto Exp	0.6749804
EZ1-Auto Exp	0.0000000
INNUPREP-Auto Exp	0.0000000
INNUPURE-Auto Exp	0.0000000
MAX-Auto Exp	0.0000000
ORGANIC-Auto Exp	0.0000000
QIAMP-Auto Exp	0.0000003
EZ1-CHELEX	0.0000002
INNUPREP-CHELEX	0.0000000
INNUPURE-CHELEX	0.0000000
MAX-CHELEX	0.0000001
ORGANIC-CHELEX	0.0000000
QIAMP-CHELEX	0.0001630
INNUPREP-EZ1	0.8562124
INNUPURE-EZ1	0.9649937
MAX-EZ1	1.0000000
ORGANIC-EZ1	0.0033866
QIAMP-EZ1	0.5845567
INNUPURE-INNUPREP	0.9999664
MAX-INNUPREP	0.8960540
ORGANIC-INNUPREP	0.1483131
QIAMP-INNUPREP	0.0381948
MAX-INNUPURE	0.9795019
ORGANIC-INNUPURE	0.0664997
QIAMP-INNUPURE	0.0911810
ORGANIC-MAX	0.0045863
QIAMP-MAX	0.5208450
QIAMP-ORGANIC	0.0000051


Differences in mean levels of all methods testeed for blood samples
CHELEX-AmbEsp
EZ1-AmbEsp
INNUPREP-AmbEsp
INNUPREABE-AmbEsp
MAX-AmbEsp
ORGANIC-AmbEsp
QIAMP-AmbEsp
EZ1-CHELEX
INNUPREP-CHELEX
INNUPREABE-CHELEX
MAX-CHELEX
ORGANIC-CHELEX
QIAMP-CHELEX
INNUPREP-EZ1
INNUPREABE-EZ1
MAX-EZ1
ORGANIC-EZ1
QIAMP-EZ1
INNUPREABE-INNUPREP
MAX-INNUPREP
ORGANIC-INNUPREP
QIAMP-INNUPREP
MAX-INNUPREABE
ORGANIC-INNUPREABE
QIAMP-INNUPREABE
ORGANIC-MAX
QIAMP-MAX
QIAMP-ORGANIC
-4
-2
0
2
95% family-wise confidence level

