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The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence.

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by

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PART A: Research protocol

Abbreviations

CODIS:	Combined DNA Index System
DNA:	Deoxyribonucleic acid
FBI QAS :	Federal Bureau Investigation Quality assurance standards
IC:	Internal control
mtDNA:	Mitochondrial Deoxyribonucleic acid
PCR:	Polymerase chain reaction
PVPP:	Polyvinil-polypyrrolidone
qRT-PCR:	Quantitative real time PCR
STR:	Short tandem repeat
SWGDAM:	Scientific Working Group on DNA Analysis Methods
TRFLP:	Terminal Restriction fragment length polymorphism

1) ***Background and significance*.**

1.1) The relevance of DNA extraction from soil in forensic investigations.

The extraction of human DNA from soil has important applications in the field of forensic science. This includes the identification of victims in mass graves from war or as a result of disasters such as tsunamis or hurricanes, where DNA isolation from decomposed remains deposited or buried in soil would be important for identification purposes. Additionally, on an outdoor homicide or rape crime scene, many circumstances may cause human biological evidence from the perpetrator or victim to be deposited in the soil (Wang *et al*. 2008; Alpay *et al*. 2008). However, soil contaminated samples are often problematic due to the presence of humic acid, which leads to the inhibition of DNA amplification and unsuccessful DNA profiling (Shahzad *et al*. 2009). As a result, soil samples containing potential blood, semen and saliva are rarely collected on homicide or rape crime scenes due to the time constraints, the high failure rate and limited biological material. This may lead to the loss of vital criminal evidence or the ability to identify victims successfully. In the case of mass graves, special extraction methods often have to be established depending upon the circumstances, which again is time consuming , costly and have less potential for automation.

1.2) Humic acid: The major soil inhibitor of DNA profiling.

Humic acid, the major organic constituent of soil (humus), peat, compost and fertilizers is formed by microbial degradation of plant and animal material (Zipper *et al*. 2003). The quantity of humic acid in the soil is dependent upon the soil type, with sandy soil having the lowest humic acid concentration. It has been found that loam sand sampled from natural botanical gardens can have a humic acid content of 25 mg/g (Lakay *et al*. 2007), while the percentage of organic content (which is proportional to humic content) is often higher for forest clay loam (44.6%) and rainforest silt loam (22.0 %) (Schneegurt & Dore 2003). Humic acid is a large aromatic complex that has several phenolic and carboxylic substituents which have chemical properties similar to double stranded DNA. Therefore humic acid may co-extract with the DNA by binding under similarly conditions to the silica column when using the standard silica based DNA extraction method (Harry *et al*. 1999). The humic acid then inhibits DNA amplification by targeting the enzyme Taq polymerase required for DNA amplification via the polymerase chain reaction (PCR). Taq polymerases are known to be inhibited by at least 1ng of humic acid in the PCR reaction (Menkings *et al*. 1999). The inhibition of Taq polymerase is also evident as increasing its amount is known to overcome humic acid inhibition (Sutlović *et al*. 2005), however this

seems to be disputed, as Opel *et al*. (2010) did not always find this to be successful. They identified that certain inhibitors can affect PCR in more than one manner, for humic acid inhibition it was determined that binding to specific DNA sequences may also affect the PCR amplification by limiting the amount of amplifiable template (Opel *et al*. 2010). Apart from humic acid, PCR inhibitors such as fulvic acid, bacteria and other phenolic compounds found in soil may also be responsible. However humic acid remains the most abundant inhibitor for several different soil types.

1.3) Humic acid contaminated forensic samples: A widespread problem.

The extent of soil and humic acid contaminated samples and thus problematic samples for DNA profiling in forensics is demonstrated by several published examples. In a recent review on 4997 medico-legal case reports from a mortuary located in the rural district of Maharashtra in India, 43 % of decomposed human remains were recovered from outdoor crime scenes (Ambade *et al*. 2011). For these local cases, recovering human remains from forest areas was particularly common. Several other cases of forest recoveries have also been recently documented, with nearly 250 dumped human foetuses aged 5-6 months discovered in the mountain region of Russia (Baklinski 2012) and mass dumping of 14 bodies, allegedly by police, in the remote Kinale forest of Kenya (The Star 2011). Forest dump sites are probably popular due to the location and dense vegetation, making discovery of the bodies more difficult. Experienced criminals may also know that forest soils are rich in diverse microbial and insect species which together increase the rate of soft tissue decomposition and reduces the chances of victim identification (Haslam & Tibbett 2009). Acidic forest soils also prevent bone preservation and may promote the rapid disintegration of human skeletal remains which leaves minimal traces behind (Carter & Tibbett 2008). Larger predators or scavengers are more likely to feast on the remains in forest locations which lead to even faster decomposition rates. Furthermore, when rain and temperature is elevated in a forest, the conditions of the environment promote the rapid decomposition of soft tissue, especially for the smaller and thinner bodies of neonates (Archer 2004).

In forensic anthropology, DNA extraction from ancient remains found at various burial sites or mass graves is crucial for the identification of war victims (Gunby 1994; Primorac *et al*. 1996). Bones and teeth are the most common ancient remains that may provide a DNA source in these cases. However the DNA extracted from bones and teeth recovered from mass graves are either badly degraded or contaminated (Primorac *et al*. 1996). Several advances in research have

overcome the issues of DNA degradation by using the more resistant and abundant Mitochondrial DNA (mtDNA) source. However the inhibition of PCR by the soil inhibitors including humic acid, is still an important factor causing failure of mtDNA typing from skeletal remains (Dhanapal 2010 ; Lee *et al*.2010).

Not only does the humic acid in the soil affect amplification of DNA from human sources, it can also affect the quality of bacterial DNA. According to the Locards exchange principle the perpetrator and the victim may exchange trace evidence between each other and with the crime scene (Ramsland 2012). When a perpetrator attempts to bury or discard human remains in remote forest locations, the transfer of soil onto the perpetrators shoes or clothing may be important evidence to link the perpetrator to the crime scene or victim. In forensic investigations soil analysis using physical observations such as colour, texture and microscopic composition have been used previously for linking the perpetrator to the crime scene (Dawson and Hillier 2010). Linking two samples of soil is also possible by characterization of the microbial population diversity and community composition between the samples. The extraction of DNA from microbial populations in the soil allows for microbial DNA profiling similar to the way human profiles are produced. For microbial typing, instead of short tandem repeat (STR) analysis, a terminal restriction fragment length polymorphism (TRFLP) is the "fingerprinting" technique of choice (Horswell *et al*. 2002). Nevertheless, to achieve successful DNA profiling from microbial species, the DNA would firstly require purification from the soil samples. Although several specialized microbial DNA soil purification kits are commercially available, it's not always guaranteed to successfully purify the DNA sample as indicated by (Meyers and Foran 2008).

1.4) Advances in forensic DNA extraction and inhibitor removal techniques.

The effective removal of inhibitors from soil contaminated samples is thus a key issue to overcome when using these valuable samples. Many advances in the purification of DNA from forensic samples have been made over the years to remove soil inhibitors. Some of these advances are incorporated into forensic human DNA extraction kits. However for some of these DNA extraction kits, the ability to remove humic acid is not clearly stated or is not fully validated in terms of the humic acid concentration, sample type and sample volumes that were used during the validation. This makes the selection of a reliable inhibitor removal extraction kit difficult, especially when only a small volume of biological sample (blood or saliva) containing soil inhibitors are available for the investigation.

Several techniques have been developed for the removal of PCR inhibitors from ancient skeletal samples, these include silica based methods (Kemp *et al*. 2006), size exclusion chromatography (Matheson *et al*. 2010) and ion exchange chromatography (Seo *et al*. 2010). Based on these principles a combination of dextran blue and a selective ethanol precipitation technique was effective in removing PCR inhibitors from 500-1200 year old human bone samples (Kalmer *et al*. 2000). Protocols using repeated silica extractions were also effective in removing PCR inhibitors from extracts of 7000-8000-year-old human skeletal remains (Kemp, Monroe & Glenn 2006). Other protocols demonstrated that using a combination of chelex resin and polyvinil-polypyrrolidone (PVPP) resin was effective in removing inhibitors from dry human bones (Stutlovi *et al*. 2007) More recently a protocol was published using an ethanol re-precipitation technique in combination with a Gene clean-BIO101 spin kit, which produced more efficient inhibitor removal compared to the spin column alone (Panday *et al*. 2011). For many of these strategies combining purification protocols provided better results, however a standardized procedure using these strategies has not yet been described in the literature, many are time consuming, labour intensive, not suitable for trace amounts of evidence and show little potential for automation. Furthermore, efficiency of inhibitor removal and successful genotyping at higher inhibitor concentrations was also a problem with some of these strategies (Stutlovi *et al*. 2007; Panday 2011 & Seo *et al*. 2010).

The invention of polystyrene beads followed by magnetization allowed for the separation of many macromolecular compounds (Kemshead & Ugelstad 1985) and yet this technology has only recently revolutionised the extraction of DNA, especially in the forensic arena. One of the first DNA extraction kits based on this technology was the ChangeSwitch™ forensic DNA extraction kit offered by Invitrogen. This kit contains the magnetic bead technology coupled with an ionisable nucleic acid binding ligand with a charge that can be switched in a pH dependent manner. This is a rapid 3 step purification technique which takes approximately 15 minutes to provide the extracted DNA. This DNA forensic kit has been around since early 2004 and has been validated on a variety of common forensic samples, including bones, teeth, cigarette buds, saliva on envelopes, and door handle swabs. It is shown to be extremely effective in extracting DNA from trace amounts of evidence, however very few validation studies are published on its ability to remove humic acid inhibitors and some indicate that it may not be effective (Johns 2006, Rawling 2010).

The other well-known Forensic DNA extraction kit, based on the magnetic bead technology, is the DNA IQ™ kit from Promega. This method is reported to be able to deal with a number of problematic forensic samples and perform well in the presence of soil inhibitors, as well as being a rapid extraction procedure (Mandrekar *et al*. 2001& 2002; Heranz *et al.* 2008). However, the majority of the validation data on inhibitor removal has not been published in peer reviewed articles, with only manufacturer reports being available. These reports lack details on the humic acid inhibitor concentration tested, volume of biological sample used and the efficiency of inhibitor removal (Mandrekar *et al*. 2001). In one of the few published reports from Promega (Heranz *et al*. 2008), a wide range of inhibitor containing samples were tested for efficiency of DNA extraction and inhibitor removal, but only one blood stained soil sample was analysed making it difficult to predict if successful STR-profiles would result from trace amounts of blood and saliva collected from humic acid rich soil.

The most widely validated extraction system, which again uses the magnetic bead principle is the PrepFiler™ DNA extraction kit from Applied Biosystems. During validation of the Prepfiler™, an inhibitor platform containing a mixture of inhibitors was introduced for the first time. It included humic acid, hematin and indigo, which was spiked into various volumes of blood. In this study by (Brevnov *et al*. 2009) the inhibitor removal abilities were evaluated using qRT-PCR and with STR genotyping. However, only one concentration of humic acid (2.5 mg/ml) was included in the inhibitor platform to give a final concentration in blood at 0.5 mg/ml, which may not represent the full extent of the possible contamination levels in common garden soil or forest soils. Unfortunately, despite the good performance of this assay in the presence of inhibitors including humic acid, the product was withdrawn from the market (October 2012) while a replacement product is being developed.

Using a very different extraction approach, a popular method for collection and extraction of DNA from forensic samples is the use of FTA cards. FTA cards are designed with a chemical formula that lyses the cell membranes upon contact, to release the DNA. It also contains a matrix which then traps the DNA on the cards (GE Healthcare life Sciences, 2010). The chemical composition of the cards matrix allows the sampling of blood, saliva, buccal swabs and other environmental DNA samples, which can be stored at room temperature for many years (Smith & Burgoyne 2004; GE Healthcare life Sciences, 2011). The extraction process is simple, fairly rapid (30 minutes for a manual extraction) and can be easily automated (Rockenbauer *et al*. 2009).The FTA purification wash reagent and the specialized FTA elute™ cards are known to

remove PCR inhibitors, including heme from the bound DNA, but the removal of soil-based PCR inhibitors such as humic acid have not yet been evaluated.

As an alternative to using specialized extraction techniques for the removal of PCR inhibitors, another approach is to improve the efficiency of the enzymes that are used for DNA amplification during the DNA profiling procedure. Several studies have identified that polymerases isolated from different bacteria can display various degrees of resistance to particular inhibitors. To this end, Eilert and Foran (2009) investigated the ability of ten commercially available Taq polymerases to amplify bone-derived DNA contaminated with various concentrations of humic acid, collagen and calcium PCR inhibitors. For most of the polymerases adding BSA was shown to overcome humic acid inhibition, but none were able to amplify effectively in the presence of 0.1 mg/ml humic acid. The authors identified that certain Taq polymerases were more resistant to particular inhibitor types and concentrations than others, they suggested that the standard Taq-polymerase commonly incorporated into DNA profiling kits may not be optimal for DNA profiling from skeletal remains that are contaminated with a cocktail of inhibitors (Eilert & Foran 2009). In a later study (Baar *et al*. 2011), a chimeric polymerase called 2D9 was isolated, which was created using molecular breeding of eight orthologous from the genus Thermus and Deinococcus. This enzyme, 2D9, has a broad spectrum resistance to various inhibitor sources, including samples from humic acid rich soils, bone dust, coprolite, peat extract, clay-rich soil, cave sediment and tar (Baar *et al*. 2011). Although this mutant polymerase shows promise in the forensic context, it will take a significant amount of development before this can be incorporated into standard operating procedures for DNA profiling and it is not yet available commercially for research. Nevertheless, there are commercially available Taq-polymerases that are resistance to multiple PCR inhibitors. This includes the OmniTaq and Omni Klentaq enzymes offered by DNA Polymerase Technology Inc. These mutant enzymes show the ability to amplify human DNA directly from blood and crude soil samples without DNA purification being required. These Taq polymerases are highly resistant to soil inhibitors, which is evident by the tolerance to humic acid concentrations as high as 0.8ug/ml, compared to 0.024ug/ml for the standard enzymes (Kermekchiev *et al*. 2009). A recent study has shown the ability of these enzymes to generate useful STR profiles from crude soil contaminated blood samples (Kermekchiev 2012), but these new inhibitor resistant enzymes have not as yet been incorporated into commercially available STR profiling kits. Therefore at this stage, the development and scientific assessment of innovative DNA purification techniques

is required, as this remains the gold standard for removal PCR inhibitors from compromised forensic sample.

Most forensic DNA samples are gleamed from very small amounts of evidence, from transfer residue on objects, saliva on cigarette butts to small drops of blood. Samples may also be heavily degraded due to environmental factors and the quantity of usable DNA extracted is often very limited. It is therefore essential that the most suitable method of extraction is used to ensure that the most information is acquired from each precious forensic sample. In the case of soil-exposed samples, this information is currently lacking. There is a dire need to validate the current forensic DNA extraction methods for use on humic acid contaminated samples and publish this information to the wider forensic community.

2) Aim and objectives

The aim is to fundamentally evaluate the ability of two forensic based DNA purification systems to overcome the soil contamination of human peripheral blood and saliva samples. The FTA Whatmans™ technologies and DNA IQ™ system will be evaluated to purify DNA from these biological samples contaminated with various concentrations of the soil inhibitor humic acid. We hope to advise the forensic community of a reliable and efficient method to extract DNA from biological material that is contaminated with soil at a potential crime scene.

The aim is divided into the following objectives:

1) To evaluate a protocol that combines the FTA elute™ cards and the FTA Whatmans™ purification reagent to remove inhibitors from DNA extracted from blood and saliva samples spiked with various concentrations of humic acid (0, 0.5, 1.5 and 2.5 mg/ml). The efficiency of inhibitor removal and DNA yield would be evaluated using the Investigator Quantiplex™ DNA quantification kit (Qiagen) (real-time PCR quantification analysis). Further evaluation will include the generation of STR genotypes using the Powerplex® 16 kit (Promega) to determine the efficiency of amplification of all the 16 loci following the extraction procedure. Samples from three separate donors will be analysed in duplicate for this analysis.

