The application and validation of direct PCR from various substrates for forensic practice

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THESIS ABSTRACT

Direct PCR is fast becoming a popular method in forensic science due to the advantages of saving time and money in the laboratory while increasing the probability of obtaining substantial results has a positive rippling effect. A laboratory is able to reduce the time spent on processing trace DNA samples, which can lead to investigators receiving important information in a timely manner that may not have been possible using standard methods. DNA extraction procedures are standard practice in the initial steps of DNA profiling when examining swabs, adhesive tapes and sections of fabric. Significant loss of DNA can occur during this process resulting in no DNA profile generated. Direct PCR circumvents the extraction process such that a DNA profile may be generated directly from the substrate. This saves time, increases the DNA yield, reduces tube changes, and minimises steps open to error or contamination in the laboratory.

To fully understand the benefits and limitations of direct PCR, several aspects of the method have been analysed in this thesis. The loss of DNA via extraction was investigated and determined for the most common extraction methods used in forensic science, as well as understanding the limit of detection for the commerical PCR human identification kits. From this, a statistical method to quantify the DNA template used in direct PCR amplifications based on the resulting magnitude of the allele peaks generated was developed. In this PhD study, trace evidence items investigated using direct PCR include: human hairs, canine hairs, fibres, human finger nails, and human teeth. Substrates have been analysed using both short tandem repeat (STR) and single nucleotide polymorphism (SNP) methods for identification. A high level of succesful profile generation has been found across all of the substrates. A successful profile produces five or more complete loci that can be up-loaded to the National Criminal Investigation DNA Database (NCIDD, Australia).

Current practice for many laboratories is that trace evidence items, such as the ones listed above, are generally not subjected to DNA testing as there is little chance of generating a meaningful DNA profile. All direct PCR methods, where applicable, have followed validated protocols as to maintain high standards and allow fast implementation within forensic laboratories. The only alteration to the PCR methods was an increase in the amount of DNA polymerase used to help overcome any potential inhibitors present on the substrate. This study highlights direct PCR sensitivity and the ability for trace DNA to be amplified without the need to increase cycle number or modify current protocols to obtain meaningful data. Based on the data presented in this thesis, a direct PCR approach is a viable option for the future of trace DNA recovery and analysis for forensic science purposes, vastly improving efficiency, sensitivity and the quality of results.

DECLARATION

I certify that this thesis does not incorporate, without acknowledgment, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Renée C. Blackie
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Chapter 7

LIST OF INFO. BOXES

LIST OF ABBREVIATIONS

ANZFSS	Australian and New Zealand Forensic Science Society
bp	Base Pair
BSA	Bovine Serum Albumin
CCD	Charged Coupled Device
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DVI	Disaster Victim Identification
FBI	Federal Bureau of Investigation
FSS	Forensic Science Service
FSSA	Forensic Science South Australia
ISFG	International Society for Forensic Genetics
LCN	Low Copy Number
$MgCl_2$	Magnesium Chloride
mtDNA	Mitochondrial DNA
NCIDD	Australian National Criminal DNA Database
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
qPCR	Real-time PCR or quantitative PCR
RFU	Relative Fluorescence Unit
SAPOL	South Australian Police
SDS	Sodium Dodecylsulfate
SNPs	Single Nucleotide Polymorphisms
STR	Short Tandem Repeat
SWFS	Society for Wildlife Forensic Science
SWGDAM	Scientific Working Group on DNA Analysis Methods
Tris-HCl	Tris(hydroxymethyl)aminomethane – Hydrochloride
UV	Ultraviolet
Y-STR	Y-chromosome Short Tandem Repeat

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Publications

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Ottens, R., et al., *Successful direct amplification of nuclear markers from a single hair follicle.* Forensic Science, Medicine, and Pathology, 2013. **9**(2): p. 238-243.
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22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Successful direct amplification of nuclear markers from fingernail clippings.*

Society for Wildlife Forensic Science (SWFS) meeting, Missoula, Montana, USA, 2015. *Successful direct amplification of nuclear markers using single dog hairs with eight DogFiler loci.*

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22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Successful direct amplification of SNP markers using single human hairs & fingernail clippings.*

Casework

Use of direct PCR on trace evidence samples

Case 1 – Seizure of Methamphetamine in South Australia

Canine DNA Detection:

Case 2 – Determining the presence of canine DNA in an extraction obtained from a glove

Case 3 – Determining if the canine DNA from Case 2 matches bone DNA from exhumed pet

Case 4 – Determining the presence of canine DNA in a sexual assault allegation

CHAPTER 1

Introduction to Forensic DNA Technology

Introduction to Forensic DNA Technology

1.1 Analysis Process

From crime scene to DNA report, a series of validated protocols are implemented in order to transform biological evidence into DNA information relevant to a case. Once biological evidence has been identified and collected, it is sent to a forensic laboratory where the DNA may be extracted, quantified, amplified and analysed.

The DNA extraction and quantification steps assist in determining the quantity and quality of the DNA recovered, such that subsequent amplification of specific regions of nuclear DNA using the enzymatic process PCR is successful. PCR creates millions of copies of the targeted DNA fragments. Once amplified, the DNA fragments are separated and detected using fluorescence methods and CE to determine the size of each fragment followed by data analysis using computer software. Once the resulting profile of a sample has been interpreted, it can then be compared to the DNA profiles from other evidentiary items or reference samples of individuals or suspects. Reports will contain evidential weights (commonly in the form of a likelihood ratio) for the samples under investigation; this determines the probability of obtaining the evidence given competing scenarios. These statistics are based on population frequencies of STR alleles, often obtained from online databases; derived from collections of profiles obtained from unrelated individuals. The comparison process can lead to several different outcomes: a known individual can or cannot be excluded as a possible contributor to the DNA profile obtained from the evidentiary item; the DNA typing results are inconclusive or uninterpretable; or the results from several evidentiary items are consistent or inconsistent with originating from a common source.

From start to finish, the analysis process requires large amounts of time, resources and human input that are not always available. The typical workflow shown in Figure 1.1 demonstrates that a minimum of 10 hours is required from start to finish. An increasing demand for DNA evidence and a push for fast results have created a bottleneck in the workflow at many laboratories [1]. The introduction of automated and robotic platforms designed to cope with a high throughput of samples has helped deal with capacity and backlog issues. The demand for DNA processing comes from two main areas: increased collection of DNA evidence in criminal cases, and increased collection of DNA samples from convicted or arrested individuals [2]. DNA evidence can easily be seen as a panacea by investigating authorities leading to the submission of multiple samples from a scene to the laboratory. Despite the adoption of automation in the laboratory, the problem still remains that the demand for DNA analysis is increasing faster than most labs can handle. There is also a public perception, known as the ‘CSI effect’, that DNA evidence always provides informative results and

is therefore expected for every sample. To push through the bottleneck, a balance between prioritising and pre-screening DNA casework samples for submission versus the efficiency and capacity of the laboratory to deal with the input needs to be an ongoing focus.

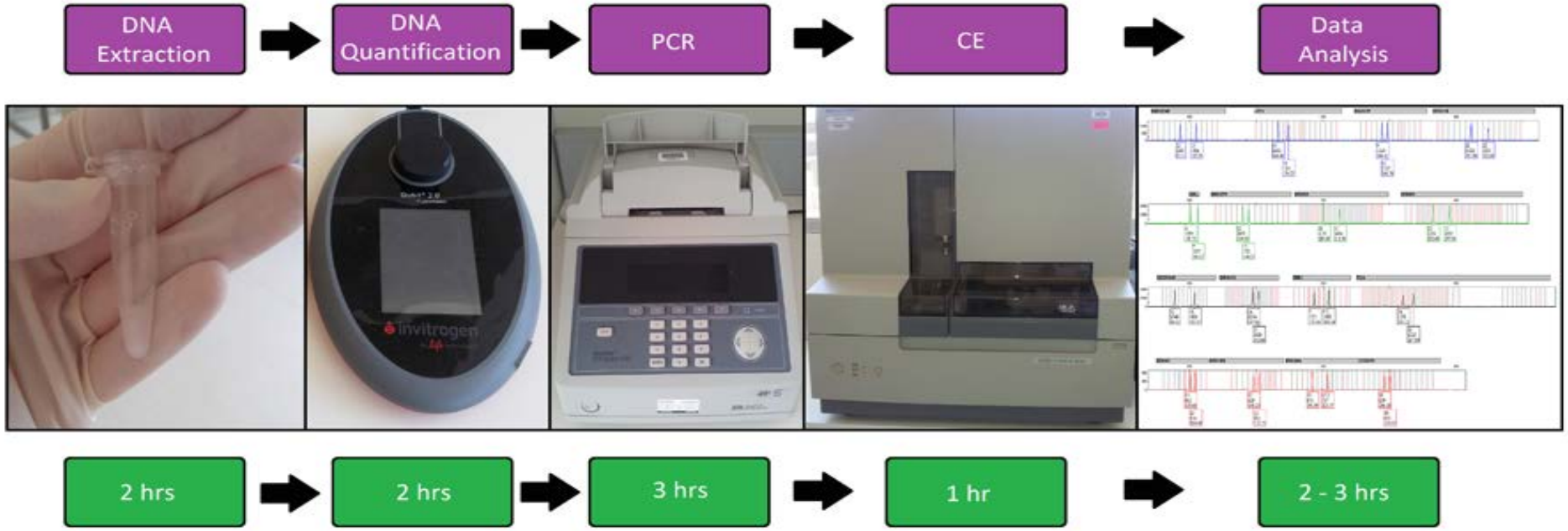

Figure 1.1 – The standard process for DNA analysis showing the time taken for each step. Typically an extraction process from blood or a buccal swab takes two hours to complete; quantification by real-time PCR takes a further two hours; amplification of STR loci a further three hours; separation on capillaries an hour per 16 samples; and finally data analysis. The total time is no less than ten hours in total. In certain circumstances, the need and advantages of obtaining DNA results quicker has lead to the development of rapid DNA instruments by biotechnology companies. The entire process can be completed in as little as 90 minutes, however the technology is considered to be in early stages and has not yet been widely adopted [3, 4].

1.1.1 Extraction Process

1.1.1.1 Importance of DNA extraction

In order to maintain optimal conditions for the downstream processes of DNA analysis, the extraction process is necessary to purify DNA from biological material and remove PCR inhibitors, such as haemoglobin, that would otherwise cause the amplification reaction to fail [5]. Multiplex PCR systems are very sensitive and rely on the correct balance of buffer constituents and reagents in order to amplify DNA successfully. The buffer is typically made up of Tris-HCl (pH 8.3), $MgCl_2$, primers, DNA polymerase, dNTPs, and DNA template at optimal concentrations; other reagents such as BSA and DMSO may also be added to help stabilise the reaction [5-10]. Inhibitors can affect PCR by interacting with the DNA, interfering with the enzyme itself, reducing the availability of cofactors or by affecting the ability of the cofactors to interact with the enzyme [11]. Depending on the type of forensic sample, specific collection techniques are implemented to avoid the unnecessary collection of inhibitors. DNA clean-up systems are marketed for this purpose, however this adds additional time to the overall process and contributes to the possibility of DNA loss and extraneous DNA contamination.

There are several methods of extraction that can be employed for minimising the transfer of inhibitors. The three most common DNA extraction techniques used in forensic laboratories, outlined in Figure 1.2, are: organic extraction, Chelex® extraction, and solid phase extraction. The choice of which extraction method to use varies greatly depending on the difficulties associated with extracting DNA from a particular substrate or sample type. The substrate type is known to affect DNA transfer and subsequently the efficiency of the DNA extraction method [12], with more difficult substrate types, such as bone, requiring further research, validation, and optimisation [10, 13-16]. The extraction method itself has also been identified as a factor of DNA yield variation [17-22], highlighting the importance for a laboratory to understand the limitations of different extraction methods. There are several studies that compare extraction method efficiency when focused on a certain substrate or sample type; this is accomplished using varying volumes of whole blood or saliva, and comparing the quantity of DNA obtained [21, 23]. These methods of comparison typically rely on the amount of DNA in whole blood or saliva to be directly proportional to its volume and does not factor in possible variance caused by the presence of cell-free DNA [24, 25]. For the most part though, a full DNA profile can be obtained from a sample typically submitted for forensic analysis; but as the demand for DNA testing increases, so does the testing of trace DNA samples. The efficiency of the extraction method for degraded or trace DNA samples needs to be high in order to avoid interpretation issues at the analysis stage, which may lead to disputes in a courtroom [19, 26].

The amount of purified DNA obtained from the DNA extraction step can vary greatly depending on the method used and the substrate that the DNA is deposited on, opening up the process to errors that could affect the overall profile quality [12]. Extractions are susceptible to exogenous DNA contamination or sample-to-sample contamination due to multiple tube changes and the extensive handling involved. This issue is amplified when dealing with samples containing minute traces of DNA, typically less than 1 ng [12, 27-29]. Multiple tube changes are often required for the removal of inhibitors via wash steps, but can also cause the loss of DNA due to tube binding, or simply by being discarded with the supernatant [24, 25]. Published studies on comparison methods and in-house validation processes have led to the common knowledge within the forensic science field that a lot of DNA can be lost to the extraction process [18, 22, 30-33]. To combat these issues and adapt to a forensic science workflow, commercial kits are evolving to include fewer steps to reduce time taken, lower contamination risk and incorporate low-DNA binding plastic-ware, with laboratories also implementing automated extraction methods to help reduce the risk of contamination and to better cope with the increased work demand [34-40].

FTA™ paper (Whatman) is a medium for DNA storage, which can be extracted by using a number of methods. It is utilised by biotechnology companies, with reference kits manufactured specifically for use with direct PCR and FTA™ punches, such as GlobalFiler® Express, to reduce the steps involved in the analysis process and decrease the overall time of analysis [41-43]. The paper is cellulose-based and contains chemicals to protect the DNA from degradation, developed by Leigh Burgoyne at Flinders University in Australia [44]. This enables DNA to be stored at room temperature for several years. The DNA in body fluids such as blood or saliva can be stored on the paper by pipetting or swab transferring to the circular area outlined on the paper. The cells begin to lyse once transferred to the paper and DNA is fixed within the matrix. Once dry, a small section of paper is punched out and transferred to a tube for extraction. Solvents, such as iso-propanol, can be used to remove haem and other inhibitors present on the paper. After cleaning, the paper punch is transferred directly to the PCR tube for amplification.

The FTA procedure provides long term stable storage at room temperature with consistent results [45]. Although the process may be automated for faster results and multi-sample processing [46, 47], automation using FTA card has not gained traction due to the effects of static electricity. The small, dry paper discs have a tendency to ‘jump’ from their tubes or wells, possibly into other sample tubes causing contamination, or lost to other surfaces resulting in loss of information [48]. FTA™ has remained a popular medium for the storage of samples due to its preservation

capabilities, particular in the collection of crime scene evidence or storage of reference DNA that may need further sampling [49-52].

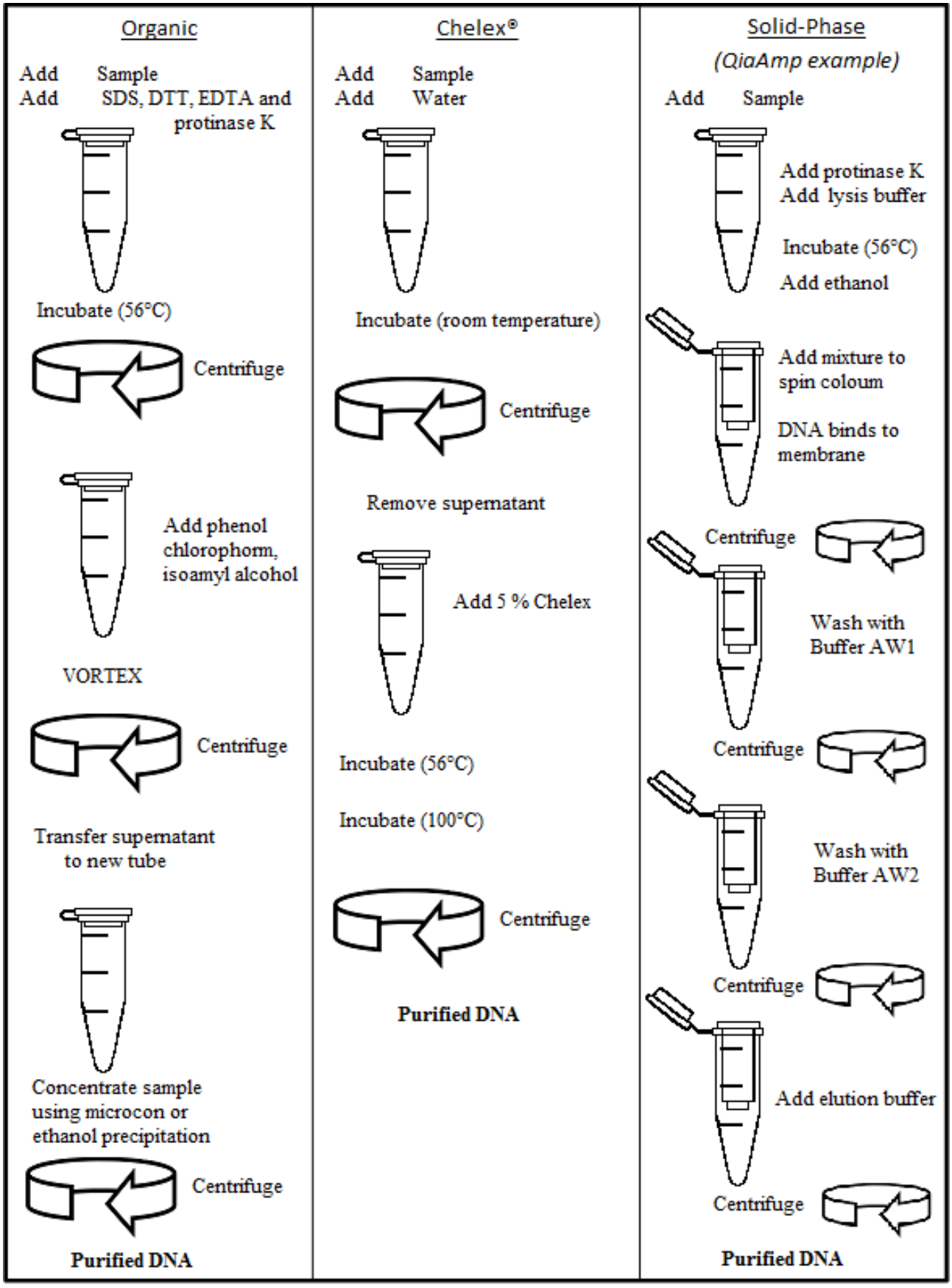

Figure 1.2 – Overview of the three most common extraction techniques.

1.1.1.2 Organic or phenol-chloroform extraction

Organic extraction is ideal for high recovery of DNA from samples, however the process is lengthy and involves the use of hazardous chemicals [10, 13]. Initially, SDS and proteinase K are added to break open the cell membrane, nuclear envelope and the proteins that encase the DNA molecules such as the histones. This is followed by the addition of a phenol/chloroform mixture to separate these proteins from the DNA molecules. This is based on the solubility of proteins into phenol under these conditions while the DNA remains soluble in the extraction buffer. Centrifugation then separates the aqueous phase containing double-stranded DNA from the dense organic phase containing cellular and protein debris, allowing for collection and transference of the DNA to a separate tube. The protocol is often repeated several times to increase the concentration and purity of the DNA yielded. Although the process is difficult to automate and requires numerous tube changes, the process does provide relatively pure DNA, depending on how it is initially collected.

1.1.1.3 Chelating resin – Chelex®

Chelex® 100 (Bio-Rad Laboratories) is more rapid than organic extraction as it involves fewer steps, and therefore fewer chances for exogenous contamination. It is also safer, simpler and overall reduces laboratory costs. It produces single stranded DNA and therefore is only suitable for PCR related analysis. A chelating-resin suspension is added directly to the sample, typically creating a 5% Chelex® suspension. It is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions that act as chelating groups in binding polyvalent metal ions such as magnesium [48, 53, 54]. This attracts and binds magnesium (Mg^{2+}), calcium (Ca^{2+}) and iron (Fe^{2+}) in the sample so that the molecules cannot activate nucleases that destroy DNA (Mg^{2+} is the DNAase co-factor and hence an inhibitor if not removed). This protects the DNA molecules within the sample. Once Chelex® resin has been added to the sample, it is boiled for several minutes to break open the cell membranes to release the single-stranded DNA. Spinning the sample in a centrifuge will move all Chelex® resin, cellular and protein debris to the bottom of the tube allowing for the supernatant containing the DNA to be removed easily. Chelex®, however, tends to be more sensitive to inhibition from certain sample types as only 2^+ ions are removed, hence other cell content that is able to co-extract with the DNA remains. This therefore affects the downstream processes and STR results, showing it to perform particularly poorly with degraded DNA samples [22, 32]. There is no purification step in this method to remove inhibitors or contaminants; wash steps may be implemented to assist in removing some inhibitors such as haem from blood, but additional tube changes may cause loss of DNA. Chelex® is a predominantly manual method, but can be modified and partially automated to assist with processing time and reduction of contamination risks [55].

1.1.1.4 Solid-phase silica

Solid-phase extraction is available in many formats of commercially manufactured kits. The process involves separating compounds, either dissolved or suspended in liquid, based on their physical or chemical properties. They are often the preferred method of extraction due to ease of use, high DNA yields, ability to be automated, removal of inhibitors and they do not use hazardous chemicals [23, 40, 56, 57]. Two companies Qiagen Inc. and Promega Corporation produce the most commonly used solid-phase extraction kits in forensic science, utilising spin columns, silica bead and magnetic bead technology.

The Qiagen QIAamp® spin columns use small glass beads to selectively absorb nucleic acids. In the presence of high concentrations of chaotropic salt, hydrogen-bonding networks in the water will be disrupted, causing denatured proteins and nucleic acids to be more thermodynamically stable than their non-denatured form [48, 58-61]. Multiple buffer washes contribute to the removal of DNA degrading cations, such as Mg^{2+}, as well as other impurities and inhibitors, leaving approximately 95 % of the DNA to be bound by charge to the silica membrane in the column when pH is less than 7.5 [48]. To release the DNA from the silica beads in the membrane, an alkaline elution buffer is used to reverse the binding.

The DNA IQ™ System (Promega) is a semi-solid-phase extraction method, employing the same wash, DNA binding and elution steps as Qiagen kits, but use silica-coated magnetic resin instead of beads [40, 62]. This approach allows the extraction to be performed in a single tube, reducing DNA loss, as DNA can bind to the surface of tubes each time a new one is used. Like the Qiagen method, DNA will bind reversibly (to the magnetic resin) when the pH is less than 7.5. A magnet is used to separate the resin from solution by drawing the resin to the side of the tube allowing for the easy removal of the solution containing impurities and cell debris. The resin is washed repeatedly to ensure the efficient removal of inhibitors. To release the DNA from the resin it is heated for several minutes. This one-tube-method is a major benefit as it is fast and simple, making it ideal for automation within forensic laboratories, enabling fast processing of large sample numbers. Although solid-phase silica extraction methods produce higher purity DNA extracts than Chelex® extraction, the commercial kits are expensive and not very efficient at binding DNA, resulting in a high loss of template DNA [23, 30].

1.1.2 Quantification of DNA

1.1.2.1 Why quantify?

DNA quantification determines how much DNA is present in an extract. This information is valuable and necessary in order to optimise downstream processes such as PCR and profile analysis. For example, commercially produced human DNA profiling kits are optimised for initial DNA starting templates typically between 0.5 – 2.5 ng of DNA at the PCR stage. The original profiling kits such as SGM® and Profiler® required 2 ng of input DNA, this reduced to 1 ng for SGM Plus® and now to 0.5 ng for newer kits as buffer constituents and polymerases become more robust, thus increasing the sensitivity. Info Box 1.1 shows how human genomic DNA quantities are calculated. Quantification allows for the optimal amount of DNA to be used; when too little or too much DNA is added to sensitive reactions, adverse outcomes may arise. Too much DNA can result in incomplete adenylation (split-peaks), off-scale peaks, and locus-locus imbalance [48] as well as excessive background noise created by too much DNA fluorescence (detected during capillary electrophoresis) making profile analysis very difficult. Too little DNA can also cause locus-locus imbalance, as well as stochastic amplification where heterozygous alleles amplify unbalanced or not at all, resulting in 'drop-out' of an allele [63-66]. Drop-out typically occurs with larger DNA fragments and can cause heterozygous loci to appear as homozygous. To reduce stochastic events, several methods have been developed for DNA quantification, with a focus on human-specific methods in forensic science [67]. Human-specific methods are important for forensic science applications, as mentioned previously, the initial starting template for optimised reactions in human identification is quite narrow. Each method for DNA quantification continues to evolve to meet the needs of the forensic laboratory: simplicity, speed, cost, accuracy, automation and adaptability. Comparison studies of quantification methods have also highlighted the need for international [68] standard methods and standard reference materials to minimise variation between laboratories, and identify the best protocols for each sample type [69, 70].

Calculation of DNA quantities in human genomic DNA:

1 bp = **618 g/mol**
A = 313 g/mol; T = 304 g/mol; A-T base pairs = 617 g/mol
G = 329 g/mol; C = 289 g/mol; G-C base pairs = 618 g/mol

1 genome copy = **~3 x 10^9 bp** = 23 chromosomes (one member of each pair)

1 mole = **6.02 x 10^{23} molecules**

1 genome copy = (~ 3 x 10^9 bp) x (618 g/mol/bp) = 1.85 x 10^{12} g/mol
= (1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
= 3.08 x 10^{-12} g = **3.08 pg** in a haploid cell

∴ a human diploid cell, containing two copies of each chromosome will contain **~ 6 pg** of genomic DNA

Human DNA kits optimised for ~ 1 ng of genomic DNA (1000 pg)
= **~333 copies** of each locus
(2 per 167 diploid cells)

Info. Box 1.1 Important values for calculating DNA quantities, adapted from Butler [48].

1.1.2.2 Slot Blot

Slot blot utilises a 40 base pair (bp) probe that binds to locus D17Z1 in humans and higher primates, showing a high degree of species specificity [67, 71]. Once extracted and denatured, the single-stranded DNA is bound to a positively charged nylon membrane. The probe is then applied and hybridises with the DNA. The hybridised complex can be detected using chemiluminescent or colorimetric signal intensities where the unknown samples are compared to a set of standards of known concentrations. The amount of DNA is estimated based on these comparisons. Chemiluminescence is more sensitive as the reaction causes the release of protons that are captured on a CCD camera and can detect down to 10-40 pg of DNA [72]. The commercially produced kit QuantiBlot® Human DNA, developed by Applied Biosystems, was short lived due to the worldwide adoption of more sensitive, faster and efficient methods, and was discontinued in 2007. A large amount of extracted DNA may be lost due to the set-up of this method, as the slot blot plate apparatus requires a minimum working volume. This is not ideal for forensic science purposes where limited extracted DNA is available, or if the extracted DNA is required for further independent testing. The method of measurement is also highly subjective as the intensity of spots for unknown samples compared to the calibration standards are estimated, with unknown samples often falling between two calibration spots. Negative controls often gave full DNA profiles, as the apparatus used is more open to cross-contamination than other methods.

1.1.2.3 Spectrophotometry & Fluorometry

UV spectrophotometry can quantify DNA by measuring the absorbance of a sample at 260 nm without the use of standard curves, detecting concentrations down to 2.5 ng/μL. Although this method is relatively fast, the limit of detection is not sensitive enough for most forensic purposes. Fluorescence tagging methods have become more popular in forensic science as the use of intercalating dyes is more sensitive than UV spectrophotometry. Intercalating dyes will bind to DNA and then fluoresce when excited. The measured fluorescence is compared to a standard curve and can detect DNA concentrations as low as 100 pg/μL. Intercalating dyes and UV spectrophotometry methods quantify the total DNA in a sample and are not human specific. The sensitivity and effectiveness of the dyes can also vary greatly depending on the dye used [73, 74]. For example ethidium bromide and PicoGreen both selectively bind to dsDNA, however PicoGreen is safer, more sensitive and has a substantially higher fluorescence than ethidium bromide [74]. The development of the Qubit® Fluorometer (Life Technologies), seen in Figure 1.3, has enabled the fast and accurate measurement of DNA, RNA and protein in the laboratory. The benefits of this small bench-top device include a small sample input, as low as 1 μL, with a total set-up and run

time being as fast as just a few minutes. The disadvantage for forensic samples is that it is not human-specific as all DNA is detected.

Figure 1.3 – A bench top UV spectrophotometer (left) and a newer style Qubit® 2.0 Fluorometer (right).

1.1.2.4 Real-time PCR

Real-time PCR or quantitative PCR (qPCR) is a fluorogenic method that measures the total amount of amplifiable genomic DNA. There are two variations of this technique used in forensic science. The first is the measurement of *Taq* polymerase activity using an intercalating fluorescent dye, such as SYBR® Green or ethidium bromide, to bind to double-stranded DNA [75]. The second is to measure the 5-nuclease activity of the *Taq* polymerase, such as TaqMan®, to cleave a target-specific fluorescent probe [76]. In both instances, the fluorescence is measured as it accumulates with each PCR cycle and compared against a standard curve with known concentrations (Figure 1.4). Human-specific regions can also be targeted during this PCR process, most commonly multi-copy Alu sequences, which appears up to one million times throughout the human genome [77, 78]. Companies such as Applied Biosystems and Promega have developed numerous commercial kits providing efficient, accurate and species-specific quantification [77]. This method is typically the preferred method for forensic samples as it can be automated allowing for high throughput

processing, adapted to simultaneously perform different qualitative analysis such as sex determination, mtDNA degradation, and cross species quantitation, detects low levels of DNA, and is human-specific [79-81].

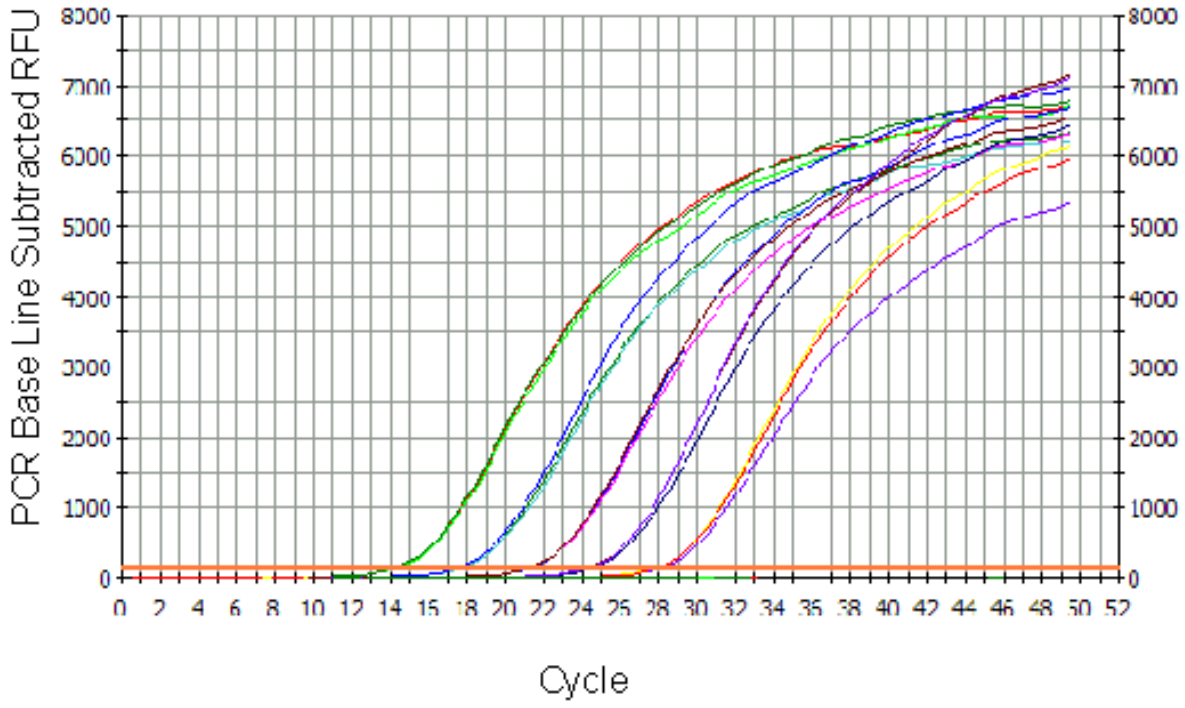

Figure 1.4 – Amplification curves obtained from serial dilutions of target DNA using real-time PCR. *Figure source: http://www.etsu.edu/com/mbcf/Services/pictures/pcr1.gif*

1.1.3 Capillary Electrophoresis

The final experimental step of the analysis process is the separation of the PCR products to obtain a DNA profile. Human identification kits target multiple STR fragments that need to be separated from each other to a one base pair resolution, such as TH01 alleles 9.3 and 10, so that individual alleles can be distinguished from one another. To ensure reproducibility between laboratories worldwide, advanced genetic analysers that utilise CE methods are used, offering high resolution and accuracy [82-84].