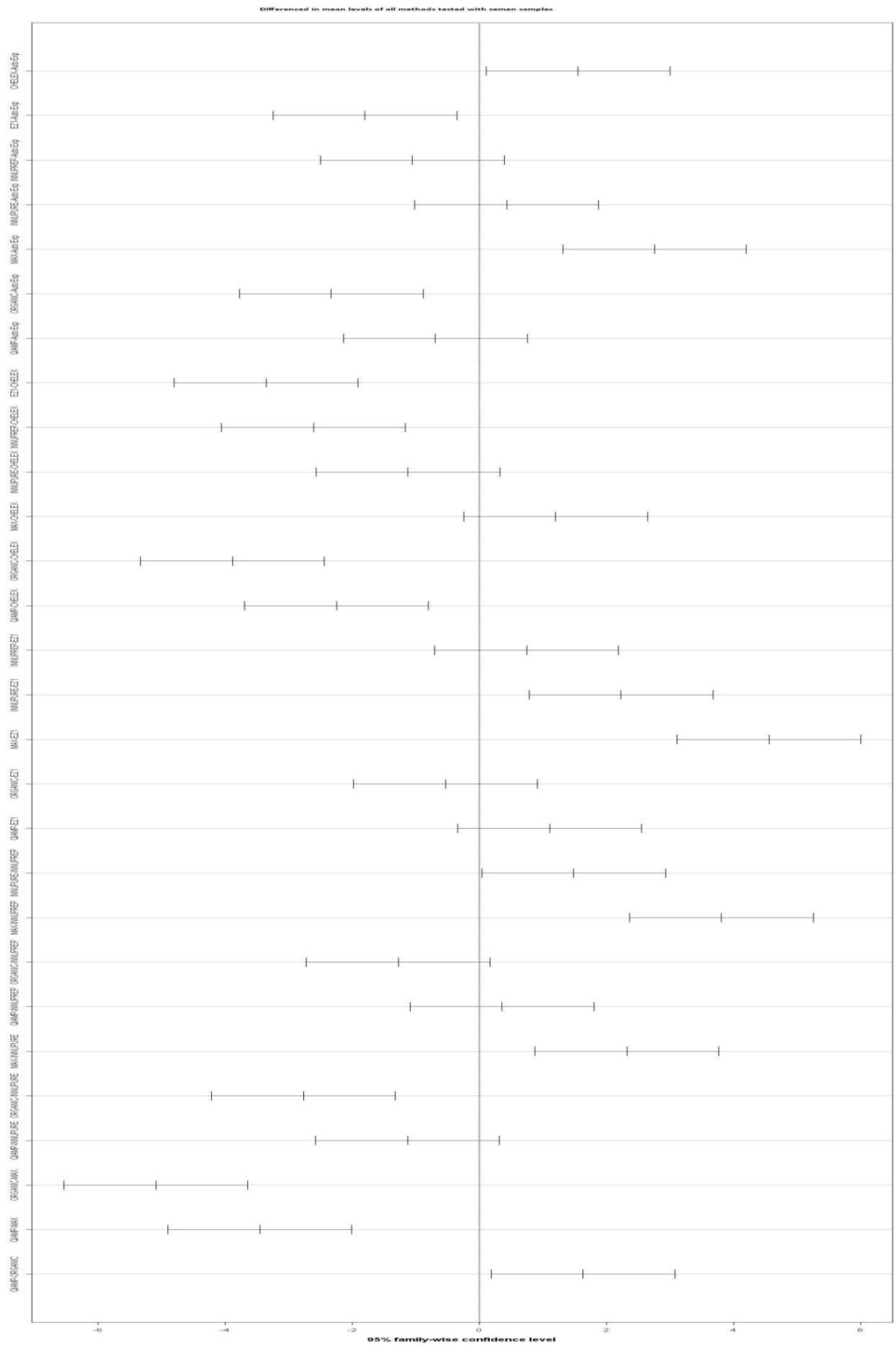
Appendix 3. A table of the pair-wise differences of mean values when the DNA yield from saliva samples was compared using 8 different extraction techniques.

Extraction Type Pairs	p-value
CHELEX-Auto Exp	0.962078
EZ1-Auto Exp	1.89E-05
INNUPREP-Auto Exp	0.000125
INNUPURE-Auto Exp	0.344853
MAX-Auto Exp	0.92311
ORGANIC-Auto Exp	0.1049188
QIAMP-Auto Exp	0.0006717
EZ1-CHELEX	0.0000004
INNUPREP-CHELEX	0.0000026
INNUPURE-CHELEX	0.0334954
MAX-CHELEX	0.3229015
ORGANIC-CHELEX	0.0058655
QIAMP-CHELEX	0.0000157
INNUPREP-EZ1	0.9994776
INNUPURE-EZ1	0.0297282
MAX-EZ1	0.0013418
ORGANIC-EZ1	0.1315821
QIAMP-EZ1	0.9708882
INNUPURE-INNUPREP	0.1103407
MAX-INNUPREP	0.0070878
ORGANIC-INNUPREP	0.3573755
QIAMP-INNUPREP	0.9996814
MAX-INNUPURE	0.9691592
ORGANIC-INNUPURE	0.9987005
QIAMP-INNUPURE	0.2939655
ORGANIC-MAX	0.7334497
QIAMP-MAX	0.0288750
QIAMP-ORGANIC	0.6673930


Differences in mean levels of all methods tested with saliva samples
95% family-wise confidence level

Appendix 4. A table of the pair-wise differences of mean values when the DNA yield from semen samples was compared using 8 different extraction techniques.