2) To validate the DNA IQ™ extraction kits (Promega) abilities to overcome humic acid inhibition efficiently and reproducibly at various humic acid concentrations (0, 0.5, 1.5 and 2.5 mg/ml) spiked into biological samples (blood and saliva). The efficiency and

reproducibility of inhibitor removal and perceived DNA yield (will be affected by inhibitor presence) would be evaluated using the Investigators Quantiplex™ DNA quantification kit (Qiagen). Further evaluation would include the generation of STR genotypes using the Powerplex® 16 kit. Samples from three separate donors will be analysed in duplicate sets for this analysis.

3) Depending upon the outcome of the above analysis of both FTA elute™ and DNA IQ™ methodologies to remove humic acid, the best methodology will be used to extract DNA from soil samples (containing varying amounts of organic material), to assess the translatability of the research into forensic field applications

3) Methodology.

3.1) Scientific design.

The scientific design for this study was planned in compliance with the declaration of Helsinki 2008, the National Health Act 61 of 2003 and the Occupational Health and Safety Act 85 of 1993 for working with human participants and their biological samples. The scientific principles and techniques to validate the effectiveness of forensic based DNA purification systems are followed in compliance with guidelines provided by international forensic societies. Such as the Scientific Working Group on DNA Analysis Methods (SWGDAM July 2003) and the FBI Quality assurance standards (QAS) 2011 for DNA typing laboratories. Aligning this study's methodology with these guidelines would assure credibility of the results which makes it applicable for forensic applications. Therefore this study would follow international accreditation standards for the DNA extraction procedures, DNA quantification and genotyping procedures.

3.2) Participants.

There is no specific study population of interest required in this study. Three healthy donors are required to donate 5-10ml of blood and 2ml of saliva for the study. Donors will be recruited from either the division of Forensic Pathology at the University of Cape Town (UCT) or other UCT divisions. Donors will be informed about the study using a flyer (see Appendix I), information sheet (Appendix II) and personal communication with the investigator. Donors are required to sign a consent form (see Appendix III) in order to participate in the study. Inclusions of participants will be based on the following criteria: nationality (South African), ability to understand and speak English and 18 years or older. Vulnerable individuals such as the elderly, mentally challenged and disable will be excluded from this study. Colleagues and staff members

based in the same laboratory (Division of Haemtology) will have to be excluded due to possible contamination issues with the DNA profiling.

3.3) Sample collection, storage and humic acid spiking.

Samples of peripheral blood (5-10ml-EDTA) from three donors will be collected by a registered phlebotomist (NHLS) on separate occasions to prevent DNA contamination. Prior to processing, a 20 µl aliquot will be set aside for white cell concentration determination using a Z2 Coulter particle count size analyser (Beckman Coulter). After collection the blood samples will be immediately spiked with various concentrations of humic acid inhibitor prepared in PBS at 0, 0.5, 1.5 and 2.5 mg/ml (final concentration), transferred onto FTA elute™ cards (100 µl of blood: humic acid mixture) and kept in FTA barrier pouches at room temperature until extraction. A 10-25 µl volume of each spiked blood sample will also be immediately extracted using the DNA IQ™ system (Promega). The DNA IQ™ and FTA extracted DNA will be stored at -20°C until required for quantification and SRT genotyping.

The saliva (2ml) samples will be collected in Oragene™ (OG-500) DNA self-collection kits. Prior to analysis a 20 µl sample will be set aside to determine the white cell count of the sample using a Z2 Coulter particle count and size analyser (Beckman Coulter). Saliva is first released from the Oragene™ storage device, spiked with humic acid (prepared in PBS at 0, 0.5, 1.5 and 2.5 mg/ml) mixed well and then transferred onto FTA cards for storage (100 µl of this saliva: humic acid mixture). Using 10 µl of this mixture transferred onto a buccal swab, samples will be immediately extracted using the DNA IQ™ system. The extracted DNA samples will then be stored as described above.

3.4) Crude soil samples

A small sample of natural humic acid rich soil can be sourced from the vegetative environments around the UCT region or from local botanical gardens. For sandy loam soils which have less organic matter than forest loam, a sample may be obtained from natural sandy garden soils. A soil with intermediate organic matter content such as silt clay loam may be sourced from a typical rose garden. To validate the adaptability of these extraction systems to forensic field work samples, blood and saliva samples are spotted onto these various soil types. The collected soil biological samples are transferred directly onto FTA cards or onto a buccal swab for DNA IQ™ extractions.

3.5) FTA DNA extractions

Following transfer of the humic acid treated blood and saliva samples or the soil-exposed samples to the FTA™ cards, the samples will be air dried overnight in a protected environment. A 3 mm Harris uni-core FTA™ punch is used to provide a sample for the extraction as the cells are lysed upon contact with the FTA elute™ cards and the DNA is trapped in the specialized matrix. The DNA containing FTA™ disc is processed in an attempt to remove inhibitors by washing several times with FTA™ purification reagent and wash buffer (1.0 M Tris-HCl, pH 8.0, 0.1 M EDTA). Elution of DNA is achieved from these discs by heating at 95°C for 30 minutes in nuclease-free water (100 µl); this elution step is also known to separate DNA from inhibitors. DNA will be stored at -20°C until required for quantification. FTA™ discs with no sample added will be processed with the samples and used as extraction blanks to ensure the integrity of the extraction process.

3.6) DNA IQ™ extractions.

DNA extraction from both the humic acid treated blood and saliva samples, as well as the soil-exposed samples will also be performed using the DNA IQ™ extraction system (Promega, USA according to the manufacturer's recommendations for each respective sample type. The DNA IQ™ kit contains a silica coated magnetic bead resin designed to capture a consistent amount of DNA. The DNA bound magnetic beads are held in place using a magnetic stand so that the contaminants in the sample are washed away. Liquid blood samples are processed by directly applying 10-25 blood to the extraction and saliva samples are processed by first transferring 100 µl saliva onto buccal swabs. The general extraction procedure includes the incubation of the samples for 30-60 minutes in a proteinase k/incubation buffer solution (Promega), to lyse the cells and degrade proteins. The samples are then mixed with the magnetic beads to specifically bind to the DNA. The DNA-magnetic bead complexes are captured as a pellet using a magnetic stand and washed several times with wash buffer (Promega) to remove contaminants. The elution buffer allows the beads to be detached from the DNA, which is then released into the buffer in the presence of the magnet. DNA will be stored at -20°C until quantification is required.

3.7) Quantiplex™ qRT-PCR assay.

The Investigators Quantiplex™ PCR assay (Qiagen,) is a forensic based human DNA quantification assay which is also designed to indicate the presence of PCR inhibitors. A specific human genetic target is amplified using real-time PCR, with the aid of novel Scorpion® primer chemistry. These primers are sequence-specific primers that are attached to a quencher which is

linked to a fluorescently labeled probe. Upon product formation, the fluorescent probe linked to the primer is released from the quencher and binds to the PCR product, releasing fluorescence and allowing monitoring of the amplification process. Through the use of a serial dilution series of provided positive control standards (DNA Z1) and the generation of a standard curve, the sample DNA is able to be quantified by comparing the crossing points, using a RotorGene Q (Qiagen) qRT-PCR platform (green channel). Amplification of the internal control 200-bp fragment is monitored using the yellow channel. This target is much more sensitive to the presence of inhibitors and the crossing point allows an assessment of the extent of the inhibition. (Pasqule *et al*. 2011 & Qiagen 2012).

For each donor, the samples will be analysed in sets, including the extraction blank and samples extracted after to exposure to 0, 0.5, 1.5 and 2.5 mg/ml humic acid. The sample with no exposure to the humic acid serves as the positive control for each set and serves as the reference point for DNA quantification and the effect on the internal control amplification. A set of DNA standards (DNA Z1) will be prepared and analysed in each run to enable quantification. For each donors blood and saliva samples, the DNA is quantified for both FTA elute™ and DNA IQ™ extraction in replicate sets, therefore inhibitor removal abilities are also interpreted for these replicate sets (see flow diagram Appendix V).Each set is to be performed independently to evaluate the efficiency and reproducibility of the results.

3.8 A non-PCR based DNA quantification assay.

Given that the presence of humic acid affects the efficiency of PCR, the primary qRT PCR quantification assay would also be negatively affected. To ensure that the extraction process itself is not affected by humic acid, a second, non PCR quantification method would be employed to quantify the DNA. The Qubit® (Invitrogen) assay is a fluorescent based assay which would also be used to quantify the DNA in the presence of humic acid.

3.9) STR genotyping (DNA profiling)

The extraction of DNA and removal of inhibitors is ultimately intended to allow the production of full DNA profiles devoid of artifacts. To evaluate the inhibitor removal ability of both purification methods, STR genotyping will be used as end-point analysis to support whether sufficient inhibitors were successfully removed. This end-point analysis is achieved by interpreting the DNA profiles as either full genotypes, partial genotypes or failed genotypes. The ability to provide DNA profiles devoid of artifacts is another benchmark to evaluate if the

purification strategy has removed sufficient inhibitors. Currently one the most discriminating and widely used DNA profiling kit is the PowerPlex® 16 kit (Promega, USA). This multiplex genotyping kit amplifies 16 loci, this includes all 13 core loci of the Combined DNA Index System (CODIS), two Penta nucleotide loci and the Amelogenein loci for gender determination. For DNA profiling, the amplification of the STR loci is achieved using the the GeneAmp® PCR 9600 thermal cycler and the amplified loci repeat lengths are analysed with capillary electrophoresis on the Applied Biosystems (ABI) 3130xl Genetic Analyser with GeneMapper® data analysis software. The internal lane standard 600 is included in each capillary injection to assign sizes to DNA fragments separated by capillaries electrophoresis and an allelic ladder mix is also electrophoresed with every genotyping experiment to ensure the correct allocation of allele sizes. For each STR experiment performed the provided human DNA control, with a known DNA profile is included, as well as the appropriate extraction blank control.

4) Impact and predicted outcomes.

Establishing a reliable, efficient and rapid DNA purification method for soil contaminated human blood and saliva samples would have the greatest impact in cases were only traces of these samples are available for the investigation. It would provide valuable knowledge to the forensic community and allow successful use of these types of samples. Using the FTA™ cards to collect these samples on the crime scene would simplify the collection and storage process. Ability of FTA™ cards to remove humic acid may also gain interest from the forensic microbiology community as an alternative to purify inhibitor free microbial DNA from soil. Altogether, the FTA system would provide a faster and economical soil inhibitor removal system as it is well established for the automation of large batches of samples. As an alternative, the DNA IQ™ system is more sensitive and has been reported to remove humic acid, however an in-depth validation study reporting the humic acid concentrations, the volume of biological sample and efficiency of inhibitor removal would provide more reliable selection criteria for using this kit for soil contaminated samples.

The intended outcomes for this research would be to make the findings available to the forensic community by publishing in a recognised forensic journal such as: “The Journal of Forensic Science”. This research is undertaken in peruse of graduating with a Masters (Msc) degree in biomedical forensic science by December 2013.

5) Ethics

Participants will be sourced using a flyer (Appendix I). The Msc student involved in this study, which has a strong molecular forensic background and knowledge of the declaration of Helsinki 2008, the National Health Act 61 of 2003 and the regulations for using human biological material for research, will discuss the details of the project. Participants will be informed that they are under no obligation to agree to participate in the study and donate samples. The participants will be informed that we will be generating DNA profiles and that these may be published, although the identity of the individuals will not be revealed. The participants are required to sign the consent form (Appendix III) before samples are donated. The identity of the individual will be immediately protected by assigning a number to the samples as soon as they are taken, and only this number used for all experimental purposes.

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Appendences :

Appendix I: flyer for participant recruitment

CRIME SCENE DO NOT CROSS

Forensic Science Research Project

You are invited to donate blood and saliva samples as a DNA source for research purpose.

Purpose of this study:

In the field of forensic science the collection of blood or saliva samples from soil found at a crime scene may contaminate the DNA sample. This contamination may either prevent identification of a victim or the perpetrator. We believe that a combination of novel sensitive purification techniques may remove these contaminants rapidly. This may provide great benefits for the investigation if only a minute amount of blood or saliva is found in soil on the crime scene.

What is required by your participation?

We require a donation of 5-10ml blood and 2ml saliva as a DNA source. For documentation we require you to provide personal infomation such as your name, contact details and signature to enrol for the study. For collection of the samples and documentation we require less than 1h:30min of your time on a date you are available.

Who may participate?

We are looking for male or female South African participants 18 years and older that understands English, willing to share personal information and have a basic understanding of DNA profiling.

If interested in the invitation please contact: Mr Mohaimin kasu from the department of Forensic Pathology and Toxicology at the University of Cape Town.
Email: Mohaimin.kasu@myuct.ac.za
Mobile : 0839661359

Appendix II: **Participant information sheet (version 1.0 May 2013)**

Researcher: Mr M.Kasu University of Cape Town department of forensic toxicology and pathology.

Project title: Using novel DNA purification technologies to remove soil PCR inhibitors from human blood and saliva samples.

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You are being invited to participate in this study by providing some blood and saliva samples as a DNA source. Your participation is completely voluntary and may choose not to participate or withdraw from the study at any point. As a potential participant you have the right to know the purpose of this study and how your biological samples containing DNA would be utilized and protected. The information presented to you here is to allow you to make an informed decision whether to accept or decline this invitation.

What is the purpose of this study?

In the field of forensic science soil-contaminated blood and saliva samples are often found. The problem is that these samples are contaminated with an inhibitor called humic acid, which prevents successful analysis of these samples in downstream applications. As a result, these important samples are often not collected from the scene and used as evidence. Our study is aimed at proving which extraction system is the best for dealing with these soil-contaminated samples, thus making the analysis of these important samples more plausible in the South African Forensic setting.

What is required by your participation in the study?

You are required to provide 5-10ml blood and 2ml saliva for the study. Collecting the blood would require insertion of a needle into your arm vein by an individual licensed to perform the procedure. For the collection of saliva you are required to fill-up 1 tube with 2ml saliva, after a fasting period of 1 hour.

What is the duration of the study?

This study would take place from May 2013 to November 2013. After this period the blood and saliva samples may not be used for further experiments or future research. The samples are discarded appropriately so that no further tests could be performed.

How would your confidentiality be maintained?

To keep the donations anonymous, during sample collection codes are provided to label the samples, labeling these samples would have no link to you directly. The DNA content provided by the sample cannot be used for any other research or diagnostic tests and cannot be used against you in any criminal investigation.

Appendix III : **Consent form**

Donating blood and saliva samples for research

Study title: ***Using novel DNA purification technologies to remove soil PCR inhibitors from human blood and saliva samples.***

Please indicate in the space provide whether you understood the following:

1) I confirm to have read and understood the information sheet version 1.0 May 2013 for the above titled study. I was able to ask questions and the answers provided were satisfactory. ☐
2) I understand that blood and saliva samples required for this study are to undergo DNA analysis. ☐
3) I understand that my blood and saliva samples would only be used for the indicated purpose and duration provided on the information sheet. ☐
4) I understand that confidentiality is to be maintained and that no diagnostic tests can be performed and reported on the samples ☐
5) I acknowledge that no personal benefits are promised and that I understood the risks involved. ☐
6) I understand that participation requires sharing of personal information such as my name and contact details and signature. ☐
7) I agree that deciding to participate was voluntary and that I was not forced to participate. ☐

Please indicate if you give consent to:

Provide 5-10ml blood and 2ml saliva as a DNA source.	☐ YES	☐ NO
Have your DNA profile as part of a scientific publication.	☐ YES	☐ NO

I..... (name of donor) consent to take part

in this study as explained to me by Mr .....