The capillaries are made of a thin fused silica (glass), with an inner diameter ranging from 50 – 100 μm and a length of 25 – 75 cm, capable of holding a sieving polymer. The properties of the capillaries allow for greater heat dispersion thus enabling higher electric fields, resulting in faster separation [85]. The polymer contains a high concentration of urea to maintain an environment within the capillary that will keep DNA molecules denatured, allowing the fragments to move consistently through the polymer matrix, as the mobility of the DNA fragments can be affected by its conformation [86, 87]. Prior to injection, PCR samples are diluted in deionized formamide with an internal size standard, and heated to 95 °C to ensure the DNA molecules are single stranded. Pores within the polymer matrix aid in the size-based separation of the PCR fragments; smaller fragments can move more freely between the pores and elute faster than the larger fragments that become entangled within the matrix [88, 89]. The PCR fragments, comprising of DNA, are pulled through the capillaries and polymer matrix using an electric current. As the phosphate groups on the DNA backbone are negatively charged, the DNA fragments can be separated over the length of the capillary using an electric current flowing from the cathode to the positively charged anode [88]. A detection window is located on the capillary just prior to the anode, where individual fluorescently labelled DNA fragments are detected by laser-induced fluorescence. Fluorescent dyes used in STR DNA labelling emit light ranging from 400 – 600 nm, in the visible region of the spectrum, allowing them to be distinguished from one another based on their specific emission wavelength. The maximum fluorescence of each dye passes through a diffraction grating and is captured by a CCD camera [48]. Raw data of the measured fluorescence signal are obtained and compared to spectral matrix calibrations for each dye signal. GeneMapper® analysis software automatically analyses the data, revealing a DNA chromatogram.

Major benefits of CE methods over slab gel methods includes the overall reduced time in the set-up of the instrument, sample preparation and separation, all of which can now be fully automated [90]. Reproducibility, resolution, cross-contamination and sample consumption are also greatly improved in the CE system.

1.1.3.1 Genetic analysers

The most commonly used CE systems in forensic science are manufactured by Applied Biosystems/ Life Technologies. The first system developed, the ABI Prism® 310 Genetic Analyser, consisted of a single capillary able to process one sample at a time. To assist with the high workflow of forensic samples, CE systems have evolved to include more capillaries per array. The ABI Prism® 3100-Avant and 3130 house a 4 capillary array, increasing to 8 capillaries in the 3500, and further increasing to 16 capillaries per array in the 3100 and 3130*xl* series. The 16 capillary array systems are the most commonly used due to the speed of processing samples (approximately 45 minutes), low contamination issues, and greatest precision and sizing accuracy when compared to other CE systems [91].

Human DNA identifying kits continue to evolve by increasing the number of loci targeted. It is important that the range of loci does not overlap in size with others; using multiple dyes in the multiplex assures there is no overlap (Figure 1.5). CE systems typically consist of 4 or 5-dye technology, where one dye is reserved as a size standard, and the remaining dyes are used to label the STR fragments. GlobalFiler® is the latest kit by Life Technologies, incorporating 6-dye, 24-locus technology offering reduced amplification time with the highest discrimination power available. With this increase in dyes, a new CE system has been developed, the 3500 Genetic Analyser, to accommodate the 6-dye technology.

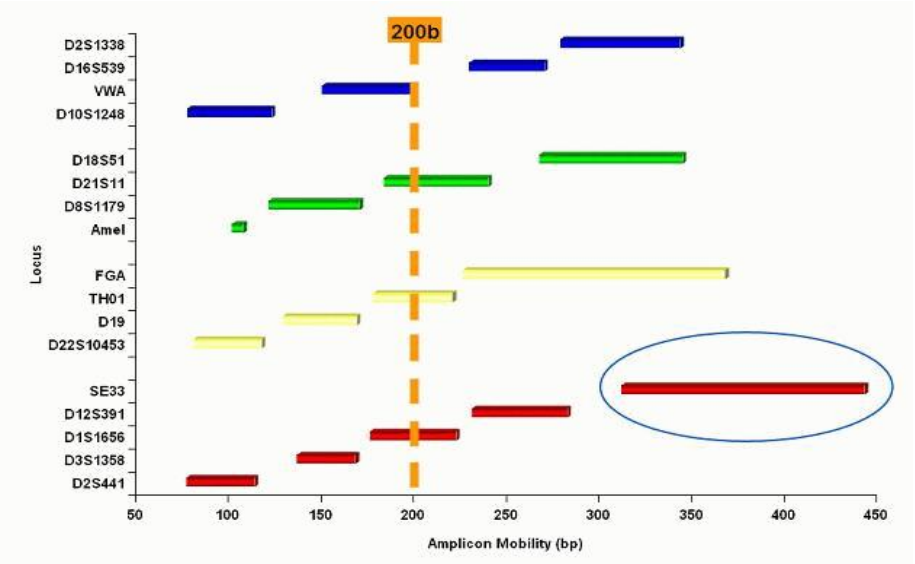

Figure 1.5 – Loci and dye arrangement for the AmpF*l*STR® NGM SElect™ (Life Technologies) human identification STR kit. Four dyes (blue, green, yellow, and red) are attached to primers for DNA STR amplification; the fifth dye (orange) is used as an internal sizing standard during CE. Circled locus indicates the additional locus between the NGM™ kit and NGM SElect™ kit.

As the demand for DNA sampling and rapid analysis continues to increase, the CE systems continue to evolve. Portable CE units developed on microchips, known as microfluidic devices, are being manufactured that can be used on-site at an investigation [92-95]. These units process the sample from DNA extraction through to STR or SNP analysis, giving results in as little as 90 minutes, thus speeding up the analysis process dramatically [96, 97]. These instruments are generally considered to be in the early stages of development, and have not been widely adopted yet.

1.1.3.2 RFU measurements

RFU are the recorded electric signals detected using software when the fluorophore attached to the DNA fragment is excited during CE. The fluorophore will absorb the laser energy and emit light at a particular wavelength. The spectral overlap of the dye sets used, as observed in Figure 1.5, is accounted for using statistical software and a preinstalled mathematical matrix that subtracts the contribution of all other dyes within a single dye measurement [48]. This ensures the results observed are from an individual dye colour and not a mixture of the spectral overlap. The stronger the signal, the brighter the fluorescence of that dye will be and a greater RFU value will be recorded. The RFU measurement is not an SI unit, but developed by ABI for use with their systems.

RFU thresholds for forensic DNA analysis are essential to maximise the detection of alleles, and to maintain universal standards for analysis methods. However, these standards may vary between each lab from 25 – 200 RFU, depending on whether the peaks are heterozygous or homozygous, and the method of threshold determination used [98]. Many laboratories are moving to continuous models for DNA interpretation and use +3 SD above the baseline to determine the stochastic RFU threshold [99-102]. The thresholds are most commonly determined by analysing the signal-to-noise ratio of blank signals after processing. The baseline noise begins to increase substantially with higher levels of DNA, causing a bleed through of the overlapping spectral colours, known as ‘pull-up’ [48, 98]. Excessive pull-up may also cause peaks to present themselves as off-ladder. Each kit identifies an optimal amount of template DNA in order to avoid these issues.

1.1.4 Profile Analysis

Once the data have been collected and processed by the relevant software, the resulting DNA profile is ready for analysis. The analysis can be affected by numerous factors, including the analysis strategy used, the nature of the DNA profile (mixed or single source), bias, and the experience of the DNA analyst [103]. Software such as FaSTR DNA offers automation of the DNA profile analysis step to increase consistency and save analysis time [104]. Statistical and continuous models, including mixture interpretation software, have been developed as a way to unify the analysis process as much as possible, and reduce variation [98, 100, 102, 105]. The factors affecting analysis therefore affect the resulting match probabilities and likelihood ratios. This leads to a concern of possibly over or understating the evidential impact of a match, particularly where kinship or mixed profiles are involved [106], having significant consequences within a courtroom [103, 107-109]. Due to the impact of different DNA interpretation methods there has been a push within the forensic science community for standardisation, with a shift from threshold-based interpretation towards continuous interpretation strategies [110].

1.1.4.1 Standards

The quality assurance standards for all types of forensic DNA analysis in the USA are governed by SWGDAM. SWGDAM are a group of forensic scientists that represent international, federal, state and local forensic DNA laboratories. They provide guidelines and documents for the direction and guidance within the community for forensic casework analysis, covering areas of nuclear DNA, mitochondrial DNA, population genetics, statistics, STRs and Y-STRs, as well as the identification and application of threshold for allele detection and interpretation, appropriate statistical approaches to interpretation of autosomal STRs and mixture interpretation. The group meet twice a year to review standard operating procedures, recommend research to be conducted or methods to be validated, and encourage laboratories to review their procedures based on the findings. In addition, any laboratory that analyses DNA samples for forensic casework purposes is required by the Quality Assurance Standards for Forensic DNA Testing Laboratories to establish and follow documented procedures for the interpretation of DNA typing results and reporting [111, 112]. The procedures are based upon validated studies, scientific literature and experience. Similarly to SWGDAM, commissions presented by the ISFG are followed by many laboratories outside of the USA [113].

The guidelines continue to evolve as new technologies emerge; however until recently (October 2014), the guidelines have not extensively addressed the validation, standardisation and interpretation of analytical results from enhanced low template DNA techniques [114]. This is

because the technology is still relatively new, with varying methods, making the overall interpretation of DNA typing results for human identification purposes particularly difficult when low-level DNA samples are involved, thus requiring professional judgement and expertise [66, 105, 115-120].

1.1.4.2 Contamination

SWGDAM and the ISFG also provide guidelines for precautions against contamination, as many studies have shown contamination to arise from various stages within the analysis process [111, 121-125]. For the most part, contamination can be avoided or greatly reduced by following the appropriate protocols and procedures and can be more easily identified with the aid of staff elimination databases, isolated laboratory sampling areas, negative controls and regular testing of lab ware and supplies [48, 126, 127]. As mentioned previously, trace DNA samples pose their own set of difficulties with interpretation and analysis. As the technology implemented in trace DNA work involves more sensitive testing, contamination is more likely to be seen and possibly go undetected, and thus compound the difficulties of the analysis process [127-129]. The impact of contamination has obvious serious ramifications when it comes to criminal investigations and prosecution [125, 127].

1.2 DNA Technology

The value of DNA evidence in a forensic investigation has increased momentously since DNA fingerprinting was first described [130], a major scientific breakthrough that allows a unique DNA profile of an individual to be recorded. The continued collaboration around the world towards DNA databases has enabled strong statistical weighting to be applied when comparing DNA samples, often making DNA the strongest link of evidence in a criminal investigation. Each one of our cells contains DNA within the nucleus and DNA within the mitochondria, except for mature red blood cells that lack a nuclear component. Investigations involving biological cellular material will utilise a set of validated protocols and steps to retrieve and analyse the important DNA information within. This DNA may be present in body fluids such as saliva, blood (white blood cells), or semen, or may be retrieved from other sources such as hair, fingernails, teeth, tissue or fingerprints.

1.2.1 Short Tandem Repeat Typing

STR markers or microsatellites are repeated DNA sequences found on a genome, typically consisting of a two – six bp motif. In forensic science, STR technology evaluates specific locations (loci) on the nuclear DNA. These areas are variable in nature (polymorphic), increasing the discrimination value between profiles of unrelated individuals [64, 65, 131-134]. The first basic multiplex, 'the quad', was developed in the UK by the Forensic Science Service (FSS) in 1994, followed quickly by the 'six-plex' Second Generation Multiplex system in 1995 and was used to create the world's first national database [135]. By 1997, the FBI has established a set of 13 core STR loci to serve as the standard for CODIS (most of which, if not all, are incorporated into currently used commercial human identification kits) [136, 137]. The aim of the core set is to ensure uniform standards and DNA database systems across the forensic science community, as well as sharing valuable forensic information.

STR human identification kits are capable of generating results from very small amounts of DNA across an increasing number of loci. The loci fragment lengths range from 100 – 450 bps. When DNA is exposed to nature's elements, arising in certain forensic situations such as DVI, degradation can occur due to bacterial, biochemical or oxidative processes [138, 139]. When degraded DNA is amplified, it is common to see dropout of the larger fragments as well as peak-imbalance [140, 141]. This is generally caused by the presence of PCR inhibitors, and DNA fragmentation that has occurred prior to amplification making full amplification incredibly difficult. To combat this issue, amplification primers are placed as close as possible to the core STR repeat to create 'mini-STR' systems that amplify smaller PCR fragments ranging from 50 – 250 bp in length [138, 142-145]. Many of the mini-STRs identified are linked to the CODIS system, enabling use of the same

databases [138]. However, a number of studies have also shown the successful analysis of degraded DNA using mini-STRs unlinked from the CODIS markers [142, 144, 146], enabling further assistance in the forensic analysis of difficult DNA samples. Human STR identification systems continue to evolve to include a greater number of loci, as well as smaller and more stable loci in order to adapt to the difficulties faced in forensic investigations, such as GlobalFiler® incorporating ten mini-STRs (Figure 1.6).

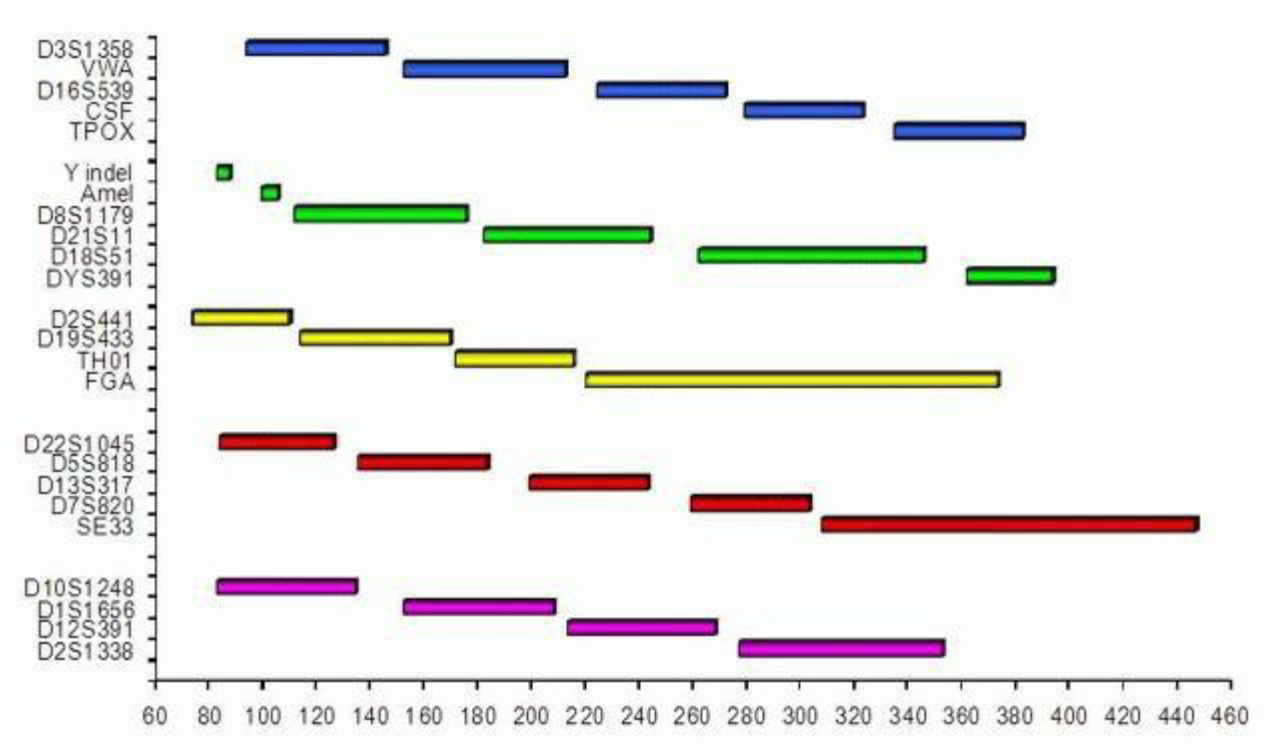

Figure 1.6 – Loci and dye arrangement for the GlobalFiler® (Life Technologies) human identification STR kit. The kit is the first to incorporate 6-Dye technology as well as ten mini-STR loci to reduce the amplification time, maximise results from degraded samples, and increase discrimination power.

1.2.2 Y-STRs

STR markers have also been identified on the Y-chromosome for forensic application, known as Y-STR markers. Y-STRs are lineage markers that are passed unchanged (except for mutation events) through the paternal line. As lineage markers remained unchanged through each generation, they are not as effective in differentiating between two individuals. However, Y-STR markers have proven to be a valuable tool in routine forensic investigations for various applications [147, 148]. Studies involving population lineage and human migration have enabled researchers to differentiate between male individuals of different paternal lineages [149-152]. As Y-STR markers target the male DNA component only of sample, they can be applied to forensic cases such as: deficiency paternity testing [153, 154]; sexual assaults where the female DNA component of a mixed

biological sample greatly outweighs the male DNA component or where there may be multiple male contributors [155-157]; and missing person or DVI investigations [148, 158]. The number of Y-STR markers used in multiplex systems has continued to increase [155, 159-161] but they are still unable to distinguish between related male individuals from the same paternal line. Due to this demand, Y-STR technology has progressed into the research of rapidly mutating Y-STRs (RM Y-STRs) to enable related individuals to be distinguished from each other [162-167].

1.2.3 Mitochondrial DNA

mtDNA is also a lineage marker that is passed unchanged each generation, through the maternal line. Autosomal STR analysis types the two copies (one paternal and one maternal) of linear nuclear DNA found in each cell, whereas mtDNA analysis types the 100 – 10,000 copies of circular DNA found in the mitochondria of each cell. The circular nature and number of copies of mtDNA means it is robust in nature, lacks recombination and is more resistant to complete sample degradation, making sequence analysis of the mtDNA hypervariable regions ideal where samples are so badly degraded that nuclear STR analysis is not possible [168-173].

Similarly to Y-STRs, the mode of inheritance means mtDNA analysis is less discriminating than nuclear STR analysis for identification purposes. However, this type of analysis still offers great information via inclusion or exclusion of individuals in forensic investigations, such as DVI scenarios, missing persons, and identifying human remains from highly degraded DNA samples such as hair, bone and teeth [174-178].

Mitochondrial analysis has several areas of limitations and important issues to consider when evaluating the results, such as: nomenclature inconsistences and the subsequent effect on reference population databases [179-181]; heteroplasmy [182-185]; paternal leakage and recombination [186-189]; and interpretation [190-192]. mtDNA testing is also a time-consuming process, more labour intensive than STR typing, not as routine or as automated in the forensic analysis process. It is recommended that laboratories undertaking mtDNA testing should have dedicated spaces, instruments, chemistry and lab wear, only tolerate low levels of contamination and be involved in regular proficiency testing programs [191]. For these reasons as well as cost, time management and resources, not all laboratories may be equipped to deal with mtDNA analysis.

1.2.4 Single Nucleotide Polymorphisms

Forensic DNA scientists often use additional markers, such as SNPs, as the need arises to obtain further information about a particular sample [193, 194]. SNP fragment sizes are similar to mini-STRs, often smaller, and are also ideal for the analysis of highly degraded DNA samples [195].

However, a SNP locus possesses only two possible alleles and therefore SNP panels require a much higher number of markers (40 – 60) to deliver a similar discriminating power of commonly used STR multiplex kits (13 – 16 loci) [193, 196, 197]. Studies are incorporating more and more SNP loci within a multiplex system, even amplifying autosomal and Y-chromosome makers together, to offer a high discriminating power to difficult forensic samples [152, 198-202]. SNPs have the added benefit of providing inference of the likely physical appearance of individuals such as hair and eye colour, as well as ancestry [194, 203-206], thus providing further means to include or exclude individuals from an investigation. As SNPs are mostly biallelic, they can be easily genotyped using the SNaPshot® Multiplex Kit (Life Technologies).

1.2.5 Low Copy Number & trace evidence

The term 'trace DNA' has many meanings and can be defined as the amount of DNA present, the quality of DNA present, DNA detected by low copy number or low template PCR, DNA that cannot be attributed to an identifiable body fluid, or the DNA transfer to surfaces of sloughed epidermal skin cells through sweat or abrasion [27, 29, 207]. Trace DNA evidence is therefore very wide-ranging and encompasses all samples that fall below recommended thresholds at any stage of the analysis process [27]. As previously stated, *Info Box 1.1*, the recommended DNA template for human identification kits is between 0.5 – 1 ng or ~ 83 – 167 diploid cells, as 1 cell contains 6 pg of DNA.

DNA is present within cellular material, but can also be found outside of the cell when cellular membranes begin to break down [208]; this is known as extracellular or cell-free DNA. Cell-free DNA can also be referred to as trace DNA as it is generally considered to be present in lower masses than cellular DNA. Cell-free DNA can be found in blood, saliva, semen and urine [25, 209, 210] as well as tested forensic samples of vomit, faeces, sweat, and buccal cells [25]. DNA can be found on a range of handled objects such as cigarette butts, clothing, nail cleaners, toothbrushes, and door handles through direct or indirect transfer, with studies showing that only minimal contact is required for skin cells and debris to be transferred to items for successful DNA results to be obtained [31, 211-214].

It has been suggested that cell-free DNA contained in sweat may contribute to the DNA profiles obtained from touched or handled items [18, 215]. This was further confirmed in a separate study where cell-free DNA was detected in the sweat of 80 % of individuals analysed [24]. In a more recent study [25], the suggestion that a substantial proportion of cell-free DNA may be lost to the extraction method, discarding potentially important forensic information, was investigated further.

This study compared the DNA profiles from the cell pellet and the concentrated supernatant, from the extraction process, and found that not all alleles present in one sample were found in the other or *vice versa* [25]. Thus, indicating that DNA information can be lost if the supernatant is discarded during extraction.

As STR PCR DNA typing technology continues to become more sensitive due to improved extraction processes, enhanced buffer systems, and greater DNA collection techniques, caution needs to be demonstrated when handling exhibits and interpreting results from trace DNA evidence, as only minimal contact can cause the transference of DNA between objects [211]. Substantial DNA transfer between individual and item (primary transfer) occurs during the initial contact [211], highlighting the importance of proper care when processing evidence and the risk of external contamination. Secondary transfer and contamination of DNA has shown to be low in several studies, indicating that although possible it is not likely to occur if proper care is taken when processing evidence [26, 29, 66, 213]. The number of factors that affect the presence or transfer of trace DNA and the ability to successfully obtain profiles from it are unknown but can include: duration of contact, the substrate surface, environmental conditions, time since depository, pressure and friction of contact, moisture, and the variation of analysis methods [26, 124, 207, 212, 216]. It has also been suggested that the success rate of DNA profiling is dependent on not only the characteristics of the DNA contributor (recently washed hands or dominant hand used), but also the activities performed by them prior to touching an item [213].

As the science of DNA developed in the late 1990s, a new analysis and interpretation of low template DNA was developed by the UK FSS, called LCN. To combat the low success rate of trace DNA evidence the PCR amplification was increased from 28 to 34 cycles [66], and is the standard for LCN PCR. LCN became a trademarked process marketed to police forces in the UK. *Info. Box 1.2* identifies important cases where LCN was implemented, with *Info. Box 1.3* explaining how new and novel methodologies is received in a court of law under the Daubert standard (applicable in the USA only). As LCN is able to detect lower thresholds of DNA, there is a real concern of incidental DNA being detected and secondary transfer therefore becomes more likely. This issue is outlined in Figure 1.7, and discussed further in *Info. Box 1.2, Reed & Reed v. R* [217]. An extensive review of LCN analysis has found the process to be a validated method, whilst also identifying advantages and limitations associated with the method and recommendations for laboratories wanting to implement the technique [218].

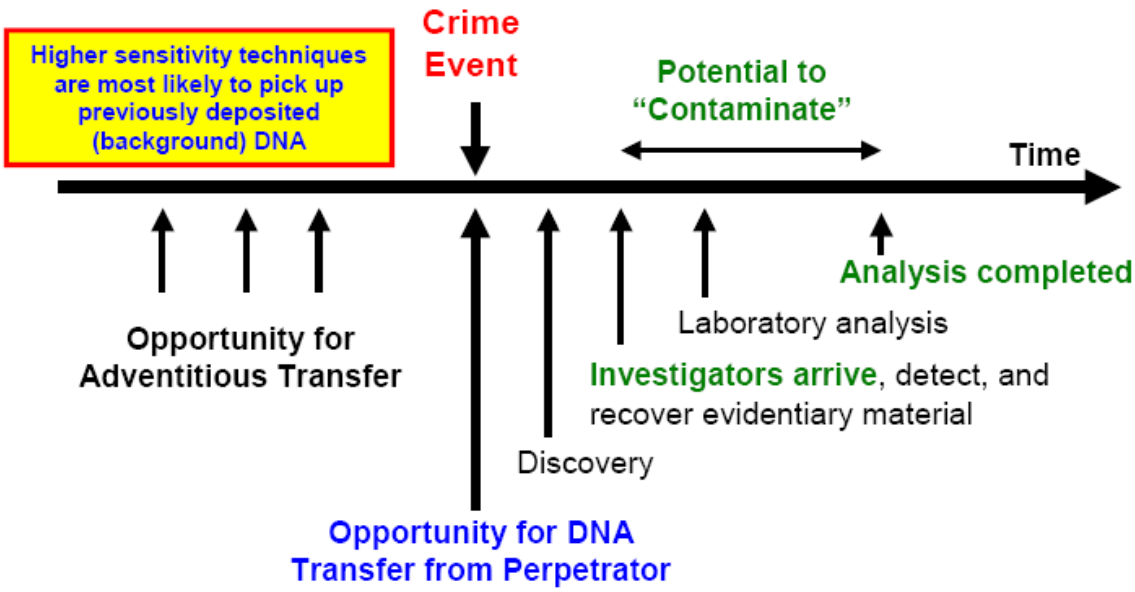

Figure 1.7 – A timeline indicating scenarios of DNA transfer pre and post crime event. LCN is capable of detecting lower thresholds of DNA, and possibly DNA transferred to items prior to a crime event. Informed comment can be made about transfer of DNA originating from certain substances such as blood or semen. However, it is much more difficult to provide comment on cell-free DNA, or low levels of DNA where limited contact may have occurred. Higher amounts of DNA present (nanograms) typically reveals greater contact or pressure between item and DNA source. The potential to contaminate evidence occurs post crime event.

Info. Box 1.2

Important LCN cases.

These cases illustrate the highly sensitive nature of the LCN technique.

Sean Hoey v. R

LCN was used to successfully generate a DNA profile from wires associated with a car bomb that killed 29 people and injured 220 others in Omagh, Northern Ireland, on 15 August 1998. The LCN profile matched Sean Hoey, a known member of the Real IRA. During the trial of Hoey (2007), questions were raised as to whether LCN is reproducible. By its very nature, it is not reproducible and Mr Justice Weir ruled the prosecution's evidence did not meet the required standard. The judge therefore removed DNA as part of the evidence, leading to the exoneration of Hoey. Hoey was later convicted of all murders in a civil trial.

Reed & Reed v. R

The appellants were convicted in August 2007 of the stabbing murder of Peter Hoe, on the basis of an analysis of LCN DNA profiles as well as other evidence. Appeals to this case have raised issues with the LCN process, including its lack of validation, limited research, absence of protocols, disputes of interpretation and the scope of the evaluation. Professor Allan Jamieson, Dr Bruce Budowle, and Dr Adrian Linacre, all holding extensive knowledge of LCN, provided the Court expert witness statements. The appellants prior to the hearing of the appeals abandoned the appeals regarding the reliability of LCN. Following further DNA testing by Dr Budowle, it was accepted that the appeal could no longer be pursued on the basis of the reliability of the LCN process. It is also worth mentioning that there was never a challenge to the fact that the profiles obtained on the knife handles matched the profiles of the appellants.

The appeal then shifted to the evidence about transfer of DNA and the various explanations that had been given by the prosecution's expert, arguing that this went beyond what was proper for an expert witness. The issue was if evidence could be admitted on the possibility of how the DNA came to be on the knife handles, and if those possibilities could be evaluated. The Court held that there was nothing wrong with the expert giving some evaluation of each of the possibilities of the circumstances of transfer and that this was indeed essential. However, the Court agreed that the expert witness went too far when she expressed the opinion that the appellants were *"handling the knives at the time when the handles broke"*, as this was not based on a reliable scientific basis. See Figure 1.7 illustrating the issues with DNA transfer.

Peter Falconio Murder: South Australia

Peter Falconio was a British tourist who disappeared while travelling with girlfriend Joanne Lees, in the South Australian outback during July 2001. Falconio's body has never been found and he is presumed dead. Bradley John Murdoch was convicted of his murder in December 2005 after LCN generated a DNA profile from the cable ties used around Joanne Lees wrists, that later found to match a DNA profile from Murdoch.

Info. Box 1.3 Admissibility of evidence based on new or novel methods in the Court.

The Daubert Standard

A Daubert standard provides a rule of evidence over the validity and admissibility of expert testimony conducted before a judge. The opposing counsel may challenge the evidence by raising a Daubert motion and the expert is required to demonstrate that their methodology and reasoning are scientifically valid and can therefore be applied to the facts of the case.

There are three U.S Supreme Court cases that articulated the Daubert standard. The phrase originated from the 1993 case *Daubert v. Merrell Dow Pharmaceuticals*. *Daubert v. Merrell Dow Pharmaceuticals*, Supreme Court held that Rule 702 of the Federal Rules of Evidence superseded Frye as the standard, incorporating a flexible reliability standard and articulating a new set of criteria for the admissibility of scientific expert testimony.

The second case, *General Electric Co. v. Joiner*, which held the judge may exclude expert testimony when there are gaps between the evidence relied on by an expert and his conclusion.

The third case, *Kumho Tire Co. v. Carmichael*, which held the judge's gatekeeping function identified in Daubert applies to all expert testimony, including non-scientific.

The Frye standard is also a test to determine the admissibility of scientific evidence derived from a new or novel scientific theory or methodology. The test stipulates expert opinion based on a scientific technique is admissible only where the technique has gained 'general acceptance' supported by a body of knowledge within the relevant scientific community. The Frye standard is limited in that it is essentially a 'counting heads' test that does not require the judge to understand the new or novel scientific theory or methodology presented to the courts. Although Frye and Daubert are not substantially different, Daubert's key subject is the scientific validity, the evidentiary relevance and reliability of the principles that underlie a proposed submission, with the focus solely on principles and methodology, not on the conclusions they generate.