Extraction Type Pairs	p-value
CHELEX-Auto Exp	0.0266886
EZ1-Auto Exp	0.0056237
INNUPREP-Auto Exp	0.3171369
INNUPURE-Auto Exp	0.9798214
MAX-Auto Exp	0.0000040
ORGANIC-Auto Exp	0.0001222
QIAMP-Auto Exp	0.8017472
EZ1-CHELEX	0.0000000
INNUPREP-CHELEX	0.0000136
INNUPURE-CHELEX	0.2437599
MAX-CHELEX	0.1694180
ORGANIC-CHELEX	0.0000000
QIAMP-CHELEX	0.0002275
INNUPREP-EZ1	0.7316634
INNUPURE-EZ1	0.0002480
MAX-EZ1	0.0000000
ORGANIC-EZ1	0.9427869
QIAMP-EZ1	0.2551869
INNUPURE-INNUPREP	0.0397385
MAX-INNUPREP	0.0000000
ORGANIC-INNUPREP	0.1220180
QIAMP-INNUPREP	0.9932312
MAX-INNUPURE	0.0001243
ORGANIC-INNUPURE	0.0000039
QIAMP-INNUPURE	0.2379942
ORGANIC-MAX	0.0000000
QIAMP-MAX	0.0000000
QIAMP-ORGANIC	0.0162448


Differenced in mean levels of all methods tested with semen samples
CHELEX-Auto Exp
EZ1-Auto Exp
INNUPREP-Auto Exp
INNUPURE-Auto Exp
MAX-Auto Exp
ORGANIC-Auto Exp
QIAMP-Auto Exp
EZ1-CHELEX
INNUPREP-CHELEX
INNUPURE-CHELEX
MAX-CHELEX
ORGANIC-CHELEX
QIAMP-CHELEX
INNUPREP-EZ1
INNUPURE-EZ1
MAX-EZ1
ORGANIC-EZ1
QIAMP-EZ1
INNUPURE-INNUPREP
MAX-INNUPREP
ORGANIC-INNUPREP
QIAMP-INNUPREP
MAX-INNUPURE
ORGANIC-INNUPURE
QIAMP-INNUPURE
ORGANIC-MAX
QIAMP-MAX
QIAMP-ORGANIC
-6
-4
-2
0
2
4
6
95% family-wise confidence level

Appendix 5)

Quantifiler® Human — **BLOOD**

DAY	Neg.	DNA amount & ct value												ADD
		Cloth	**SD**	*ct Value*	*SD*	**Metal**	**SD**	*ct Value*	**SD**	**Glass**	**SD**	*ct Value*	**SD**	
1	0	**0.38**	0.14	***29.3***	*0.45*	**0.53**	0.15	***27.8***	0.17	**0.35**	**0.27**	***28.1***	*2.51*	0
3		**0.27**	0.13	**29.8**	*0.74*	**0.33**	0.1	***27.4***	0.58	**0.31**	**0.05**	***27.6***	*0.69*	85.25
5		**0.25**	0.23	***33.8***	*4.97*	**0.91**	0.21	***26.8***	0.09	**1.07**	**0.37**	***27***	*0.11*	164
7		**0.03**	0.01	***30.8***	*3.32*	**0.53**	0.32	***26.9***	0.16	**0.97**	**0.15**	***27.1***	*0.35*	250.5
9		**0.14**	0.1	***30.7***	*0.69*	**0.41**	0.18	***26.9***	0.14	**0.49**	**0.5**	***26.7***	*0.15*	338.5
11		**0.19**	0.22	***30.8***	*2.14*	**0.29**	0.08	***26.8***	0.06	**0.59**	**0.17**	***26.9***	*0.18*	428
13	0	**0.21**	0.08	***30.1***	*0.45*	**0.12**	0.04	***26.7***	0.05	**0.3**	**0.06**	***26.7***	*0.12*	515.25
15		**0.27**	0.03	***29.7***	*0.14*	**0.2**	0.03	***26.6***	0.03	**0.34**	**0.15**	***26.9***	*0.25*	599
18		**0.34**	0.06	***30.7***	*4.15*	**0.08**	0.03	***26.9***	0.07	**0.14**	**0.05**	***27.6***	*0.62*	724.5
21		**0.31**	0.19	***31.5***	*0.69*	**0.1**	0.04	***26.8***	0.04	**0.14**	*0.09*	***30.5***	*3.48*	852.75
24		**0.23**	0.08	***29.4***	*1.55*	**0.07**	0.01	***26.9***	0.17	**0.09**	**0.02**	***27.3***	*0.53*	986.5
27		**0.26**	0.03	***28.8***	*2.86*	**0.05**	0.02	***27.2***	0.64	**0.07**	**0.01**	***29***	*0.9*	1118.5
30		**0.22**	0.07	***26.9***	*0.02*	**0.03**	0.02	***28***	1.46	**0.05**	**0.03**	***28.5***	*0.66*	1250.25
33		**0.15**	0.02	***26.9***	*0.08*	**0.08**	0.12	***29.6***	4.03	**0.07**	**0.09**	***31.3***	*6.6*	1381
36	0	**0.13**	*0.09*	***28.6***	*1.8*	**0.01**	*0.01*	***32.2***	*6.4*	**0.02**	**0.03**	***34.1***	*5.57*	1523.25
39		**0.15**	0.06	***27.9***	*0.75*	**0.01**	0.01	***27.2***	0.27	**0.01**	**0.01**	***30.7***	*3.86*	1660. 5
42		**0.19**	0.03	**27.5**	0.35	**0.01**	0.01	**26.8**	0.08	**0.01**	0.002	**27.4**	0.71	1793.25
45		**0.09**	0.08	**27.4**	0.27	**0.004**	0.001	**26.8**	0.06	**0.01**	0.01	**28.7**	1.8	1926.25
48		**0.11**	0.04	**27.5**	0.18	**0.01**	0.01	**27**	0.24	**0.01**	0.01	**28.1**	0.89	2062. 5
51	0	**0.08**	0.05	**27.7**	0.28	**0.004**	0.002	**27.1**	0.19	**0.01**	0.01	**28.9**	2.15	2201.5