..... (signature of donor) ..... (date)

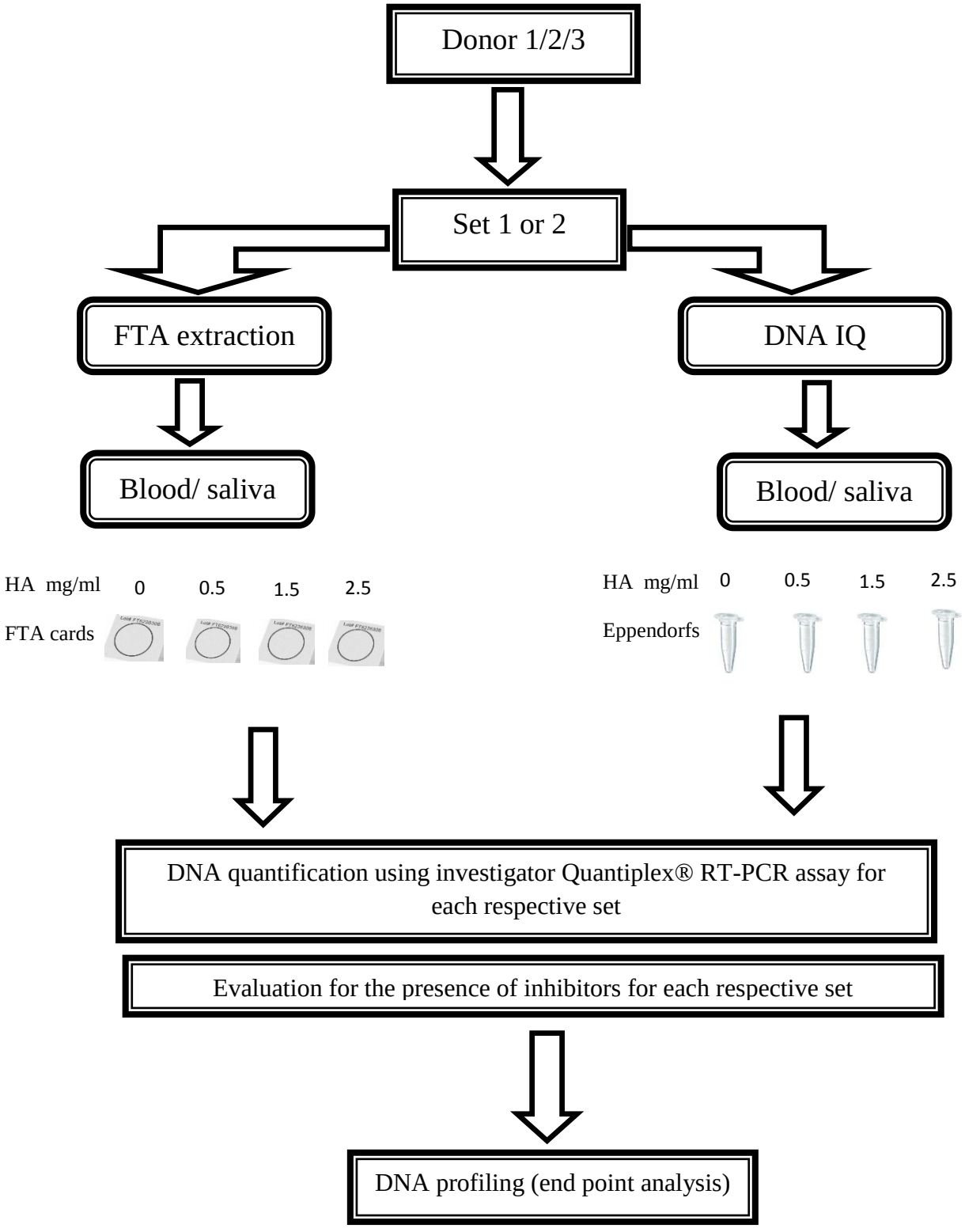
..... (signature of researcher) ..... (date)

..... (signature of witness) ..... (date)

Mr Mohaimin kasu and Dr Karen Shires
Department of Clinical Laboratory Sciences
Division of Haematology
Mobile: 0839661359 /Office tel: 0214066673

Human Research Ethics Committee
Professor Marc Blackman
Office tel: 0214066492

Appendix V: **Flow diagram showing experimental sets for each donor.**



Budget and Manufacturers/distributors contact details.

Materials	Price	Manufacturers/Distrabutors
1) FTA technologies:		
FTA purification reagent(500ml)	R 3 998.22	**Sigma-Aldrich** Pty. Ltd. Johannesburg, South Africa Phone: 27 11 979 1188 Fax: 27 11 979 1119 E-mail: rsa@sial.com or Leisl.brand@sial.com
2X (25 FTA micro elute cards)	R 3 011.38	
1.0 M Tris-HCl, pH approx. 8.0	R 263.38	
containing 0.1 M EDTA		
Harris cutting mat	R 441.07	
Harris 3mm FTA punch	R 1 301.02	
100 multi barrier pouches	R 729.84	
FTA tech Total :	**R 9 744.91**	
2) Sample collection, storage and prep		
Humic Acid (10g)	R 320.70	**SIGMA-ALDRICH**
25 Oragene™ Saliva collection kit(OG-500)	R 3 433.06	**DNA GenoTek** Lauren Scully [lauren.scully@dnagenotek.com]
3) Quantification assay		**QIAGEN**: Whitehead Scientific (Pty) Ltd Brackenfell Cape Town Tel: +27 21 944 6460 email: whitesci@whitesci.co.za
2X Investigators Quantiplex® kit (200)	R 15 742.10	
4)DNA IQ system:		**Promega:** Anatech Instruments (Pty) Limited Distributor Cape Town Fax: (27) (0) 21 946 4276 Tel: (27) (0) 21 946 2207 E-mail Address: sales@anatech.co.za Web Address: www.anatech.co.za
2 XDNA IQ kit 100 RXN	R 9 132.00	
Proteinase K and incubation buffer (100)	R 3 493.00	
Total	**R 41 865.77**	

PART B: Literature Review

1) Soil contaminated evidence in forensic science.

1.1) The relevance of extracting DNA from soil.

The extraction of human / non-human DNA from soil samples is of relevance to a diverse forensic community. On an outdoor homicide or rape crime scene, especially taking place on an open field or vegetative catchment, the deposit of human blood, saliva or semen is likely to occur in soil (Wang *et al*. 2008; Alpay *et al*. 2008 and Shahzad *et al*. 2011). Additionally, dumping of human bodies or human remains in forest locations is frequently observed in homicide and rape cases in an attempt to prevent the discovery of the deceased by the authorities (Ambade *et al* 2011). In forensic anthropology, the victims recovered from the mass graves of war, or as a result of disasters such as tsunamis or hurricanes can be identified using DNA isolation from decomposed remains or skeletal remains deposited or buried in soil (Kontsevaia 2013; Niemcunowicz-Janica *et al*. 2006). Additionally, in forensic microbiology, the transfer of soil between the perpetrator and the crime scene or with the victim can also be matched using microbial DNA typing from the soil sample (Horswell *et al*. 2002). In all these situations the potential contamination with various inhibitory compounds in soil remains a huge problem for production of full DNA profiles.

1.2) The scope of soil contaminated evidence in rape and homicide investigations.

In countries such as South Africa, India and Zimbabwe were rape remains a major crisis and informal settlements (townships/slums) are frequently observed, improving the use of soil contaminated biological samples may be particularly important. The International Criminal Police Organization (INTERPOL) has allegedly recently named South Africa the rape capital of the world, with an incidence of rape believed to occur every 17 seconds (SABC 2012, http://www.sabc.co.za). Additionally, the incidence of child rape and gang rape in South Africa has been recognized for many years as one of the highest in the world (Hirschowitz & Orkin 2000; Jewkes *et al*. 2009).According to national crime surveys for South Africa, sexual offences including underage offences are frequently reported to take place on an isolated field, in the park or in some cases along the streets of residential areas (33.6% - 34.2%) (Statistics South Africa 2011 and 2012). Likewise, multiple perpetrator rape (gang rape) in South Africa is more often committed in isolated outdoor locations such as on an open field, in an alley or road (47.6%) rather than in the victim or perpetrators home (24.7%). As a result, in gang rape

the victim may frequently be abducted (60%) so that the sexual offence is committed in an isolated location (Jewkes *et al*. 2012).

In investigations of homicide, it is common criminal behaviour to bury or discard the remains of the victim at a secondary crime scene (Turvey 2008). Although the secondary crime scene may be any particular location, in cases of murder, the victim is often dumped in an isolated forest type environment (Ambade *et al*. 2011), as the degree of isolation and amount of vegetation may significantly influence discovery of the victim before decomposition. Informed criminals may also know that forest soils are rich in diverse microbial and insect species which together increases the rate of soft tissue decomposition and reduces the chances of victim identification (Haslam & Tibbett 2009). Acidic forest soils also prevent bone preservation and may promote the rapid disintegration of human skeletal remains, leaving behind minimal evidences (Carter & Tibbett 2008). Larger predators or scavengers are more likely to feast on the remains in forest locations which lead to even faster decomposition rates. Besides, when rain and temperature is elevated in a forest, the conditions of the environment promote the rapid decomposition of soft tissue, especially for the smaller and thinner bodies of neonates (Archer 2004). Provided with these motivations, the perpetrator may deposit evidence from the victim or from themselves in the topsoil either during the act of the crime or when attempting to discard the human remains. In cases were an abduction takes place and the victim struggles, biological evidence deposited potentially on outdoor locations from either party can be essential to lead the investigation from the primary abduction location. Although the time period after depositing blood in topsoil significantly influences its visibility and whether DNA integrity is suitable for profiling, it has been established that dried blood can be detected in the soil using Luminol (Stene *et al*. 2004) and that full STR profiling can be achieved using dried blood stained soils as old as 10 days (Shahzad *et al*. 2009).

1.3) Identification of human remains from mass graves and mass disasters.

The issue of soil contamination still remains a problem in the forensic anthropology arena. Identifying victims of war recovered from mass graves are prioritised in forensic anthropology, not only to return the remains and provide closure to siblings, but also to pay respect towards the soldiers and civilians of war crimes and crimes against humanity (Kontsevaia 2013). The application of DNA profiling in this regard has evolved through investigations of some of the most devastating genocide crimes committed against humanity. In the Cambodian massacre (1975-1979), where approximately 1.7 million victims were executed and dumped in more than 20 000 different mass graves, DNA analysis proved to be critical for identifying missing persons in the masses (Menzel 2007). DNA profiling from skeletal remains was also used extensively to identify missing persons of the Bosnia-Herzegovina war (1992-1995), which involved more than 20 different massacres including the Srebrenica massacre (Primorac *et al*.1996; Alonso *et al*. 2001; Herman 2006), for which, according to the International Commission of Missing Persons (ICMP) more than 6000 out of the 8000 missing were identified using DNA profiling (ICMP July 9, 2009). DNA from skeletal remains was also used by the ICMP to identify 902 bodies from the Thailand tsunami (2004), 350 people from Hurricane Katrina (2005) and many other mass disaster recovery initiatives (ICMP, 2008)

The revolution of DNA profiling in the field of forensic anthropology would not have been as successful if innovative DNA purification techniques were not continuously being developed. Apart from designing optimal protocols to extract DNA from bones and teeth, DNA purification methods were also continuously adapted to remove various contaminants in soil. The contaminants known as fulvic acid and humic acid were in particularly being targeted for removal, as many reported it to co-extract with DNA recovered from ancient skeletal remains (Tuross 1994 Stutlovic 2005, 2007 & Kemp *et al*. 2006).

1.4) DNA typing of soil microbial organisms.

According to Locards exchange principle originally postulated by Edmond Locard (1877-1966), the perpetrator and the victim may exchange trace evidence between each other and with the crime scene. When a perpetrator attempts to bury or discard human remains in remote forest locations, the transfer of soil onto the perpetrators shoes or clothing may be important evidence to link the perpetrator to the crime scene or victim. In forensic investigations soil analysis using physical observations such as colour, texture and microscopic composition have been used previously for linking the perpetrator to the crime scene (Dawson and Hillier 2010). Linking two samples of soil is also effective by characterization of the microbial population diversity and community composition between the samples (Horswell *et al*. 2002). The extraction of DNA from microbial populations in the soil allows for microbial DNA profiling, where a terminal restriction fragment length polymorphism (TRFLP) is the "fingerprinting" technique of choice (Horswell *et al*. 2002). However, to achieve successful DNA profiling from microbial species, the DNA would firstly require purification from the soil samples. Although several specialized microbial DNA soil purification kits are commercially available, it is not always guaranteed to provide a contaminant free and useful DNA sample as experienced by Meyers and Foran (2008).

2) Humic acid: The major organic constituent of soil.

Humic acid, which is the main organic component of humus in soil, peat, compost and fertilizers forms part of many important biochemical pathways. Its formation naturally is not restricted to a single mechanism, instead several theories are postulated describing its complex formation from the decay of both animal and plant matter in the soil. As the key ingredient of a productive soil, humic substances may stimulate the growth of plants, promote the survival of microbial organisms and are important components of the soil carbon cycle. Humic acid forms part of the life cycle of most plants by recycling the carbon components of degraded matter through the soil and back into the life cycle (Chen *et al*. 2004). It achieves this effectively by having a large organic structure constituting a mixture of various molecules, usually aromatic phenolic and carboxylic substituents, which may be conjugated with polysaccharides (sugars) and polypeptides (proteins) (see Figure 1).

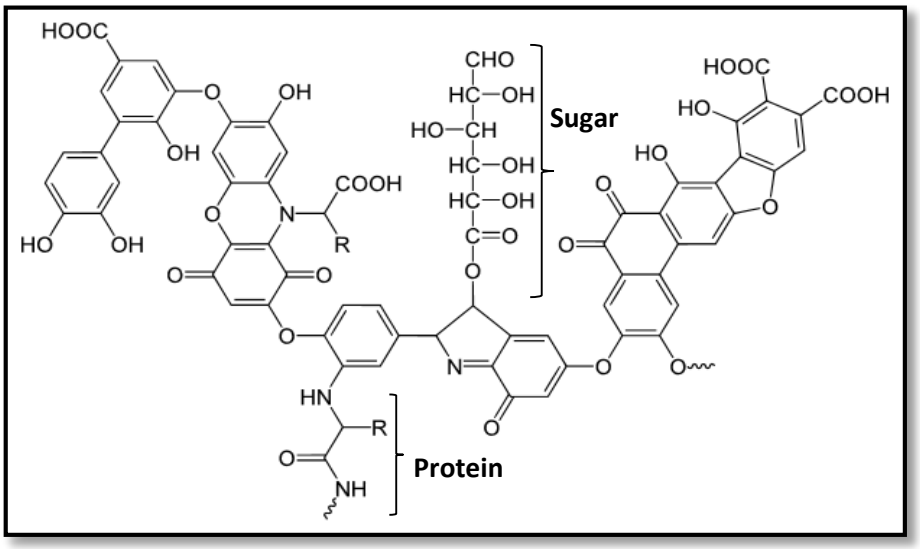


Figure 1: Humic acid:

A representative chemical structure of humic acid indicating interactions with polysaccharides (sugars) and polypeptides (proteins) (taken from Stevenson 1994).

The ability to interact with proteins and sugars also allow humic acid to host chemically active functional groups such as carboxyl, carbonyl, phenolic and hydroxyl moieties, which altogether defines its diverse characteristics and function in the soil (Stevenson 1994; Trevisan *et al*.2010). The numerous carbon bonds in humic acid provide a source of energy for many soil microbes, especially for the non-photosynthetic microbial species (Sellamuthu & Govindaswamy 2003; Qi *et al*.2004). The large organic structure of humic acid provides a large surface area which allows effective water retention, a property exploited for growing crops during drought periods (Rawls *et al*. 2003; Hueso *et al*. 2012). The charged species

associated with humic acid makes it a powerful chelating agent, binding to metal ions such as magnesium (Mg), iron (Fe), copper (Cu) and calcium (Ca) allows these essential minerals to be exchanged with the plants roots via the chelation exchange reaction (Wallace 1963; Akinci *et al*.2009). Furthermore, with its chelating and powerful reducing abilities, humic acid prevents the plant from taking up various toxic pollutants from the soil. (Livens 1991; Davis *et al*. 2001).

Although the humic acid content of a given soil is largely dependent on the type of soil and its regional location, one study demonstrated a humic acid concentration range between 5.0-7.63mg/g for natural soil samples (Tebbe & Vahjen 1993). Loam sand sampled from natural botanical gardens was however reported to have a humic acid content as high as 25 mg/g (Lakay *et al*. 2007) and it is expected to be even higher in other loam samples with a higher organic content i.e. forest clay loam (44.6%) and rainforest silt loam (22.0 %) (Schneegurt & Dore 2003). Samples taken from arid forest environments showed lower humic acid concentrations of 2.0- 2.6mg/g, but this was still more abundant than the fulvic acid content (Abril *et al* 2013). Humic acid may therefore be considered the most organic rich compound found in various natural soils and are key compounds for the survival of various soil organisms.