The Queen v. Bonython

This case brought to question the qualifications of a witness, Sergeant Daly, to express his opinion on handwriting and the identification of signatures, and if his opinion is admissible in the Court. The Judge ruled the question regarding Sergeant Daly's expertise, designed to establish that the materials upon which the witness formed his opinion were inadequate, related to the weighting of the opinion and not to the admissibility of the opinion. A witness may give evidence only as to matters observed by him or her. Opinions are not admissible. The exception to this is the opinion of an expert. An expert witness must prove that the body of knowledge or experience which is sufficiently organised or recognised to be accepted as a reliable body of knowledge or experience are not, or are not wholly, within the within the knowledge or experience of ordinary persons. The judge must be satisfied that the witness possesses the necessary qualifications, whether they are from study, experience or both. In this case, the witness was able to satisfy the Judge of his knowledge, and was considered an expert witness.

LCN also presents interpretational issues: more extreme heterozygous peak imbalance, increased stutter, and increased laboratory based contamination (or allelic drop-in). All samples amplified using 34 cycles are processed in duplicates or triplicates, counting the alleles that appear twice, creating a consensus profile to assist with profile interpretation (Figure 1.8). Consensus profiles however, may also cause important data to be discarded from the final resulting profile if alleles are not observed in multiple replicates (Table 1.1). The incidences of increased artefacts in low-level DNA samples compromise the quality of the profile. The quality of a DNA profile is typically based on the number of loci, in which alleles are present, as well as the height based on relative fluorescence units (RFU) value of those alleles. With low levels of template DNA, allele peak heights fall and alleles begin to drop-out; making it difficult for an analyst to interpret the results especially when there may be more than one DNA contributor to the sample (mixed profile).

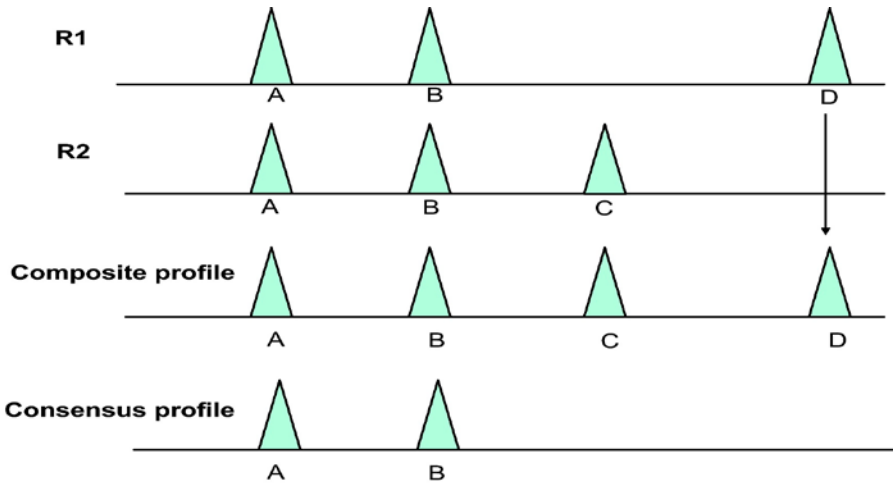

Figure 1.8 – Understanding the difference between composite and consensus profiles by comparing two replicates, R1 and R2. The consensus profile only contains alleles A and B as they appear in both replicates, whereas the composite profile contains all alleles (A, B, C and D) observed in both replicates. *Figure source: Bright [219]*.

Table 1.1 – A hypothetical example of a replicated sample showing the consensus profile. Observed donor alleles may be discarded as unconfirmed and not used as evidence with the consensus method (D8, allele 13). *Table derived from Cowen [220]*.

	AMEL	vWA	TH01	D8	FGA	D21
Replicate 1	X	15, 17	6, 9	10, 13	–	28, 31, 32
Replicate 2	X	14, 15, 17	6	10, 11	20	28, 31
Consensus	X, F	15, 17	6, F	10, F	F, F	28, 31
Unconfirmed donor alleles				13, 11	20	
Drop-in		14	9			32
Donor Profile	X, X	15, 17	6, 6	10, 13	20, 23	28, 31

The issues discussed surrounding trace DNA decrease the confidence of the analyst to correctly interpret a resulting profile, and why some laboratories are opposed to processing trace evidence. The value of trace DNA in an investigation is viewed differently depending on its application. It can be highly significant in terms of intelligence applications [131], where partial profiles are typically generated, they can aid in the inclusion or exclusions of a suspect. However, the weighting or value of trace DNA is viewed to decrease significantly if it is to be relied upon as evidence in a court of law [124]. It should also be noted that although analysis errors are rare due to contamination, they are possible and can have very serious consequences. For example, six murders were incorrectly linked to a hypothesised unknown female serial killer dubbed the "Phantom of Heilbronn" or the "Woman Without a Face" after DNA evidence linked crime scenes in Austria, France and Germany from 1993 to 2009 [221]. The source of the DNA was eventually found to have already been present on the cotton swabs used for collecting DNA samples, contaminated accidentally by a woman working at a factory in Bavaria. Human error and sample contamination are the most common cause of error rates in relation to the laboratory process [131], and highlights the need to investigate all aspects of the analysis process to reduce the number of contaminations and errors made.

1.3 Direct PCR

The process of direct PCR enables a DNA profile to be generated from a sample without the use of the extraction step, the sample instead is placed directly into the PCR reagents and then amplified. In microbiology, this technique is commonly known as 'colony PCR', and has been widely used since the early 1990s [222-224]. Colonies of bacteria yeast are directly amplified with specific primers as a rapid test to determine if the cloning process was successful [225-228]. In forensic science, existing standard operating procedures describe the necessity of extraction methods to remove potential PCR inhibitors, thus allowing the downstream processes to occur. In regards to trace DNA samples, however, where the starting DNA amount may already be incredibly low, the potential for DNA to be lost through sample collection and the extraction process can be highly detrimental as it could dramatically reduce the likelihood of obtaining a meaningful DNA profile [229]. Significant resources are often used to process trace DNA samples, with commonly submitted samples such as handled items typically returning the least successful profiles [230]. The LCN technique was developed to combat the low success rate of obtaining meaningful data from trace DNA samples, but as previously outlined LCN has its own set of disadvantages associated with the validation of the technique, as well as the analysis and interpretation of the results.

To use direct PCR with forensic trace DNA samples would mean that the downstream processes are open to the effects of inhibition, however, common forensic inhibitors such as haem compounds from blood, bile salts and complex polysaccharides, urea, and collagen and certain dyes found in clothing, are not commonly present in touch or trace DNA samples [231]. The type of extraction method used is also known to play a significant role in the loss of template DNA due to number of tube transfers required [30, 31]. Therefore, circumventing the extraction process would save time and money by speeding up the processing procedures and potentially increasing the DNA template of challenging and difficult samples. It is hypothesised that during direct or colony PCR, the initial polymerase activation step (typically 95°C) is hot enough to disrupt the membranes of the cells, releasing the DNA into the master mix making it available for amplification [18, 232]. Similarly, any cell-free DNA present on the surface of touched samples could also be released into the PCR mix during this direct process [31, 215, 232].

Direct PCR was first investigated in forensic science in 2010; swabs, cotton, nylon, polyester and denim fabrics that had been in direct contact with a person were amplified directly (placed within the PCR mastermix) following manufacturer's instructions [18]. Complete and partial DNA profiles were obtained from these samples without initially extracting the DNA, reducing the processing time required by up to two hours. Circumventing the extraction process resulted in no tube changes,

thus reducing the chances of DNA contamination from external sources, as well as reducing the amount of DNA lost due to tube retention. As more DNA was made available to the amplification process, the sensitivity of the test improved without the need to alter the manufacturer's protocol by increasing the number of cycles [18]. The overall improved time and cost reduction, as well as the increased sensitivity and results of these difficult trace samples is a positive and promising step forward in the forensic science community. These conclusions were further supported by comparative studies of direct PCR to traditional DNA extraction methods where direct PCR consistently generated higher quality and more complete DNA profiles [232, 233]. The studies found that the type of extraction technique used as well as the type of DNA substrate used, directly influenced the amount of DNA lost during the collection and amplification steps, subsequently affecting the generation of a DNA profile.

1.4 Aims of Thesis

This thesis aims to further investigate the use of direct PCR in forensic casework by exploring a wider range of forensic relevant substrates and samples, optimisation and validation of the technique, addressing limitations and concluding with implementation recommendations.

Optimisation of direct PCR will include testing different polymerases and PCR buffer constituents, to determine the best combination to overcome any potential inhibitors present on the samples. Due to the nature and variation of crime scenes and sample types, samples may come into contact with inhibitors ranging from components found in whole blood (such as haem) to soils and dirt, dyes found in fabrics, or other chemicals (such as house hold cleaners). Surface substrates, such as brick, glass, plastic, or fabric type may also affect the efficiency in which DNA is collected, transferred or extracted due to their particular characteristics. The efficiency of DNA collection may be further inhibited by the method of extraction and concentration used when processing evidence.

To reduce the effects of these variables and maximise the potential of the direct PCR technique, this thesis will focus on trace evidence sample types that are known to be difficult in consistently producing useable profiles, and samples that also lack potential inhibitors to ensure there is little to no processing of the samples prior to amplification.

Chapters of this thesis consist of published articles, short communications or technical notes, casework involvement, conference proceedings, or data presented at conferences in the form of posters or abstracts, with all remaining data and discussion formatted for submission to scientific journals. Chapters also contain manuscripts formatted to an appropriate journal, but are not yet ready for submission; these manuscripts may require additional data or research to be collected outside of this PhD candidature. An additional two manuscripts are currently under review. Each chapter will begin with a short preface to outline the data presented, followed by concluding remarks and appropriate appendices.

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CHAPTER 2

Direct PCR Enhancement

Manuscript prepared as: a letter to the editor, International Journal of Legal Medicine.

The efficacy of DNA extraction methods in forensic science

Manuscript prepared as: a letter to the editor, Australian Journal of Forensic Sciences.

PCR buffer enhancement of STR kits used for human identification

Ottens, R., et al., *Application of direct PCR in forensic casework.* Forensic Science International: Genetics Supplement Series, 2013. **4**(1): p. e47-e48.

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A method for the DNA quantification of direct PCR samples

Use of direct PCR on trace evidence samples

Case 1 – Seizure of Methamphetamine in South Australia

Direct PCR Enhancement

2.1 Preface

To identify the capabilities of direct PCR in a forensic setting, different steps of the DNA analysis process were investigated further for areas of improvement. Firstly, *section 2.2* explores the efficacy of DNA extraction methods. As the DNA extraction and isolation step is skipped during direct PCR, it is important to determine how this step may impact on the resulting DNA profiles of trace evidence samples. The results are also dependant on variables such as sample type, collection method and extraction method used. Three common extraction methods were used, keeping other variables constant, to determine how much DNA is lost during the process, which method produced consistent results, and which method was most suitable for trace evidence samples. Following the extraction step, PCR buffer constituents were explored in *section 2.3* to identify additives that may assist in overcoming PCR inhibition during the direct process. Known PCR enhancers were used in varying combinations and concentrations between two commonly used human identification kits, with improvements determined by an increase in the quality and number of alleles obtained, as well as the intensity or height of the resulting DNA profiles. *Section 2.4* applies the preliminary findings of extraction kit efficacy and buffer enhancements to a wider range of forensic relevant samples, as well as determining the limit of detection for direct PCR methods. To address concerns that may arise over the inability to quantify DNA during direct PCR, as the extraction step (and consequently quantification step) is skipped, *section 2.5* investigates a mathematical-based method to quantify the input DNA of samples post amplification. The knowledge obtained throughout these investigations enabled the direct PCR technique to be applied to difficult casework samples *in section 2.6*. Results were obtained from a variety of samples where traditional methods had failed to produce DNA information.

Statement of authorship

The efficacy of DNA extraction methods in forensic science

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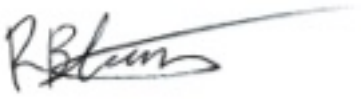
Date: March 2016

Renée Blackie (Candidate)

Performed all laboratory work (DNA extractions and quantification), data analysis and interpretation, created data table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

2.2.1 *Manuscript prepared as: a letter to the editor, International Journal of Legal Medicine.*

The efficacy of DNA extraction methods in forensic science

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Dear Editor,

In forensic science, the occurrence of trace DNA at crime scenes is common and sometimes may be the only evidence available to an investigation. Trace evidence presents its own set of difficulties; too little DNA will result in a partial profile, providing reduced significance, or more commonly no profile at all. The extraction method is the first step involved in DNA analysis, and is open to variation in DNA yield depending on the method used. It is an important step for the isolation and purification of DNA as it aims to remove inhibitors permitting downstream processes to work effectively. This process typically involves several wash steps and tube changes, each allowing for DNA retention to tubes and pipette tips, resulting in the loss of information. Extractions routinely work well for certain sample types such as blood, tissue and saliva where a large number of cells are typically present. Trace evidence such as single hairs, fibres, or swabs of touch DNA routinely fails to produce DNA profiles of significance. This poses great difficulty for investigations as these are often found at crime scenes and may be the only physical evidence available. We report on the retention and loss of DNA using three commonly used extraction methods in forensic analysis. DNA mass was measured prior and post extraction using the Qubit® 2.0 Fluorometer, and compared to assess the percentage lost.

Three common extraction methods were tested to determine the average loss of DNA from each: QIAamp® DNA Micro Kit (QIAGEN), DNA IQ™ System (Promega) and Chelex®. Previously extracted DNA (from buccal swabs using QIAGEN Micro Kit) was quantified on a Qubit® 2.0 Fluorometer (Invitrogen™) and used as the control in this experiment. Control DNA ranging from 35 – 39 ng was used as the starting concentration for each extraction. Extractions were performed following the manufacturer's instructions for each kit and were eluted in a final volume of 30 μL for the DNA Micro Kit and 100 μL for Promega IQ™ and Chelex®. Chelex® extraction involved adding 200 μL of 5 % Chelex® to the DNA in a 1.5 mL tube and incubating at 56 °C for 20 mins

with shaking, followed by 100 °C for 8 mins with shaking. Sample was centrifuged at max speed (13000 g) for 3 mins. Liquid was removed without disturbing the Chelex® pellet and transferred into a new sterile 1.5 mL tube. Extractions were repeated eight times for each method used. Each extraction sample was quantified using the Qubit® following manufacturer's instructions, using 10 μL from each sample for quantification. The quantification results were compared against the initial input DNA to determine percentage lost.

DNA loss ranged from 36.5 % to 96.9 % across all methods, with QIAamp® performing the best with the lowest average of 53.4 % DNA loss (Table 1). Promega IQ™ and Chelex® were highly consistent with the results obtained, showing approximately 3 % variation between the highest and lowest losses observed. Although the results varied quite significantly for the QIAamp® Micro Kit, it still out-performed the other kits with the highest DNA loss obtained (82.6 %) being less than the lowest percentages observed for the other two kits (89 % and 94.3 %). These three common methods of DNA extraction are very inefficient with their average loss ranging from 53.4 – 95.8 %. The currently used extraction methods are not ideal for trace evidence samples where minimal initial DNA is present, and methods should be adjusted for trace evidence samples in order improve results and reduce time and money wasted on the inefficiency of current methods.

Table 1: DNA lost from standard extraction methods

DNA Extraction Kit	Starting Mass (ng)	Average Final Mass (ng)	Average Percentage Lost (%)	Lowest/Highest Percentage Observed (%)
Promega IQ™	36	1.5	95.8	94.3 / 96.9
QIAamp® Micro	39	17.1	53.4	36.5 / 82.6
Chelex®	35	3	91.5	89.0 / 92.3

Acknowledgements

Funding was provided by the Attorney General's Department of South Australia.

Statement of authorship

PCR buffer enhancement of STR kits used for human identification

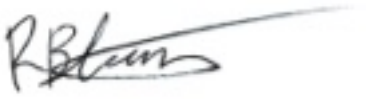
Manuscript prepared as: a letter to the editor, Australian Journal of Forensic Sciences.

Date: March 2016

Renée Blackie (Candidate)

Performed all laboratory work (PCR amplification, capillary electrophoresis), data analysis and interpretation, statistical analysis, created the figure, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Jennifer Templeton

Provided preliminary laboratory results for the use of Triton X as a buffer enhancer, and edited the paper.

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Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

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Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

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Signed  Date March 2016

2.3.1 *Manuscript prepared as: a letter to the editor, Australian Journal of Forensic Sciences*

Title: PCR buffer enhancement of STR kits used for human identification

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Abstract

We report on the significant improvement of DNA profiles by the addition of standard chemicals to the polymerase chain reaction (PCR) buffer provided in commercially available human identification kits. Triton® X-100, BSA and DMSO were added to the PCR buffer, both singly and in combinations, provided in the AmpFλSTR® Profiler Plus® or NGM SElect™ kit (Life Technologies, Victoria, Australia). PCRs were performed in 0.2 mL thin walled tubes following the standard protocol for a 25 μL volume reaction, following all manufacturers' conditions for amplification. PCR products were analysed using a 3130*xl* (ABI) and GeneMapper® ID v3.2 (Life Technologies) with a threshold of 50 relative fluorescence units (RFU) for allele assignment. The average RFU was calculated for each profile obtained where the buffer was adjusted. The values were compared against positive and negative controls where no buffer adjustments were made. A 70 % increase in RFU value was observed with the addition of a combination of DMSO with either BSA or Triton® X when using Profiler Plus®, and a 35.7 % increase was observed with the singular addition of BSA for NGM SElect™. Our data indicate the clear improvement in the quantity of profiles obtained across both kits, offering a fast and cost effective way to boost the results. This enhancement may be beneficial in cases where limited or degraded DNA is present, offering further assistance in difficult investigations.

Key words: forensic science, DNA typing, direct polymerase chain reaction, buffer enhancement, human identification

Dear Editor,

We show how the addition of standard chemicals to the PCR buffer provided in commercially available STR kits improve the quality of DNA profiles by significantly increasing the RFU value. The factors that affect the quantity of PCR product include the initial quantity of DNA template and the presence of inhibitors of the amplification and the buffer components. Triton® X-100, BSA and DMSO have been shown to increase the amount of PCR product produced and overcome inhibitors to the amplification [1, 2] and hence these standard materials were added to the PCR buffer, both singly and in combinations, provided in two commercially supplied STR kits, and the resulting data analysed. Although this experiment uses control DNA, in which there are no inhibitors present, the effect of each buffer adjustment should be documented first to indicate which addition will potentially have the greatest impact for the use of more difficult sample sets such as trace DNA or overcoming inhibitors in direct PCR.

PCRs were performed using either the AmpFλSTR® Profiler Plus® or NGM SElect™ kit (Life Technologies, Victoria, Australia) in 0.2 mL thin walled tubes following the standard protocol for a 25 μL volume reaction. Positive and negative controls acted as the standards in this experiment with no additions made to their buffers. Final concentrations of Triton® X (0.004 %), DMSO (4 %), BSA (0.008 μg) were added to PCR buffers in the following combinations: Triton® X (T), DMSO (D), BSA (B), B/D, T/D, and T/B/D. All PCRs contained 1 ng of control DNA (2800M, Promega, Victoria, Australia). Amplification conditions for all samples followed the manufacturer's instructions at 29 cycles using a GeneAmp® 9700 96-well thermal cycler (Life Technologies). PCR products were analysed using a 3130*xl* (ABI) and GeneMapper® ID v3.2 (Life Tehcnologies) with a threshold of 50 RFU for allele assignment. The average RFU value of a profile (9 loci Profiler Plus® or 15 loci NGM SElect™) for each modified PCR was compared against the average RFU value of the standard (positive control) to determine the effect of the buffer adjustments. For each kit, each buffer addition (six combinations), and positive and negative controls was set up three times and run in triplicate on the 3130*xl*, resulting in a total of 144 samples used in this experiment. No other combinations were trialled in this experiment.

The effect of the buffer adjustments is illustrated in Figure 1, which shows the increase in RFU values for both STR kits using the additions to the buffer. PCR products obtained using the Profiler Plus® kit showed the greatest increase in RFU value across all buffer adjustments compared to the NGM SElect™ kit. The combinations of BSA/DMSO and Triton® X/DMSO resulted in an RFU increase of approximately 70 % across all nine loci. All RFU increases using this kit were statistically significant ($p = < 0.001$). Using NGM SElect™, the average RFU increase

ranged from 1.4 % to 35.7 %. The only statistically significant increase was observed with the singular addition of BSA ($p = < 0.05$). Overall, an increase in RFU value was seen for each addition and combination across both kits.

Our data indicate the clear improvement in the quantity of STR products produced with the addition of a combination of DMSO with either BSA or Triton® X when using Profiler Plus®, and the addition of BSA for NGM SElect™. This offers a fast and cost effective way to boost the quality of results obtained for these kits. Such an enhancement may be crucial in cases where there is trace DNA. Direct PCR incorporates limited DNA template into the buffer to enhance the profiles obtained [3, 4], coupled with the buffer additions outlined in this study could further aid difficult investigations.

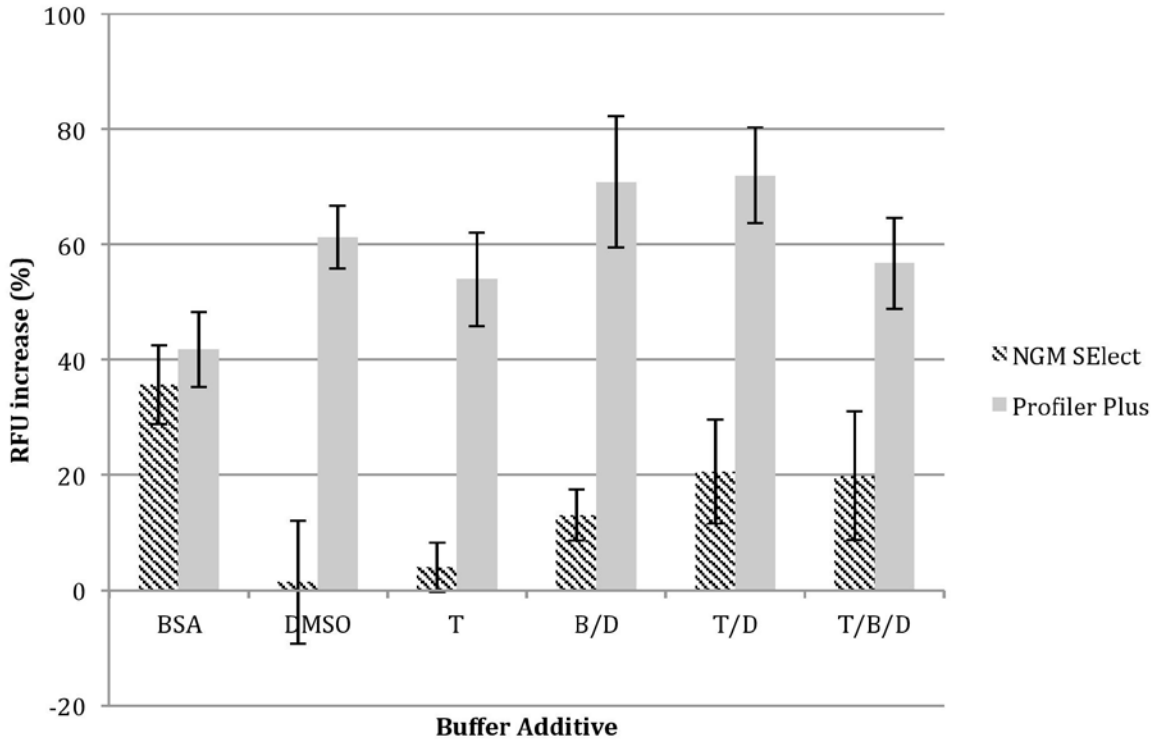

Figure 1. Known buffer enhancers BSA, DMSO and Triton® X were added individually and in combination to two standard STR kit buffers. The average RFU value across all loci for a sample was compared against the RFU value of the positive control sample (no buffer additions). The increase in RFU value from the positive control was recorded in per cent (%) and shown above across all enhancement types for both STR kits. Standard Error (SE) bars are displayed for n = 9 replicates of each buffer additive.

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We thank members of Forensic Science South Australia and the School of Biological Sciences at Flinders University for assisting with reagents and providing helpful advice. Funding was provided by the Attorney General's Department of South Australia.

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Statement of authorship

Application of direct PCR in forensic casework

Published in Forensic Science International: Genetics Supplement Series

Date: October 2013

Renée Blackie (Candidate)

Performed laboratory work for determining the DNA lost via extraction, quantification of DNA results using the Qubit® fluorometer, performed direct PCR amplification and capillary electrophoresis for hair, fibre and tape samples, performed laboratory work for the limit of detection of direct PCR, as well as all relating data, interpretation and statistical analysis for these sections. Created the table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Jennifer Templeton

Performed direct PCR amplification and capillary electrophoresis for swabs on different surfaces and glove samples, as well as providing all relating data, interpretation and statistical analysis for these sample types. Edited the paper.

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Signed  Date March 2016

Viviana Paradiso

Provided preliminary laboratory direct PCR results for the swab samples.

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Damien Abarno

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Duncan Taylor (Co-Supervisor)

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Adrian Linacre (Supervisor)

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Application of direct PCR in forensic casework

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ABSTRACT

Direct PCR is fast becoming a popular method in forensic science due to the advantages of saving time and money in the lab while increasing the probability of obtaining substantial results has a positive rippling effect. A laboratory is able to reduce the time spent on processing trace DNA samples, which can lead to investigators receiving important information in a timely manner that may not have been possible using standard methods. This study highlights the benefits of direct PCR in forensic casework by analysing trace and touch DNA on a range of substrates and exploring the loss of initial DNA due to extraction.

1. Introduction

Direct PCR can generate full STR DNA profiles from trace samples without using extraction or quantification processes [1]. A previous study reports a loss of up to 76% of initial DNA due to the multiple wash steps and tube changes involved in the extraction process [2], ultimately affecting the quality of the DNA profile obtained. Samples containing less than 100 pg are considered to be trace DNA [3], and generally require an increase to 34 cycles to obtain meaningful data. This study highlights direct PCR sensitivity and the ability for trace DNA to be amplified without the need to increase cycle number or modify current protocols to obtain meaningful data. A direct PCR approach is a viable option for the future of trace DNA recovery and analysis for forensic science purposes, vastly improving efficiency, sensitivity and the quality of results.

2. Materials and method

2.1. Determining loss of DNA via extraction

QIAamp DNA Micro Kit (QIAGEN) and DNA IQ™ System (Promega) were used to determine the average loss of DNA. A total of 20 ng of control DNA (2800 M, Promega) was used as the starting concentration for each extraction. Extractions were performed followed the manufacturer's instructions for each kit and were eluted in a final volume of 30 μL for maximum DNA yield. Extractions were repeated five times for each kit used. Each extraction sample was quantified on a Qubit® 2.0 Fluorometer (Invitrogen™) following manufacturer's instructions, using 5 μL from each sample for quantification. The quantification results will be used to determine the loss of DNA. The quantification results will be compared against the initial input DNA to determine percentage lost.

2.2. Direct PCR

DNA extraction and quantification are bypassed. The range of samples tested include: single hairs in anagen and telogen growth phases, fibres from swabs used on plastic and glass surfaces, single fibres from clothing, latex gloves, and a range of plastic tapes. Each sample is placed directly into a prepared 0.2 mL thin walled tube containing 25 μL of reagents from either the AmpFℓSTR® Profiler Plus® or NGM™ kit (Life Technologies, Victoria, Australia). Sample sizes were approximately 5 mm in length for hair and fibres, and 5 mm × 5 mm for tapes, swabs and gloves. Amplification conditions for all samples followed the manufacturer's instructions at 29 cycles. PCR was performed on a GeneAmp® 9700 96-well thermal cycler (Applied Biosystems) following manufacturer's instructions. PCR products were analysed using a 3130*xl* (ABI) and GeneMapper® ID v3.2 with a threshold of 50 RFU for allele assignment.

2.3. Limit of detection for PCR

A series of positive control (DNA 2800M) samples were created to determine the lowest concentration of DNA needed to produce a

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Table 1
DNA lost from standard extraction.

DNA extraction kit	Starting concentration (ng)	Average final concentration (ng/30 μL)	Average percentage lost (%)
Promega IQ	20	3.3	83.5
QIAGEN Micro	20	5.7	71.5

full DNA profile. Positive control PCRs were set up in triplicates in the following DNA concentrations: 0.05 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.8, 0.9, and 1 ng. Each sample was amplified and analysed in the same way as the direct PCR method, except 1 μL of each DNA concentration was used.

3. Results and discussion

3.1. Loss of DNA

The QIAGEN Micro DNA extraction kit yielded a higher DNA concentration overall, retaining on average 28.5% of the original amount. Although the QIAGEN kit performed better, both methods lost a substantial amount of DNA ranging from 71.5% to 83.5% (Table 1). Validated STR kits recommend using 1 ng of input DNA to optimise PCR, thus from a 30 μL elution, 9.1 μL would be required from the Promega kit and 5.3 μL from the QIAGEN kit. The average DNA lost when combining both kits is 77.5%.

3.2. Direct PCR

Profiles 'up-loadable' (≥12 alleles) to the Australian National DNA Database were obtained from the following substrates:anagen and telogen hairs, single fibres from worn or touched fabric, plastic tape, and single fibres from a range of swabs used on glass and plastic surfaces. Less than 12 alleles were observed from latex glove samples.

3.3. Limit of detection

Using standard PCR cycling conditions, the lowest initial template concentration of DNA at which a full DNA profile could be obtained was 100 pg. With a reduction to 50 pg, up-loadable profiles were obtained, with some allelic drop-out occurring. A single diploid human cell contains ~6 pg of DNA [4], therefore 100 pg equates to ~17 cells (assuming there is no contribution from cell-free DNA). If a sample lost 80% of its DNA via an extraction method (based on a 30 μL elution), a starting concentration of 500 pg (~84 cells) is needed in order to obtain a template of 100 pg for the PCR, and hence a full profile. However, most kits allow up to a maximum of 10 μL of input DNA for a standard 25 μL reaction. Therefore, to maintain this reaction volume size, the true starting DNA concentration using standard extraction procedures needs to be approximately 1.5 ng (250 cells), with an extraction loss no greater than 80%.

4. Conclusion

As DNA is not lost during an extraction process when using direct PCR methods, only 17 cells are required to obtain a full STR profile compared to 250 cells when using standard DNA extraction methods; thus making direct PCR up to ~15 times more sensitive than conventional extraction methods. No modifications are made to STR kit protocols allowing for quick and easy implementation into forensic laboratories. The results obtained by this laboratory are encouraging when typing trace amounts of DNA, and potentially are a vast improvement when compared to current industry methods.

Role of funding

Funding was provided by the Department of Justice, South Australia.

Conflict of interest

None.

Acknowledgements

We thank members of Forensic Science South Australia and the School of Biological Sciences at Flinders University for assisting with reagents and providing helpful advice.

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[4] J.M. Butler, Forensic DNA Typing, second ed., Elsevier, 2005.

Statement of authorship

A method for the DNA quantification of direct PCR samples

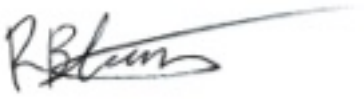
Manuscript prepared as: a technical note, Australian Journal of Forensic Sciences.

Date: March 2016

Renée Blackie (Candidate)

Performed all laboratory work (PCR amplification and capillary electrophoresis), data analysis and interpretation, statistical analysis, created the table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, performed data and statistical analysis, created the figure, commented on data, and edited the paper.