SEMEN

DAY	Neg.	DNA amount & ct value												ADD
		Cloth	**SD**	***ct value***	**SD**	**Metal**	**SD**	***ct value***	**SD**	**Glass**	**SD**	***ct value***	**SD**	
1	0	**7.46**	2.16	***27.1***	*0.16*	**13.2**	5.19	**30.1**	2.41	**10.9**	4.9	**32.4**	5.63	0
3		**14.9**	0.77	***28.9***	*1.38*	**6.72**	2.94	**32.8**	3.82	**7.91**	3.96	**27**	0.68	85.25
5		**9.75**	1.98	***29.8***	*1.91*	**4.95**	1.6	**26.7**	0.19	**3.6**	0.07	**30.6**	1.46	164
7		**4.1**	2.6	***27.7***	*0.65*	**7.41**	0.2	**26.7**	0.18	**5.12**	1.01	**27.4**	0.71	250.5
9		**5.42**	0.74	***28.6***	*1.73*	**12.2**	2.09	**27**	0.2	**7.93**	5.36	**26.7**	0.17	338.5
11		**8.73**	0.71	***27.4***	*0.11*	**10.5**	2.15	**26.8**	0.36	**9.38**	3.01	**26.9**	0.45	428
13	0	**7.38**	3.7	***27.2***	*0.32*	**6.98**	1.49	**26.9**	0.14	**8.69**	1.63	**27.4**	0.37	515.25
15		**4.94**	2.91	***29***	*2.99*	**4.15**	1.88	**27.2**	0.3	**7.91**	2.38	**27.8**	0.78	599
18		**2.72**	2.1	***32***	*6.78*	**1.85**	0.84	**27.3**	0.66	**5.83**	1.02	**27.1**	0.1	724.5
21		**3.58**	0.76	**27.7**	*0.27*	**1.52**	0.75	**27**	0.03	**4.93**	0.79	**26.9**	0.2	852.75
24		**2.27**	0.32	***27.5***	*0.18*	**1.68**	0.16	**26.9**	0.15	**4.11**	1.71	**26.9**	0.12	986.5
27		**2.71**	0.79	***27.3***	*0.18*	**0.91**	0.22	**26.8**	0.07	**3.13**	1.08	**27**	0.05	1118.5
30		**1.73**	0.23	***26.9***	*0.1*	**0.37**	0.1	**26.8**	0.08	**0.92**	0.54	**27.6**	0.77	1250.25
33		**0.93**	0.35	***27.5***	*0.96*	**0.13**	0.12	**27**	0.19	**1.16**	0.47	**27.5**	0.43	1381
36	0	**0.59**	0.25	***26.9***	*0.04*	**0.09**	0.05	**27.2**	0.2	**0.83**	0.44	**28.4**	0.86	1523.25
39		**0.5**	0.003	***27***	*0.03*	**0.05**	0.032	**27.7**	0.77	**0.53**	0.23	**27.9**	0.61	1660. 5
42		**0.35**	0.02	***27.1***	*0.17*	**0.06**	0.01	**27.2**	0.15	**0.28**	0.21	**27.3**	0.17	1793.25
45		**0.2**	0.04	***27.1***	*0.17*	**0.03**	0.01	**27.1**	0.17	**0.07**	0.08	**27.7**	1.11	1926.25
48		**0.18**	0.04	***27.1***	0.03	**0.01**	0.002	**27.4**	0.58	**0.02**	0.002	**27.3**	0.08	2062. 5
51	0	**0.1**	0.04	**27**	0.03	**0.01**	0.01	**27.1**	0.1	0.01	0.01	**27.4**	0.27	2201.5