3) The inhibitory effect of humic acid on DNA profiling.

3.1) Targeting components of the Polymerase chain reaction.

The development of the polymerase chain reaction (PCR) to amplify DNA (Mullis & Faloona 1987) quickly enabled the development of highly sensitive and discriminative DNA profiling systems for human identification. In most forensic DNA testing laboratories, short tandem repeat (STR) markers, which are repeated regions of non-coding DNA, are amplified using the principle of PCR to produce a unique allele pattern (DNA profile) by separation of the STR fragments using capillary electrophoresis. Unfortunately humic acid, so abundantly present in soil, inhibits the production of full DNA profiles, which in turn severely restricts the use of soil contaminated evidence.

Firstly, to understand the inhibitory mechanisms of humic acid on DNA profiling, it is essential to briefly indicate the components of a PCR reaction integrated with DNA profiling assays. Basically the DNA template (STR repeat region) is amplified using the enzyme Taq polymerase isolated from the bacterium *Thermus aquatics* (Chien *et al.* 1976).This is achieved using a fluorescently labelled primer that is designed to bind to the 5' region flanking the STR repeat sequence and a second unlabelled primer which binds to the 3'region (see Figure 2). Taq polymerase would then extend the double-stranded primed region in the 5' to 3' direction, by incorporating the building blocks of DNA known as deoxyribonucleotide triphosphates (dNTP's). Furthermore, for this to occur successfully Taq polymerase requires Mg^{2+} as a co-factor for the extension of the DNA template and for the efficient binding of the primers.

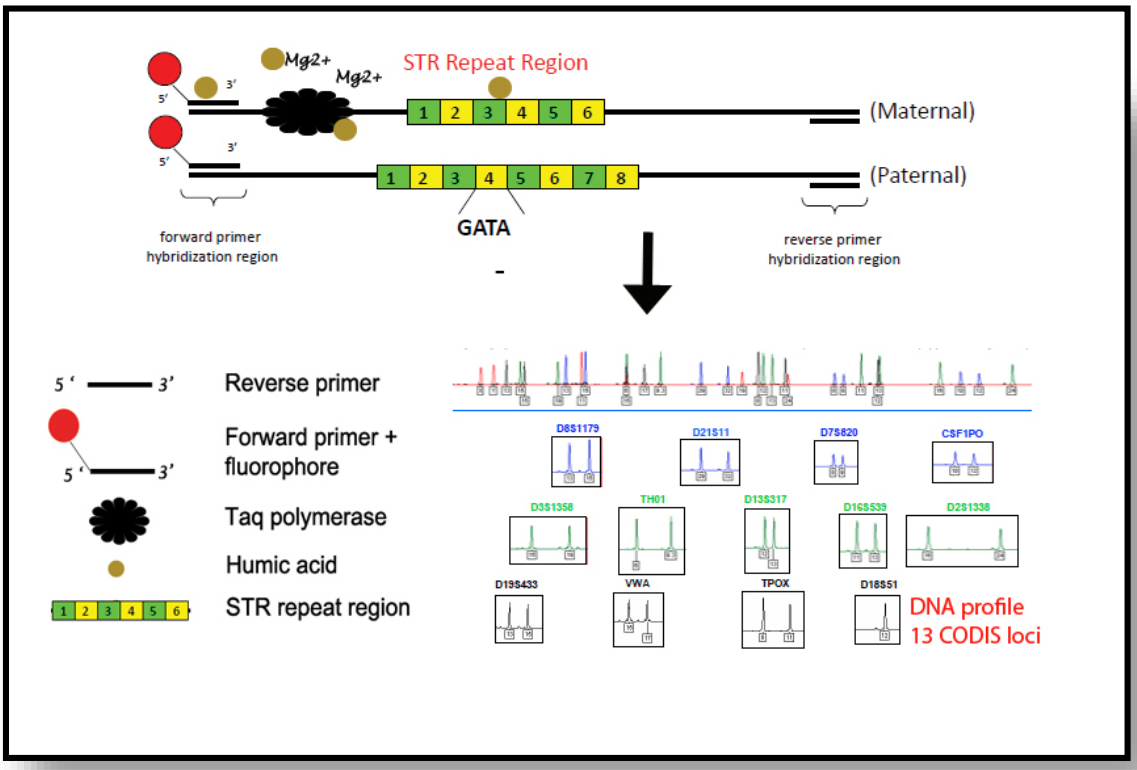


Figure 2: The DNA binding abilities of humic acid affects most PCR components of DNA profiling assays. Humic acid prevents effective amplification of the STR region in several ways. A) Humic acid can bind directly to the DNA STR repeat region and prevent amplification. B) Humic acid bound to Taq polymerase prevents DNA synthesis. C) Humic acid chelating effect on Mg^{2+} prevents extension of the STR region by Taq polymerase and D) Humic acid binds to primer linked flourophores and prevents STR amplification.

It has been shown that humic acid affects DNA amplification by interacting with most of these PCR components (Tsia & Olson 1992; Matheson *et al*. 2010; Hedman 2011). Humic acid has similar physiochemical properties to double stranded DNA (Harry *et al*. 1999). This similarity

allows it to compete with the DNA for silica binding and actually co-elutes with the DNA under similar conditions, when using the standard silica based DNA extraction techniques (Harry *et al*. 1999; Miller *et al*.1999). Once co-eluted, the chelating abilities of humic acid may chelate Mg^{2+} in the PCR reaction, which affects the activity of Taq polymerase (Tsia & Olson 1992). Due to its protein and nucleotide binding abilities, humic acid can also interact directly with Taq polymerase to cause enzyme inactivation and bind directly to the STR repeat region and the primers to inhibit DNA amplification (Tsia & Olson 1992: Matheson *et al*.2010; Opel *et al*.2010) (see Figure 2). To achieve successful DNA profiling from humic acid contaminated evidence, it is therefore essential to try to remove as much of the humic acid as possible during the DNA extraction process, because as little as 1 ng/µl of humic acid in the PCR reaction is known to inhibit DNA amplification (Randstrom *et al*.2004 ; Menkings *et al*.1999).

3.2) Effect on accurate DNA quantification.

Humic acid further influences the success of DNA profiling by interfering with the quantification of the extracted DNA. The process of DNA profiling, which includes both PCR and capillary electrophoresis is sensitive to the amount of DNA used, and by introducing too much/ too little DNA, the chances of a partial or failed DNA profile significantly increases. Quantification of the DNA prior to STR analysis thus plays an important role in generating a useful profile and is particularly important when extracting DNA from trace evidence. Unfortunately, the presence of humic acid in the extracted DNA sample affects most DNA quantification systems. Ultraviolet (UV) DNA quantification techniques (i.e.: spectrophotometry) cannot be utilised in the presence of humic acid, as it also absorbs in the UV spectrum and as a consequence the calculated DNA concentration is usually overestimated (Cullen *et al*. 1999). Using fluorescent DNA intercalating dyes such as picogreen and hoechst to quantify the DNA in the presence of humic acid is not specific enough for human DNA, and is therefore not ideal for forensic applications (Zipper 2003; Bachoon *et al*. 2001). Furthermore, humic acid also affects DNA quantification when using Real-Time PCR (RT-PCR) techniques through similar PCR inhibitory mechanisms indicated in figure 2 (Opel *et al*. 2010). However, due the superior sensitivity and specificity offered by this technique, most forensic based RT-PCR quantification assays are adapted to detect the presence of various PCR inhibitors and at least allow the investigator the opportunity to attempt inhibitor removal prior to STR profiling (Green *et al*. 2005; Qiagen 2011).

The Investigators Quantiplex® RT-PCR assay (Qiagen) is a forensic based human DNA quantification assay which is also designed to indicate the presence of PCR inhibitors. This multiplex assay amplifies the 146-bp human specific multicopy gene 4NS1C (~ 40 copies per cell) present in the sample and co-amplifies a 200-bp internal control(IC) DNA fragment (present in the PCR mastermix) to provide an assay with remarkable sensitivity and specificity (Qiagen 2011; Shewale & Liu 2013). Made available by Qiagen in March 2011, this RT-PCR assay was designed specifically to benefit the workflow of a forensic DNA laboratory. Based on the novel fast Scorpion® primer chemistry and a self-probing system, its design is intended to prevent the formation of less desirable elements such as primer dimers and secondary structures (Whitecombe *et al*. 1999). This is achieved using a sequence specific forward primer that is covalently bonded to a DNA probe which is linked to a fluorophore that is initially quenched (see Figure 3A). The probe is designed complementary to a DNA sequence on the human target so that it becomes part of the amplicon. Following the first round of annealing and extension, the newly formed dsDNA incorporated with the primer-probe complex is denatured to give single stranded DNA (see Figure 3B).The primer-probe complex as part of the amplicon then forms a "scorpion tail", which hybridizes to the homologous region on the amplified PCR product to release the fluorophore from the quencher (see Figure 3C). Once activated by a laser, this molecule fluoresces and allows monitoring of the amplification process through a fast unimolecular binding event. This design makes the reaction instantaneous and fluorescent signal stronger than the bimolecular collisions of Taqman® probes (Arya *et al*. 2005), as a result scorpion chemistries currently provide the fastest and most sensitive RT-PCR quantification assays. For the investigators quantiplex assay the 146-bp human target is quantified by measuring the fluorescence using the green channel (470/510nm) as depicted in figure 3D on either the Rotogene 6000 (Corbett) or the Rotogene Q (Qiagen). Using a serial dilution of the positive control DNA Z1 the sample DNA concentration is calculated with aid of a standard curve, which is capable of giving linear readings over the rage of 20– 0.005 ng/µl DNA.

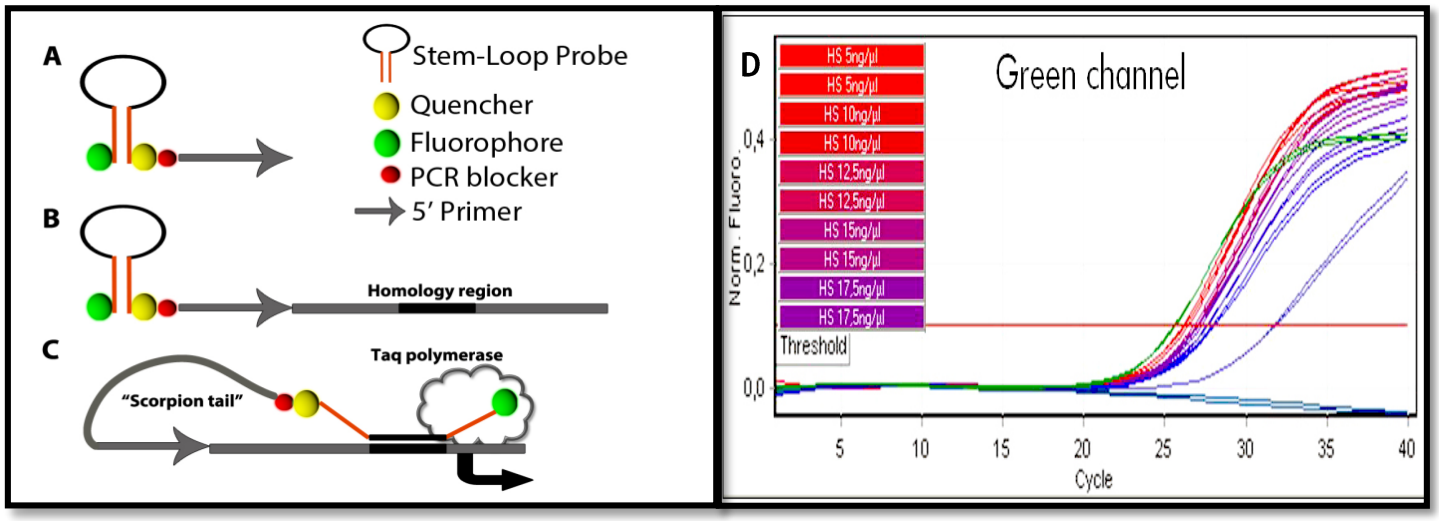


Figure 3: Scorpion primer chemistry used for the investigators Quantiplex RT-PCR assay.
A) The fluorescent labeled 5' primer is covalently linked to a DNA probe via the quencher. B) At the end of the first round of annealing and extension the primer-probe complex in incorporated into the newly synthesized 146-bp dsDNA human target. C) After the subsequent round of denaturation the DNA probe hybridizes to the homology region on the human target and fluorescence is released through a unimolecular binding event. D) The increase in fluorescence is detected in the green channel using a real-time PCR instrument (i.e: RotorGene, Qiagen).

Using a second independent set of scorpion primers, the amplification of the 200-bp internal control (IC) DNA template is monitored using the yellow channel. It is the amplification of this synthetic DNA fragment which allows the detection of potential inhibitors that co extract with the DNA. During the developmental validation of the Investigators Quantiplex RT-PCR assay, the DNA Z1 standard was spiked with humic acid concentrations from 0 to 50 ng/µl .It was established that DNA quantification using this methodology is reliable up to a final humic acid concentration of 20 ng/µl in the DNA sample (Qiagen 2011). At humic acid concentrations above 20 ng/µl the amplification of the IC is inhibited, which is reflected in an increase in the IC Ct value. This system to identify the presence of inhibitors has also been validated for the forensic based Quantifiler kit and Quantifiler Duo kit from Applied Biosystems (Green *et al*. 2005 ; Barbisin *et al*. 2009). Thus this RT-PCR system allows for the detection of significant amounts of humic acid contamination, allowing the investigator a chance to improve sample purity. It also allows for a certain degree of surety in the DNA quantification process if IC Ct values are not adversely affected.

4) Advances in humic acid removal.

4.1) Humic acid removal through DNA extraction.

There is clearly a need to improve the use of soil contaminated forensic samples, as they can form a critical sample set, especially in the South African setting . However, in order to use them effectively, inhibitors such as humic acid should be effectively removed during the DNA extraction process, as we have previously described the negative effect this acid has on molecular processing, especially DNA profiling . Many advances in the purification of DNA from forensic samples have been made over the years to specifically remove soil inhibitors. Some of these advances are incorporated into sophisticated DNA extraction kits specifically intended for forensic casework samples and are discussed below. However, the validation of some of these systems are not very comprehensive in terms of extracting DNA from a limited biological source contaminated with high concentrations of humic acid as expected in a natural soil.

4.2) Techniques to remove PCR inhibitors from buried skeletal remains.

DNA extraction from ancient remains such as bones and teeth found at various burial sites or mass graves is crucial for the identification of war victims (Gunby 1994; Primorac *et al*. 1996). However the DNA extracted from bones and teeth recovered from mass graves are either badly degraded or contaminated (Primorac *et al*. 1996). Several advances in research have overcome the issues of DNA degradation by using the more resistant and abundant mitochondrial DNA (mtDNA) source. However the inhibition of PCR by the soil inhibitors including humic acid is still evidently an important factor causing failure of mtDNA typing from skeletal remains (Dhanapal 2010; Lee *et al*.2010). Several novel techniques have been developed for the removal of PCR inhibitors from ancient skeletal samples, these include silica based methods (Kemp *et al*. 2006), size exclusion chromatography (Matheson *et al*. 2010) and ion exchange chromatography (Seo *et al*. 2010). Other successful attempts have been achieved by combination of various extraction techniques. Ethanol precipitation in combination with chromatography was effective in removing PCR inhibitors from 500-1200 year old human bone samples (Kalmer *et al*. 2000). Using a combination of Chelex resin and polyvinil-polypyrrolidone (PVPP) resin for removing inhibitors from dry human bones was also effective (Stutlovic *et al*. 2007). For these strategies, combining purification protocols provided better results, however a standardized procedure has not yet been described in the literature and many are time consuming, labour intensive, are not suitable for trace evidence and show little

potential for automation. Furthermore efficiency of specific inhibitor removal and successful genotyping at higher inhibitor concentrations was also found to be a problem with some of these strategies (Stutlovic 2007 ; Panday 2011; Seo *et al*. 2010). Nevertheless, an effective system to extract DNA rapidly from a limited calcified tissue source with removal of PCR inhibitors is offered in the Prepfiler BTA Kit from Applied Biosystems. Based on sophisticated magnetic bead chemistry and an innovative BTA® (bone, teeth, adhesive) lysis buffer provides a system that is now automated for DNA extraction from these challenging sources(Stray *et al*. 2009; Applied Biosystems, 2012; Davis *et al*. 2012).