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Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

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Signed  Date March 2016

2.5.1 *Manuscript prepared as: a technical note, Australian Journal of Forensic Sciences*

Note: manuscript requires additional data from another research project not yet undertaken

Title: A Method for the DNA Quantification of Direct PCR Samples

Authors: R. Blackie[1], D.A. Taylor[1,2], A. Linacre[1]

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Abstract

We report on a method to quantify the DNA template used in direct PCR amplifications based on the resulting magnitude of the allele peaks generated. Direct PCR has the advantage of using all genetic material available from a sample thus increasing the information obtained from trace evidence. A potential criticism of direct PCR is that quantification of the initial starting template DNA is not possible. To counter this, we demonstrate a method for determining the starting DNA concentration based on the profile obtained. We start on single source pristine samples ranging from 0.05 ng to 1 ng to mimic the range of DNA concentrations likely to be encountered in casework. We proceed to casework samples and demonstrate how to take into account complicating factors such as degradation, inhibition and contribution from multiple sources. The resulting method demonstrates how, using peak heights from the DNA profile, the original template DNA can be calculated with a desired quantile of interest allowing an estimate of the number of cells from a sample processed via direct PCR to be provided.

Key words: DNA profiling, direct PCR, DNA quantification, reverse quantification, confidence intervals

Introduction

The benefits of direct PCR have been greatly highlighted by its successful application to trace DNA samples, such as touch DNA, where conventional DNA extraction prior to quantification and amplification failed to routinely generate a DNA profile[1-7]. The technique is gaining this interest due this increase in sensitivity by omitting the standard DNA extraction process, with the associated benefits or reduced time and costs. DNA extraction processes typically result in an 80 % loss of starting DNA[8, 9] such that at the conclusion of the process, the subsequent quantification may indicate that there is insufficient genetic material to generate a DNA profile. DNA profiles can be generated from single hairs[2], and touch DNA when submitting the same samples to DNA extraction leads to very little chance of generating a meaningful DNA profile[6]. While direct PCR has these clear advantages there are some disadvantages such as lacking in reproducibility and the circumventing of the quantification step. Quantification of the mass of DNA in an extract is a requirement of the SWGDAM recommendations[10].

Quantification is a common step in generating a DNA profile as it informs the analyst how much DNA has been recovered from an extraction and hence the mass of DNA to be added to a PCR; this being typically 1 ng to 500 pg using commercially available STR DNA profiling kits. It also has the dual purpose of providing some important investigative information to analysts as to how much cellular material was recovered initially. Such information may assist the analyst in assessing whether there was a large amount of DNA present or only trace levels[11]. A method of DNA quantification for direct PCR would allow for estimations to be made on the level of cellular material found on substrates prior to direct PCR typing.

The level of fluorescence in a treated DNA sample can be used to indicate how much DNA is present[12, 13]. Conventional quantification methods such as RT-PCR and the Qubit® Fluorometer are based on the measurement of fluorescent probes against known standards[13, 14], whereas continuous

systems of DNA interpretation use peak height or area, measured in relative fluorescent units (RFU), information to determine the quantity of DNA in their calculations[15].

In the downstream process of DNA analysis, during capillary electrophoresis (CE), the measurement of laser-induced fluorescence of dye-labelled PCR products is used to produce a DNA profile with peak heights measured in RFU. There are several factors that affect fluorescence emission and the RFU value obtained. Using validated kits and techniques, these factors are: initial DNA available in a PCR, environmental conditions such as temperature, pH and salt concentration[16], and DNA sample factors such as degradation, inhibition, locus amplification efficiency and contribution of DNA by multiple individuals. An expectation is an approximately linear correlation between DNA mass and the RFU value should be observed within a single CE run. A linear correlation between these two factors would allow for calculations to be made of DNA mass based on the resulting RFU value. Here we report on a method to estimate the mass of the initial DNA template used in a direct PCR amplification using single source samples. We start with simple, single source, pristine samples and then demonstrate how to adapt the method for more complex situations.

Materials and Methods

DNA Amplification – pristine DNA

DNA was amplified using the NGM SElect™ kit (Life Technologies, Victoria, Australia). This kit amplifies 16 STR loci plus amelogenin using 29 cycles of amplification, as validated by the manufacture if starting with 500 pg of DNA as template. A known mass of starting DNA 2800 (Promega, Victoria, Australia) template was used ranging from 0, 0.05, 0.1, 0.2, 0.5 and 1 ng of DNA. Each series of known mass of DNA (0 – 1 ng) was prepared three times for a total of 18 samples. Each sample was prepared in a 0.2 mL thin walled tube containing 25 µL of reagents following the manufacturer's instructions. A total of 29 PCR cycles was performed on a GeneAmp® 9700 96-well thermal cycler (Life Technologies) following manufacturer's instructions. PCR products (1 µL) were

combined with 0.5 µL Liz 500™ (Life Technologies) and separated using a 3130*xl* Genetic Analyser (Life Technologies). The data were analysed using GeneMapper® ID v3.2 (Life Technologies) with a threshold of 50 RFU for allele assignment. Samples were run in triplicate on the 3130*xl* for a total data set of 54 complete DNA profiles (nine data series of 0 – 1 ng).

DNA Amplification – Casework DNA samples

100 samples were chosen from criminal cases at Forensic Science SA (FSSA). DNA samples were extracted using the DNA IQ™ system (Promega), and PCR amplifications carried out on a 9700 using GlobalFiler™ (Thermo Fisher Scientific) as per manufacturer's instructions. Amplification fragments were resolved using a 3130*xl* Genetic Analyser and analysed in GeneMapper® ID-X to obtain peak height information for each profile using a detection threshold of 30 RFU.

Reverse Quantification Analysis

A method was required that could take into account complicating factors that affect peak height such as degradation of DNA, PCR inhibition, locus amplification efficiencies and contribution of DNA from multiple individuals. Combinations of these points (particularly the contribution of DNA from multiple individuals), means that the heterozygote or homozygote status of any individual allele in the DNA profile can be uncertain. We employ the idea of Total Allelic Product (TAP) when considering fluorescence. TAP theory suggests that from a starting amount of template DNA we expect a total amount of fluorescence, however this will be split across allele and stutter peaks. The same theory can be applied to mixed DNA profiles deriving from a number of individuals; we expect a total amount of fluorescence from this starting template, it will just be split across a number of peaks. To deal with this, we sum the heights of all peaks at each locus (which we call T_l for the *T*otal fluorescence at locus *l*). This means that the total template DNA is being considered, regardless of number of contributors, the presence of stutter artefacts or the heterozygous or homozygous nature of any particular peak.

Given T_l there is still the issue of accounting for degradation, inhibition and locus specific amplification efficiencies. There are a number of options that can be considered when regressing fluorescence against DNA concentration:

- Average over profile – ($T_{l,1}$) – This may overcome amplification efficiency imbalances across the profile
- Choosing the loci with the lowest molecular weight – ($T_{l,2}$) – This method should overcome the issue of degradation
- Choosing the loci with the largest T_l value – ($T_{l,3}$) – This method should overcome degradation and inhibition (important if the lower molecular weight loci are those that are affected by inhibition)

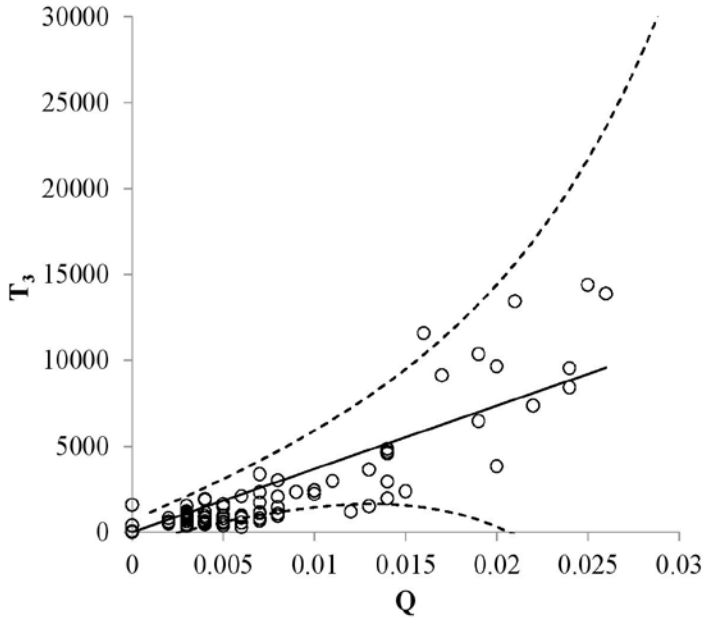
We trial each of these three methods by carrying out regression of DNA amount added to the PCR, Q, (in ng) against $T_{l,i}$, using R.

$$Q = \beta T_{l,i}$$

Note that the above regression forces the data through the origin as we assume that a DNA extract with no DNA would yield a profile with no peaks.

We first apply the regression to pristine data. We then split the 100 casework samples into two groups of 50. The first group we consider a training group, used to generate the regression parameter values and the second group we consider the test group, to which we apply the regression results. This was done for each of the three fluorescence measurement options in order to determine their portability across datasets. Q values of greater than 0.04 were omitted from the analysis as this corresponds to the point at which the peaks produced from PCR reach saturation on the 3130*xl*.

Heteroscedasticity was observed in the data with respect to *T* values, namely as *T* increased so too did the size of the residuals. We fit an exponential function to the standard deviation in T value for Q brackets of 0.005 and use this to plot confidence intervals from the regression.



T3 V Q showing regression line (solid) and 95% confidence intervals (dotted). Based on all casework data at this stage (training and test)

Results and Discussion

Pristine DNA

The three regression methods gave roughly equivalent results for the pristine data, although this is expected given that the pristine data was single sourced, non-degraded, no-inhibited and of known genotypic origin. The results of the regression are given in Figure 1, which shows the expected vs known DNA amounts and the 95% confidence interval on the estimation. The reverse quantification method relies on the assumption that a linear relationship exists between template DNA mass and the fluorescence of the resulting DNA profile. The DNA amounts estimated by the regression generally underestimated the amount of DNA in the 1 ng standard, which would be expected if it were subject to saturation, which affects the linear relationship between DNA amount and fluorescence.

Conclusion

Previously there has been no means of estimating the initial amount of DNA used in a direct PCR. The statistical method described in this study enables the calculation of the DNA mass. The data indicate a high correlation between DNA mass and average RFU value, allowing for DNA mass calculations to be made within a factor of 1.57 of the actual value at a 95% confidence level. Not only will this provide knowledge of how much DNA template was probably present in direct PCR sample, but it can help identify how much DNA was within a substrate prior to the amplification. It is noted that this preliminary study illustrates the potential for the accurate estimation of the quantification DNA present using single source DNA and therefore applicable to use with single hairs or areas where a single person has made contact with a substrate. The application would therefore be beneficial in processing touch and trace DNA from fingermarks, single hairs, and surface swabs as this technique provides an informative estimation of initial amount of template DNA. The estimate of quantification is accurate even at low levels of starting DNA. As the standards required to calculate the DNA mass of samples are equivalent to the positive and negative control samples of a PCR setup, no additional laboratory work is required to implement this technique.

Acknowledgements

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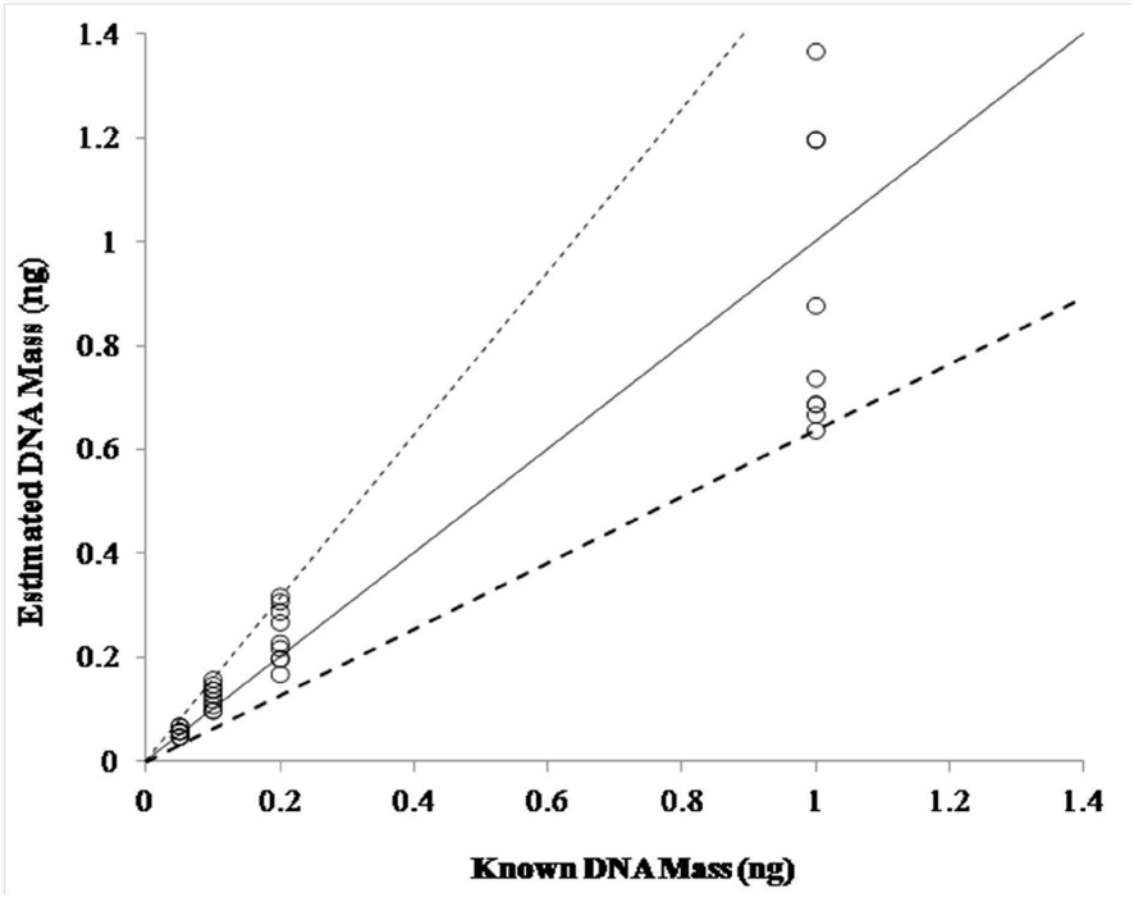

Figure 1: The known DNA mass compared to the estimated mass using the reverse quantification method (raw data seen in Table 1). The solid line represents the space where observed and expected values are equal and the dotted lines show the 95 % confidence interval boundaries on DNA mass prediction.

Table 1: Calculated DNA mass (ng) for each series versus the actual DNA mass (ng) with corresponding R^2 values. Note the 0.5ng value is omitted as it was used to create the calibration curve for each series of data.

	Calculated DNA Mass (ng)								
Actual DNA Mass (ng)	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6	Series 7	Series 8	Series 9
1.0	1.37	1.2	1.2	0.64	0.69	0.88	0.74	0.69	0.67
0.2	0.27	0.22	0.23	0.31	0.29	0.32	0.17	0.2	0.2
0.1	0.16	0.14	0.14	0.13	0.12	0.15	0.1	0.11	0.1
0.05	0.07	0.05	0.05	0.06	0.05	0.06	0.07	0.06	0.06
R^2 Value	0.9758	0.9901	0.9897	0.9451	0.9626	0.9334	0.9925	0.9994	0.9994

2.6 Application

2.6.1 Case 1 – Seizure of Methamphetamine in South Australia

March 2013 saw 7.33 kg of pure methamphetamine uncovered by police in one of the state's biggest drug hauls, valued at more than AU $20 million. The drugs were discovered roadside when two men were acting suspiciously and drew the attention of police officers. The men were interrupted as they were digging up containers filled with the drug, as pictured in Figure 2.1.

Figure 2.1 – Several containers were discovered partially buried roadside in South Australia, containing a total of 7.33 kg of pure methamphetamine.

Although two suspects were arrested and charged following the find, a drug operation of this scale would involve a larger network of individuals. Forensic Science SA (FSSA) were tasked with processing the evidence in hopes of obtaining DNA profiles, and other valuable information, to link to the apprehended suspects, and possibly other suspects to the crime. Standard procedures were unable to provide meaningful DNA information from the evidence sampled. To obtain DNA from the evidence would be highly challenging, as many factors would impede the results. Firstly, DNA present on the containers pictured would most likely be found in trace amounts in areas such as the underside of the tape, particularly the tape ends. DNA present on the outside of the containers would be exposed to environmental elements such as bacteria in the soil and varying temperatures that contribute to DNA degradation. Lastly, a high percentage of DNA is lost to the extraction process, as mentioned previously.

South Australian Police were referred to the DNA laboratory at Flinders University by FSSA, due to the recent success and promising preliminary results the direct PCR technique had with trace DNA samples. A range of evidence samples was selected by Renée Blackie, Jennifer Templeton and Adrian Linacre, to be processed using the direct PCR method. Samples included empty containers and a range of adhesive tapes – identified on the tapes for further processing included several fibres and single hairs. Tapes and containers were also swabbed at various locations, with the fibres from the swabs processed directly. A total of 24 samples were processed.

Swab tips were moistened with 2 µL of pre-heated (50°C) Triton® X (Sigma-Aldrich) at 0.1 % concentration. Moderate to heavy pressure was used on samples. Swab tip fibres were cut directly into pre-labelled 0.2 mL thin walled tubes ready for processing. All samples were amplified using AmpFℓSTR® NGM SElect™ kit (Life Technologies, Victoria, Australia) in 0.2 mL thin walled tubes following the standard protocol for a 25 µL volume reaction. An additional 1 µL of AmpliTaq Gold® DNA polymerase was added to overcome inhibitors that may have been present. Amplification conditions followed the manufacturer's instructions at 29 cycles using a GeneAmp® 9600 thermal cycler (Life Technologies). PCR products were analysed using an ABI 3130*xl* Genetic Analyser (Life Technologies) and GeneMapper® v3.2.

Alleles were obtained from all 24 samples processed, ranging from 4 – 42 alleles, with 17 samples resulting in 10 or more alleles. As expected, due to the sensitivity of the technique, 14 profiles contained alleles from two or more contributors (casework item can be observed in Figure 2.2, with resulting profiles in Figures 2.3 – 2.7). All data obtained was forwarded to FSSA for further analysis.

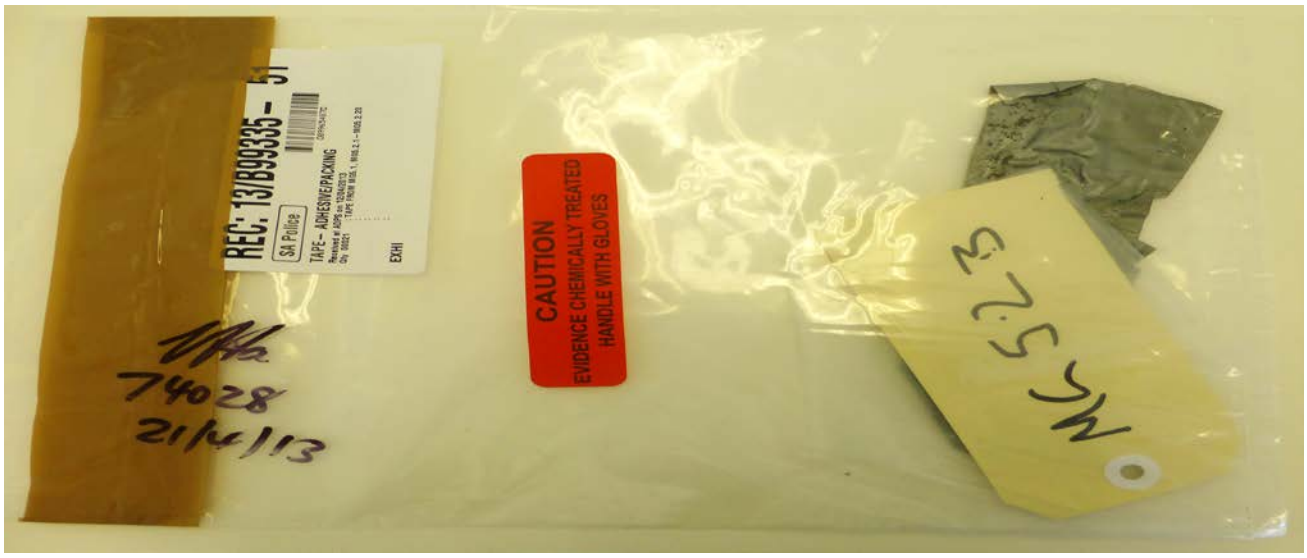

Figure 2.2 – Case item MG523.B, grey duct tape removed from the lid of a container holding methamphetamine.

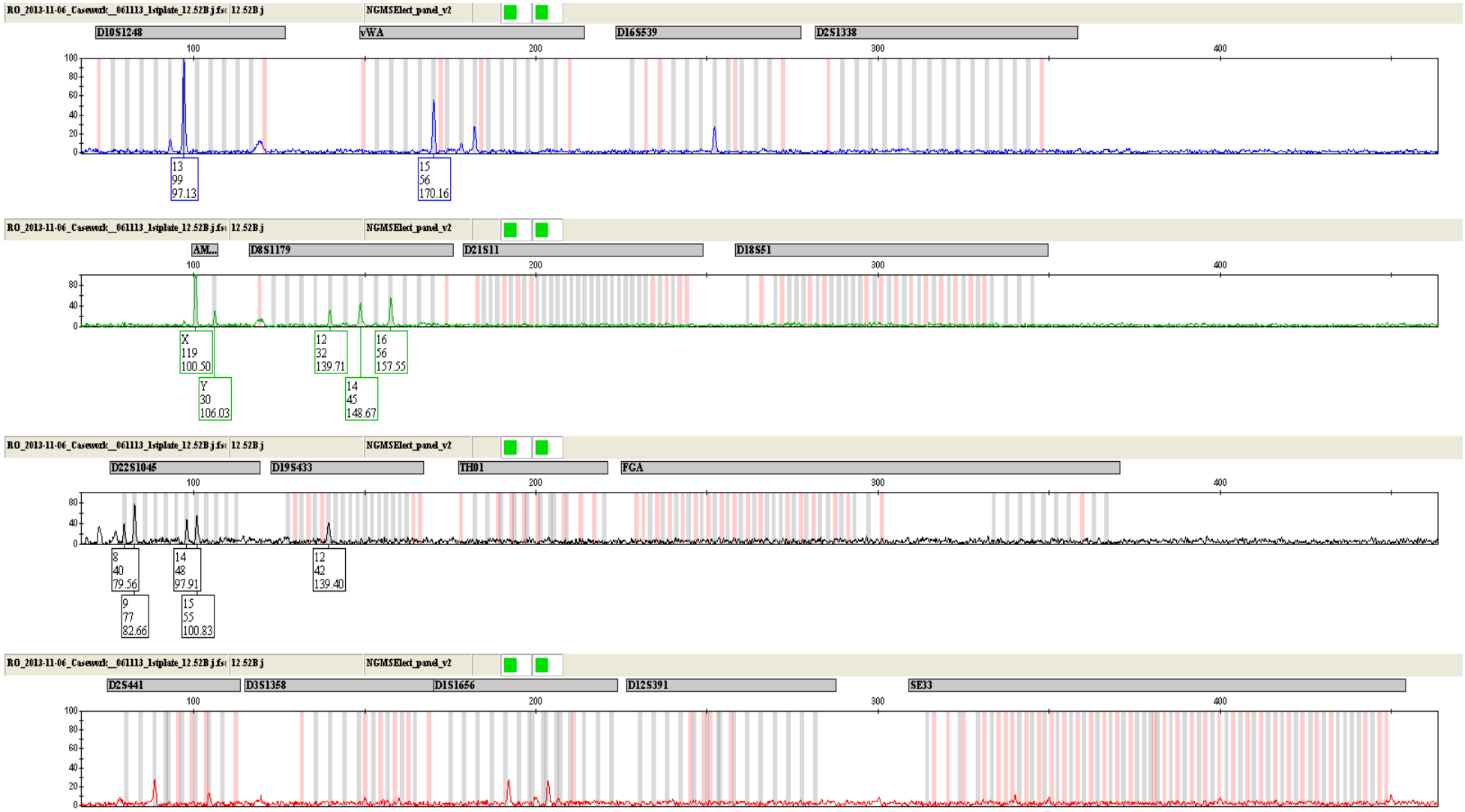

Figure 2.3 – NGM SElect™ kit DNA profile obtained from case item MG523.B using direct PCR from swab fibres at 29 cycles. PCR tube label: j52B.

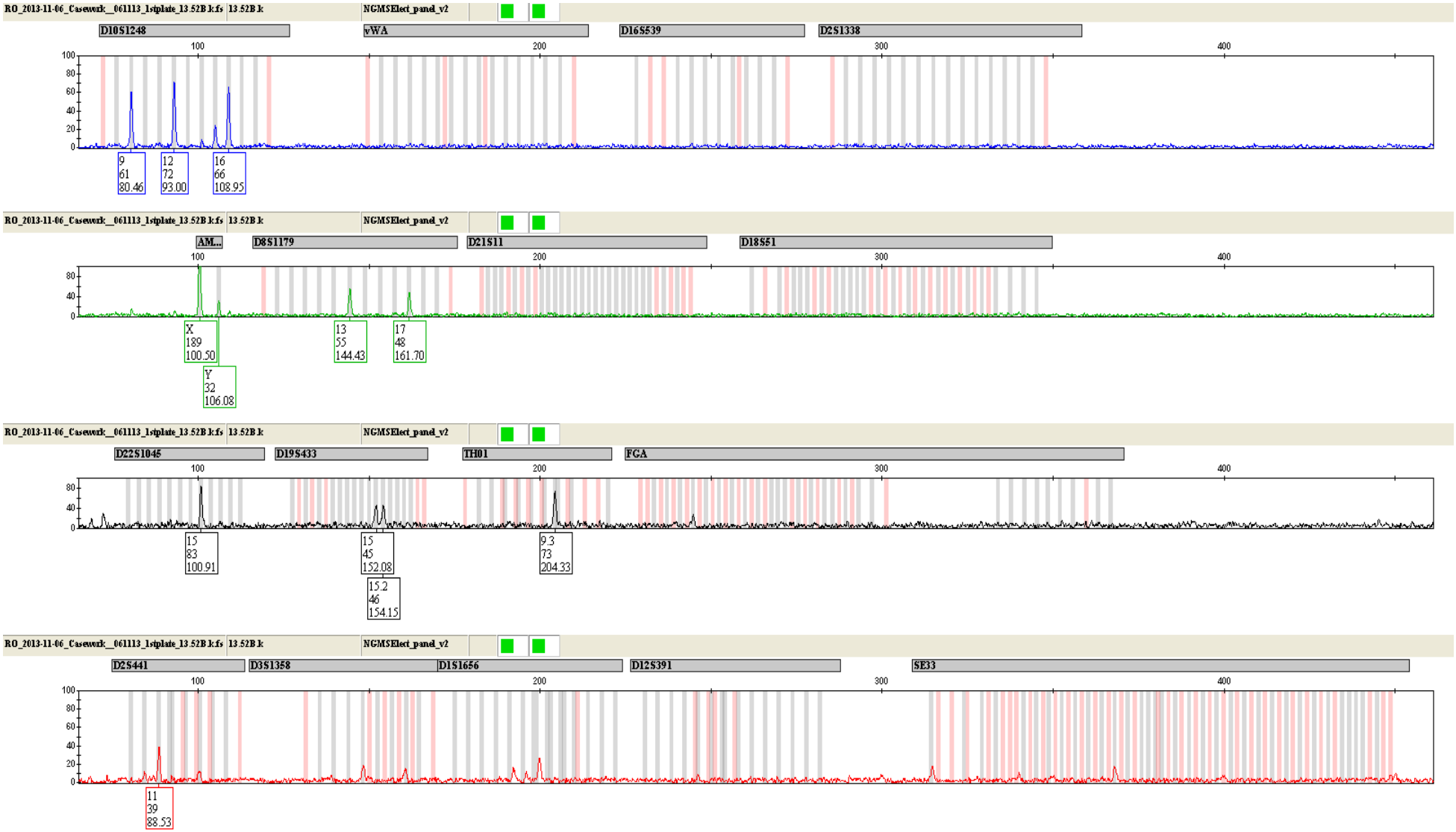

Figure 2.4 – NGM SElect™ kit DNA profile obtained from case item MG523.B using direct PCR from swab fibres at 29 cycles. PCR tube label: k52B.

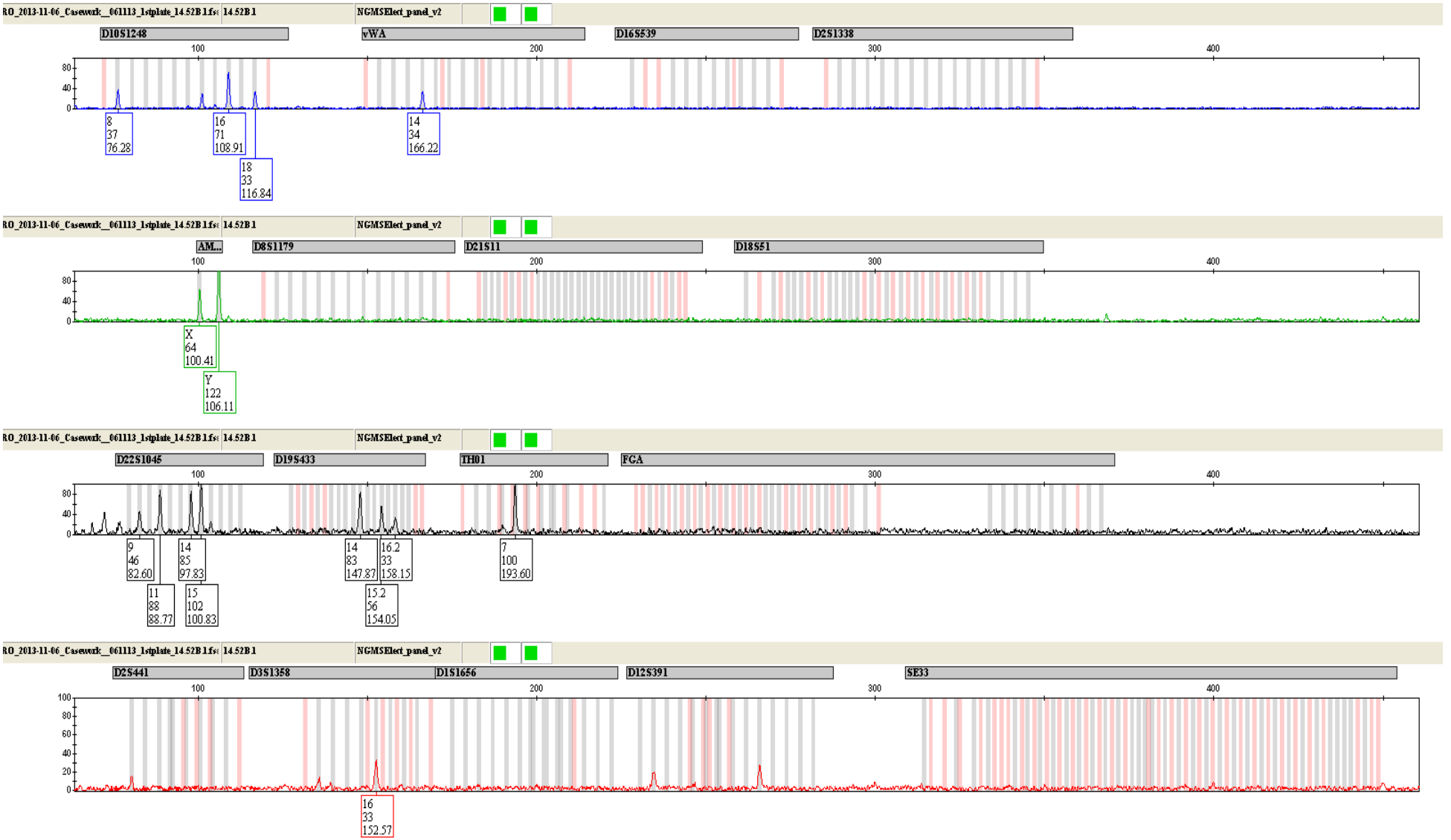

Figure 2.5 – NGM SElect™ kit DNA profile obtained from case item MG523.B using direct PCR from swab fibres at 29 cycles. PCR tube label: l52B.