SALIVA

DAY	Neg.	DNA amount & ct value												ADD
		Cloth	**SD**	***ct value***	**SD**	**Metal**	**SD**	***ct value***	**SD**	**Glass**	**SD**	***ct value***	**SD**	
1	0	**0.03**	0.02	***28.2***	**0.14**	**0.02**	0.01	27.2	**0.23**	**0.08**	0.06	**26.9**	0.17	0
3		**0.023**	0.01	***27.8***	*0.17*	**0.0024**	0.003	27.1	**0.37**	**0.02**	0.01	**27.4**	0.6	85.25
5		**0.024**	0.01	***27.5***	*0.13*	**0.004**	0.005	26.7	**0.07**	**0.013**	0.012	**26.7**	0.03	164
7	0	**0.0013**	n/a	***26.8***	*0.3*	**0.001**	0.0003	26.6	**0.04**	**0.01**	0.01	**27.1**	0.34	250.5
9		**0.0012**	0.001	***26.8***	*0.34*	**0.007**	0.006	26.7	**0.24**	**0.003**	0.002	**27**	0.21	338.5
11		**0.02**	0.03	***27.6***	*0.99*	**n/a**	n/a	26.7	**0.04**	**0.57**	0.98	**26.8**	0.25	428
13	0	**n/a**	n/a	**27**	0.33	**n/a**	n/a	26.7	**0.08**	**n/a**	n/a	**26.9**	0.07	515.25
15		**0.0005**	n/a	***26.8***	*0.03*	**n/a**	n/a	26.9	**0.15**	**n/a**	n/a	**26.8**	0.03	599