4.3) Magnetic bead purification technologies.

The invention of micro polystyrene beads of uniform size followed by magnetization has revolutionised several research fields. These micro magnetic beads are designed with a unique outer layer which may target particular cellular compounds of interest (Ugelstad *et al*. 1988). Using an external magnetic source these compounds are rapidly isolated from the biological specimen and separated from the undesired components with remarkable enrichment. For example, in cancer research, magnetic beads coated with antibodies have for many years simplified the extraction and enrichment of specific tumour cell from bone marrow (Kyalheim *et al*. 1987) and circulating tumour cells from peripheral blood(Yu *et al*. 2011). Despite revolutionising medical research in diagnosis of disease and the study of its progression, these technologies only entered the forensic arena around 2001 (Madrekar *et al*. 2001). One of the first commercially available forensic DNA extraction kits based on this technology was the Change Switch™ DNA extraction kit offered by Invitrogen 2004. The magnetic bead in this case was coupled with an ionisable nucleic acid binding ligand with a charge that can be switched in a pH dependent manner. This kit has been validated on a variety of common forensic samples, including bones, teeth, cigarette buds, saliva stains and touched DNA samples (Johns 2006; Barbaro *et al*. 2008). It is shown to be extremely effective in extracting DNA from trace amounts of evidence (Johns 2006), however very few validation studies are published on its ability to specifically remove humic acid inhibitors and some indicate that it may not be effective in this case (Rawling 2010).

The first official forensic magnetic bead extraction kit which is currently one of the most developed for automation , is the DNA IQ™ kit from Promega 2001. Its magnetic bead is coated with a silica matrix which has an affinity for the DNA molecule. The magnetic bead bound DNA is separated from the unbound components using an external magnetic source followed by washing away contaminants and finally releasing the DNA from the beads

(Frégeau*et al*. 2010). This system is reported to deal effectively with blood, saliva, bone, decomposed tissue and various trace evidence with effective separation of the DNA from several PCR inhibitors, including humic acid (Mandrekar *et al*. 2001& 2002; Heranz *et al*.2008). However, the majority of these studies are manufacturer's validation reports which are not published in peer reviewed articles. These reports lack details on the humic acid inhibitor concentration tested, the volume of biological sample used and the efficiency of inhibitor removal (Mandrekar *et al*. 2001). In one of these reports (Heranz *et al*. 2008), a wide range of inhibitor containing samples were validated for efficiency of DNA extraction and inhibitor removal on a robotic workstation, but only one blood stained soil sample was analysed. The humic acid concentration of the soil sample was not reported and the amount of blood deposited in the soil was not communicated. Although some validation studies of the magnetic bead system have artificially spiked biological samples with humic acid (Brevnov *et al*. 2009), the humic acid concentrations in some natural soils may be much higher than the concentrations introduced by these platforms (Matheson *et al*. 2010; Lakay *et al*. 2007). Unfortunately, as humic acid can also bind to the silica coated matrices not only will it lower the amount of DNA that can be extracted from a sample (competition for binding sites), but the contaminate will also co-elute with the DNA (Harry *et al*. 1999; Miller *et al*.1999), making it questionable weather sufficient decontamination can be achieved with the DNA IQ™ silica magnetic system.

The most widely validated extraction system, which again uses the magnetic bead principle is the PrepFiler™ DNA extraction kit from Applied Biosystems 2008. This magnetic bead of the Prepfiler ™ kit which uses multicomponent surface chemistry to trap the DNA on a polymer is recognized as one of the most effective inhibitor removal magnetic extraction systems (Applied Biosystems 2008; Barbaro *et al*. 2010). During validation of the Prepfiler™, an inhibitor panel containing a mixture of inhibitors was introduced for the first time to validate its magnetic bead systems (Brevnov *et al*. 2009). It included a mixture of humic acid, hematin and indigo, which was spiked into trace amounts of blood. In this study by Brevnov *et al* (2009), the inhibitor removal abilities were evaluated proficiently using RT-PCR and STR genotyping. However, only one concentration of humic acid (2.5 mg/ml) was included in the inhibitor platform to give a final concentration in blood at 0.5 mg/ml, which may not represent the full extent of the possible contamination levels in common garden soil or forest soils. The Prepfiler™ has also been established on several automated extraction systems, including the AutoMate Express™ DNA Extraction System (Applied Biosystems) and HID EVOlution™ System (Applied Biosystems), which are commonly featured in forensic DNA laboratories. Unfortunately, despite the good performance of this kit in the presence of multiple inhibitors, the product was

discontinued in November 2012 and has been replaced recently by the PrepFiler® Forensic DNA Extraction Kit (Updated) version.

4.4 FTA purification technologies.

The Flinders Technology Associates (FTA™) is another popular forensic based DNA extraction and inhibitor removal technology. The system is designed to store, extract and rapidly remove PCR inhibitors using specialized cellulose cards (FTA paper) in combination with a propriety FTA reagent. These FTA™ cards have been used to collect blood, buccal cells (Park *et al*. 2008), plants (Lin *et al*. 2000) and many other wildlife DNA sources (Smith & Burgoyne 2004). In addition to its adhesive nature, the surface of the FTA™ cards is designed with a chemical formula that lyses the cells membranes so that the DNA is released and trapped in a cellulose matrix on the cards (GE Healthcare life Sciences 2010). Its chemical formula prevents enzymatic damage and inhibits the growth of bacteria, which altogether allow the sample to be stored at room temperature with the preservation of the DNA for several years.

Standard protocol with the FTA™ cards is to take a single punch sample, wash away contaminants in a solution known to remove hematin inhibitors and insert the discs directly into the PCR reaction (GE Healthcare life Sciences BD08). However, the FTA™ systems ability to capture a crude soil sample and remove specific soil-based PCR inhibitors has not yet been evaluated. The DNA extraction procedure is also one of the most simple, fairly rapid and easily automated systems (Rockenbauer *et al*. 2009), which may be of interest to a diverse forensic community with regard to processing soil contaminated evidence.The FTA™ system is also robust with regard to DNA profiling, as processing the same FTA™ sample disc repetitively though the extraction can improve DNA yield from nanograms to micrograms (Stangegaard *et al*. 2011). The primary advantage of the FTA™ system is that samples are protected from degradation for years and can be extracted and analysed at a later date, as opposed to standard DNA extraction methods which require immediate processing. Furthermore, given its adhesive characteristics these cards may provide an effective “on the scene” collection and preservation strategy for soil contaminated biological evidence.

5) Advances in PCR technology to overcome humic acid contamination and inhibition.

Apart from adapting DNA extraction systems to maximize inhibitor removal, molecular modifications made to quantitative RT-PCR and STR profiling systems have also been introduced to tolerate a broad spectrum of PCR inhibitors. A major focus has been the introduction of inhibitor tolerant Taq polymerases into DNA quantification and DNA profiling kits to allow these systems to tolerate specific inhibitor concentrations (Eilert & Foran 2009; Barbisin *et al*.2009; Applied biosystems, Rev D 2012).

It has been identified that Taq polymerases isolated from different bacteria can display various degrees of resistance to particular inhibitors. To this end, Eilert and Foran (2009) investigated the ability of ten commercially available Taq polymerases to amplify bone-derived DNA contaminated with various concentrations of humic acid, collagen and calcium PCR inhibitors. The authors identified that certain Taq polymerases were more resistant to particular inhibitor types and concentrations than others (Eilert & Foran 2009). This finding led to efforts to genetically engineer enzymes that had a broader spectrum of inhibitor resistance. Successful products displaying this feature are the recently developed OmniTaq and Omni Klentaq enzymes offered by DNA Polymerase Technology Inc (Kermekchiev *et al*. 2009). These mutant enzymes are resistance to multiple PCR inhibitors and may amplify human DNA directly from blood and crude soil samples without DNA purification being required (Kermekchiev *et al*. 2012). These Taq polymerases are highly resistant to soil inhibitors, which is evident by the tolerance to humic acid concentrations as high as 0.8ug/ml, compared to 0.024ug/ml for the standard enzymes (Kermekchiev *et al*. 2009). Recently these enzymes were shown to generate full STR profiles from blood stained crude soil samples and several other inhibitory sources without requiring DNA extractions (Kermekchiev *et al*. 2012). However, these multi inhibitor resistant enzymes have only recently been validated using a commercially available STR-profiling kit, and much more validation is required before it can be officially incorporated in forensic DNA profiling kits or RT-PCR quantification kits.

Indeed, the future developments of STR-profiling kits containing multi inhibitor resistant Taq polymerases would significantly improve the success rate of DNA profiling from highly contaminated DNA samples, such as soil-contaminated samples. It is evident that in the future these molecular modifications may even negate the necessity of DNA purification when processing traces of compromised DNA evidence

6) Conclusion.

The implication of DNA profiling to associate the accused with the crime scene or with the victim is key during investigations of rape and homicide. In particular, the DNA obtained from blood, saliva or semen deposited in the soil of an outdoor crime scene may even become the primary evidence to lead the investigation when a body is absent. However, the biological evidence collected from soil is often contaminated with substances that interfere with the process of DNA profiling. Substances such as humic acid, abundant in various natural soils, are the major cause of partial or failed DNA profiles. This contamination is particularly problematic when only a limited amount of biological sample is available for the investigation. Therefore, to limit the loss of precious trace evidence a reliable inhibitor removal technique would often be selected based on previous validated studies. Although innovative systems are continuously being developed to purify DNA from various problematic forensic samples, for many of these systems, peer-reviewed publications detailing the validation for the removal of soil-based inhibitors is either lacking or is not very comprehensive. Due to the high failure rate of DNA profiling from soil evidence and the lack of effective collection strategies, soil-contaminated biological samples from outdoor homicide or rape crime scenes are often not used for analysis. The biological evidence in such cases is usually also limited and both time and resources are not conducive to allow for experimental optimisation. There is therefore a dire need to validate an effective and reliable DNA purification system to improve the use of soil contaminated evidence, which can be communicated to our own forensic community.

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PART C: Publication-ready Manuscript

The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence.

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Abstract:

The production of full DNA profiles from biological evidence found in soil has a higher failure rate due to the inhibitory substance, humic acid. Abundant in various natural soils, humic acid co-extracts with DNA during extraction and inhibits DNA profiling by binding to the molecular components of the genotyping assay. To successfully utilize traces of soil contaminated evidence a reliable humic acid removal extraction system would often be selected based on previous validation studies. However, for many standard forensic DNA extraction systems, peer-reviewed publications detailing with the validation on soil evidence are either lacking or are not very comprehensive. The aim was to validate the common forensic DNA extraction systems, namely DNA IQ™ and FTA elute™ Nucleosave™ for processing blood and saliva from soil. To achieve this, a forensic appropriate volume of biological evidence was spiked with humic acid (0, 0.5, 1.5 and 2.5 mg/ml) and processed through each extraction protocol for the evaluation of humic acid removal using real-time PCR and STR-genotyping. While the DNA IQ™ magnetic bead and FTA elute™ systems effectively removed humic acid from highly contaminated blood and saliva, only the DNA IQ™ system allowed the generation of full STR profiles from both biological evidence types. The DNA IQ™ system also proved superior for use on DNA extractions from crude soil field samples and is thereby recommended for use in this forensic setting.

KEYWORDS: Forensic, DNA IQ™, FTA elute™, humic acid, real-time PCR, DNA profiling.

1) Introduction

On an outdoor crime scene of rape or homicide, using blood, saliva, or semen deposited in the soil to identify the perpetrator and even the victim may be crucial for the investigation (Alpay *et al*. 2008; Wang *et al*. 2008; Shahzad *et al*. 2011). The development of the polymerase chain reaction (PCR) to amplify DNA (Mullis & Faloona 1987) enabled the development of highly sensitive and discriminative DNA profiling systems. Short tandem repeat (STR) markers, which are repeated regions of non-coding DNA, are amplified using PCR and separated with capillary electrophoresis to provide a unique DNA profile. However, the probative value of this unique DNA match is contested when the DNA integrity and purity is less than optimal (Semikhodskii 2007). In the case of soil contaminated biological evidence, various contaminants present in the soil inhibits DNA amplification to the degree which

relegates the use and even collection of these potentially valuable soil forensic samples (Sutlovic *et al*. 2005; Matheson *et al*. 2010 and Opel *et al*. 2010).

Although soil hosts several PCR inhibitors, the primary contaminant responsible for the high failure rate in DNA profiling is humic acid (HA) (Matheson *et al*. 2010). HA is formed naturally by the degradation of plant and animal remains and is the chief organic compound in various soils. With a large organic structure that possesses sugar and protein binding abilities, HA provides sustenance to the diverse soil life (Livens 1991; Sellamuthu & Govindaswamy 2003; Hueso *et al*. 2012). The HA concentrations in agricultural soils are reported in the range of 5.0-7.63mg/g (Tebbe & Vahjen 1993) and 15.0-25.0mg/g for suburban garden soils (Lakay *et al*. 2007), while lower for soils of arid climates 2.0- 2.6mg/g (Abril *et al*. 2013) and considerably higher in forest soils where the amount of organic material is much higher (Schneegurt & Dore 2003).

The complication with regards to DNA profiling is that HA has physiochemical properties that resemble double stranded DNA (Harry *et al*. 1999). It co -extracts with DNA, either by binding to components of the extraction system (silica) or by direct binding to the DNA molecule itself (Harry *et al*. 1999; Miller *et al*. 1999 and Opel *et al*. 2010). Once co-eluted, HA binds to most components of the PCR reaction and inhibits DNA amplification (Tsia & Olson 1992; Matheson *et al*. 2010 and Opel *et al*. 2010). It further affects DNA profiling indirectly by affecting the accuracy of a variety of DNA quantification methodologies (Cullen *et al*. 1999; Bachoon *et al*. 2001; Zipper *et al* 2003 and Opel. 2010). Therefore, to utilize soil contaminated trace evidence, it is important to use DNA extraction technologies that are known to remove as much HA as possible, so as to maximise downstream application success. Unfortunately, although many innovative forensic based DNA purification technologies exist, peer-reviewed publications detailing the validation of the removal of soil-based inhibitors are either lacking or not very comprehensive. This provides a significant problem when faced with a limited sample volume and the use of a system that potentially will lead to DNA profiling failure.

The Flinders technology associates filter paper (FTA™ card system, Whatmans GE Healthcare Life Sciences) is one of the most widespread DNA extraction and preservation systems which may be utilized on the crime scene. FTA cards are designed with a unique surface chemical that causes cell lysis on immediate contact with the biological material. The DNA is then trapped in a cellulose matrix which can preserve the sample at room temperature for several years. The FTA cards are also designed to capture various types of crude biological evidence

(Smith & Burgoyne 2004; Lin *et al*. 2000; Park *et al*. 2008) and to endure washing in a FTA purification reagent for the removal of PCR inhibitors (GE Healthcare Life Sciences BD08). In particular, the FTA elute™ cards provide rapid decontamination by retaining inhibitors such as haematin in the matrix while the DNA is eluted (GE Healthcare Life Sciences 2010). The evaluation of this system to deal with HA contamination and the assessment of biological samples present in soil has not yet been reported. Alternatively, the Nucleosave™ card (Macherey–Nagel), which uses a similar cell lysis mechanism, is often used in a forensic environment as an alternative due to a cost saving. However, they are mainly used for collecting reference blood samples and depend on a secondary extraction system for inhibitor removal. Recent advancements in this particular technology paired a modified Nucleosave card with a silica spin column extraction system (Nucleospin Tissue®), but it was not found to be ideal for HA removal (Faber *et al*. 2013). Combining these cards with the efficient FTA purification reagent has not yet been tested in a soil environment and may provide a suitable alternative.