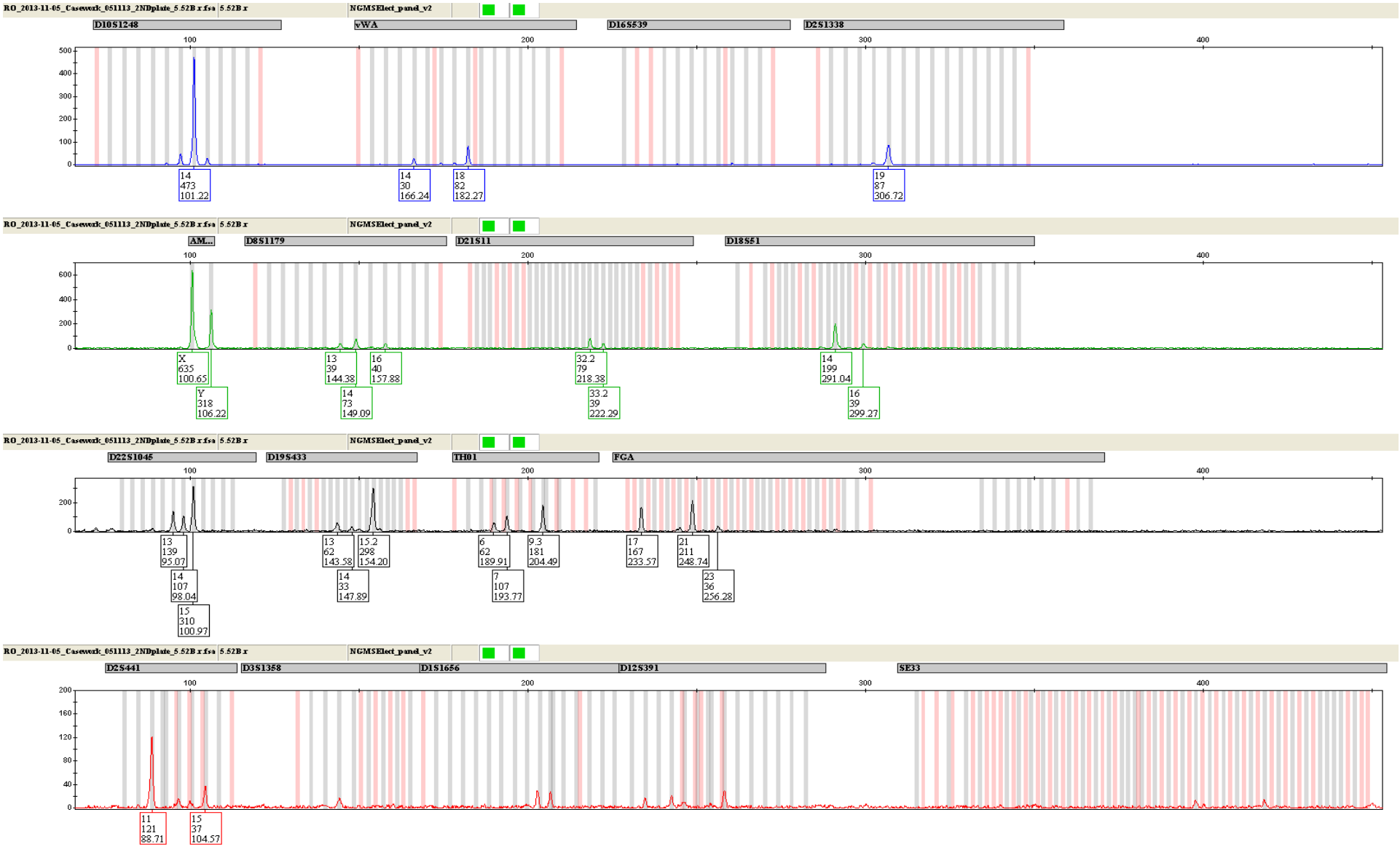

Figure 2.6 – NGM SElect™ kit DNA profile obtained from case item MG523.B using direct PCR from swab fibres at 29 cycles. PCR tube label: r52B.

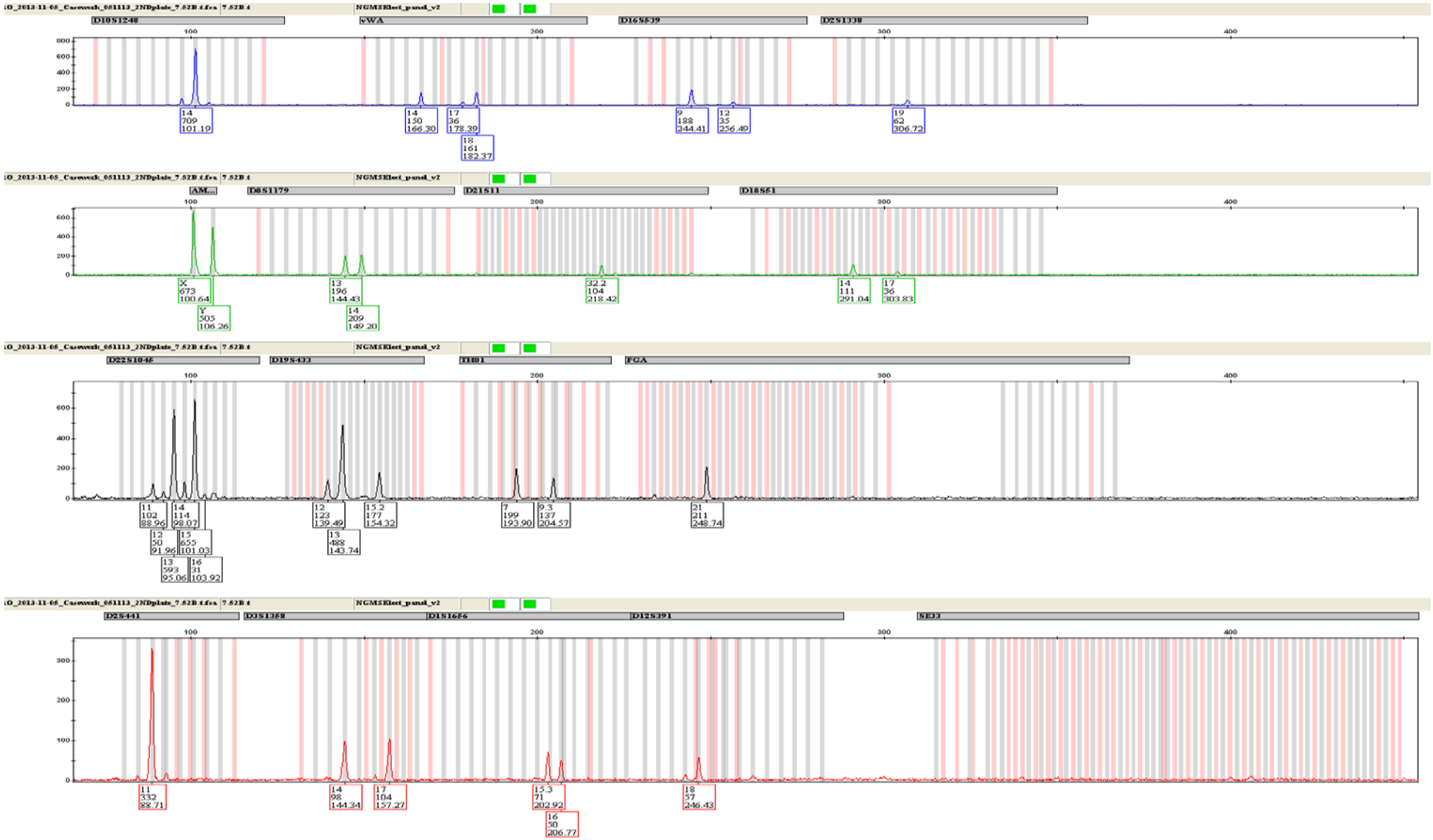

Figure 2.7 – NGM SElect™ kit DNA profile obtained from case item MG523.B using direct PCR from swab fibres at 29 cycles. PCR tube label: t52B.

2.7 Concluding Remarks

The investigation into each step of the DNA analysis process has enabled the direct PCR process to be enhanced and optimised to suit forensic relevant samples. The data obtained has identified that up to 97 % of DNA is lost to the extraction step depending on the method used. This extreme loss is highly detrimental when processing trace DNA samples, indicating a massive downfall in the current methods and that most commercial kits are not designed to process such samples. With the process circumvented, the DNA is not only retained for amplification, but there is less handling involved, decreasing not only time and costs, but risk of contamination. Buffer enhancements such as the addition of extra AmpliTaq Gold®, Triton® X, and DMSO has also shown to assist in overcoming inhibition, and enabling better DNA collection when using swabs.

Supplementary Material

a. The efficacy of DNA extraction methods in forensic science

Preliminary data

Two common buccal swabs, FLOQSwabs™ (COPAN) and Fitzco (Pathtech) were tested using the QIAamp® DNA Micro Kit (QIAGEN) following manufacturer's instructions to determine if a particular swab yielded significantly higher DNA concentrations and to provide control DNA for further testing. Using the Qubit® 2.0 Fluorometer (Invitrogen™), the FLOQSwab™ resulted in 39.45 ng of DNA and the Fitzco swab resulted in 35.25 ng of DNA.

Raw data

Three extraction methods were carried out following manufacturer's instructions, using control DNA obtained in the preliminary testing. Each extraction method was tested for eight samples.

Table 2a.1 – DNA lost from three standard extraction methods, showing final percentage lost for eight samples per extraction method.

Extraction Method	Input DNA mass (ng)	Recovered DNA mass (ng)	Percentage Lost (%)
QIAGEN	39	19.10	45.81
		22.40	36.45
		22.30	36.73
		21.10	40.14
		6.87	82.57
		7.12	81.93
		22.40	43.21
		15.70	60.20
Promega IQ	36	2.00	94.44
		2.02	94.38
		1.12	96.88
		2.02	94.38
		1.30	96.38
		1.12	96.88
		1.10	96.94
		1.20	96.66
Chelex®	35	3.84	89.02
		2.68	92.34
		2.78	92.05
		2.86	91.82
		2.92	91.65
		2.80	92.00
		3.08	91.20
		2.82	91.94

b. PCR buffer enhancement of STR kits used for human identification

Raw data

Table 2b.1 – NGM SElect™ allele call and RFU value of positive control DNA samples.

NGM SElect Loci	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
Pos1_a	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	3263	3282	2486	3078	3290	3013	2846	2625	5411	4250	5140	4650	4648	4958	3753	4119	4936	3976	4568	3857	5775	4088	3464	2676	1693	2005	1729	2332	2032	1739	1597	1416	1848	2116
Pos1_b	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	2427	2451	1836	2286	2445	2227	2078	1925	3565	2851	3434	3113	3102	3317	2529	2760	3412	2762	3220	2771	3916	2800	2404	1851	1151	1376	1133	1557	1402	1202	1099	979	1267	1441
Pos1_c	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	2964	3003	2292	2897	3109	2832	2671	2470	4418	3517	4276	3867	3950	4227	3205	3578	4224	3466	4081	3475	4997	3600	3128	2379	1385	1647	1408	1926	1732	1484	1368	1210	1615	1868
Pos2_a	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	3714	2978	3590	3284	3863	3128	3580	3036	5752	4437	4802	4327	4502	4238	3818	4116	5371	4014	4456	4252	4982	4793	2733	3407	1727	2084	2249	2212	1727	1374	1472	1751	2133	1568
Pos2_b	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	3050	2431	2934	2696	3212	2606	2999	2532	4362	3444	3682	3325	3471	3262	2995	3190	4137	3060	3366	3257	3775	3652	2045	2612	1261	1580	1722	1675	1294	1038	1129	1329	1627	1198
Pos2_c	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
	2454	1963	2332	2132	2503	2023	2354	2012	3402	2676	2900	2592	2703	2525	2307	2461	3247	2418	2668	2540	2993	2854	1603	2006	1028	1284	1388	1368	1040	830	891	1057	1295	948

Table 2b.2 – NGM SElect™ allele call and RFU value of positive control DNA samples with added BSA at 0.008 µg.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
B1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3354	3185	3553	3579	3489	4398	2673	3107	4615	4705	5379	5892	5127	5734	4976	4990	5183	3755	5141	4939	5043	4056	3445	3324	1488	2217	1793	1889	1757	1490	1645	1630	1722	1904
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2407	2287	2462	2491	2430	3056	1877	2196	3663	3726	4263	4678	3983	4434	3879	3845	4201	2965	3872	3777	3919	3157	2652	2574	1274	1857	1536	1594	1414	1146	1321	1309	1377	1479
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2277	2156	2294	2315	2285	2902	1736	2012	2759	2803	3202	3467	2968	3318	2915	2900	3326	2338	3076	2989	3051	2458	2089	2026	1022	1471	1196	1242	1095	890	1023	1025	1086	1200
B2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2425	2255	2373	2383	2677	2553	2217	1930	3299	3220	3008	3092	3373	3459	3128	2774	3034	3005	3280	2786	3074	3277	2151	2165	1112	1283	1594	1328	1158	1246	1225	1084	1435	1182
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3831	3526	3857	3901	4473	4267	3747	3257	4642	4645	4362	4499	5004	5128	4729	4229	4311	4411	5078	4335	4561	4808	3308	3338	1084	1386	1556	1284	1428	1839	1516	1343	1817	1527
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2892	2669	2860	2883	3220	3081	2694	2366	4000	3965	3698	3832	4185	4272	3903	3482	3691	3642	4038	3419	3842	4025	2692	2698	1355	1552	1963	1648	1433	1532	1508	1341	1787	1500

Table 2b.3 – NGM SElect™ allele call and RFU value of positive control DNA samples with added DMSO at 4 %.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
D1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		1868	2232	2881	2265	2504	2051	1515	1115	2609	1833	3424	3116	3657	3035	2393	1799	3211	2958	3258	3046	3010	2673	1586	1654	1468	1474	1506	1474	890	1044	887	837	587	590
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		1817	2258	2756	2240	3180	2653	2919	2320	2477	1750	3410	3123	4087	3584	3778	3020	2986	2810	3410	3220	3077	2763	2104	2304	1401	1436	1503	1483	898	1085	1138	1098	1404	1655
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2067	2544	3011	2451	3524	2947	3296	2642	2947	2086	4111	3738	4867	4209	4475	3553	3657	3310	3908	3653	3653	3290	2506	2701	1645	1719	1867	1849	1069	1277	1342	1288	1682	1940
D2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2429	2367	2201	2611	2483	2761	2795	2144	2130	2044	3393	3056	3466	3312	3098	3274	3157	2616	3054	3252	3529	2962	1898	2175	1297	1135	1688	1475	1184	1102	1035	971	1932	1699
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2791	2718	2560	3043	2896	3214	3283	2537	2488	2370	3946	3585	4056	3890	3645	3872	3718	3103	3598	3830	4196	3520	2246	2591	1473	1303	1943	1711	1350	1271	1193	1117	2269	2007
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3023	2910	2740	3248	3072	3390	3440	2613	2617	2479	4107	3758	4213	4053	3793	4006	3972	3278	3863	4076	4434	3760	2367	2763	1607	1406	2108	1832	1452	1357	1271	1181	2385	2127

Table 2b.4 – NGM SElect™ allele call and RFU value of positive control DNA samples with added Triton X at 0.004 %.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
T1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3005	2708	3280	2678	3018	3001	2956	2354	3486	3020	4373	3527	3186	4005	3455	3588	3501	3204	3154	2886	4930	3088	2898	2525	1448	1525	1555	1501	1605	1130	970	1207	1337	1489
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2866	2590	3126	2534	2832	2802	2732	2191	3316	2833	4075	3322	2994	3754	3215	3292	3351	3055	3009	2694	4633	2895	2675	2342	1408	1489	1510	1452	1561	1092	929	1155	1260	1399
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2688	2430	2981	2426	2723	2719	2665	2121	3128	2706	3912	3187	2899	3653	3193	3275	3156	2880	2885	2599	4500	2803	2621	2312	1398	1471	1505	1460	1566	1103	954	1183	1319	1464
T2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		4009	3699	3173	3449	4519	3474	3275	3159	4608	4538	5697	6312	6213	5642	4327	5755	5011	5404	5377	4859	4738	5355	4158	3268	1573	1986	2136	1921	1633	1735	1851	1749	1839	2212
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2635	2464	1996	2172	2812	2206	2124	2050	3373	3278	4220	4558	4425	4026	3068	4038	3688	3916	3897	3403	3324	3850	2929	2286	1222	1524	1598	1501	1190	1223	1359	1284	1365	1603
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2632	2473	2000	2172	2867	2243	2065	1995	2695	2639	3319	3662	3520	3210	2474	3244	3071	3284	3256	2847	2793	3162	2421	1918	1020	1295	1307	1233	985	1008	1124	1056	1131	1343

Table 2b.5 – NGM SElect™ allele call and RFU value of positive control DNA samples with added BSA and DMSO.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
BD1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2612	2917	3722	4195	4417	4160	3306	3356	2675	3036	5792	4131	4433	4873	5393	5340	4513	4285	5546	4494	4697	4394	3424	3035	1803	2100	1941	1641	1538	1538	1473	1289	1974	1835
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2021	2233	2726	3102	3265	3079	2485	2516	2296	2533	4842	3448	3657	4030	4425	4378	3900	3606	4866	3650	3857	3701	2806	2513	1588	1875	1634	1484	1296	1250	1233	1097	1668	1523
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		1907	2110	2566	2912	3123	2939	2336	2386	1718	1913	3651	2602	2767	3034	3354	3325	3019	2794	3833	2915	3018	2881	2215	1975	1255	1458	1264	1143	1010	977	973	863	1333	1228
BD2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		1992	1781	2306	1858	2228	2083	1981	1994	2008	1768	3002	2028	2452	3101	2596	2391	2575	2543	2373	2418	2491	2830	1743	1770	1448	962	1718	1128	872	899	935	737	1618	992
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3190	2872	3768	3023	3639	3421	3217	3250	2900	2612	4485	3022	3637	4611	3870	3595	3783	3903	3915	3834	3749	4215	2667	2682	1627	1094	1847	1116	1088	1409	1168	920	2004	1233
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3015	2760	3658	2922	3549	3315	3172	3157	3199	2820	4906	3280	4081	5152	4338	4053	4042	4082	3839	3891	4119	4657	2930	2954	2314	1537	2795	1827	1442	1487	1548	1218	2700	1675

Table 2b.6 – NGM SElect™ allele call and RFU value of positive control DNA samples with added Triton X and DMSO.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
TD1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2233	1725	2146	2437	2326	3167	2562	2717	2048	2079	3623	3471	3594	2927	3725	3378	3154	2968	3321	3012	3109	3005	2286	2572	1437	1266	1672	1508	1203	866	971	947	1550	1342
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2857	2201	2761	3158	3015	4108	3293	3496	2680	2676	4737	4563	4724	3832	4886	4439	4087	3871	4346	3890	4095	3994	3018	3407	1804	1628	2159	1953	1542	1115	1248	1209	2004	1748
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3151	2398	3109	3517	3399	4588	3688	3873	2901	2900	5104	4905	5139	4190	5361	4883	4469	4217	4808	4332	4552	4437	3390	3816	2034	1797	2408	2163	1722	1254	1403	1357	2272	2001
TD2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2866	2718	3269	3314	4667	3813	3758	3200	2603	1963	3739	3860	4075	3690	3147	3845	4273	3100	4345	3634	3964	4266	3198	2547	1681	1411	2019	2137	1200	1385	1092	1270	1900	1861
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2476	2370	2793	2832	3877	3163	3118	2662	2203	1663	3169	3224	3384	3070	2602	3154	3670	2658	3710	3103	3302	3523	2659	2087	1469	1243	1758	1883	1040	1189	930	1075	1606	1566
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2263	2144	2568	2615	3580	2906	2858	2417	2054	1549	2954	2996	3137	2872	2410	2941	3409	2497	3430	2867	3071	3287	2475	1953	1438	1204	1722	1824	1007	1159	914	1049	1568	1519

Table 2b.7 – NGM SElect™ allele call and RFU value of positive control DNA samples with added Triton X, BSA and DMSO.

NGM SElect Loci		D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
Control DNA 007		12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
TBD1	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		1915	1647	1841	2060	2267	2393	1837	1766	1774	1520	2646	1907	1929	2744	2342	1997	2017	2296	2403	2075	2235	2391	1475	1445	851	1269	1320	1078	741	714	815	629	1409	886
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3466	2985	3417	3835	4250	4404	3356	3223	2938	2570	4463	3216	3258	4622	3983	3390	3365	4009	4391	3747	3843	4048	2553	2507	1036	1669	1560	1263	1052	1269	1166	878	2005	1245
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3282	2817	3309	3756	4196	4348	3385	3269	3211	2760	4853	3534	3690	5224	4582	3949	3527	4117	4447	3825	4293	4573	2853	2827	1525	2313	2463	2023	1402	1354	1564	1209	2779	1744
TBD2	A	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3447	2752	4291	3699	4522	3478	3666	3609	3508	2748	4419	5061	4618	4007	5851	4436	3833	4140	4196	4558	5447	4967	2826	2900	1964	1875	2613	2048	1502	1413	1444	1506	1815	2099
	B	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		2570	2048	3173	2705	3266	2541	2664	2619	2727	2138	3444	3948	3569	3082	4463	3342	2893	3173	3205	3451	4061	3744	2111	2167	1545	1472	2070	1609	1162	1097	1108	1164	1371	1573
	C	12	15	14	16	9	10	20	23 X	Y		12	13	28	31	12	15	11	16	14	15	7	9.3	24	26	14	15	15	16	13	15.3	18	19	17	25.2
		3514	2802	4331	3702	4558	3507	3748	3681	4089	3206	5093	5844	5351	4650	6832	5140	4326	4616	4001	4508	6064	5574	3218	3331	2228	2121	3234	2499	1689	1606	1655	1742	2084	2393

Table 2b.8 – Profiler Plus® allele call and RFU value of positive control DNA samples.

Profiler Plus Loci	D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
Pos1_a	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	2762	2459	3940		2746		6650		2217	2611	3103		1146	996	3294		1266	1284	900	893
Pos1_b	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	1325	1181	1868		1373		3059		1038	1213	1395		565	491	1454		588	600	418	415
Pos1_c	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	1165	1037	1641		1242		2807		977	1129	1322		544	471	1401		571	593	415	413
Pos2_a	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	4529	3479	5439		4501		6877		2343	2920	3188		1761	1624	5035		1812	1566	1084	952
Pos2_b	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	3093	2405	3695		3039		4549		1536	1940	2128		1112	1040	3583		1290	1093	750	651
Pos2_c	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
	3407	2664	4089		3395		4890		1689	2114	2328		1259	1164	3437		1220	1054	728	623

Table 2b.9 – Profiler Plus® allele call and RFU value of positive control DNA samples with added BSA at 0.008 µg.

Profiler Plus Loci		D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA		14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
B	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2510	2150	3278		2352		5424		1691	1612	3092		1114	967	3065		1341	928	724	804
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2994	2574	4534		3313		6407		1843	1820	4225		1535	1347	3687		1803	1228	955	1071
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2754	2386	4185		3098		6214		1721	1680	4071		1498	1291	3578		1747	1186	936	1042
B1	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4846	4694	7331		6027		7062		3246	3055	6095		2297	1780	4802		1795	1584	1749	1313
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4284	4127	6531		5452		7634		3021	2851	5679		2182	1689	4574		1740	1539	1680	1258
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3579	3479	5460		4524		7429		2705	2553	5057		1951	1529	4268		1608	1412	1558	1183
B2	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5967	5289	7939		7240		6632		3564	3906	7293		2440	2820	7039		3119	2093	1934	1320
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5241	4570	7166		6356		6896		2968	3272	6163		2022	2356	6726		2814	1891	1733	1180
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5327	4639	7292		6587		7045		2991	3309	6289		2120	2479	5905		2521	1705	1558	1076
B3	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3509	2943	4708		4683		6568		2551	2659	4660		2006	1610	3905		1464	1464	1112	1081
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4677	3929	6442		6403		7521		3287	3421	6157		2724	2180	4771		1814	1830	1388	1374
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4273	3633	5817		5809		7417		3197	3318	5952		2596	2081	5030		1892	1905	1478	1435

Table 2b.10 – Profiler Plus® allele call and RFU value of positive control DNA samples with added DMSO at 4 %.

Profiler Plus Loci		D3S1358		vWA	FGA		AM		D8S1179		D21S11		D18S51	D5S818	D13S317		D7S820	
Renee Control DNA		14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
D	1	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		3367	2810	4936	2304		6958		4205	4591	4178	1203	1428	6373	1568	1030	1300	1212
	2	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		2428	2047	4226	2047		4899		2703	3045	3447	1030	1227	4497	1236	810	1012	952
	3	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		2103	1751	3667	1769		4369		2439	2745	3097	932	1105	4147	1151	741	952	887
D1	1	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		5512	5422	8819	5763		6668		6111	5827	7464	2671	2658	6476	2463	2417	1908	2016
	2	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		4912	4807	8063	5187		6881		5293	5017	6506	2276	2258	6401	2305	2263	1751	1866
	3	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		4614	4563	7765	4900		7322		4854	4641	6019	2183	2172	6812	1870	1834	1451	1533
D2	1	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		3917	3425	6079	4349		7671		3980	4355	5040	1929	1796	6801	1605	1669	1414	1208
	2	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		4940	4290	7595	5526		7152		4807	5207	6206	2401	2218	6978	1842	1945	1650	1399
	3	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		4792	4177	7440	5368		6915		4936	5383	6407	2455	2279	6813	2067	2181	1844	1569
D3	1	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		5357	5148	8136	6355		6521		7105	6373	7042	2667	2729	6580	2342	2294	1856	1569
	2	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		3844	3722	6454	4590		7323		5388	4843	5369	2018	2054	7030	1912	1846	1500	1269
	3	14	15	16	25	X			13	15	31.2	14	18	11	9	14	10	11
		5748	5476	7791	5863		6226		7285	7032	6782	2489	2509	6435	2277	2214	1762	1483

Table 2b.11 – Profiler Plus® allele call and RFU value of positive control DNA samples with added Triton X at 0.004 %.

Profiler Plus Loci		D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA		14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
TX	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4943	4990	7310		5276		6668		3176	4360	6843		2496	2572	6386		3098	2476	1292	1361
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3349	3378	5641		4034		6934		1986	2752	5190		1903	1945	4460		2364	1859	962	1021
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2232	2267	3679		2630		4588		1262	1770	3292		1201	1237	2947		1546	1206	608	641
T1	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3663	3524	4491		4055		6260		2191	2222	4741		1501	1652	4215		1794	1362	1332	1060
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4983	4799	6321		5766		7706		2888	2946	6438		2112	2279	5267		2300	1746	1711	1351
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4635	4439	5798		5197		7492		2841	2851	6247		2016	2173	5629		2429	1827	1783	1416
T2	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5372	4844	8510		6674		6648		3481	3303	7596		2960	2678	6628		2892	2322	1928	1241
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3902	3529	6774		4795		7568		2650	2466	5679		2221	1993	5311		2310	1838	1524	980
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5955	5364	8409		6896		6360		4139	3830	7605		3071	2779	6850		3065	2458	2056	1340
T3	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4489	3659	4985		4392		6699		3115	2285	5001		1705	1569	4867		1970	1412	1175	1349
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6163	5025	6928		6063		7085		4235	3109	6841		2316	2146	6379		2565	1867	1534	1765
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6046	4944	6910		6114		7096		4286	3129	7085		2427	2268	6651		2713	1976	1620	1884

Table 2b.12 – Profiler Plus® allele call and RFU value of positive control DNA samples with added BSA and DMSO.

Profiler Plus Loci		D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA		14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
BD	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3198	2715	4304		2525			5071	3674	3687	3695		1298	1267	5665		1226	1008	895	841
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2685	2265	4214		2580			4470	2990	3036	3737		1330	1311	5938		1454	1186	1058	984
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		2827	2416	4545		2800			4724	3168	3263	4071		1493	1482	5508		1383	1137	1012	942
BD1	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3722	2966	4638		3175			6598	5830	4634	4617		1249	1303	5287		1491	1350	1046	987
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4613	3666	5728		3948			7758	6953	5520	5475		1501	1585	5960		1681	1514	1191	1119
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6815	5479	8444		6683			6522	6444	6984	6975		2775	2941	6317		3203	2947	2384	2246
BD2	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		7494	7130	9101		8635			6036	6221	6489	6445		4418	3913	6061		3669	3526	3079	2756
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3991	3724	6862		4527			7021	5999	5836	6024		2185	1897	7055		2006	1898	1632	1450
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6872	6392	9026		7531			5900	6356	6506	6698		3729	3258	5972		3229	3064	2675	2359
BD3	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4345	4774	6004		4238			7431	4730	5733	5364		1566	1362	7088		1812	1534	1303	1274
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6699	7358	8580		6605			6259	7103	7061	7589		2428	2102	6246		2655	2246	1950	1904
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6385	7058	8344		6362			6307	7072	7289	7752		2429	2142	6332		2691	2286	1972	1927

Table 2b.13 – Profiler Plus® allele call and RFU value of positive control DNA samples with added Triton X and DMSO.

Profiler Plus Loci		D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA		14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
TD1	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5642	4878	8519		5493		6572		5861	5140	7012		2539	2432	6653		2346	2265	1750	1448
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4069	3501	6696		3917		7640		4398	3833	5190		1874	1790	6880		1833	1762	1373	1128
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6426	5552	8870		6119		6510		7110	6157	7544		2864	2727	6305		2697	2651	2045	1694
TD2	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4042	3447	6464		3516		7617		4936	6015	5104		1659	1541	6752		1598	1475	1576	1161
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5567	4748	8242		4851		6669		6664	7426	6859		2225	2091	6683		2089	1943	2045	1517
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5458	4661	8253		4868		6674		6776	7500	7180		2359	2211	6652		2205	2037	2167	1623
TD3	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4236	4006	7597		4366		7615		6541	5428	5954		1749	1785	6040		1885	1609	1154	1377
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3678	3444	6659		3794		7091		5900	4929	5415		1573	1618	5612		1756	1500	1089	1286
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3034	2871	5456		3111		6199		5161	4325	4765		1395	1434	5077		1589	1365	993	1169

Table 2b.14 – Profiler Plus® allele call and RFU value of positive control DNA samples with added Triton X, BSA and DMSO.