Quantifiler® Trio

BLOOD

DAY	Neg.	DNA amount & DI value												ADD
		Cloth	**SD**	***DI***	**SD**	**Metal**	**SD**	***DI***	**SD**	**Glass**	**SD**	***DI***	**SD**	
1	0	**0.12**	0.16	***0.04***	*0.06*	**0.64**	0.15	***1.04***	*0.13*	**0.53**	0.16	***1.29***	*0.07*	0
3		**0.04**	0.03	***0.04***	*0.03*	**0.38**	0.1	***1.52***	*0.14*	**0.39**	0.02	***1.39***	*0.04*	85.25
5		**0.12**	0.17	***0.19***	*0.3*	**1.06**	0.23	***0.86***	*0.17*	**1.01**	0.28	***0.73***	*0.02*	164
7		**0.02**	0.004	***0.59***	*1*	**0.59**	0.35	***0.9***	*0.06*	**0.9**	0.24	***0.89***	*0.08*	250.5
9		**0.03**	0	***0.04***	*0*	**0.48**	0.38	***0.83***	*0.06*	**0.46**	0.48	***1.24***	*0.22*	338.5
11		**0.15**	0.22	***0.49***	*0.8*	**0.39**	0.1	***1.19***	*0.22*	**0.61**	0.18	***1.19***	*0.18*	428
13	0	**0.1**	0.07	***0.22***	*0.17*	**0.1**	0.04	***1.18***	*0.3*	**0.35**	0.09	***1.62***	*0.03*	515.25
15		**0.12**	0.07	***0.39***	*0.26*	**0.19**	0.05	***2***	*0.21*	**0.4**	0.17	***1.94***	*0.04*	599
18		**0.19**	0.07	***0.82***	*0.52*	**0.11**	0.03	***2.59***	*0.71*	**0.15**	0.06	***3.74***	*0.63*	724.5
21		**0.22**	0.03	***1.3***	*0.09*	**0.08**	0.04	***3.23***	*0.68*	**0.27**	0.04	***3.35***	*0.47*	852.75
24		**0.19**	0.06	***1.47***	*0.5*	**0.06**	0.01	***2.2***	*0.48*	**0.09**	0.03	***2.33***	*0.54*	986.5
27		**0.23**	0.01	***1.55***	*0.75*	**0.03**	0.01	***1.59***	*0.21*	**0.1**	0.05	***2.83***	*0.89*	1118.5
30		**0.34**	0.14	***1.87***	*0.63*	**0.04**	0.01	***1.99***	*0.65*	**0.06**	0.03	***2.56***	*1.14*	1250.25
33		**0.22**	0.03	***2.11***	*0.28*	**0.05**	0.04	***52***	*84.5*	**0.04**	0.02	***2.53***	*0.71*	1381
36	0	***0.15***	*0.07*	***1.84***	*0.19*	**0.03**	0.01	***2.36***	*1.3*	**0.06**	0.03	***3.31***	*0.39*	1523.25
39		**0.26**	0.09	***2.82***	*0.79*	**0.01**	0.01	***1.65***	*1.01*	**0.04**	0.02	***4.8***	*3.84*	1660. 5
42		**0.27**	0.01	**2.8**	0.14	**0.02**	0.001	**6.79**	2.82	**0.03**	0.002	**14.9**	10.3	1793.25
45		**0.19**	0.11	**5.4**	0.85	**0.01**	0.001	**11**	8.49	**0.03**	0.01	**28.4**	24.6	1926.25
48		**0.21**	0.12	**6.36**	1.13	**0.01**	0.01	**11**	5.51	**0.02**	0.02	**17**	16.4	2062. 5
51	0	**0.14**	0.02	**5.94**	1.82	**0.005**	0.003	**8.51**	4.85	**0.02**	0.004	**9.16**	3.64	2201.5

SEMEN

DAY	Neg.	DNA amount & DI value												ADD
		Cloth	**SD**	***DI***	**SD**	**Metal**	**SD**	***DI***	**SD**	**Glass**	**SD**	***DI***	**SD**	
1	0	**28.1**	14.6	***1.3***	*0.3*	**21.7**	7.72	**1**	0.01	**39.5**	32.6	**1.13**	0.16	0
3		**15.7**	1.96	***1.3***	*0.25*	**9.09**	2.81	**1.66**	0.36	**15.2**	6.05	**1.49**	0.31	85.25
5		**9.93**	1.28	***2.18***	*0.27*	**6.61**	2.28	**2.64**	0.26	**5.79**	1.05	**2.23**	0.43	164
7	0	**3.71**	1.67	***5.84***	*0.9*	**8.3**	0.58	**6.67**	0.82	**7.67**	6.07	**3.5**	0.89	250.5
9		**5.43**	1.87	***3.25***	*1.52*	**8.37**	5.22	**3.55**	1.18	**6.93**	3.11	**4.3**	3.65	338.5
11		**7.21**	1.78	***9.31***	*2*	**6.78**	1.73	**8.42**	4.24	**7.14**	3.23	**8.02**	4.15	428
13	0	**5.05**	2.36	***12.6***	*2.19*	**4.71**	1.75	**15.4**	9.01	**6.14**	1.76	**10.4**	7.96	515.25
15		**3.4**	2.05	***36.1***	*28*	**4.32**	2.27	**38.6**	33.5	**6.29**	3.5	**20.1**	16.1	599
18		**2.45**	1.8	***62.3***	*58.8*	**2.64**	2.1	**292**	309.5	**4.44**	2.38	**49.5**	53.3	724.5
21	0	**3.81**	1.72	**47834.8**	*82698.2*	**1.71**	1.3	**584.8**	570.5	**3.8**	0.36	**25.5**	3.45	852.75
24		**0.81**	0.09	***159.1***	*108.1*	**0.92**	0.18	**576.9**	398.6	**2.69**	1.19	**35.6**	6.46	986.5
27		**1.22**	0.41	***581.2***	*521.7*	**0.42**	0.07	**1462.1**	323	**2.14**	1.47	**54.8**	21.1	1118.5
30		**0.67**	0.03	***1257.9***	*492.8*	**0.19**	0.07	**678.6**	253.4	**0.39**	0.29	**255.5**	289.1	1250.25
33		**0.41**	0.03	***1296.4***	*376.5*	**0.05**	0.05	**137.7**	171.2	**0.52**	0.15	**145.2**	75.9	1381
36	0	**0.27**	0.13	***5314.4***	*3023.9*	**0.03**	0.19	**154.3**	93	**0.46**	0.19	**96.5**	24.4	1523.25
39		**0.23**	0.05	***4669.6***	*1415.3*	**0.01**	0.002	**279**	193	**0.27**	0.08	**310**	230.8	1660. 5
42		**0.12**	0.008	***7074.2***	*1970.1*	**0.01**	0.001	**120**	59	**0.13**	0.11	**337.8**	197.1	1793.25
45		**0.07**	0.003	***n/a***	*n/a*	**0.009**	0.004	**144.2**	118.7	**0.04**	0.04	**902**	283.8	1926.25
48		**0.07**	0.02	***206.3***	0	**0.006**	0.002	**79.4**	92	**0.008**	0.005	**94.7**	5.57	2062. 5
51	0	**0.02**	0.007	**n/a**	n/a	**0.006**	0.004	**678.6**	253.4	0.009	0.001	**365.1**	498.5	2201.5