Another popular forensic system designed to rapidly separate DNA from various contaminants is the DNA IQ™ extraction system (Promega). The DNA IQ™ system which is based on silica coated magnetic beads is well established in forensic laboratories (Lett *et al*. 2010). Its magnetic bead chemistry is reported to extract DNA with separation from various contaminants, including HA (Mandrekar *et al*. 2001 & 2002; Heranz *et al*.2008). However the majority of these reports fail to communicate the volume of biological evidence used and the concentrations of HA tested. It therefore remains unknown if these validation studies exercised HA levels that are more likely to occur naturally. Furthermore, peer-reviewed protocols on its use on crude soil applications are currently lacking. Indeed, validation of this system with an inhibitory platform that better represents natural HA concentrations is required as to improve its reliability for processing soil evidence.

The aim of this study was to validate the DNA IQ ™, FTA elute™ and Nucleosave ™ systems for HA removal from highly contaminated blood and saliva. In validation, an inhibitor platform was established using biological evidence spiked with a final HA concentration of 0.5, 1.5 and 2.5 mg/ml. For each of the systems the removal of HA was assessed using a forensic quantitative real-time PCR (QPCR) and STR-genotyping assay. In turn, for evaluating field applications the most efficient inhibitor removal systems were tested with crude soil of high and low HA content.

2) Materials and methods

2.1) Sample preparation

2.1.1) Biological sample collection

Peripheral blood (10ml) from 2 donors was collected in separate EDTA vacutainers, while a 2ml saliva sample was also collected from each donor using the Oragene™ OG500 kit (DNA Genotek, Canada). The saliva was incubated at 50˚C for 1 hour according to the manufacturer's protocol and processed immediately. Blood and heat-treated saliva was spiked with the relevant HA preparation, incubated at room temperature for 10 minutes with vortex mixing and processed immediately using each extraction system. All samples were acquired with informed consent and the study was approved by the Faculty of Health Science Ethics Committee (HERC REF: 282/2013).Collection of the blood and saliva in this manner was intended for sample preservation as to limit DNA degradation. Although this does not truly constitute an outdoor crime scene equivalent, its purpose was to control for DNA degradation influencing the DNA profiling results, to provide an independent inference on HA.

2.1.2) Crude soil sample preparation

A humus rich soil sample (mushroom potting compost) and a white sand sample (in-land Philippi sand, no obvious humus) were collected and 100 µl blood or pure saliva was deposited in each soil type. The samples were incubated in the soil at room temperature for 10 minutes and a 0.5ml of the stained soil was used for the extraction. Phosphate buffer saline (PBS) (100 µl) was added to the preparation, mixed well and the samples were centrifuged at 14000 rpm for 10 seconds. A 50 µl volume of the supernatant was used for extraction using the DNA IQ and FTA DNA extraction methods.

2.1.2) HA preparation.

HA (Sigma Aldrich, South Africa) was dissolved in nuclease free water with the aid of NaOH pellets and buffered to pH 7. The stock HA was spiked into blood and saliva at a ratio of 1:5(v/v) to give the final concentration of 0, 0.5, 1.5 and 2.5 mg/ml HA. The inhibitory platform for blood was prepared using 100 µl blood and 20 µl of HA (0, 3, 9 and 15 mg/ml) to provide the respective final concentrations. A 120 µl pure saliva equivalent released from the

Oragene OG-500 device (240 µl) was spiked with 24 µl stock HA (0, 5.5, and 16.5, 27.5 mg/ml) to provide the respective final concentrations in saliva. For all the uninhibited biological spiked evidence a volume equivalent of nuclease free water was added to account for the dilution.

2.2) DNA extraction

2.2.1) DNA IQ™ extraction: blood and saliva.

DNA IQ™ (Promega, Madison USA) extractions for inhibited and uninhibited blood samples were performed according to the manufacturer's protocol using 25 µl of treated blood. Samples were incubated for 56˚C for 1 hour in the proteinase K 1.8mg/ml lysis buffer and the standard DNA IQ extraction protocol was followed. The saliva extractions were performed according to the manufacturer's protocol using a 50 µl treated saliva volume, with incubation at 70˚C for 30 min in the lysis buffer containing dithiothritol (1 M) and the standard DNA IQ™ extraction procedure was followed. All extractions were performed in duplicate at each spiked HA concentration for blood and saliva.

2.2.2) FTA elute™ and Nucleosave™ extraction: blood and saliva

A 50 µl volume of HA treated blood and saliva was transferred immediately onto FTA elute™ (non-indicating) and FTA elute™ (indicating) cards respectively (Whatmans, Germany), as well as Nucleosave cards (Macherey-Nagel, Germany). The cards were allowed to dry completely at room temperature before extractions. All cards were processed using the combined methodology of FTA classic cards and FTA elute™ cards. A 3 mm Harris micro punch (Whatmans) was used to sample 2 X 3 mm discs for each card type. The discs were washed 3 times by pulse vortex 5 times per minute for 5 min each in FTA purification reagent (Whatmans) and in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) (Sigma Aldrich, South Africa). Discs were rinsed 3 X 5seconds in sterile water and the DNA was eluted in sterile water by incubation at 95˚C for 30 min. The discs were pulse vortexed 60 times and removed, the eluted DNA was stored at -20 ˚C.

2.2.3) DNA IQ™ and FTA elute™ combined extractions

A 50 µl volume of HA treated blood samples were transferred onto FTA elute™ cards and 2 X 3 mm discs were sampled for each extraction. The discs were washed in FTA purification

reagent and TE buffer as described above. Discs were incubated at 90˚C for 30 min in the DNA IQ™ lysis buffer containing dithiothritol (1M) (Promega) and removed, with the lysate then processed using the standard DNA IQ™ protocol.

2.4) DNA quantification and inhibitor detection.

All extracted DNA samples were quantified by QPCR using the Investigators Quantiplex kit (Qiagen, Germany) on a Rotogene 6000 (Qiagen, Germany). Quantification was achieved by amplification of the human specific 4NS1C multi copy gene and a standard curve generated using a serial dilution of the reference standard DNA Z1 (20 ng/µl) in the range 20 - 0.005 ng/µl. The amplification was performed with 2step cycling through 40 cycles as outlined in the manufacture's protocol. The PCR amplification was detected using the green channel on the Rotogene 6000 with data analysis software version 1.7.

Co-amplification of the 200-bp internal control (IC) allowed for the detection of PCR inhibitors (amplification detected on the yellow channel). As controls for PCR inhibition, all QPCR experiments were performed with a control consisting of HA negative DNA extract (0HA) from either saliva or blood (where appropriate) and a post extracted DNA sample of the same source spiked with HA to a final concentration of 100 ng/µl.

The extracted DNA was also quantified for selected samples using the Qubit™ dsDNA HS assay (Invitrogen, USA) on the Qubit fluorometer 1.0, according to the manufacturer's instructions.

2.6) DNA profiling

DNA profiling was performed using STR genotyping methodology. The PowerPlex® 16 HS system (Promega, USA) was used for this purpose, using previously validated methodology (Molecular Haematology, Groote Schuur Hospital, NHLS), according to the manufacturers recommendations. For each extraction system, the 200 pg DNA extracted from the 0 and 2.5 mg/ml HA spiked biological evidence and from crude soil fractions was genotyped using The Powerplex 16® HS methodology (Promega, USA). The amplification of the STR loci was achieved using the GeneAmp® PCR 9600 thermal cycler and the amplified loci repeat lengths were analysed with capillary electrophoresis on the Applied Biosystems (ABI) 3130xl Genetic Analyser, with GeneMapper® data analysis software version 4.1 and data collection software version 3.1.1.

3) Results

The forensic DNA extraction techniques based on magnetic beads (DNA IQ™) and cellulose cards (FTA elute™ and Nucleosave™) were compared for their abilities to deal with HA contaminated blood and saliva and yield DNA that was useful in DNA profiling. The performance of these systems was evaluated by assessing the extracted DNA samples for residual inhibitor, DNA yield and the ability to generate useful STR DNA profiles. Blood and saliva were treated with 0, 0.5, 1.5 and 2.5mg/ml HA and then extracted using each of the systems simultaneously (in duplicate), using 2 separate donors.

3.1) The efficiency of HA removal

The ability to decontaminate HA contaminated biological evidence was evaluated by monitoring the amplification and cycle threshold (Ct) values of a synthetic IC DNA fragment using the Investigators Quantiplex QPCR assay (Qiagen). In each assay, the uninhibited biological evidence (0 HA) was used as a control, along with a HA spiked version of the same extract (added post-extraction at 100 ng/µl HA). Figure 1 and Table A of the supplementary data shows the results of this analysis. The reported Ct value of an uninhibited sample for this assay is *30±1*.0 according to the manufacturers (Qiagen 2011) The analysis of 20 separate extractions of a HA free sample, via all 3 methodologies revealed an average Ct value of *29.8±0.8* with the highest value being 31.06. Thus all of the extraction methods yielded inhibitor-free DNA (including heme) in the absence of HA addition. The average IC Ct values obtained from all positive HA control samples (20 replicates of 100 ng/µl HA) was *32.7±1.4*, which correctly indicated the presence of an inhibitor and potentially a problematic sample (Ct>31.06).

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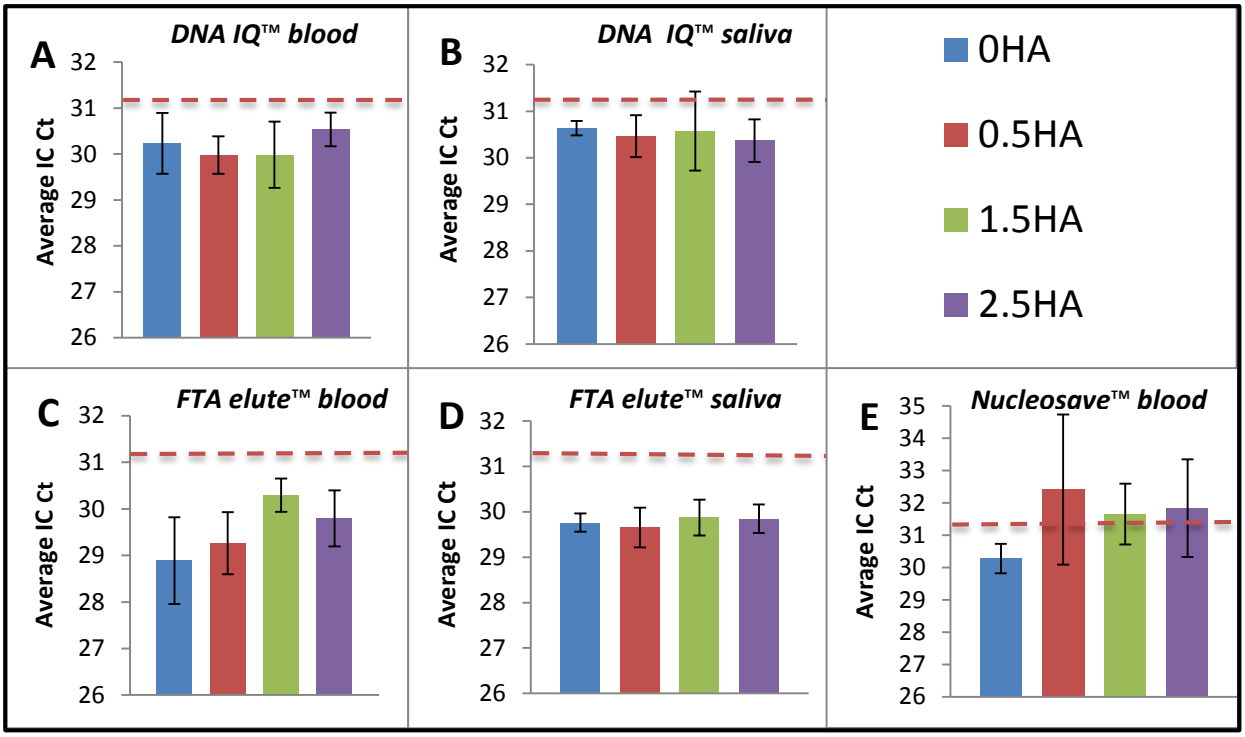


Figure 1: IC Ct values for evaluating humic acid removal.

Blood and saliva spiked with HA at 0, 0.5, 1.5 and 2.5 mg/ml were extracted and assessed for inhibitor removal with QPCR. The average IC Ct data for DNA IQ™, FTA elute™ and Nucleosave™ systems are presented for duplicate extractions for 2 donors. Error bars indicate the standard deviation between the 4 samples. The redline indicates the HA detection threshold (31.06).

Both the DNA IQ™ and FTA elute™ systems generated DNA where the IC Ct values showed effective HA removal (<31.06), from both blood and saliva samples (Figure 1). While the Nucleosave™ and FTA elute™ cards were processed through the same thorough disc washing regime in FTA purification reagent to remove inhibitors, the efficiency and reproducibility of inhibitor removal for HA spiked blood stored on Nucleosave™ cards was inferior to the FTA elute™ cards (non-indicating) (Figure1E). The IC Ct values for Nucleosave™ extractions for 67% (8/12) of the spiked samples were above the 31.06 inhibitor threshold (Table A of supplementary data, highlighted in red) and 42% (5/12) indicated that inhibitor levels were above 100 ng/µl in the final DNA extracts. The Nucleosave™ extractions also gave a slightly worse reproducibility profile, showing 7% variation between replicates, while all the DNA IQ™ and FTA™ elute extractions fell with a 3% error. Due to the poor performance of the Nucleosave™ system, analysis of HA spiked saliva was not performed.

3.2) DNA quantification.

Apart from achieving sufficient HA removal, the performance of the extraction system to yield sufficient DNA for forensic applications in the presence of this soil contaminate is also an important issue to address. In addition, the ability to quantify the DNA accurately in the presence of potentially low levels of co-extracting HA is also of importance for successful STR DNA profiling.

Table 1 show the results of this analysis, which was achieved by quantifying the extracted DNA using a DNA concentration standard curve and QPCR approach. The average DNA yield achieved using the DNA IQ™ system was 121.77±*30.86ng* from a 25µl treated blood sample. The extraction from saliva was less efficient and also appeared to be donor-dependant, with donor 1 yielding an average of 53.75 ±*22.60ng* and donor 2 only 4.60±*2.06ng*. The DNA yield achieved from the spiked biological evidence correlated well with the yields of uninhibited (0HA) blood and saliva for the magnetic bead system (Table 1), indicating that HA did not negatively affect the DNA yield.

The DNA yield from the FTA elute™ cards and the Nucleosave™ cards was expected to be lower than the DNA IQ™, as potentially only a 1/ 10^{th} of the treated biological evidence is used at any one time for extraction purposes using these systems, with only the equivalent of 5 µl of blood being analysed by the cards in comparison to the 25 µl sample using the DNA IQ™ system. However, the yield from blood samples stored on FTA elute™ cards was 300 fold less than the DNA IQ™ equivalent extractions (Ave: 0.37±*0.22ng*), showing a significantly inferior performance compared to the magnetic bead system (Table 1). Despite this, the DNA yield achieved from the spiked biological evidence correlated with the yields of uninhibited (0HA) blood and saliva, indicating that HA did not negatively affect the DNA yield during this extraction and quantification process. In an attempt to improve the DNA yield from blood stored on FTA cards, with the assumption that while DNA was effectively trapped in the FTA matrix it was not being effectively released, the discs were incubated in the DNA IQ™ lysis buffer and processed through the magnetic bead system. The average DNA yield from 0, 0.5, 1.5, 2.5 mg/ml HA spiked blood stored on FTA elute™ cards were significantly improved to 134.65, 80.98, 73.95, 81.77ng respectively.

Table 1: DNA yield for the DNA IQ™, FTA elute™ and Nucleosave™ extractions.