Profiler Plus Loci		D3S1358		vWA		FGA		AM		D8S1179		D21S11		D18S51		D5S818		D13S317		D7S820	
Renee Control DNA		14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
TBD	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3800	3310	5392		2935		7128		4930	5347	4431		1676	1219	6527		1350	1255	1702	1183
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		1981	1731	3360		1900		3900		2501	2807	2946		1142	849	3844		906	850	1141	791
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3056	2684	5197		3001		5803		3771	4215	4284		1679	1228	5889		1368	1276	1742	1215
TBD1	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		3301	3660	6241		3524		7633		5058	5830	4176		1767	1461	6662		1736	1192	1260	1131
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4625	5145	8179		4903		6617		6851	7511	5698		2419	2028	6670		2269	1591	1657	1488
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4272	4668	7793		4508		6870		6539	7463	5420		2330	1983	6818		2206	1523	1624	1454
TBD2	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		6019	5014	7848		5008		6596		5798	6532	5726		2310	1802	6924		1839	1653	1304	1291
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5396	4486	7271		4446		7001		5438	6120	5330		2156	1656	6731		1781	1581	1249	1226
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4764	3960	6479		4094		7373		5154	5890	5225		2142	1673	6584		1790	1602	1286	1280
TBD3	1	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		7508	6355	7976		6278		6429		7312	7513	6997		2449	2140	6560		2686	2367	1756	1790
	2	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		4186	3537	4556		3321		6579		3957	4057	3639		1238	1074	5722		1464	1284	911	943
	3	14	15	16		25		X		13	15	31.2		14	18	11		9	14	10	11
		5037	4265	5704		4436		7592		4748	4918	4644		1666	1486	6085		1657	1483	1083	1133

c. A method for the DNA quantification of direct PCR samples, and limit of detection for PCR

Raw data

The following raw data sets were used to determine the limit of detection for PCR of the NGM SElect™ human identification kit, as well as the basis for the statistical quantification of direct PCR samples. Raw data for single hairs, fibres, and tapes can be found in the relevant chapters of this thesis.

Table 2c.1 – NGM SElect™ allele call and RFU value of positive control DNA samples at concentrations 50 pg, 100 pg, 200 pg, 500 pg and 1 ng. Replicate 1.

NGM SElect loci	D1051248		vWA		D165539		D251338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
control	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
1a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	2437	1839	1500	1808	2100	1761	2069	1846	2454	2838	1935	2601	1421	1827	1849	1859	5611		2717	3999	2555	3389	1894	1346	1080	909	1163	1212	1140	863	938	993	1267	1328
05a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	709	638	607	477	697	608	678	759	968	821	1062	955	725	902	698	982	2097		1007	1237	973	1114	497	472	338	337	436	384	397	289	323	249	568	523
02a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	264	301	339	375	427	278	604	444	410	319	413	644	226	286	454	394	1140		514	438	495	525	521	127	177	170	292	122	185	165	248	169	358	301
01a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	117	285	270	141	226	203	328	73	287	199	74	226	248	216	326	164	698		325	621	468	570	295	249	89	52	209	193	83	92	116	81	156	115
005a		15													16							9.3	20											
		142													146							81	63											
1b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	3018	2763	1889	2780	2669	2747	2676	2374	2954	3416	2430	3243	1760	2739	2334	2348	6333		3311	4917	3194	4243	2308	1672	1317	1105	1494	1564	1404	1067	1164	1708	1630	1697
05b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	951	853	826	657	974	855	954	1059	1284	1107	1452	1305	1006	1242	945	1367	2809		1365	1702	1332	1545	681	647	460	445	623	538	529	387	440	336	796	736
02b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	315	357	396	439	498	320	702	519	482	388	496	764	263	335	518	459	1388		619	506	591	618	610	146	211	206	367	149	216	191	282	193	427	348
01b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	146	346	322	172	262	247	393	90	345	236	86	272	288	253	385	187	860		383	730	549	659	342	290	103	61	267	238	91	107	137	97	179	140
005b	13	15					22				14						16					9.3	20											
	51	171					59				60						152					100	75											
1c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	3207	2406	2020	2439	2877	2427	2665	2387	3167	3668	2600	3478	1920	2461	2330	2366	6183		3579	5248	3461	4577	2559	1825	1398	1186	1605	1661	1531	1155	1262	1340	1632	1694
05c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	993	902	868	682	1021	897	975	1083	1361	1165	1517	1388	1043	1307	959	1374	2966		1442	1781	1408	1608	737	700	487	478	649	564	566	409	467	357	812	742
02c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	357	407	447	502	559	360	742	544	549	437	556	861	292	384	524	468	1607		702	586	653	719	684	169	230	226	411	175	244	218	327	219	435	363
01c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	149	347	324	171	264	238	376	85	339	240	84	270	292	251	359	182	860		393	745	557	665	341	298	104	61	270	238	95	109	137	98	177	135
005c	13	15					22				14						16					9.3	20											
	55	186					64				61						179					103	73											

Table 2c.2 – NGM SElect™ allele call and RFU value of positive control DNA samples at concentrations 50 pg, 100 pg, 200 pg, 500 pg and 1 ng. Replicate 2.

NGM SElect loci	D1051248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D2S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391		SE33	
control	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
1a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	915	1271	878	887	1043	769	1490	931	1040	939	1048	852	1012	857	720	991	2243		1189	1369	1740	1598	868	730	596	351	777	487	355	419	427	311	701	655
05a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	1110	975	630	674	969	816	961	859	977	754	778	744	551	691	717	693	1839		938	1362	1037	614	677	790	418	480	448	317	258	402	343	351	393	267
02a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	677	429	425	457	322	309	264	385	409	678	527	373	569	862	927	397	996		1051	545	716	819	541	361	264	274	142	168	187	202	155	120	199	180
01a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3		23		14	17	18	12	13	18	23	15	16
	231	53	171	97	223	266	139	154	136	155	220	100	284	519	78	159	421		247	300	256	580		197		203	111	94	97	133	61	81	136	87
005a	13			19	9	13		X			14		29	31.2	16					14			20	23										
	107			69	171	67			90		64		140	53	51					134			60	77										
1b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	1239	1735	1211	1215	1469	1071	2073	1301	1380	1275	1455	1173	1372	1170	994	1372	3071		1586	1850	2393	2185	1188	999	792	467	1097	686	486	569	571	429	1006	922
05b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	1366	1214	786	836	1221	1029	1232	1090	1202	941	981	919	692	852	902	866	2340		1151	1698	1326	768	829	980	515	591	600	422	322	503	429	434	509	340
02b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	791	498	498	528	384	359	321	457	471	796	616	436	659	990	1096	456	1163		1196	622	859	944	633	404	304	324	173	207	213	242	174	140	247	217
01b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3		23		14	17	18	12	13	18	23	15	16
	261	59	193	110	253	305	161	174	152	177	248	118	329	602	89	182	508		276	337	290	684		222		225	137	109	111	155	72	90	157	102
005b	13			19	9	13		X			14		29	31.2						14			20	23										
	117			75	177	67			86		68		141	54						145			63	77										
1c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	1315	1812	1282	1302	1573	1162	2231	1383	1475	1346	1537	1237	1473	1252	1064	1488	3257		1684	1964	2546	2342	1299	1093	834	505	1162	732	531	610	619	468	1076	1005
05c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	1188	1030	667	713	1035	874	990	888	1021	782	826	788	592	724	725	700	2007		990	1433	1114	638	712	849	449	506	506	360	271	422	364	372	401	276
02c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23	15	16
	742	467	465	492	364	345	295	419	448	743	565	409	623	947	999	426	1093		1104	588	797	902	606	393	278	303	167	196	194	221	172	130	222	198
01c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3		23		14	17	18	12	13	18	23	15	16
	264	63	202	118	263	321	160	177	161	189	261	121	356	636	84	174	532		302	353	304	729		240		237	140	119	116	160	77	100	153	104
005c	13			19	9	13		X			14		29	31.2	16					14			20	23										
	113			82	136	78			97		68		150	56	56					149			66	84										

Table 2c.3 – NGM SElect™ allele call and RFU value of positive control DNA samples at concentrations 50 pg, 100 pg, 200 pg, 500 pg and 1 ng. Replicate 3.

NGM SElect loci	D10S1248		vWA		D16S539		D2S1338		AM			D8S1179		D21S11		D18S51			D22S1045		D19S433		TH01		FGA			D2S441		D3S1358		D1S1656		D12S391		SE33	
control	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
1a	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1438	1627	1350	1380	1899	1477	1538	2200	1808	1681		2046	1193	1274	1944	1398	1091	3801		1860	2272	2236	2168	1174	1400		852	778	828	812	609	691	817	658	1006	837	
05a	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1422	1092	964	1035	1170	759	1348	1377	1064	1238		1071	1103	852	1040	1126	1159	2217		1437	1444	1680	1725	1204	870		306	354	498	526	472	442	326	343	544	403	
02a	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	251	491	394	134	402	232	424	310	303	236		329	255	168	319	506	522	1022		909	566	383	754	328	121		224	170	241	179	267	170	171	61	162	221	
01a	13	15	16	19	9	13	22	25 X	Y			14	15	29		16	18	16			14	6	9.3	20			10	14	17	18	12	13	18		15	16	
	287	231	135	187	177	174	375	288	295	158		378	258	193		232	214	676			391	240	92	105			60	66	108	75	98	130	139		127	186	
005a		15	16		9	13		25 X	Y			14	15		31.2	16	18	16		13	14	6	9.3		23											
		123	119		72	105		76	114	136		248	142		91	178	120	216		207	129	152	217		55											
1b	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1605	1776	1511	1561	2205	1721	1816	2599	2004	1874		2330	1326	1461	2229	1604	1775	4308		2092	2579	2576	2537	1379	1628		950	885	993	979	687	784	937	745	1210	1015	
05b	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1717	1325	1175	1268	1455	960	1685	1742	1275	1493		1320	1338	1019	1771	1381	1425	2721		1735	1736	2060	2105	1464	1065		355	470	635	661	567	530	382	420	691	513	
02b	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	356	679	553	191	593	342	637	460	410	307		461	365	237	447	720	739	1425		1247	789	539	1069	458	174		306	234	355	266	373	230	245	87	238	331	
01b	13	15	16	19	9	13	22	25 X	Y			14	15	29		16	18	16			14	6	9.3	20			10	14	17	18	12	13	18	23	15	16	
	370	298	174	247	230	236	495	379	374	204		480	330	250		295	278	855			503	312	107	135			78	82	152	97	125	162	177	71	166	259	
005b		15	16		9	13		25 X	Y			14	15		31.2	16	18	16		13	14	6	9.3		23											
		132	126		76	108		78	120	147		267	142		91	178	122	232		205	136	156	223		52											
1c	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1574	1781	1483	1504	2119	1642	1720	2470	1970	1844		2281	1315	1422	2162	1556	1217	4281		2085	2574	2480	2473	1368	1592		938	864	962	945	671	755	907	725	1165	974	
05c	13	15	16	19	9	13	22	25 X	Y			14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		10	14	17	18	12	13	18	23	15	16	
	1755	1349	1184	1275	1455	948	1597	1636	1308	1540		1334	1373	1052	1287	1284	1341	2802		1798	1790	2092	2150	1476	1073		369	436	632	663	572	537	394	430	634	477	
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	367	695	569	197	589	344	606	436	434	311		463	369	243	459	683	702	1469		1277	786	544	1095	467	177		318	247	349	262	372	236	249	84	225	309	
01c	13	15	16	19	9	13	22	25 X	Y			14	15	29		16	18	16			14	6	9.3	20			10	14	17	18	12	13	18	23	15	16	
	346	282	160	233	215	221	455	346	359	188		452	313	238		275	259	812			457	292	99	131			71	79	143	93	114	153	171	64	147	231	
005c		15	16	19	9	13		25 X	Y			14	15		31.2	16	18	16		13	14	6	9.3		23						12					
		144	137	53	82	122		89	130	155		293	159		101	194	137	266		236	149	180	240		56						51					

Appendix

i. **Poster Presentation** 22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *An investigation of the efficacy of DNA extraction methods.*

An investigation of the efficacy of DNA extraction methods

Renée Blackie (nee Ottens)[1], Duncan Taylor[2], Adrian Linacre[1]

[1]School of Biological Sciences, Flinders University
[2]Forensic Science South Australia, Adelaide

Flinders University
Government of South Australia
Forensic Science SA

Forensic Biology Lab
Flinders University
South Australia

Presented at the 2014 ANZFSS Conference Adelaide, Australia

Funding was provided by the Department of Justice, South Australia

Introduction

In forensic science, trace DNA is common and sometimes may be the only evidence available to an investigation. Trace evidence presents its own set of difficulties; too little DNA will result in a partial profile, providing reduced significance, or more commonly no profile at all. The extraction method is the first step involved in DNA analysis, and is open to variation in DNA yield depending on the method used. It is an important step for the isolation and purification of DNA as it aims to remove inhibitors permitting downstream processes to work effectively. This process typically involves several wash steps and tube changes, each allowing for DNA retention to tubes and pipette tips, resulting in the loss of information. We report on the retention and loss of DNA using three commonly used extraction methods in forensic analysis. DNA concentrations were measured prior and post extraction using the QUBIT® fluorometer, and compared to assess the percentage lost.

DNA Extraction Methods Investigated

QIAamp Micro | Chelex | Promega IQ

Methods

- **Extraction:** Three common extraction methods were tested to determine the average loss of DNA from each: **QIAamp DNA Micro Kit** (QIAGEN), **DNA IQ™** System (Promega) and **Chelex**.
- Previously extracted DNA (from buccal swabs using QIAGEN Micro Kit) was quantified on a Qubit® 2.0 Fluorometer (Invitrogen™) and used as the control in this experiment. Control DNA ranging from 35 – 39 ng was used as the starting concentration for each extraction. Extractions were performed following the manufacturer's instructions for each kit and were eluted in a final volume of 30 □L for the DNA micro kit and 100 □L for Promega IQ and chelex.
- Chelex extraction involved adding 200 □L of 5 % chelex to the DNA in a 1.5 mL tube and incubating at 56 ℃ for 20 mins with shaking, followed by 100 ℃ for 8 mins with shaking. Sample was centrifuged at max speed (13000 g) for 3 mins. Liquid was removed without disturbing the chelex pellet and transferred into a new sterile 1.5 mL tube.
- Extractions were repeated eight times for each method used. Each extraction sample was quantified on the Qubit® following manufacturer's instructions, using 10 □L from each sample for quantification. The quantification results were compared against the initial input DNA to determine percentage lost.

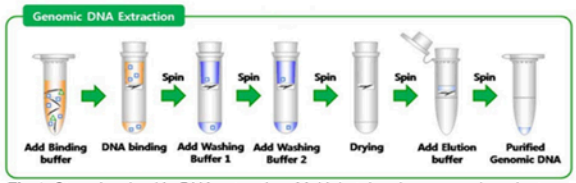

Fig 1. Steps involved in DNA extraction. Multiple tube changes and wash steps can contribute to exogenous DNA contamination as well as DNA loss. The extraction process is beneficial in removing inhibitors of downstream processes.

Image from Bioneer http://eng.bioneer.com

Results

DNA loss ranged from 36.5 % to 96.9 % across all methods, with QIAamp performing the best with the lowest average of 53.4 % DNA loss. Promega IQ and chelex were highly consistent with the results obtained, showing approximately 3 % variation between the highest and lowest losses observed. Although the results varied quite significantly for the QIAamp Micro kit, it still out performed the other kits with the highest DNA loss obtained (82.6 %) being less than the lowest percentages observed for the other two kits (89 and 94.3 %).

Table 1. Comparison of standard extraction methods, showing the range of DNA lost and kit efficiency across three methods.

DNA Extraction kit	Starting Mass (ng)	Average Final Mass (ng)	Average Percentage Lost (%)	Lowest/Highest Percentage Observed (%)
Promega IQ	36	1.5	95.8	94.3 / 96.9
QIAamp Micro	39	17.1	53.4	36.5 / 82.6
Chelex	35	3	91.5	89.0 / 92.3

Concluding Remarks

- Three common methods of DNA extraction are very inefficient with average loss ranging from 53.4 – 95.8 %
- DNA extraction is not ideal for trace evidence samples where minimal initial DNA is present
- Methods should be adjusted for trace evidence samples to reduce time and money wasted and improve results
- QIAamp Micro kit performed the best overall

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Corresponding abstract for poster presentation:

An investigation of the efficacy of DNA extraction methods

Renée Ottens[1], Adrian Linacre[2]

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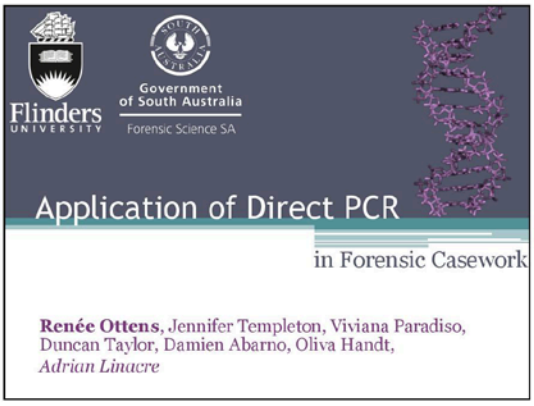
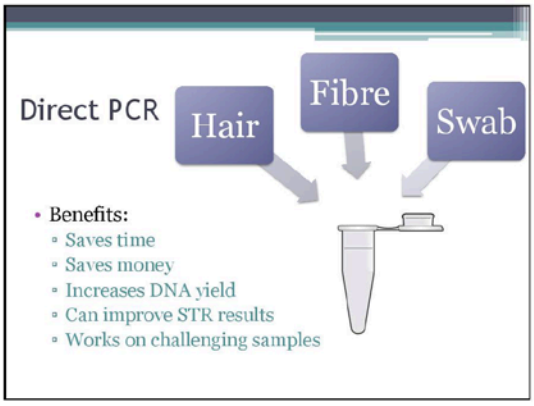
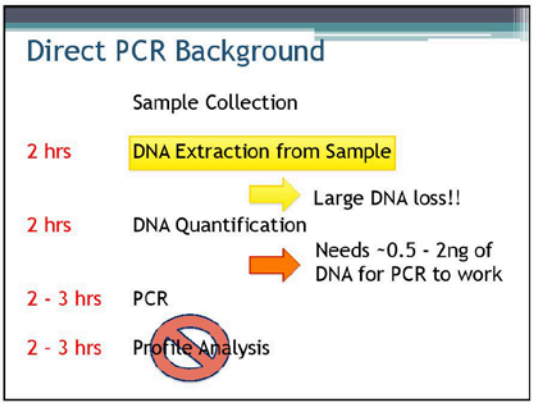
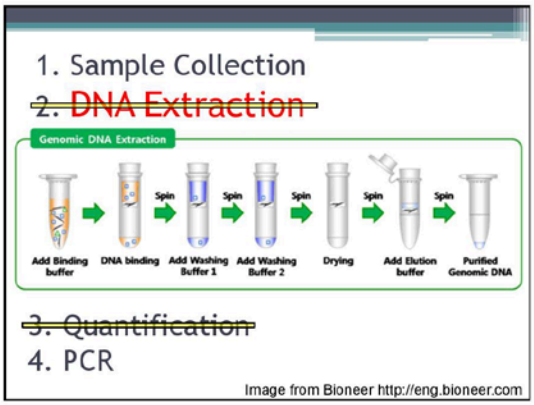
In forensic science, trace DNA is common and sometimes may be the only evidence available to an investigation. Trace evidence presents its own set of difficulties; too little DNA will result in a partial profile, providing reduced significance, or more commonly no profile at all. The extraction method is the first step involved in DNA analysis, and is open to variation in DNA yield depending on the method used. It is an important step for the isolation and purification of DNA as it aims to remove inhibitors permitting downstream processes to work effectively. This process typically involves several wash steps and tube changes, each allowing for DNA retention to tubes and pipette tips, resulting in the loss of information. We report on the retention and loss of DNA using three commonly used extraction methods in forensic analysis. Known concentrations of DNA were extracted using Chelex®, DNA IQ™ (Promega), and QIAamp DNA Micro kit (Qiagen). DNA concentrations were measured prior and post extraction using the QUBIT® fluorometer, and compared to assess the percentage lost. DNA loss ranged from 36.5 % to 96.9 % across all methods with QIAamp performing the best with an average of 53.4 % DNA loss.

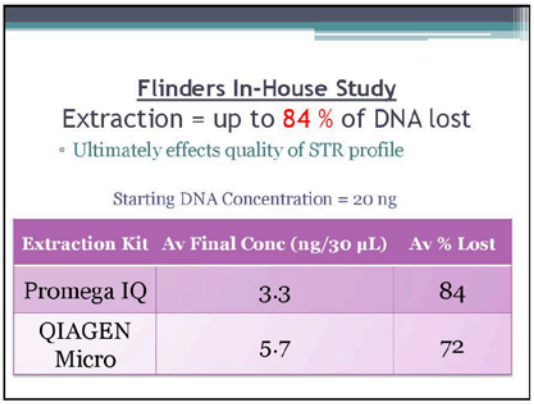
ii. **Oral Presentation** 25th World Congress of the International Society for Forensic Genetics (ISFG), Melbourne, Australia, 2013. *The development and implementation of direct PCR in casework.*











Flinders In-House Study

Direct PCR sensitivity

- Up to **15 x** higher than standard extraction PCR
- Ability to detect low levels of DNA without the need to modify validated protocols

Assuming no input from cell-free DNA

Direct PCR needs 17 cells (100 pg)

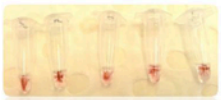
VS

Standard PCR needs 250 cells (1.5 ng)

(prior to extraction)

Flinders Direct PCR Projects

- Optimising:
  - Hairs
    - Human and animal
  - Fibres
    - Fibre types
    - Dyes
    - Single Fibre Vs Direct Tape Lift
  - Trace DNA samples:
    - Touch/fingerprints,
    - Swab comparison
    - Surface comparison
  - Enzyme comparison
    - Phusion, AmpliTaq Gold 360
  - + many more substrates and sample types



Hair Study

- ~5 mm of hair from root end removed
- Placed directly into PCR tube
- NGM™ human identification kit used
- Standard 29 cycles (NO increase or adjustment!)
- 5 particpants
- Over 60 hairs, anagen and telogen
- Aged hairs also tested

Hair - Anagen (forcefully removed)

Based on **Full** Profile results

	NGM™ Direct	Profiler Plus® Standard
	Flinders Study†	**Forensic Science SA***
	100 %	53 %

Over 30 anagen hairs used from 5 individuals

Worked on 'aged' hairs... cold case potential.

† Ottens, R., et al., *Successful direct amplification of nuclear markers from a single hair follicle*. Forensic Science, Medicine, and Pathology. 2013. 9(2): p. 238-243

* White, E. et al. Evidence Recovery Report, *Comparison of Hair Roots to DNA Profiles Obtained*, Forensic Science SA, 2007.

Hair - Telogen (shed naturally)

	NGM™ Direct	Profiler Plus®
Profile Type	**Flinders Study†**	**FSSA***
Complete or Up-loadable (≥ 5 complete loci)	33.3 %	16 %
Incomplete	66.7 %	84 %

Over 30 telgoen hairs used from 5 individuals

† Ottens, R., et al., *Successful direct amplification of nuclear markers from a single hair follicle*. Forensic Science, Medicine, and Pathology. 2013. 9(2): p. 238-243

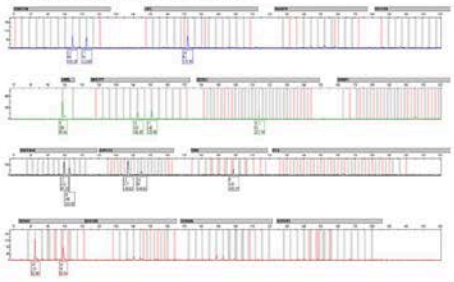
* White, E. et al. Evidence Recovery Report, *Comparison of Hair Roots to DNA Profiles Obtained*, Forensic Science SA, 2007.

Fibre Study

- ~5 mm of fabric, single fibre or square used
- Placed directly into PCR tube
- NGM™ human identification kit used
- Standard 29 cycles (NO increase or adjustment!)
- Items worn by participants or held/touched

Fibre - Touched 15 seconds - NGM™

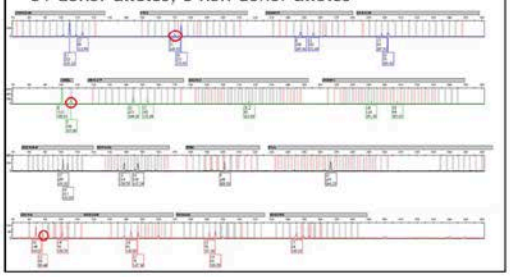
8 complete loci from donor



Fibre - Worn 12 hrs - NGM™

single fibre from cuff

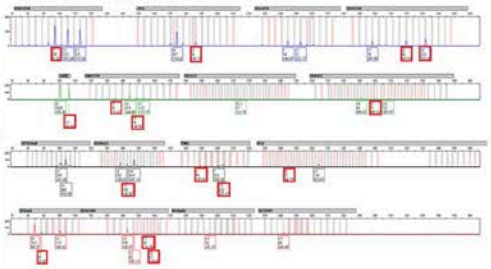
31 donor alleles, 3 non-donor alleles



Fibre - Direct Tape lift - NGM™

Worn 12 hrs, tape lift from cuff

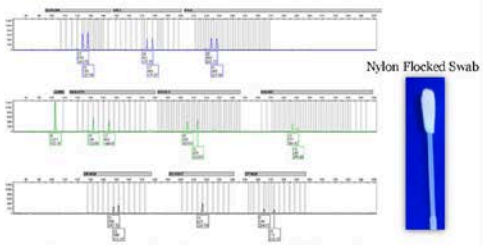
28 donor alleles, 15 non-donor alleles



Touch DNA

- Participants asked to wash hands
- Place finger on plastic, glass or brass substrate
- Hourly intervals
- Fingermark swabbed with different swab types
- Tip of swab removed (~ 5 mm x 5 mm)
- Placed directly into PCR tube
- NGM™ and Profiler Plus® kits used
- Standard 28 or 29 cycles
  - (NO increase or adjustment!)

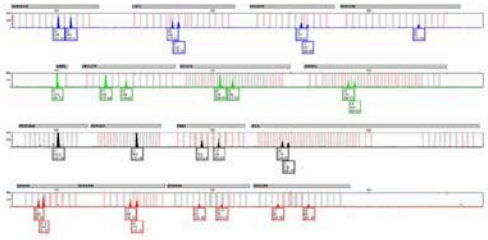
Swabs from touch DNA - Profiler Plus®



Nylon Flocked Swab

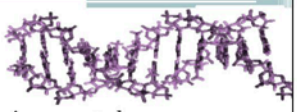
Chromatogram of nuclear DNA collected from a plastic substrate touched by the left index finger, 1 hr after washing their hands.

Swabs from touch DNA - NGM™



Chromatogram of nuclear DNA collected from a plastic substrate touched by the left index finger, 2 hrs after washing their hands.

Summary

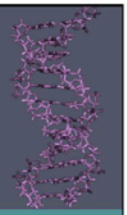


- Extraction process circumvented
  - DNA not lost via wash steps or tube changes
  - Greater DNA yield
- Cost effective and time saving
- Minimal to no changes made to **protocols** of already validated STR human identification kits
  - Easy & quick implementation in forensic labs

Flinders UNIVERSITY

Government of South Australia

Forensic Science SA



Thank You

Partial funding provided by the *Department of Justice SA*

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C R I M T R A C

Corresponding abstract for oral presentation:

Title: The development and implementation of direct PCR in casework

R. Ottens[1], J. Templeton[1], D. Taylor[2], D. Abarno[2], O. Handt[2], A. Linacre[1]

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DNA isolation and purification procedures are standard practice in the initial steps of DNA profiling when examining swabs, adhesive tapes and sections of fabric. Significant loss of DNA occurs during this process resulting in no DNA profile generated. Direct PCR circumvents the extraction process such that a DNA profile may be generated directly from the substrate. This potentially saves time, increases the sensitivity, reduces tube changes, and minimises steps open to error or contamination in the laboratory.

We report on the generation of DNA profiles from a range of substrates such as hairs, fibres, and swabs taken from touch substrates. Sections of hair shafts were placed in the reaction solution with no prior treatment. Fibres from clothing were treated likewise. Individual fibres from swabs used to remove latent DNA on plastics and metals were removed and placed directly in the reaction tube. The number of amplification cycles remained as recommended by the supplier.

The only alteration required to generate DNA profiles that can be uploaded to the Australian National Criminal Investigation DNA Database (NCIDD) was to increase the amount of DNA polymerase. The result is that DNA profiles can be generated from single hair shafts, single fibres and substrates touched for 5 seconds.

iii. **Poster Presentation** 22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Quantifying DNA from direct PCR samples.*

Quantifying DNA from direct PCR samples

Renée Blackie (nee Ottens)[1], Duncan Taylor[2], Adrian Linacre[1]

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Forensic Biology Lab
Flinders University
South Australia

Presented at the 2014 ANZFSS Conference Adelaide, Australia

Funding was provided by the Department of Justice, South Australia

Introduction

Direct PCR in forensic science has the advantage of using all genetic material available from a sample as none is lost during the extraction process, saving time, money and increasing the magnitude of profiles obtained from trace evidence. Quantification of the DNA however, is not possible as all genetic material is used in direct amplification. A potential criticism of this method is that there is no knowledge of the amount of DNA in the tested sample. We report on an accurate method to quantify the DNA template used in direct PCR amplifications for the first time. The resulting magnitude of the alleles generated (relative fluorescence units or RFU value) can be used to calculate the original mass of DNA template.

Method

- **Amplification:** DNA was amplified using the NGM SElect™ kit (Life Technologies, Victoria, Australia) using a known mass of starting DNA template of 0, 0.05, 0.1, 0.2, 0.5 and 1 ng of DNA (2800M, Promega, Victoria, Australia). Each series of known mass of DNA (0 – 1 ng) was prepared three times for a total of 18 samples. Each sample was prepared in a 0.2 mL thin walled tube containing 25 □L of reagents following the manufacturer's instructions. A total of 29 PCR cycles was performed on a GeneAmp® 9700 96-well thermal cycler (Life Technologies) following manufacturer's instructions. PCR products (1 µL) were combined with 0.5 µL Liz 500™ (Life Technologies) separated using a 3130*xl* Genetic Analyser (Life Technologies). The data were analysed using GeneMapper□ ID v3.2 (Life Technologies) with a threshold of 50 RFU for allele assignment. Samples were run in triplicate on the 3130*xl* for a total data set of 54 profiles (nine data series of 0 – 1 ng).

- **DNA Analysis:** NGM SElect™ amplified 17 STR loci resulting in a possible 34 alleles. The average RFU value was calculated for each profile by dividing the sum of all RFU values of that profile, by the number of alleles obtained, counting a single allele for a homozygous locus. This average was recorded against the known DNA mass of the samples. Confidence intervals of 95 % were calculated by multiplying or dividing the expected DNA mass by 1.57

- **Reverse Quantification Analysis:** For each electrophoresis run, a calibration curve was produced using the positive control (0.5 ng) as the known upper standard and forcing the linear regression through the point of origin (making the assumption that no DNA will lead to no fluorescence). The average RFU of each sample on that run was then converted to an initial DNA mass using the calibration curve. This process was carried out on the DNA profiles generated from the prepared DNA dilution series (0 – 1 ng), to determine the accuracy of the reverse-quantification method.

Results

The reverse quantification method relies on the assumption that a linear relationship exists between template DNA mass and the fluorescence of the resulting DNA profile. This assumption was tested using the DNA dilution series (0 – 1 ng).

The 0.5 ng standard was used to generate the calibration curves and produced more evenly spread data around the expected value. The linear correlation of DNA mass and RFU value was determined using the coefficient of determination (R^2 value), for each series. The R^2 values ranged from 94.5 % to 99.9 %, with an average correlation of 97.6 % across all nine series (Table 1).

The estimated mass values obtained using the reverse quantification method can be seen in Figure 1. The log base 10 of the ratio of observed to expected mass values was modelled with a normal distribution that had a mean of 0 and a standard deviation of 0.1. This lead to 95% confidence intervals being calculated for expected mass being determined by a factor of 1.57 so that the intervals in Figure 1 were obtained by multiplying or dividing the expected DNA mass by 1.57. The 95% bounds covered 94% of observed data.