SALIVA

DAY	Neg.	DNA amount & DI value												ADD
		Cloth	**SD**	***DI***	**SD**	**Metal**	**SD**	***DI***	**SD**	**Glass**	**SD**	***DI***	**SD**	
1	0	**0.04**	0.03	***0.96***	*0.22*	**0.04**	0.02	1.15	**0.12**	**0.11**	0.04	**1.28**	0.09	0
3		**0.02**	0.006	***7.81***	*1.06*	**0.005**	0.005	21.9	**3.8**	**0.04**	0.02	**7.01**	0.97	85.25
5		**0.02**	0.006	***56.6***	*38.8*	**0.006**	0.01	32	**17.5**	**0.02**	0.02	**6.74**	1.41	164
7	0	**0.001**	0.0006	***n/a***	*n/a*	**0.0005**	0.0003	n/a	**n/a**	**0.003**	0.004	**41.6**	31.1	250.5
9		**0.006**	0.005	***113.6***	*n/a*	**0.001**	0.0003	8.43	**n/a**	**0.02**	0.007	**26**	12.7	338.5
11		**0.0008**	0.0002	***4.53***	*n/a*	**0.004**	0.006	12.6	**n/a**	**0.008**	0.008	**68.6**	73.3	428
13	0	**0.0002**	0.00007	**n/a**	n/a	**0.0003**	0.0003	n/a	**n/a**	**0.0007**	0.0005	**5.43**	n/a	515.25
15		**0.0003**	0.00004	***4.77***	*0.67*	**0.002**	n/a	3.01	**n/a**	**0.0004**	0.0004	**4.01**	3.36	599