Blood DNA yield (ng)	DNA IQ™#						FTA elute™*						Nucleosave™					
	Donor 1		Donor 2		Ave	SD	Donor 1		Donor 2		Ave	SD	Donor 1		Donor 2		Ave	SD
	R 1‡	R 2	R 1	R 2			R 1	R 2	R 1	R 2			R 1	R 2	R 1	R 2		
0HA	107.15	92.05	113.74	83.67	**99.16**	**13.75**	0.14	0.15	0.36	0.37	**0.26**	**0.11**	3.76 2.82	3.57 2.05	4.32 4.14	1.79 2.35	**3.36**	**1.09**
0.5HA mg/ml	158.40	129.77	156.65	184.65	**157.37**	**22.41**	1.34	0.67	0.63	0.09	**0.68**	**0.51**	4.65 4.26	10.79 6.45	3.32 5.82	1.93 5.89	**5.17**	**3.90**
1.5HA mg/ml	96.73	151.85	56.70	148.40	**113.42**	**45.45**	0.26	0.17	0.08	0.23	**0.19**	**0.08**	42.09 9.80	51.32 11.86	1.22 3.13	1.31 3.34	**24.0**	**26.50**
2.5HA mg/ml	55.83	134.50	128.59	149.65	**117.14**	**41.83**	0.60	0.64	0.13	0.08	**0.36**	**0.30**	48.61 9.35	52.62 9.50	2.02 1.84	2.20 2.49	**26.4**	**28.10**
Saliva DNA yield (ng)																		
0HA	26.03	40.65	1.62	2.77	**29.50**	**18.95**	2.50	2.28	1.96	1.77	**2.13**	**0.28**						
0.5HA mg/ml	31.82	75.72	4.51	6.09	**15.01**	**28.69**	2.36	2.66	1.35	6.04	**3.10**	**2.04**						
1.5HA mg/ml	64.00	35.81	5.36	3.46	**27.16**	**41.58**	29.01	47.58	4.09	3.12	**20.95**	**21.4**						
2.5HA mg/ml	72.23	83.78	4.75	8.26	**42.26**	**1.12**	6.18	11.15	9.21	5.13	**7.92**	**2.76**						

‡: R1 and R2 represent duplicate extractions performed at the same time.

#: HA-spiked blood from either donor was processed using all three methodologies simultaneously.

*: FTA elute™ non-indicating cards was used for blood and indicating cards used for saliva.

Note: Qubit DNA yield indicated by values highlighted in red.

The results of the IC amplification from Nucleosave™ extracted samples proved the co-elution of HA and PCR inhibition. This would then affect the QPCR method chosen for quantification of these samples. To adjust for this potential problem, all Nucleosave samples were also quantified using the Qubit™ dsDNA HS assay, which we have shown to be less affected by the presence of low HA concentrations (Figure A supplementary data). In Table 1 the results of the QPCR quantification and the corresponding Qubit analysis are presented for the Nucleosave DNA extractions (highlighted in red). Although the Qubit and QPCR results correlated in terms of DNA yields for 0 and 0.5HA, the presence of HA significantly affected accurate quantification via real-time methodology for donor 1 at 1.5 and 2.5HA respectively (donor 1 - 46.70 vs 10.8 ng) and (donor 1- 50.6 vs 9.4 ng). The Qubit analysis of the Nucleosave samples however, again showed that the presence of HA contamination did not affect DNA yield directly, and that the extraction of DNA was actually more efficient than the FTA elute™ system.

3.3) DNA profiling

The ability to produce DNA profiles devoid of artefacts and allele drop out is important for accurate human identification and is the end-point in the processing of biological evidence at a forensic crime scene. It was thus important to evaluate the efficiency of the extraction systems in this PCR environment, to produce quality DNA.

Initial DNA profiling was performed as recommended by the manufacturers (Promega) using 1ng and 500 pg DNA for the 0HA and 2.5HA blood samples extracted with the DNA IQ™ system and full profiles were obtained (data not shown). This was expected, as it has been reported that the Powerplex® 16HS system is tolerant to HA below 100 ng/µl when 500 pg or more DNA is used for the analysis, while at HA of 125 ng/µl 50% of alleles fail to amplify using the same amount of DNA (Ensenberger and Fulmer 2009). Our local genotyping system appeared to be more robust, allowing generation of full profiles with 125 ng/µl HA spiked DNA using both 500 pg and even 200 pg of DNA. Instead we identified that 200 ng/µl HA was required to cause a 50% allele amplification failure using 500 pg DNA (Figure B supplementary data). We chose to thus use the 200 ng/µl HA spike as a positive control for inhibitor interference and a reduced 200 pg input DNA as a more representative sample that one may encounter in the soil environment at a crime scene.

DNA profiles were thus obtained using a total of 200 pg DNA, extracted from HA-spiked blood and saliva (where appropriate) (0 and 2.5HA), using the DNA IQ™, FTA elute™ and Nucleosave™ systems. The results of this analysis are represented graphically in Figure 3. For the HA free samples (0HA) extracted with the DNA IQ™ system, 100% (23/23) of alleles were called for blood and 96.5% (22/23) of alleles were correctly called for the HA free saliva samples (identical duplicate profiles). The DNA IQ™ extracted 2.5HA blood and saliva samples also allowed correct allele calling with no skewing of loci amplification or artefacts, for 96.5 % (22/23) of the alleles (performed in duplicate). The alleles that were not amplified above the 50 rfu threshold in each case were the larger loci, which also failed in the respective HA free biological samples.

Similar results were obtained for the FTA extracted DNA from blood, with 21/23 and 21/23 alleles called in the duplicate 0HA samples and 23/23 and 22/23 called correctly for the 2.5HA DNA sample (Figure 3). However, the results for the saliva analysis were unexpected, with an average of only 65% (15/23) of the alleles being correctly called for the 0HA samples and only 34.8 % (8/23) alleles called for each duplicate of the 2.5HA saliva samples (Figure 3). STR amplification using the Nucleosave extracts was unsuccessful as expected for the 2.5HA blood samples, with only 8.7 % (2/23) and 0% (0/23) alleles being amplified.

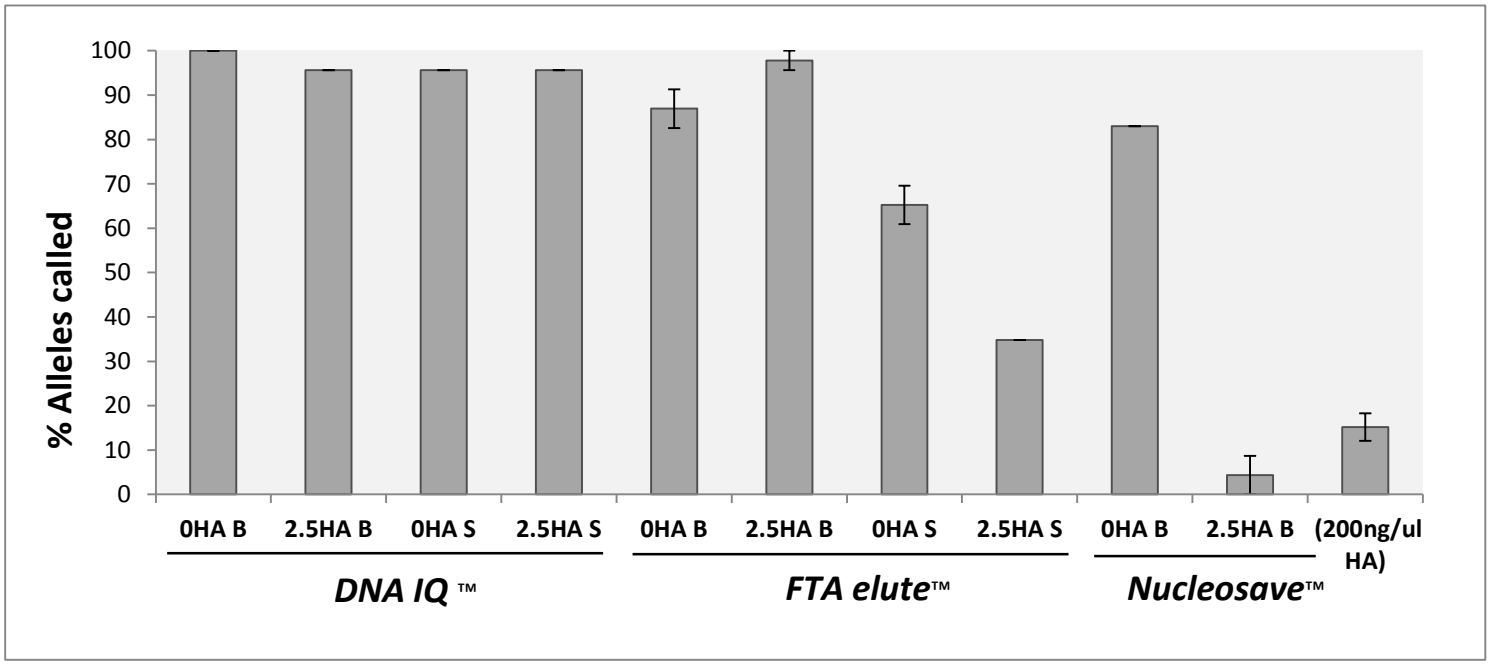
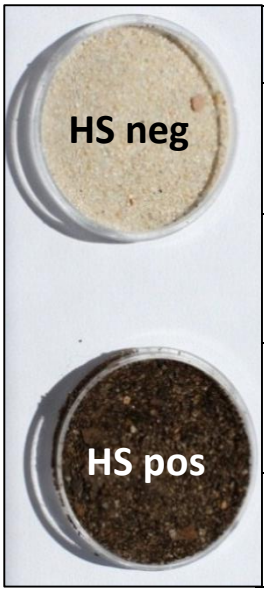


Figure 3: The percentage of alleles amplified using STR profiling of 200 pg DNA contaminated with HA. The STR profiles were performed for a single donor blood (B) and saliva(S) sample spiked at (0 & 2.5mg/ml) HA, profiles were performed in duplicate for each extraction and the average percentages of alleles called were reported with the standard error between duplicates. The HA control (200 ng/µl HA) was prepared by spiking 200 ng of a post extracted DNA sample.

3.4) Crude soil applications

The superior performance of the DNA IQ™ and FTA elute™ cards for blood extraction in the presence of rich HA levels encouraged field application testing for these systems. It was also an opportunity to establish if the experimental system used for saliva collection (Oragene OG 500™) was the cause of the poor performance of the FTA-extracted saliva samples. For this analysis 2 different soil types were tested, which showed apparent differences in humus content (HS) and thus HA levels: white in-land Philippi sand and rich potting compost, respectively (see table 2 for pictures). The samples were assessed for IC amplification, DNA yield using QPCR and STR profiling (% of alleles called). Table 2 shows the results of this analysis. It must be noted, that due to the particulate nature of the sample, certain steps needed to be modified to ensure optimal DNA extraction. This included the vigorous shaking during the washing of the FTA elute™ discs , as well as the introduction of a brief centrifugation step after lysis treatment to separate the lysate from large soil particles before adding the magnetic beads of the DNA IQ™ system.

Table 2: Crude soil QPCR and STR genotyping data for the DNA IQ™ & FTA elute™ systems.



	DNA yield (ng)	IC Ct	% alleles called	
Blood DNA IQ™			R1	R2
HS neg‡	12.5	29.26	100%	100%
HS pos	16.59	29.42	100%	100%
Blood FTA elute™				
HS neg	44.6	30.38	96%	96%
HS pos	8.69	29.31	100%	100%
Saliva DNA IQ™				
HS neg	34.86	27.6	100%	100 %
HS pos	108.95	29.64	92.3%	100 %
Saliva FTA elute™				
HS neg	0.95	30.23	84.6%	53.8%
HS pos	2.70	30.76	50%	76.9%

‡: Humus negative (HS neg): white in-land Philippi sand; Humus positive (HS pos): rich potting compost.

Blood was stored on FTA elute™ non-indicating cards & saliva on FTA elute™ indicating cards.

R1 & R2 represents duplicate DNA profiles.

The IC Ct values showed that the extracts were inhibitor free as all values were below the inhibitor threshold (Ct values < 31.06) (Table 2). While the extractions were inconsistent with regards to DNA yield, the yields from both systems, using either blood or saliva was more than the amount recommended for DNA profiling (>50 pg/µl).

The DNA IQ™ system was capable of providing full DNA profiles (26/26 alleles called, donor 2) for blood and saliva samples deposited in both sand types. For the FTA system, full DNA profiles were only obtained for blood samples. Blood samples that were collected from the humus (HS) positive soil and stored on FTA elute™ cards provided full DNA profiles for each attempt. Although the HS negative soil sample in this case provided 96% (25/26) of called alleles, the same 2 alleles failed at the Penta E loci in each duplicate, and was therefore not observed as a major inhibitory effect. As for the saliva samples captured on FTA elute™ cards, only partial DNA profiles were obtained, as previously observed with the spiked experimental samples. Partial profiles containing an average of 69% of the alleles were obtained for the sand sample (HS neg), with a similar profile of an average of 63% of alleles being called for the humus rich sample (HS pos). Altogether, the DNA IQ™ system was more efficient and reproducible in providing high quality full DNA profiles for the blood and saliva deposited in each soil type.

4) Discussion:

Utilizing biological evidence from soil on a crime scene of rape or homicide can be of importance for identifying the perpetrator or victim with DNA profiling. However, HA which is abundant in various soils, co-extracts with the DNA and impairs the production of full DNA profiles by affecting the PCR quantification and STR amplification processes. In this study, the forensic DNA IQ™, FTA™ elute and Nucleosave™ systems were assessed with QPCR and STR-genotyping to validate their use on biological evidence in soil. A final inhibitor concentration of 0.5-2.5 mg/ml HA was selected with the initial concentrations spiked between 3.0-27.5 mg/ml to provide a highly contaminated platform with respect to the natural HA levels in soil. Using a limited volume of highly contaminated evidence was also intended to represent a more appropriate field case sample. This was important to consider as samples providing minute DNA amounts with potentially high levels HA are more likely not to give full DNA profiles (Faber *et al*. 2013). Finally systems with satisfactory performance on the simulated inhibitor platform were exercised using crude soils of high and low humic acid content.

Using the Investigators Quantiplex QPCR assay with a set of HA negative and positive controls provided an interface for evaluating HA removal. The IC Ct values for DNA IQ™ silica magnetic bead system and the FTA elute™ cards indicated that HA was efficiently removed from blood and saliva to yield DNA extracts with less than 100 ng/µl HA in the sample. These systems provided better decontamination capabilities than the Nucleosave™ cards, for which HA removal was not always sufficient and reproducible. Despite the optimal purity provided by the DNA IQ™ and FTA elute™ system, obtaining sufficient DNA yields in the presence of HA was of equal importance. According to the results, HA did not negatively affect the binding of DNA to the silica magnetic bead or the matrix of the FTA cards. Although, the DNA IQ™ system provided much better yields than the FTA elute™ cards, the major rate limiting step with the FTA system was considered to be the physical heat elution of the DNA from these cards, as using a lysis buffer for elution improved the yields dramatically. Although the DNA profiles from 2.5 mg/ml HA spiked blood for both the DNA IQ™ and FTA elute™ provided amplification of all 13 CODIS loci, only the DNA IQ™ provided reasonable DNA profiles for saliva.

Finally testing the crude soil samples showed that the DNA IQ™ system once again gave superior performance for both blood and saliva contaminated soil, allowing the generation of full profiles from potentially heavily HA contaminated biological samples (natural garden soil >15mg/g HA, Lakay *et al*. 2007).

The DNA IQ™ magnetic bead has recently been identified as a binding target of 2 common forensic PCR inhibitors, namely (haematin and black denim dye), which outcompetes the DNA for binding sites particularly at higher inhibitor levels (Fregeau and Moors, 2012). We established that at high levels, HA had not outcompeted DNA for binding sites on the silica bead to negatively affect the DNA yield. With remarkable selectivity this system offers a greater benefit for processing soil evidence appose to silica column extractions, which can be deficient in DNA purification from HA and in addition challenging to automate (Harry *et al*. 1999; Miller *et al*.1999; Lee *et al*.2010, Faber *et al*. 2013). In our study, using the standard DNA IQ™ protocol, the bulk of the HA was efficiently removed from blood and saliva spiked samples, but additional processing was required for processing a natural soil sample, where the presence of large soil particles prevented thorough bead washing due to clumping of the beads. In this case, a brief centrifugation was implemented to separate the lysate from the large soil particles before adding the beads, which proved crucial for optimal results.