Results

Table 1: Calculated DNA mass (ng) for each series versus the actual DNA mass (ng) with corresponding R^2 values. Note the 0.5ng value is omitted as it was used to create the calibration curve for each series of data.

	Calculated DNA Mass (ng)								
Actual DNA Mass (ng)	*Series 1*	*Series 2*	*Series 3*	*Series 4*	*Series 5*	*Series 6*	*Series 7*	*Series 8*	*Series 9*
1.0	1.37	1.2	1.2	0.64	0.69	0.88	0.74	0.69	0.67
0.2	0.27	0.22	0.23	0.31	0.29	0.32	0.17	0.20	0.20
0.1	0.16	0.14	0.14	0.13	0.12	0.15	0.10	0.11	0.10
0.05	0.07	0.05	0.05	0.06	0.05	0.06	0.07	0.06	0.06
R^2 Value	**0.976**	**0.990**	**0.989**	**0.945**	**0.963**	**0.934**	**0.993**	**0.999**	**0.999**

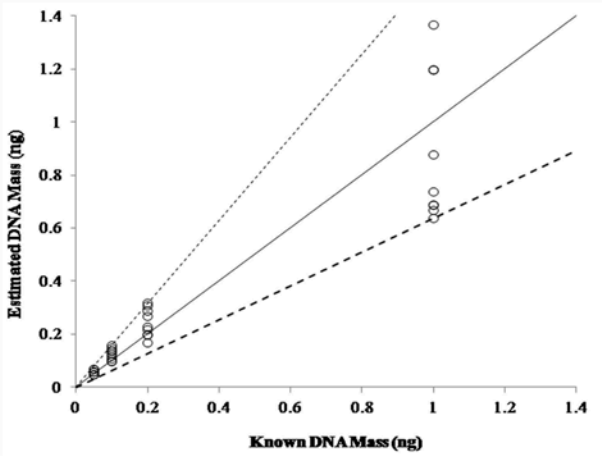

Figure 1: The known DNA mass compared to the estimated mass using the reverse quantification method (raw data seen in Table 1). The solid line represents the space where observed and expected values are equal and the dotted lines show the 95 % confidence interval boundaries on DNA mass prediction.

Concluding Remarks

- Previously there has been no means of estimating the initial amount of DNA used in a direct PCR.
- The statistical method described in this study enables the calculation of the DNA mass.
- The data indicate a high correlation between DNA mass and average RFU value, allowing for DNA mass calculations to be made within a factor of 1.57 of the actual value at a 95% confidence level.
- Can provide knowledge of amount of DNA template in direct PCR sample.
- Can help identify how much DNA was within a substrate prior to the amplification.
- Application beneficial in processing touch and trace DNA such as fingermarks and surface swabs as this technique is provides an informative estimation of DNA amount, even at low levels of starting DNA.
- As the standards required to calculate the DNA mass of samples are equivalent to the positive and negative control samples of a PCR setup, no additional laboratory work is required to implement this technique.

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Corresponding abstract for poster presentation:

Quantifying DNA from direct PCR samples

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Direct PCR in forensic science has the advantage of using all genetic material available from a sample as none is lost during the extraction process, saving time, money and increasing the magnitude of profiles obtained from trace evidence. Quantification of the DNA however, is not possible as all genetic material is used in direct amplification. A potential criticism of this method is that there is no knowledge of the amount of DNA in the tested sample. We report on an accurate method to quantify the DNA template used in direct PCR amplifications for the first time. The resulting magnitude of the alleles generated (relative fluorescence units or RFU value) can be used to calculate the original mass of DNA template. A number of standards of known DNA quantity were amplified using the NGM SElect™ STR kit, and were separated on a 3130*xl*. The data was plotted on a graph to assess linearity, and therefore calculate mass based on the RFU value. The data indicated that the original template DNA could be calculated within ± 64 pg of the known DNA mass. This method can be used to report the estimated number of cells from a sample processed via direct PCR.

iv. Published Conference Proceeding of the 2013 ISFG, Melbourne, Australia (2nd Author).

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Genetic profiling from challenging samples: Direct PCR of touch DNA

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ABSTRACT

We report on a novel direct PCR approach for retrieving touch DNA from handled items. Trace levels of DNA on handled items can be difficult to recover and analyse using standard methodology. The lack of a full nuclear STR DNA profile may be related to insufficient DNA being present initially, the presence of PCR inhibitors, or the loss of nucleic acid during sample collection and processing. The aim of this study was to optimise the swabbing method used to collect DNA and to improve the success rate of STR-based DNA profiling from touched items. A comparison was carried out to evaluate the use of three common swab types, nylon, cotton and foam at retrieving and releasing template DNA for amplification by direct PCR. To test the effectiveness of our method at processing low levels of DNA, complete STR DNA profiles were obtained from fingerprints deposited onto sterile plastic slides, which were swabbed and subjected to direct PCR. Our findings indicate the potential use of direct PCR when analysing samples that contain limited DNA.

1. Introduction

Low levels of DNA recovered from touched items can be close to the limit of detection for current STR-based DNA profiling kits. The success of a DNA profile depends largely on how much DNA is retrieved from the evidence. Cotton swabs, pre-moistened with distilled water, are used routinely in forensic laboratories. However, other swab types should also be considered due to the low success rate of current methods. Following DNA collection, current methodology subjects the swab to a DNA extraction to isolate and purify the target DNA, typically using a solid phase substrate, prior to amplification. The DNA extraction process contributes to a significant loss (20–70%) of sample DNA [1] and has the potential to introduce extraneous DNA into the reaction [2]. Direct PCR circumvents the need for a DNA extraction. For direct PCR, the biological material is transferred directly into the PCR tube with no prior extraction or purification steps. Previous studies report full STR DNA profiles obtained from trace evidence using direct PCR [3,4]. The aim of this study was to examine aspects of sample collection, apply the method of direct PCR to low level DNA templates, and to improve the success of STR DNA profiling. In this study we report the ability of direct PCR, using 29 cycles, to generate full STR DNA profiles from fingerprints deposited on plastic substrates.

2. Materials and methods

Control DNA (2800 M, Promega) of a known concentration (1 ng/μL) was deposited onto 3× sterile plastic microscope slides and swabbed using either foam (Whatman, USA), DNA-free nylon FLOQswabs™ (Copan Industries, Vic) or cotton swabs (Livingstone, NSW). For direct PCR, a 2 mm^2 portion of each swab tip was cut using a sterile scalpel blade and placed directly into a 0.2 mL PCR tube. Amplification conditions followed the Profiler Plus® STR kit (ABI) guidelines in a final 25 μL reaction volume and standard 28 cycles. For fingerprint deposition, three donors placed the index finger of their dominant hand onto a sterile plastic slide 2 h after hand washing. For direct PCR, a 2 mm^2 portion of the swab tip was cut using a sterile scalpel blade and placed directly into a 0.2 mL PCR tube. Amplification followed the NGM™ STR kit (ABI) guidelines in a final 25 μL reaction volume and 29 cycles. PCR products were run on the 3130*xl* (ABI) and profiles analysed using GeneMapper™ ID v3.2 software and a peak amplitude threshold of 30 RFU was used to assign alleles.

3. Results and discussion

Nylon swabs generated the highest DNA yield, as assayed by the relative fluorescence units (RFU) of peak heights, followed by foam and then cotton swabs (Fig. 1). Full STR DNA profiles were

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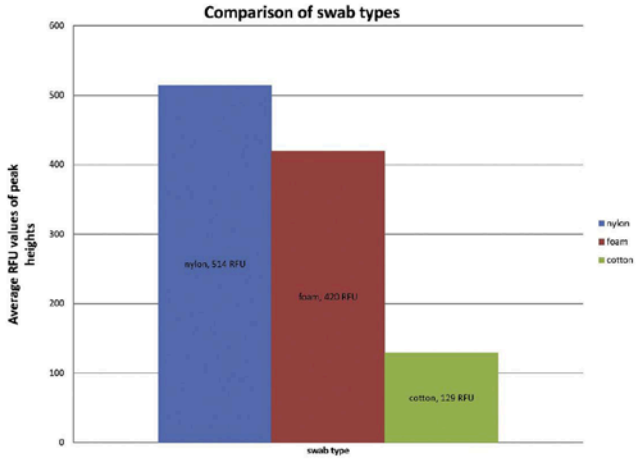

Fig. 1. A comparison between swab types, using control DNA deposited on a plastic slide, prior to swabbing, PCR and STR DNA profiling.

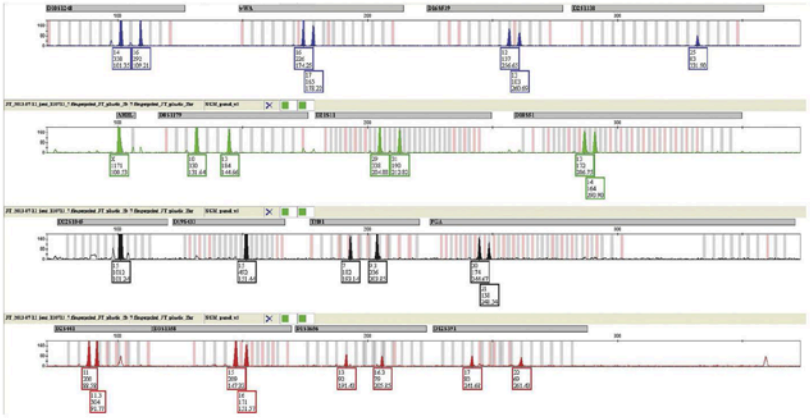

Fig. 2. Example of an STR DNA profile generated, using direct PCR, from a fingerprint deposited on a plastic slide prior to swabbing and processing (29 cycles of PCR, using NGM™ STR kit (ABI)).

obtained, using nylon flocked swabs and direct PCR, from all three fingerprints deposited on plastic substrates 2 h after the donor washed their hands. No allele drop-in or allele drop-out was observed (Fig. 2). Laboratories may benefit from using direct PCR on handled items due to its high-throughput potential and increased sensitivity. However, the success and quality of DNA profiles obtained using direct PCR will be dependent on the nature of the material examined and the presence of PCR inhibitors; which are generally eliminated during the extraction process.

4. Conclusion

Nylon flocked swabs (Copan) provided the optimum method of DNA recovery from plastic slides. Nylon flocked swabs and direct PCR was used to generate full STR DNA profiles from three fingerprints deposited on plastic substrates 2 h after the donor washed their hands. No allelic drop-in was detected and only 29 cycles were used in the PCR process. The method of direct PCR should be considered as an alternative method for analysing samples that contain low amounts of DNA for use in a high-throughput environment. A validated study is required to assess the limitations of the direct PCR approach. However, we anticipate the method to have future niche applications in analysing latent DNA recovered from touched items that face the limits of detection when using standard protocols.

Role of funding

Funding was provided by the Department of Justice, South Australia.

Conflict of interest

None.

Acknowledgements

We thank members of Forensic Science South Australia and the School of Biological Sciences at Flinders University for assisting with reagents and providing helpful advice.

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CHAPTER 3

Direct PCR from Human Hair

Ottens, R., et al., *Successful direct amplification of nuclear markers from a single hair follicle.* Forensic Science, Medicine, and Pathology, 2013. **9**(2): p. 238-243.
http://dx.doi.org/10.1007/s12024-012-9402-6
Citations: 19

Ottens, R., et al., *Optimising direct PCR from anagen hair samples.* Forensic Science International: Genetics Supplement Series, 2013. **4**(1): p. e109-e110.
http://dx.doi.org/10.1016/j.fsigss.2013.10.056
Citations: 8

Direct PCR from Human Hair

3.1 Preface

Single human hairs may be encountered in a forensic investigation in one of three growth stages: anagen hairs are in the active growth phase and maintain a follicular tag or skin sheathing at the root end, to be found loose would suggest forceful removal; telogen hairs have completed the growth cycle, shedding themselves naturally once skin sheathing at the root end has finished degrading; catagen hairs represent the transitional growth phase between the active (anagen) and dormant (telogen) growth phases. DNA is intrinsic to the hair, associated higher with the active growth phase where skin cells and sheathing material are still present, and is hypothesised that cell-free DNA is present on the shaft of the hair. Human hair represents an on going challenge in forensic casework due to the low amounts of DNA associated with a single hair and are notoriously difficult to consistently obtain DNA information from, if at all [1-5]. This is particularly true for telogen hairs that lack sheathing material, and as they shed naturally they are more commonly found in a forensic investigation [5-7]. Although a shift from standard STR typing to mini-STR nuclear DNA analysis has improved the chances of obtaining alleles from single hairs [4, 8-10], improvised pre-treatment methods of hairs such as multiple wash steps, certain staining techniques and extraction methods have failed to make a significant difference in success rates [7, 11, 12].

Human hairs are an ideal substrate for direct PCR as inhibitors, such as humic acid (found in soil) or haem (found in blood), are unlikely to be found on the surface of the hair. Melanin (a PCR inhibitor found within the hair) is unlikely to be released or broken down during the amplification stage as the hairs are not digested during this process, the hairs remain intact. The following sections investigate the application of human hairs as a substrate for direct PCR (*3.2*), the optimisation of the substrate (*3.3*), and further testing and implementation of the technique with human hairs (*3.4*).

Statement of authorship

Successful direct amplification of nuclear markers from a single hair follicle

Published in Forensic Science Medicine, and Pathology

Date: December 2012

Renée Blackie (Candidate)

Performed all laboratory work (sample collection, DNA extractions and quantifications, PCR amplification, capillary electrophoresis), data analysis and interpretation, created data table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Damien Abarno

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, co-authored and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

3.2.1 *Published manuscript, a technical report, Forensic Science Medicine, and Pathology.*

Forensic Sci Med Pathol (2013) 9:238–243
DOI 10.1007/s12024-012-9402-6

TECHNICAL REPORT

Successful direct amplification of nuclear markers from a single hair follicle

Renée Ottens · Duncan Taylor · Damien Abarno · Adrian Linacre

Accepted: 15 December 2012 / Published online: 30 December 2012

Abstract We report on successful amplification of DNA profiles from a single hair. Direct amplification was used on the root tip of both anagen and telogen hairs using a kit to amplify 15 STR loci. All 30 anagen hairs tested from five different people gave full DNA profiles after 29 cycles with no allelic drop-in or heterozygous imbalance. Six of the 30 telogen hairs tested resulted in a full DNA profile, and a further four telogen hair samples tested produced a DNA profile of five or more complete loci that could be uploaded to the National DNA Database (Australia). A full DNA profile was also obtained from the shaft of an anagen hair. Current practice for many laboratories is that a single hair may not be subjected to DNA testing as there is little chance of success, hence this 100 % success rate from anagen hairs is a significant advancement. A full DNA profile was obtained from a 5 year-old single hair illustrating the success when using direct PCR rather than attempting an extraction prior to the amplification step. The process described deliberately uses current DNA profiling methods with no increase in cycle number, such that the methodology can be incorporated readily into operational practice. For the first time in the field of human identification, single hairs can be analyzed with confidence that a meaningful DNA profile will be generated and the data accepted by the criminal justice system.

Keywords Hair · DNA · PCR · Identification · Profile

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Introduction

Direct PCR of samples has gained much interest in a forensic context due to the potential saving in time [1–4], increase in sensitivity, and minimizing of steps open to error or contamination [5]. Much of the focus has been on direct amplification from a stain [6] with an emphasis also on the speed of the DNA typing [7] and the types of DNA polymerase used. A previous report illustrated the potential benefit of direct amplification from fibers [5] and touch DNA [8]. To date there has been no testing of human hairs with direct PCR despite the prevalence of hair sampling during forensic examination [9]. The potential to generate a profile from hair follicles is dependent on the presence of nucleated cells such as within a follicular tag, as such hairs in the growth phase (anagen) being more likely to generate a result than hairs in the dormant phase (telogen). In many forensic laboratories, nuclear DNA profiling is not attempted on hairs when there is no indication of a root sheath at the proximal tip; leading to a potential loss of information. Mitochondrial DNA testing is possible from single hairs for animal testing [10] and human identification [11–13] but requires specialist equipment and skills. If nuclear DNA testing is required then there may be a need to use low template DNA typing methods [14]. Trace amounts of nuclear DNA limit the possibility of generating meaningful DNA profiles from single hairs or hairs in the telogen state. Current practice at Forensic Science South Australia (FSSA) is that hairs are examined by microscopy for the presence of sheath material. If there is no sheath material the hair is not submitted for DNA analysis as there is a low probability of obtaining an interpretable DNA profile. While the majority of hairs on the scalp are in the anagen phase, it is telogen hairs that are found more frequently in forensic investigations as these are the hairs that

are shed naturally, thus the ability to obtain nuclear DNA profiles from these samples would be of great benefit.

We report on the first use of direct amplification of a DNA from single hair in the anagen and telogen growth phases. As a single hair is used in the analysis it is assumed that any resulting DNA profile will be from a single source. The aim was to develop a simple, operational method that could be used routinely in forensic science casework with no further modification and a greater DNA profiling success rate than standard extraction methods on this type of sample. For ease of implementation, the process should also adhere to standard methods with no increase in the cycle number and also generate DNA profiles from hairs of unknown age.

Materials and methods

Samples

A buccal swab and scalp hairs were collected from both male and female donors working at FSSA. DNA was extracted from the buccal swabs using the QIAGEN (Doncaster, Victoria, Australia) Mini DNA extraction kit following the manufacturer's protocol. The resulting DNA profiles were used in subsequent comparisons.

Donors were asked to pluck a number of hairs from their scalp; as well as collect loosely/naturally shed hairs. Each hair was examined microscopically to determine its growth phase. Hairs were categorized as either anagen or telogen (with one example of catagen). A total of 30 hairs in the anagen growth phase and also 30 hairs in the telogen growth phase were analyzed. Two examples of body limb, pubic, and eyebrow hair samples (six in total), that had been stored in sealed plastic bags and kept at room temperature since 2007, were also supplied by FSSA. The shaft of three anagen hairs from one individual was also analyzed.

Direct PCR amplification and conditions

Direct PCR was conducted by removing approximately 5 mm of the proximal tip using sterile scissors and tweezers. For the shaft samples, once the proximal tip had been cut for use, the next 5 mm was cut for use. The hair fragment was placed into a 0.2 mL thin walled tube containing 10 μL of PCR master mix from either the AmpFℓSTR® NGM™ or NGM SElect™ kit (Life Technologies, Victoria, Australia) along with 5 μL of the primer mix and 1 μL of AmpliTaq Gold® DNA polymerase. A further 9 μL of sterile H_2O were added to make the final volume 25 μL. The amplification was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the manufacturer's recommended conditions. The standard 29 cycles was used for all reactions. There was no alteration to the methodology of amplification deliberately so as to ensure that the process could be adopted readily by the forensic science community. The NGM™ and NGM Select™ kits amplify 15 STR loci plus the amelogenin locus.

Capillary electrophoresis

Capillary electrophoresis was performed on an ABI 3130*xl* Genetic Analyser (Life Technologies) using POP-4™ polymer (Applied Biosystems). An aliquot of either 1 μL or 1 μL of a 1 in 50 dilution into H_2O of the PCR sample was added to a solution of 0.5 μL of ABI GeneScan-600 LIZ® Size Standard and 9.5 μL of Hi-Di™ Formamide. Samples were then denatured at 95 °C for 3 min. Electrophoresis was conducted at 3 kV with a 10 s injection. The data were analyzed using Genemapper® v3.2. The detection threshold was set at 50 relative fluorescence units (RFU).

Data analysis

The data were tabulated based on the number and percentage of alleles generated from the 15 STR loci amplified by the NGM™ and NGM SElect™ kits. Currently FSSA use Profiler Plus (Life Technologies), which amplifies 9 STR loci plus amelogenin. Five complete STR loci are required to up-load to the Australian National DNA Database, therefore a profile that can be uploaded if there are greater than five complete loci whereas an incomplete profile is when less than five loci are generated.

Results and discussion

Anagen and catagen hairs

A total of 30 hairs (29 anagen and 1 catagen) from five individuals were amplified directly using the NGM™ and NGM SElect™ kits and the data analyzed. A full profile, comprising all 15 STR loci and the amelogenin locus, was obtained for every individual hair sample (Fig. 1). In 30 instances, the DNA profile generated from the anagen hair section matched the DNA profile of the donor with no additional alleles and no allelic drop-out. Initially, most of the samples yielded DNA profiles with RFU values above 10,000 for some loci; this is typical of over amplification. We recommend a dilution of the final PCR product for anagen hair samples. For example, a 1 in 50 dilution of the anagen samples decreased the average RFUs from 4,217 to 1,240 (Table 1). Allele pull-up was not observed in the

diluted samples, and background noise was also greatly decreased. The average heterozygous peak balance (defined as the lower intensity peak divided by the higher intensity peak) was observed to be 84.8 % for neat samples and 86.6 % for diluted samples. There was no increase in stochastic effects compared to DNA profiles generated using the standard extraction methods. Heterozygous balance was good (with the two peak heights being within 80 % of each other in all direct amplification experiments) and dropout occurred in the expected range.

Telogen hairs

A total of 30 telogen hairs from five individuals were amplified directly and the data analyzed. Full DNA profiles were obtained from six samples (Fig. 2), and up-loadable profiles (with five or more complete loci) were observed in a further four samples. The remaining twenty samples yielded profiles containing eleven alleles or less. In all 30 instances, the DNA profile generated from the telogen hair section matched the DNA profile of the donor with no additional alleles. In total, the 30 samples had an average RFU value of 1,202 and heterozygous peak balance of 82.4 %.

Aged hairs

Two hairs of different somatic origin (pubic, eyebrow, and body limb) were amplified directly using the NGM™ kit and the data analyzed. The growth stages of the hairs were not identified prior to amplification to avoid the risk of transferring and losing any DNA onto the microscope slides. The hairs had been stored in sealed plastic bags for use in microscopic hair training without taking measures to preserve the DNA at the root. As the objective was to establish if any meaningful DNA profiles could be obtained via direct PCR, a comparison to a reference profile was not required. The complete 15 STR loci were observed for each of the six samples and the two samples from each hair type revealed identical profiles as each other. In total the six samples had an average RFU value of 2,929 and heterozygous peak balance of 82.9 %.

Anagen hair shaft

Standard procedure at FSSA for anagen hair extraction includes using a section of the hair shaft as the negative control. The first 5 mm from the proximal tip is removed

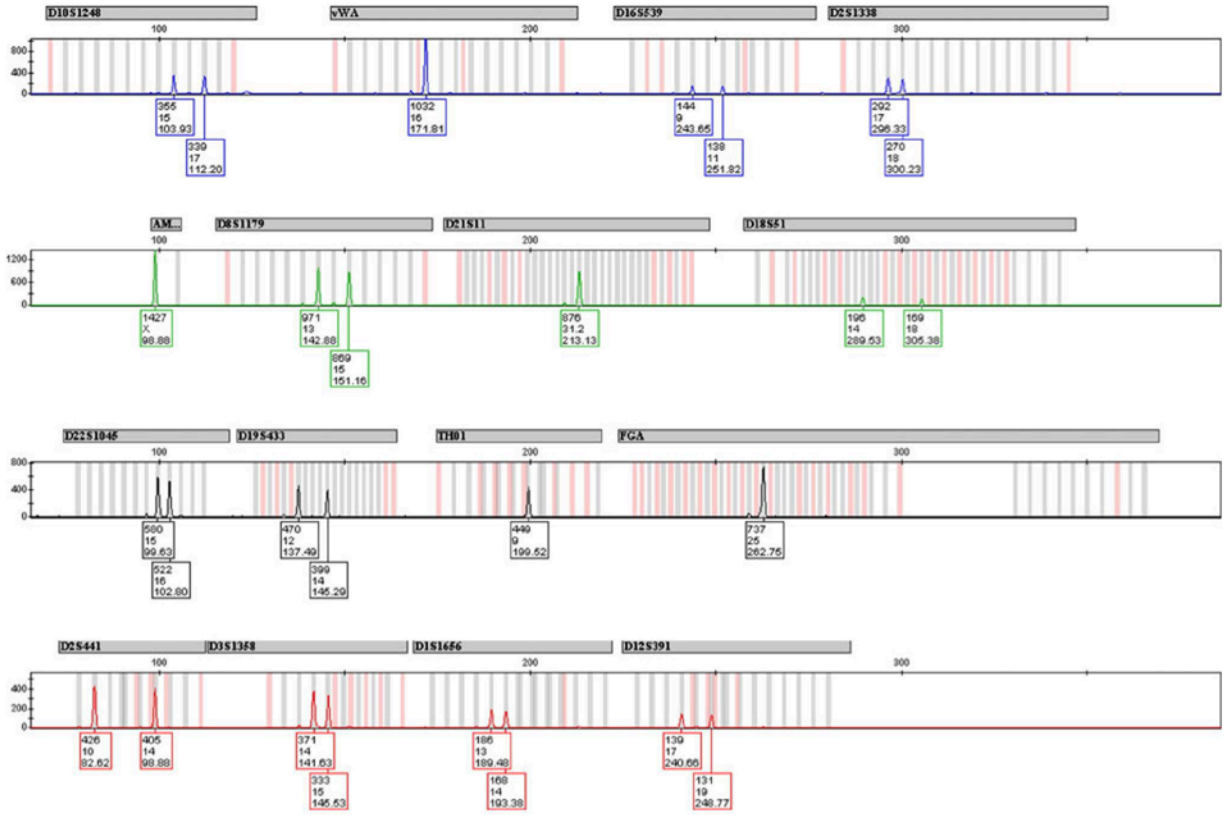

Fig. 1 Chromatogram of nuclear DNA from a single anagen hair root, amplified using AmpFℓSTR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Sample was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s

Table 1 Comparison of DNA profiles obtained from different growth phases of hair, showing the success rate (out of 30 STR alleles for NGM and 18 STR alleles for Profiler Plus)

Hair type	NGM profile type (%)			Average RFU
	Complete	Incomplete and up-loadable	Incomplete and not up-loadable	
Anagen	100	0	0	4,217 (1,240 diluted)
Telogen	20	13.3	66.7	1,202
Aged	100	0	0	2,929

Hair type	Profiler plus profile type (%)		
	Complete	Incomplete but up-loadable	Incomplete and not up-loadable
Anagen	53	12.5	34.5
Telogen	10	6	84

An incomplete and up-loadable profile is when less than all the alleles, but more than 5 complete loci, were generated. The average RFU values for hairs tested using direct PCR are also shown

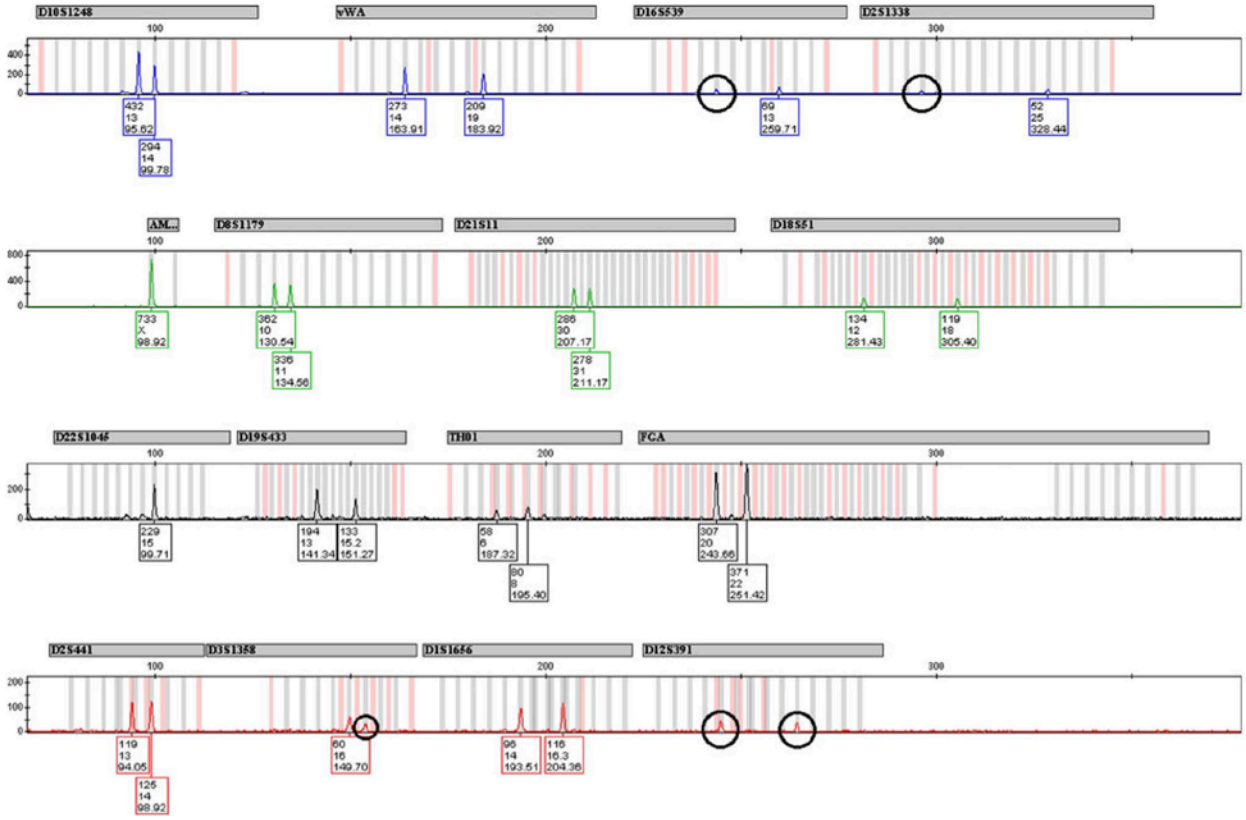

Fig. 2 Chromatogram of nuclear DNA from a single telogen hair root, amplified using AmpF*ℓ*STR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Sample was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. Allelic drop out can be observed at the *circled* loci D16S539, D2S1338, D3S1358, and D12S391

for extraction, and the following 5 mm is used as the negative control. This method was adapted using direct PCR for three anagen hair shaft samples. A full profile belonging to the individual was obtained from one hair sample. The remaining two shaft samples yielded eight alleles each.

FSSA comparison

FSSA have previously conducted an in-house study to compare the growth phase of a hair sample against the DNA profile obtained in order to determine which category of hair yields the most complete DNA profile. The hair type and percentage of profile obtained can be observed in Table 1. A profile of value (up-loadable to the National DNA Database, Australia) was obtained in 33.3 % of telogen samples in this study compared to 16 % by FSSA. Similarly, the success for obtaining meaningful profiles from anagen samples has increased from 65.5 to 100 % in this study. However, telogen hairs in forensic practice are typically not processed for DNA due to the low success rate. For example, based on the FSSA standard operating procedure for hair analysis, a telogen hair would not be submitted for further DNA analysis. There is no standard operating procedure nationwide for hair analysis; however there are numerous studies detailing the analysis on single telogen hairs for forensic purposes resulting in limited success and inconsistent results [9, 15].

Conclusion

We demonstrated a 100 % success rate for the generation of complete profiles (15 STR loci and amelogenin) from a single hair in the anagen growth phase. This is a significant increase compared to the process of performing standard DNA extraction then amplification of the extract. It is noted that some of the differences between the results from the standard extraction (using Profiler Plus) and the direct amplification (using NGM) could be attributed to improvements in the kit chemistry, although the commercial amplification kit used in the direct amplification is not designed primarily for this application.