Quantus®

BLOOD

DAY	Neg.	DNA amount						ADD
		Cloth	**SD**	**Metal**	**SD**	**Glass**	**SD**	
1	0	**0.24**	0.05	**0.13**	0.07	**0.15**	**0.1**	0
3		**0.62**	0.19	**0.18**	0.07	**0.21**	**0.02**	85.25
5		**0.55**	0.27	**0.17**	0.01	**0.19**	**0.05**	164
7		**0.54**	0.48	**0.34**	0.14	**0.39**	**0.08**	250.5
9		**0.49**	0.1	**0.34**	0.12	**0.26**	**0.27**	338.5
11		**0.44**	0.18	**0.35**	0.09	**0.36**	**0.2**	428
13	0	**0.4**	0.07	**0.16**	0.1	**0.3**	**0.3**	515.25
15		**0.32**	0.09	**0.2**	0.2	**0.28**	**0.04**	599
18		**0.26**	0.11	**0.09**	0.01	**0.15**	**0.05**	724.5
21		**0.27**	0.01	**0.07**	0.07	**0.15**	*0.04*	852.75
24		**0.18**	0.03	**0.12**	0.09	**0.12**	**0.02**	986.5
27		**0.16**	0.06	**0.12**	0.1	**0.21**	**0.09**	1118.5
30		**0.15**	0.08	**0.08**	0.01	**0.13**	**0.05**	1250.25
33		**0.11**	0.08	**0.1**	0.02	**0.57**	**0.68**	1381
36	0	**0.12**	*0.17*	**0.1**	*0.05*	**0.19**	**0.1**	1523.25
39		**0.14**	0.11	**0.09**	0.03	**0.14**	**0.15**	1660. 5
42		**0.24**	0.02	**0.06**	0.053	**0.08**	0.06	1793.25
45		**0.13**	0.06	**0.03**	0.02	**0.13**	0.1	1926.25
48		**0.09**	0.05	**0.04**	0.04	**0.1**	0.04	2062. 5
51	0	**0.08**	0.05	**0.02**	0.02	**0.07**	0.06	2201.5

SEMEN

DAY	Neg.	DNA amount & SD						ADD
		Cloth	**SD**	**Metal**	**SD**	**Glass**	**SD**	
1	0	**4.12**	0.51	**2.48**	0.59	**2.49**	**0.11**	0
3		**13**	1	**3.01**	1.1	**4.03**	**3.39**	85.25
5		**9.87**	0.98	**2.76**	0.94	**2.3**	**0.23**	164
7		**3.96**	2.19	**5.09**	0.48	**3.74**	**0.99**	250.5
9		**4.55**	1.11	**5.59**	1.23	**3.83**	**1.67**	338.5
11		**5.68**	0.85	**4.98**	1.64	**4.65**	**1.35**	428
13	0	**4.64**	2.33	**4.01**	0.62	**3.48**	**0.74**	515.25
15		**4.22**	0.51	**3.15**	0.63	**4.47**	**1.68**	599
18		**4.2**	0.43	**1.73**	0.76	**2.96**	**0.37**	724.5
21		**3.29**	0.29	**1.78**	0.57	**2.3**	*0.58*	852.75
24		**2.71**	0.34	**1.89**	0.34	**2.4**	**0.79**	986.5
27		**3.46**	0.37	**2.03**	0.56	**2.32**	**0.76**	1118.5
30		**2.87**	0.26	**1.53**	0.46	**1.23**	**1.02**	1250.25
33		**2.68**	0.22	**1**	0.67	**1.19**	**0.64**	1381
36	0	**1.76**	*0.67*	**1.19**	*0.34*	**1.35**	**0.41**	1523.25
39		**2.48**	0.14	**1.35**	0.87	**1.1**	**0.41**	1660. 5
42		**1.99**	0.49	**1.25**	0.33	**0.65**	0.26	1793.25
45		**1.33**	0.31	**0.5**	0.13	**0.44**	0.29	1926.25
48		**1.63**	0.08	**0.37**	0.26	**0.55**	0.63	2062. 5
51	0	**0.63**	0.23	**0.7**	0.1	**0.23**	0.08	2201.5

SALIVA

DAY	Neg.	DNA amount & ct value						ADD
		Cloth	**SD**	**Metal**	**SD**	**Glass**	**SD**	
1	0	**0.06**	0.01	**0.09**	0.07	**0.08**	**0.04**	0
3		**0.07**	0.02	**0.15**	0.03	**0.16**	**0.01**	85.25
5		**0.07**	0.01	**0.01**	0.004	**0.03**	**0.02**	164
7		**0.03**	0.02	**0.35**	0.12	**0.21**	**0.15**	250.5
9		**0.04**	0.03	**0.2**	0.1	**0.34**	**0.19**	338.5
11		**0.01**	0.01	**0.35**	0.09	**0.29**	**0.08**	428
13	0	**0.01**	0.01	**0.2**	0.07	**0.35**	**0.11**	515.25
15		**0.02**	0.01	**0.26**	0.12	**0.22**	**0.14**	599

Appendix 6. Published paper based on Chapter 4 of this study