The manufacturer's validation reports for the DNA IQ™ have been somewhat inadequate with regard to processing soil evidence. These reports do not communicate the volume of biological sample used or the levels of HA tested, which is important information to provide in order to establish the decontaminating capabilities of the system. As for the more comprehensive soil inhibitor platforms, as that used by Brevnov *et al*. (2009), 1ul of an inhibitor mixture containing haematin (0.5 mM); indigo (12.5 mM); humic acid (2.5 mg⁄mL) was spiked into 5 µl blood and a 2 µl sample was transferred onto cotton cloth. Another platform for validating the DNA IQ™ system was prepared using a soil fraction (3ml soil: 3ml H_20) into which a swab was dipped and dried. Blood was transferred onto the dried swab and processed on an automated platform for the DNA IQ™ system (Frégeau *et al*. 2010). In these studies HA was not explored independently at elevated levels and these platforms had not considered processing a dried blood stained crude soil sample. In our study the DNA IQ™ system validated for the first time using HA levels in relation to a natural soil sample and for processing crude soil samples without the transfer onto cotton material or buccal swabs.

The potential disadvantage of the DNA IQ™ system is that the sample can only be preserved after it has been collected and transported to the laboratory for extraction (not on site). Alternatively, a blood stained soil sample can be collected on FTA cards for immediate DNA preservation. The FTA cards are well recognized to capturing DNA from field samples, such as from the muscle tissue of abalone, crab and fowl (Smith and Burgoyne 2004); for the storage of plant DNA (Karle *et al*. 2003) and in forensic entomology for capturing insect DNA (Dickey *et al*. 2012). This study represents the first to report that these cards can be used successfully to extract blood deposited in crude soil by the exploitation of its adhesive properties. Furthermore, using a highly contaminated HA platform has not been validated for these cards to establish its decontamination capabilities. Due to the superior durability and robust properties offered by the FTA cards, the discs were able to endure vigorous washing in the FTA purification reagent which was effective for flushing out HA and adhering soil particles. These robust properties were not observed for the Nucleosave system, for which the sample discs often disintegrated and presented fibres in the DNA extract, and as a result sufficient purity was not always guaranteed. This improved DNA purity offered by the FTA system compared to Nucleosave cards has also been shown previously (GE Healthcare life Sciences, 2010). Nevertheless, the major disadvantage of the FTA system is that the yields appear limited. We found that this is due to the elution extraction step and that this can be overcome by the processing of these discs through a secondary extraction protocol such as the DNA IQ™ system. While others have shown that yields can be improved by processing the FTA sample disc repetitively through the extraction, although this is time-consuming (Stangegaard *et al*. 2011).

The FTA system could not however provide full DNA profiles for both the spiked saliva samples and for pure saliva deposited in crude soil. Although, the IC Ct values indicated that the purity of these sample were sufficient, only partial profiles were obtained. The conventional method with these cards is to use take a buccal swap from the inside of the cheek for transfer onto the FTA cards (Park *et al*. 2008; Stangegaard *et al*. 2013). Very few studies have attempted to use pure saliva captured on FTA cards and the studies that have achieved full DNA profiles from “saliva” on FTA cards have not clearly distinguished a saliva sample from a buccal sample (Hansen *et al*. 2006). In our case, a saliva sample from the Oragene OG500™ device or a pure saliva soil sample was used.

It is possible, that due to the much higher content of amylase present in a saliva sample compared to a buccal swab, that the DNA is not completely protected against this enzyme and that a period of DNA degradation persists on these cards. The DNA purity would not be affected by this and the QPCR would not be grossly affected due to the amplification of a very small 200-bp DNA fragment. However, larger PCR amplicons would be affected, which was evident with the lack of amplification of the larger loci in the STR profiling of these samples.

Our results indicate that the DNA IQ™ magnetic bead system is effective for purifying the DNA from blood and saliva, contaminated with a wide HA inhibitor concentration range. This was confirmed in a field application, with the DNA IQ™ system being particularly efficient for processing crude soil samples of high and low humus content for blood and saliva, following removal of the large soil particles. This applied to the quantity, quality of DNA as well as the ability to produce full quality STR profiles from the extracted DNA. As for the FTA elute™ system, although proven superior to the Nucleosave cards, this system was only effective for processing contaminated blood samples and not saliva. In this study we have comprehensively assessed a reliable HA inhibitor removal extraction system that was proven effective for crude soil applications. We advise that on encountering a blood or saliva stain that is limiting, the sample should be collected in a suitable plastic container and processed in the laboratory using the DNA IQ™ system.

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Supplementary data

Table A: IC Ct values for evaluating humic acid inhibitor removal.

Blood IC Ct values	DNA IQ™#						FTA elute™						Nucleosave™					
	Donor 1		Donor 2		Ave	SD	Donor 1		Donor 2		Ave	SD	Donor 1		Donor 2		Ave	SD
	R 1‡	R 2	R 1	R 2			R 1	R 2	R 1	R 2			R 1	R 2	R 1	R 2		
0HA	29.8	29.61	31.06	30.46	**30.2**	**0.66**	27.34	29	29.68	29.53	**28.9**	**0.93**	30.81	30.06	29.78	30.46	**30.3**	**0.45**
0.5HA mg/ml	29.62	29.79	29.94	30.56	**30.0**	**0.41**	28.65	28.74	29.98	29.68	**29.3**	**0.67**	34.77	33.93	29.83	31.13	32.4	**2.32**
1.5HA mg/ml	29.7	29.09	30.59	30.55	**30.0**	**0.72**	30.06	30.26	30.81	30.05	**30.3**	**0.36**	33.01	31.41	31.35	30.84	31.7	**0.94**
2.5HA mg/ml	30.95	30.56	30.06	30.57	**30.5**	**0.37**	28.96	29.74	30.25	30.23	**29.8**	**0.6**	33.36	32.91	30.45	30.63	31.8	**1.51**
Saliva IC Ct values																		
0HA	30.62	30.55	30.51	30.86	**30.6**	**0.16**	29.83	29.44	30.00	29.78	**29.8**	**0.20**						
0.5HA mg/ml	30.93	30.05	30.77	30.11	**30.5**	**0.45**	29.06	30.05	29.90	29.60	**29.7**	**0.44**						
1.5HA mg/ml	30.10	31.79	30.50	29.90	**30.6**	**0.85**	29.42	29.95	30.36	29.76	**29.9**	**0.39**						
2.5HA mg/ml	30.51	29.80	30.26	30.89	**30.4**	**0.46**	30.13	29.48	29.69	30.09	**29.8**	**0.32**						

‡: R1 and R2 represent duplicate extractions performed at the same time.

#: HA-spiked blood from either donor was processed using all 3 methodologies simultaneously.

FTA elute™ non-indicating cards was used for blood and indicating cards used for saliva.

IC values > 31.06 threshold are highlighted in red.

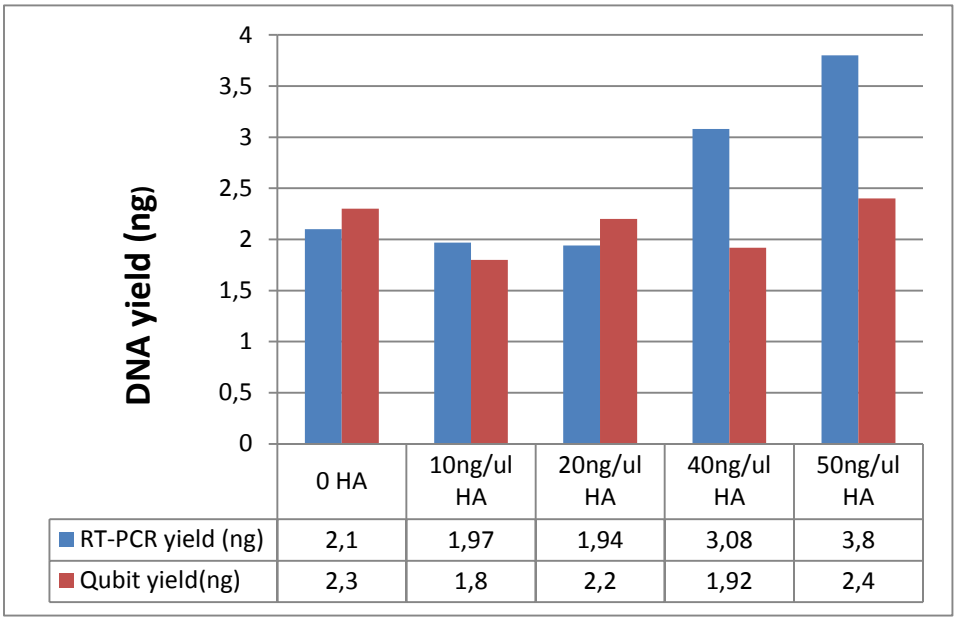

Figure A: Dosage response data for QPCR and Qubit DNA quantification.

A 2 ng DNA sample (OHA, DNA IQ™ saliva sample) was spiked with HA (0, 10, 20, 40, 50 ng/µl) to evaluate the accuracy of DNA quantification in the presence of low HA levels.

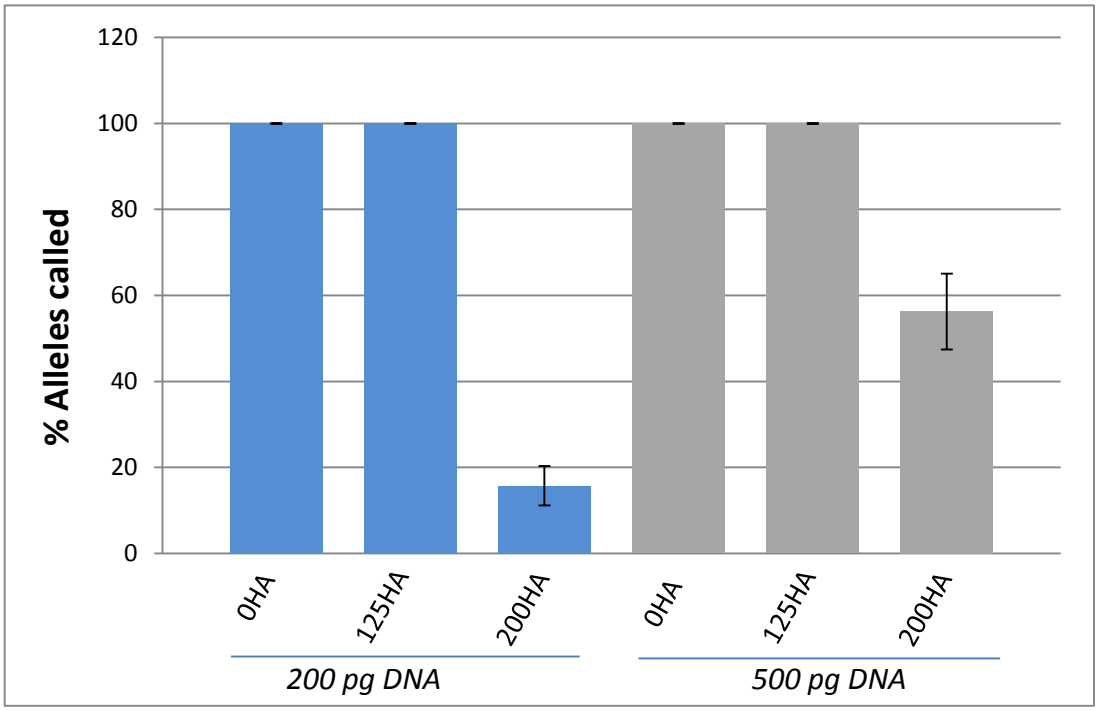

Figure B: The dosage effect of humic acid in relation to amount of input DNA.

A total of 200 pg and 500 pg of DNA (OHA, DNA IQ™, blood) sample was spiked with 0, 125 and 200 ng/µl HA and analysed using the Powerplex 16 HS STR profiling kit. The average percentage of alleles called is reported for duplicate preparations. The error bars represents the deviation between duplicate profiles.

PART D : Appendices

Conflict of interest statement.

We wish to confirm that there are no known actual or potential conflicts of interest associated with this publication.
We confirm that no financial, personal or other relationships with other people influenced or perceived to influence this publication.

Manuscript Title:

The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence.

Author name:	Signature:	Date:
_____	_____	_____
_____	_____	_____

Submission Declaration.

We confirm that this work has not been published previously as an abstract, a published lecture, an academic thesis or as an electronic preprint. And that this work is not under consideration for publication elsewhere. We confirm that all contributing authorities and authors are in agreement with the contents and approve this work for release.

Author name:	Signature:	Date:
	Signed by candidate	

Role of the funding source

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Official ethics approval letter

UNIVERSITY OF CAPE TOWN

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15 May 2013

HREC REF: 282/2013

Mr M Kasu
c/o Dr K Shires
Department of Clinical Laboratory Sciences
Division of Haematology
FHS

Dear Mr Kasu

PROJECT TITLE: USING NOVEL DNA PURIFICATION TECHNOLOGIES TO REMOVE SOIL PCR INHIBITORS FROM HUMAN BLOOD AND SALIVA SAMPLES

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year till the 28 May 2014.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

sAriefdien

Consent form : Donating blood and saliva samples for research

Study title: ***Using novel DNA purification technologies to remove soil PCR inhibitors from human blood and saliva samples.***

Please indicate in the space provide whether you understood the following:

1) I confirm to have read and understood the information sheet version 1.0 May 2013 for the above titled study. I was able to ask questions and the answers provided were satisfactory. ☑
2) I understand that blood and saliva samples required for this study are to undergo DNA analysis. ☑
3) I understand that my blood and saliva samples would only be used for the indicated purpose and duration provided on the information sheet. ☑
4) I understand that confidentiality is to be maintained and that no diagnostic tests can be performed and reported on the samples ☑
5) I acknowledge that no personal benefits are promised and that I understood the risks involved. ☑
6) I understand that participation requires sharing of personal information such as my name and contact details and signature. ☑
7) I agree that deciding to participate was voluntary and that I was not forced to participate. ☑

Please indicate if you give consent to:

Provide 5-10ml blood and 2ml saliva as a DNA source.	☑ YES	☐ NO
Have your DNA profile as part of a scientific publication.	☑ YES	☐ NO

I ROLANDA LONOT ..... (name of donor) consent to take part in this study as explained to me by Mr Mohaimin Kasu.

..... (signature of donor) 10/07/2013 (date)

..... (signature of researcher) 10/07/2013 (date)

..... (signature of witness) 10/07/2013 (date)

Mr Mohaimin kasu and Dr Karen Shires
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Mobile: 0839661359 /Office tel: 0214066673

Human Research Ethics Committee
Professor Marc Blockman
Office tel: 0214066492

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7) I agree that deciding to participate was voluntary and that I was not forced to participate. ☑

Please indicate if you give consent to:

Provide 5-10ml blood and 2ml saliva as a DNA source. ☑ YES ☐ NO

Have your DNA profile as part of a scientific publication. ☑ YES ☐ NO

I..Rose Tooste..... (name of donor) consent to take part in this study as explained to me by Mr ..Mohaimin Kasu.....

R Tooste..... (signature of donor) 21/08/2013..... (date)

..... (signature of researcher) 21/08/2013..... (date)

..... (signature of witness) 21.8.2013..... (date)

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7) I agree that deciding to participate was voluntary and that I was not forced to participate. ☑

Please indicate if you give consent to:

Provide 5-10ml blood and 2ml saliva as a DNA source.	☑ YES	☐ NO
Have your DNA profile as part of a scientific publication.	☑ YES	☐ NO

I. GRANT GODSMARK ..... (name of donor) consent to take part in this study as explained to me by Mr Mohaimin Kasu

..... (signature of donor) 08/10/13 ..... (date)

..... (signature of researcher) 08/10/13 ..... (date)

..... (signature of witness) 08/10/13 ..... (date)

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6) I understand that participation requires sharing of personal information such as my name and contact details and signature. ☑
7) I agree that deciding to participate was voluntary and that I was not forced to participate. ☑

Please indicate if you give consent to:

Provide 5-10ml blood and 2ml saliva as a DNA source.	☑ YES	☐ NO
Have your DNA profile as part of a scientific publication.	☑ YES	☐ NO

I..Calvin Mole..... (name of donor) consent to take part in this study as explained to me by Mr ...Kasu.....

..... (signature of donor) ....19/07/2013..... (date)

..... (signature of researcher) ....19/07/2013 (date)

..... (signature of witness) ....19/07/2013 (date)

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