A complete DNA profile at all fifteen STR loci were also obtained from hairs that have been stored for 5 years. It was found that direct PCR of single anagen hairs produced overloaded DNA profiles after the standard 29 amplification cycles and we recommend a dilution of the product prior to electrophoresis of the sample. The heterozygote balance was typically greater than 80 % and in no case was below 60 %. The profiles indicated no stochastic effects beyond what is expected from standard DNA profiling techniques. No allelic drop-in or any indication of contamination was noted in any of the 66 samples tested.

Direct PCR produced DNA profiles that could be uploaded to the National DNA Database (Australia) from a single hair in the telogen growth phase in ten of the 30 samples tested. Twelve telogen hairs produced partial profiles with eleven alleles or less, and the remaining eight samples produced no alleles. In all cases the profiles obtained were more complete and of greater quality than profiles obtained using standard extraction methods.

The generation of DNA profiles from telogen hairs was not always dependent on initial visualization of a follicular sheath. It may be that free-DNA on the surface of the hair, or associated loosely with the hair shaft, is that source of the DNA. Free-DNA has been suggested as a source of DNA from a variety of sources previously such as fabrics [5] and sweat [16].

There is the possibility of detecting a DNA profile from a hair that is not from the donor of the hair. The introduction of non-donor DNA to a hair can occur prior to the hair being examined in the laboratory and secondly during the laboratory process. The first is due to extraneous DNA from another individual being deposited on the hair while the second can be more formally referred to as contamination of the hair during laboratory procedures.

The deposition of a second individual's DNA onto a hair could be determined readily if the resultant profile is from more than one individual. This indicates multiple donors to the hair and would readily be assessed by the analyst. The potential risk is that a single source profile is obtained, which has not originated from the DNA of the hair donor, and would not be flagged by an analyst. This type of result from a single hair is potentially possible, however as it is proposed that nuclear DNA on the surface of the hair is the predominant source of DNA, then a second contributor (i.e. not from the hair donor) is only possible if a source of DNA comes into contact with the hair surface and no profile is obtained from the donor of the hair. We believe this possibility to be unlikely. Alternatively this may result from the hair being contacted with a biological fluid such as semen or blood. Prior microscopic examination of a hair by a trained analyst would determine the presence of a biological fluid on the hair shaft or bulb.

We propose that contamination of a single hair during the laboratory process is less likely to occur from this direct approach compared to standard extraction procedures. Standard DNA extraction procedures involve numerous wash methods and tube changes, each being a possible step where contamination may occur before the amplification of the DNA. It is also these same steps where the initial DNA can be lost, either via washing or retained on the silica membrane. The method described in this paper avoids the extraction process and thus greatly reduces DNA loss and potential contamination prior to amplification.

This simple process of direct PCR from single hairs can be readily adopted for use into forensic DNA practice and we demonstrate that the process has the capability of generating full DNA profiles from anagen hairs, aged hairs, and partial profiles from single telogen hairs. Hairs that would otherwise not be tested, as there was little chance of gaining a meaningful profile, can now be profiled.

Key Points

1. A successful and novel method for obtaining DNA profiles from single hairs using direct PCR.
2. The DNA extraction process was completely circumvented. This allows for a greater yield of DNA from hair, as DNA is not lost via wash steps or tube changes.
3. Full profiles were obtained in 100 % of anagen hair samples. Profiles 'up-loadable' to the Australian National DNA Database were obtained in 33.3 % of the telogen hair samples.
4. Allelic drop-in or contamination was not observed in any of the 66 hair samples tested.
5. Standard protocol for DNA amplification from the NGM™ and NGM SElect™ STR typing kits was not modified, allowing for easy and quick implementation into forensic laboratories.
6. This method is cost-effective and time saving in forensic casework, as a lengthy extraction process is not required.

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Statement of authorship

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Renée Blackie (Candidate)

Performed all laboratory work (sample collection, DNA extractions and quantifications, PCR amplification, capillary electrophoresis), data analysis and interpretation, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Damien Abarno

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

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Optimising direct PCR from anagen hair samples

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ABSTRACT

Anagen hairs are in the active growth phase, and when forcefully removed, may contain an intact root or sheathing. The hair root or sheathing is a source of nucleic DNA and can be amplified using direct PCR. Human identification STR kits are optimised to a small range of input DNA for PCR. Anagen hairs are unable to be quantified prior to amplification and can exhibit characteristics of an over-loaded DNA sample when analysed. The aim of this study was to optimise direct PCR for anagen hair sampling. Two separate modifications to the downstream processes were carried out in order to determine the most effective method at minimising PCR artefacts. Decreasing the cycle number from the standard 29 cycles to 27 cycles when using the NGM™ kit displayed the best results for this method. However, decreasing the cycle number may increase allelic drop-out and would be costly for laboratories to perform an in-house validation. Diluting the PCR product during electrophoresis analysis minimises the effects of PCR artefacts in the same way decreasing the cycle number does. Diluting the PCR product is the most cost-effective method and does not increase the chance of allelic drop-out.

1. Introduction

Due to the nature of direct PCR, a sample is unable to be quantified prior to amplification. The extraction step is bypassed and therefore quantification is not possible. This increases the sensitivity of PCR, highlighting its benefit for latent DNA samples. However, the recommended input of template DNA for most human identification kits is between 0.5 and 2 ng [1]. A single anagen hair sample processed directly can sometimes produce chromatograms with characteristics of an overloaded PCR sample [2], suggesting that a single anagen hair contains greater than 2 ng of nucleic DNA. Characteristics of adding too much DNA include split peaks, uneven heterozygous peaks, and increased "stutter" and "pull-up" [3,4]. This study identifies two methods that can be applied in the downstream processes of direct PCR for the optimisation of anagen hair sampling that minimises the effects of PCR artefacts.

2. Materials and methods

2.1. Decreasing PCR cycle number

A total of 20 anagen hairs were used to assess any benefits associated with decreasing the PCR cycle number to 28 or 27. Amplification of the samples was performed on the GeneAmp® 9700 96-well thermal cycler (Applied Biosystems) following the AmpFℓSTR® NGM® kit (Applied Biosystems, USA) manufacturer's instructions. The cycle number was decreased from the standard 29 to either 28 or 27 cycles. Amplified PCR product (1 μL) was run with 9.5 μL Hi-Di™ Formamide (Life Technologies, Victoria, Australia) and 0.5 μL LIZ-600™ size standard (GeneScan™, Applied Biosystems, USA). Samples were injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. The data were analysed using GeneMapper® software v3.2.

2.2. PCR product dilution

A total of 30 anagen hairs were used for PCR product dilution. Amplification of the samples was performed on the GeneAmp® 9700 96-well thermal cycler following the AmpFℓSTR® NGM® kit manufacturer's instructions. Each amplified PCR product from a single anagen hair was diluted into concentrations of 1:10, 1:30 and 1:50, in which 1 μL from a dilution was combined with 9.5 μL Hi-Di™ Formamide and 0.5 μL LIZ-600™ size standard. Samples were injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. The data were analysed using GeneMapper® software v3.2.

3. Results and discussion

3.1. Decreasing PCR cycle number

Decreasing the cycle number to 27 cycles for the NGM® kit provided the best results in minimising PCR artefacts. Although

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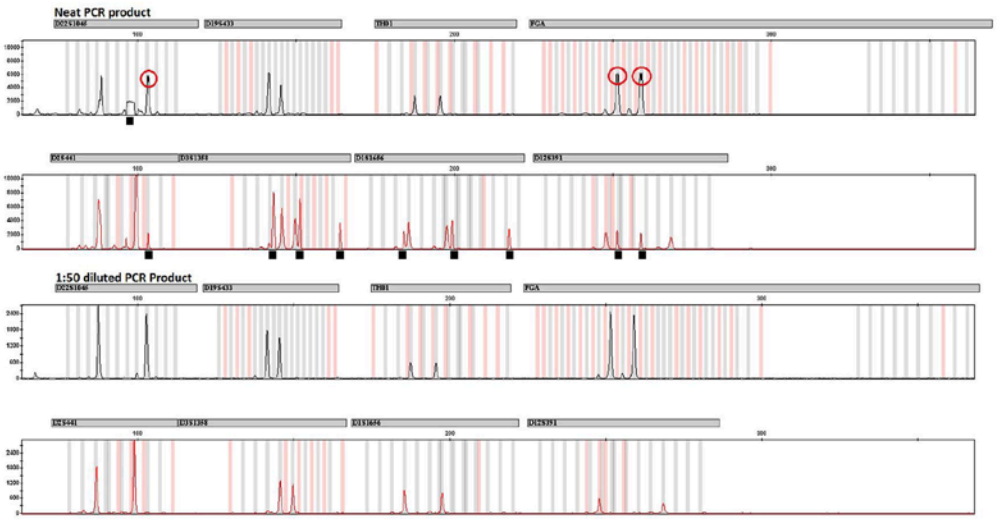

Fig. 1. Chromatogram of nuclear DNA from a single anagen hair root, amplified using AmpFℓSTR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Figure shows 8 loci from NGM™ kit. PCR sample was analysed neat and diluted to a concentration of 1:50 before it was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. Red circles indicate split peaks and black squares indicate pull-up in the neat sample. Split peaks and pull-up is not observed in the diluted sample.

100% success rate was maintained for obtaining a full profile from a single anagen hair, a decrease in cycle number has not been validated by the manufacturer and can be time consuming and expensive for a forensic laboratory to complete in-house.

As not every anagen hair contains the same amount of DNA, the decrease in cycle number could potentially decrease the success rate of obtaining a full DNA profile.

3.2. PCR product dilution

It was found that a dilution of 1:50 was the most efficient in reducing the effects of PCR artefacts without losing alleles to over-dilution (Fig. 1). It is recommended that several dilutions, including the original PCR product, are analysed on the same run to save time. There was a decrease of 29.4% in the average RFU when anagen hair samples were diluted to a 1:50 concentration.

4. Conclusion

Anagen hair samples when amplified using direct PCR methods can exhibit PCR artefacts that are consistent with overloading a PCR with too much DNA. Our recommendation is to use the lowest cycle number that has been validated by the STR kit in use, or to dilute the final PCR product to minimise the effects of pull-up, split peaks and stutter.

Role of funding

Funding was provided by the Department of Justice, South Australia.

Conflict of interest

None.

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3.4 Applications

In February 2013, I travelled to the Life Technologies Training Laboratory in Melbourne (VIC, Australia) to investigate and compare anagen and telogen hair samples between two human identification kits: AmpFℓSTR® NGM SElect™ PCR amplification kit and the GlobalFiler™ Express PCR amplification kit (Life Technologies), with and without modified PCR amplification conditions (decreased cycle numbers, and additional AmpliTaq Gold® DNA polymerase). The GlobalFiler™ Express kit incorporates the use of a 3500*xL* Genetic Analyser (Life Technologies), which is required to analyse 6-dye data. GlobalFiler™ Express is an STR multiplex assay optimised to allow direct amplification from the following types of single-source samples:

- Blood and buccal samples on treated paper substrates without the need for sample purification.
- Blood and buccal samples collected on untreated paper substrates and treated with Prep-n-Go™ Buffer.
- Buccal samples collected on swab substrates and treated with Prep-n-Go™ Buffer.

The kit amplifies 21 autosomal STR loci, Amelogenin, one Y-STR locus, and one Y insertion/deletion (Y indel) locus in a single PCR (24 loci total).

A total of 72 hairs (36 anagen and 36 telogen) from six individuals were amplified directly using the NGM SElect™ and GlobalFiler™ Express kits. Approximately 5 mm of the proximal tip of each hair was removed and placed into a 0.2 mL thin walled tube containing the PCR buffer constituents for the respective kits. NGM SElect™ required 10 µL of PCR master mix, 5 µL of primer mix, and either 10 µL of Low-TE Buffer or 9 µL with 1 µL of AmpliTaq Gold®, making a total volume of 25 µL. GlobalFiler™ Express required 6 µL of PCR master mix, 6 µL of primer mix and either 3 µL of Low-TE Buffer or 2 µL with 1 µL of AmpliTaq Gold®, making a total volume of 15 µL. Amplification was conducted in a GeneAmp® System 9700 thermal cycler using the manufacturer's recommended conditions for each kit. The standard 29 cycles was used for all NGM SElect™ reactions, and 27, 28 or 29 cycles for GlobalFiler™ Express reactions.

Results indicated that without additional AmpliTaq Gold®, resulting DNA profiles displayed signs of high inhibition typical of over amplification and were unable to be analysed. Profiles presented with split-peaks or incomplete adenylation, imbalanced heterozygous peaks, imbalanced loci (complete locus drop out of larger fragments), and increased baseline noise (Figures 3.1 and 3.2).

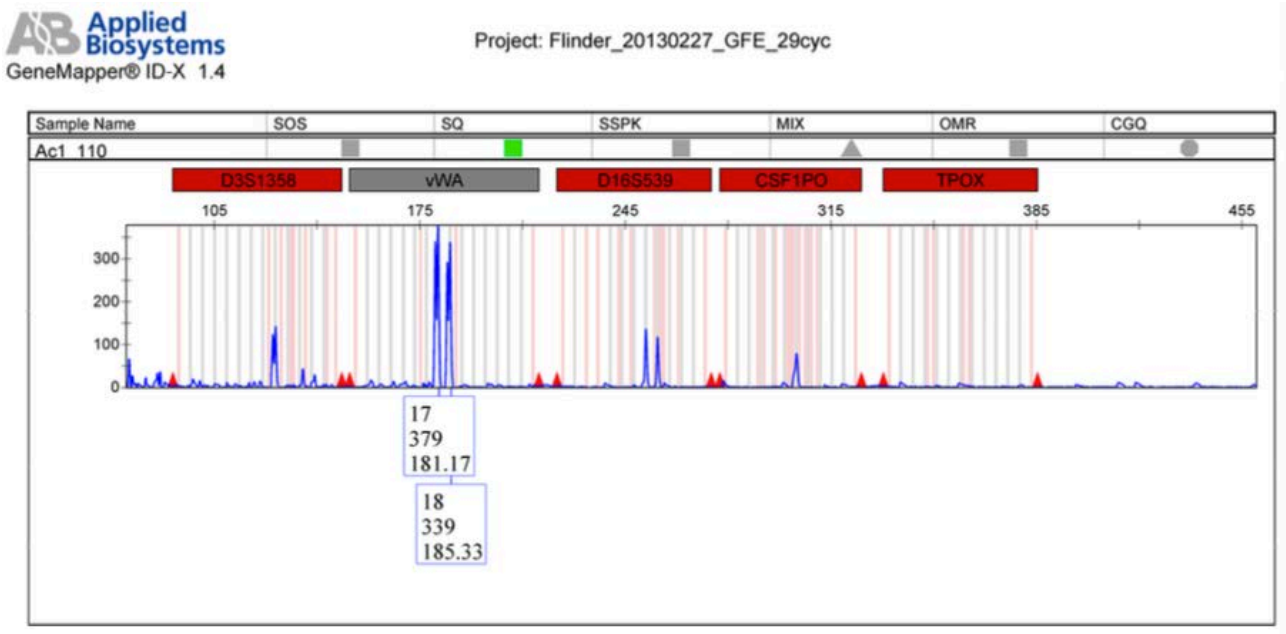

Figure 3.1 – Chromatogram of nuclear DNA from a single anagen hair root amplified using GlobalFiler™ Express PCR kit at 29 cycles on a GeneAmp® System 9700 thermal cycler, without additional AmpliTaq Gold®.

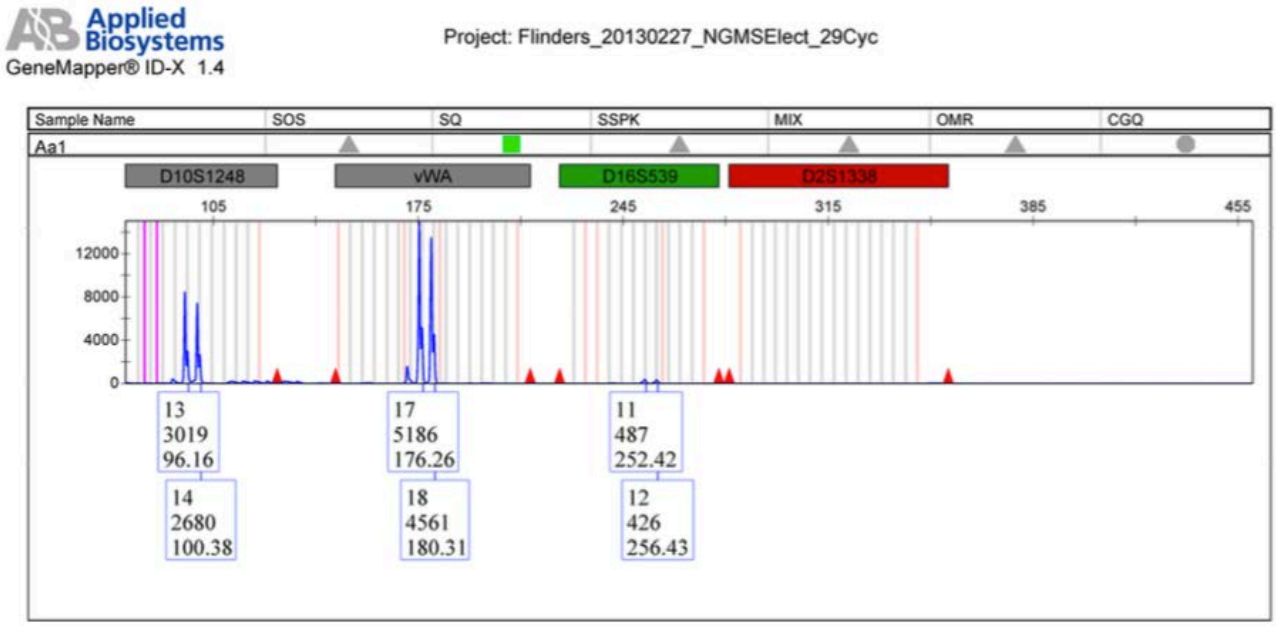

Figure 3.2 – Chromatogram of nuclear DNA from a single anagen hair root amplified using NGM SElect™ PCR kit at 29 cycles on a GeneAmp® System 9700 thermal cycler, without additional AmpliTaq Gold®.

The quality of profile increased when the cycle number was decreased to from 29 to 28 and 27 for GlobalFiler™ Express, and additional AmpliTaq Gold® DNA polymerase was added to the master mix (Figures 3.3 and 3.4).

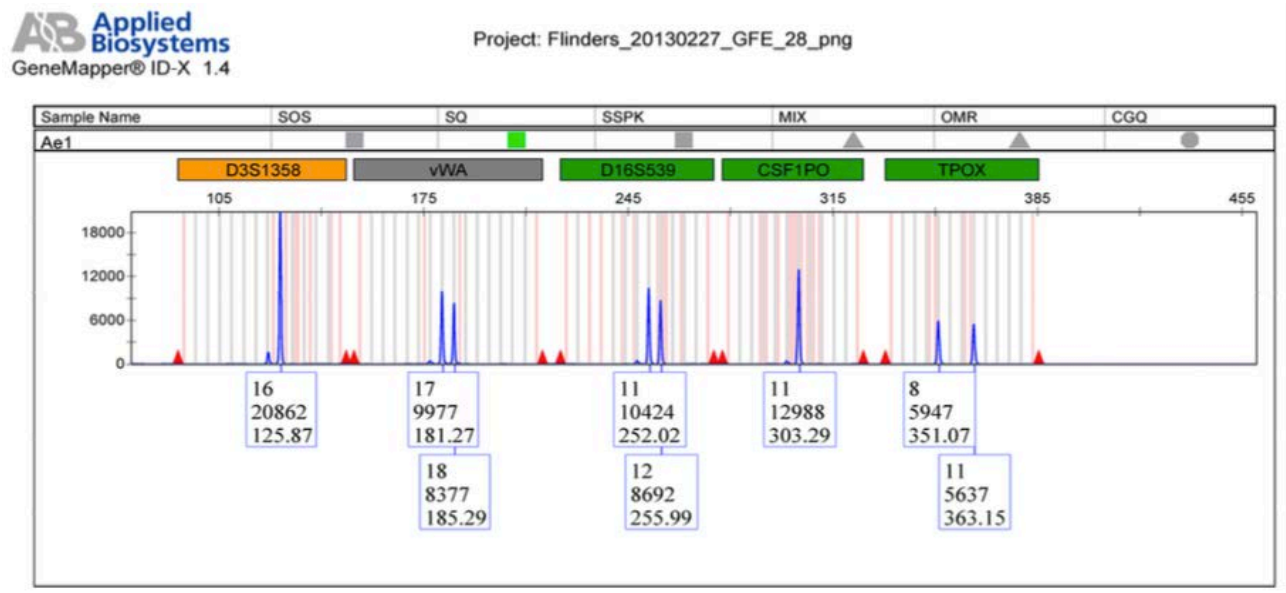

Figure 3.3 – Chromatogram of nuclear DNA from a single anagen hair root amplified using GlobalFiler™ Express PCR kit at 28 cycles on a GeneAmp® System 9700 thermal cycler, without additional AmpliTaq Gold®.

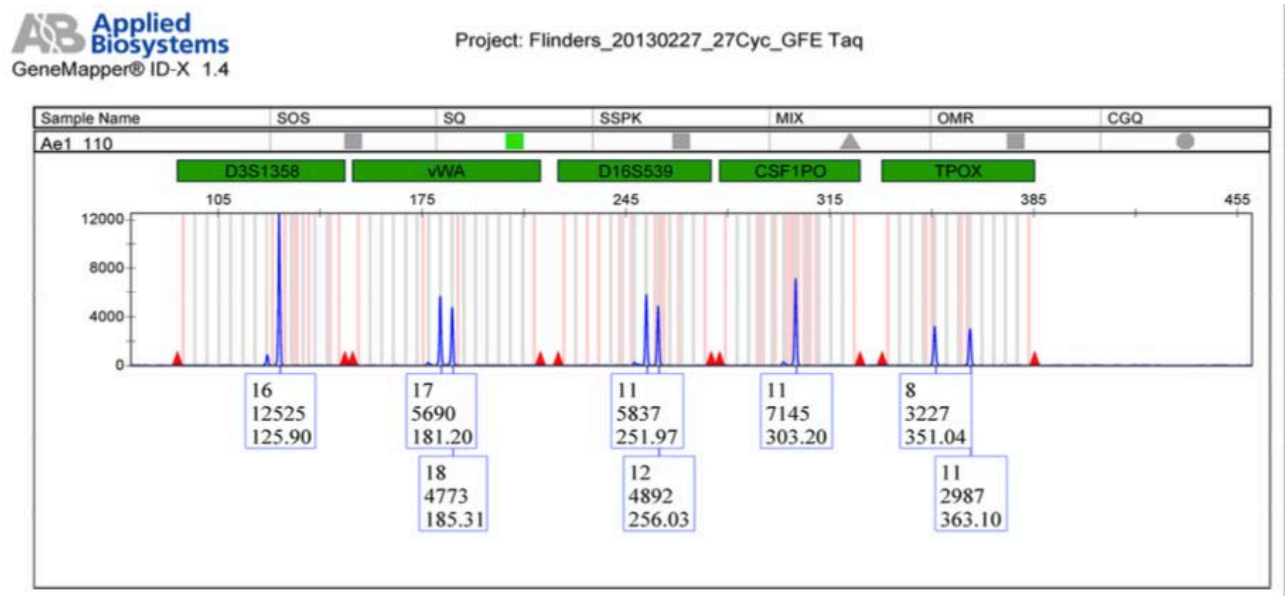

Figure 3.4 – Chromatogram of nuclear DNA from a single anagen hair root amplified using GlobalFiler™ Express PCR kit at 27 cycles on a GeneAmp® System 9700 thermal cycler, with additional AmpliTaq Gold®.

Twelve telogen hairs were sampled with each kit at 29 cycles and there was no significant difference between resulting profiles for either kit used. Using NGM SElect™, one full profile was obtained with an additional four samples returning between 8 – 10 alleles. Likewise for GlobalFiler™ Express, one full profile was obtained with an additional four samples returning between 8 – 11 alleles.

Single anagen and telogen hair results aligned with the data obtained from *section 3.2*, however GlobalFiler™ Express kit has a higher discrimination power with an increased number of loci compared to NGM, with full profiles obtained at a lower cycle number (27) for anagen hairs, therefore reducing the time taken to process samples. Based on the data obtained at the Life Technologies Training Laboratory, telogen hairs still prove to be a challenging sample type from which to consistently obtain meaningful DNA information. Forensic Science SA have also begun to validate the direct PCR technique using hair samples and GlobalFiler™ Express, as the 6-dye technology has become more available.

To assist in improving results obtained from telogen hairs, I travelled to Canberra (ACT, Australia) in February 2015, to explain and help implement the direct PCR technique at the Australian Federal Police forensic laboratory and the National Centre for Forensic Studies (University of Canberra). International forensic student Linda Kron led an experiment under the supervision of Dr. Dennis McNevin to determine the best method for obtaining consistent and meaningful DNA data from telogen hairs.

In order to optimise the success rate, telogen hairs were microscopically classified as either root type 1: the club root without any soft tissue present (most common), or type 2: the club root with a small amount of soft tissue present. Samples were either digested or incubated in TE buffer prior to direct PCR amplification. Other factors were noted to determine if there was an impact on the STR results, such as whether the hairs had been washed or unwashed at time of collection, whether hair roots or shafts were used, or if there was a difference between donors. All samples were amplified using the PowerPlex® 21 System (Promega) following manufacturer's procedures.

Telogen root type 1 hairs (n = 120) produced profiles with six or more alleles in 5.8 % of samples, with only one hair resulting in a full profile. Telogen root type 2 hairs (n = 20) produced profiles with six or more alleles in 20 % of samples, with no samples resulting in a full profile. This gives an average of 12.9 % compared to the 33.3 % success described in *section 3.2.* Although the root types were not classified in *section 3.2*, and may have contained a number of type 3 roots (club root with a large amount of soft tissue present), the large discrepancy between the results is more likely due to the variation in the methods. Additional units of AmpliTaq Gold® was not used in this study, and the direct method does not involve any wash, digestion or incubation step prior to amplification. The addition of these steps is likely to increase the chance of DNA being washed away or lost to the process. It was also observed in this study that the success rate was lower for samples incubated in TE buffer compared to complete digestion.

3.5 Concluding Remarks

Using the direct PCR technique on single human hairs has shown to greatly improve the likelihood of routinely obtaining meaningful DNA information. The simplicity of the technique means validation and implementation within forensic science laboratories can be fast. Significant benefits include the reduction of time and costs involved in the analysis process, allowing for criminal investigations to proceed faster. The preliminary data using aged hair samples demonstrates that this technique can be also applied to cold cases where single hairs have not already been processed for DNA. The investigations conducted at the Life Technologies Training Laboratory and at the University of Canberra strongly indicated that the optimal conditions for sampling human hair using direct PCR should include using additional AmpliTaq® Gold DNA polymerase with no treatments to be made to the hairs prior to amplification. Using direct PCR methods on single telogen hairs, profiles that resulted in enough alleles to be uploaded onto the Australian National Criminal DNA Database (NCIDD) were obtained in approximately 33.3 % of samples, whereas when additional treatment or extraction methods are implemented this is greatly reduced. The study conducted at the University of Canberra included digestion and incubation steps prior to using direct PCR methods, thus decreasing the number of up-loadable profiles to 12.9 %. When extraction and staining methods are implemented, we see a further reduction to 4 % [12] and just 1 % [7] for profiles containing more than eight alleles. As a result of this work, FSSA have verified the same process in-house with an aim to implement the technique into active casework. The technique was officially implemented in July 2015, and approximately 50 hairs have been processed to date, increasing every day.

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Supplementary Material

a. Successful direct amplification of nuclear markers from a single hair follicle

Raw data examples

Table 3a.1 – NGM™ allele call, RFU value, profile percentage and heterozygous peak balance for ten anagen hair samples for IND 1.

		D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Control		15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		1531, 1498	3045	1792, 1804	2480, 2127	4224	1795, 1870	2490	1100, 1024	1690, 1900	2518, 2051	3637	3642	1004, 995	1121, 954	721, 648	645, 649
Code	Profile %	97.8		99.3	85.8		96		93.1	88.9	81.5			99.1	85.1	89.9	99.4
A1	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		5201, 4762	11383	2447, 2318	5660, 5059	10318	9463, 9521	9567	2399, 2113	5017, 5797	6031, 4272	6021	5406	6770, 10988	6196, 6533	3721, 3402	2818, 2591
		91.6		94.7	89.6		99.4		88.1	86.5	70.8			61.6	94.8	91.4	91.9
A2	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		4640, 4157	12353	2595, 2308	4920, 4456	10499	9527, 9555	9572	3804, 3273	5809, 6655	6291, 4801	6990	5870	5231, 10482	4875, 4702	2916, 2676	2400, 2234
		89.6		88.9	90.6		99.7		86	87.3	76.3			49.9	96.5	91.8	93.1
A3	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		5609, 5027	11102	2663, 2467	5785, 5156	9826	8951, 9015	9018	3073, 2758	4893, 6112	5801, 4640	5941	5513	5993, 11005	6401, 7151	4482, 4211	3146, 2964
		89.6		92.6	89.1		99.3		89.7	80	80			54.5	89.6	94	94.2
A4	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3194, 3026	11080	2898, 2733	5493, 4946	10430	8537, 8264	9285	2085, 1878	5643, 5187	5348, 4794	7142	6361	3607, 8151	3588, 3481	2281, 2117	2069, 2007
		94.7		94.3	90		96.8		90.1	91.9	89.6			44.3	97	92.8	97
A5	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		4549, 4212	10907	2416, 2242	6564, 5740	10070	9077, 9076	9125	2282, 2017	5655, 6700	6238, 4910	6258	6105	5140, 10462	5265, 4848	2936, 2699	2402, 2208
		92.6		92.8	87.5		100		88.4	84.4	78.7			49.1	92.1	90.9	91.9
A6	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		2509, 2297	8246	2323, 2215	3080, 2798	9432	6073, 5939	8184	1991, 1813	3817, 3354	3670, 3418	3702	6979	2266, 5157	2368, 2282	1810, 1725	1534, 1535
		91.6		95.4	90.8		97.8		91.1	87.9	93.1			43.9	96.4	95.3	100
A9	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3195, 2993	11655	2349, 2245	5241, 4684	10732	8867, 8482	9548	1989, 1739	5284, 4913	4545, 4093	6892	6807	3647, 8342	3679, 3375	2147, 1949	1879, 1782
		93.7		95.6	89.4		95.7		87.4	92.3	90.1			43.7	91.7	90.8	94.8
A10	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		4490, 3862	11982	156, 139	2098, 1893	10240	9197, 9214	9313	744, 645	5586, 6633	4005, 3308	3141	6515	6577, 10666	3631, 3060	1904, 1716	1129, 1077
		86		89.1	90.2		99.8		86.7	84.2	82.6			61.7	84.3	90.1	95.4

Table 3a.2 – NGM™ allele call, RFU value, profile percentage and heterozygous peak balance for ten telogen hair samples for IND 1.

		D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl		15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		1531, 1498	3045	1792, 1804	2480, 2127	4224	1795, 1870	2490	1100, 1024	1690, 1900	2518, 2051	3637	3642	1004, 995	1121, 954	721, 648	645, 649
Code	Profile %	97.8		99.3	85.8		96		93.1	88.9	81.5			99.1	85.1	89.9	99.4
T1	0																
T2	0																
T3	0																
T4	53.1	15, 17	16			x	13, 15	31.2		15, 16	14		25	10, 14			
		188, 208	233			182	217, 171	89		263, 222	58		88	138, 113			
		90.4					78.8			84.4				81.9			
T5	34.38	15, 17				x	13, 15			15, 16	12			10, 14			
		58, 54				247	54, 75			176, 96	168			112, 67			
		93.1					72			54.5				59.8			
T6	87.5	15, 17	16	9, 11	..	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	..
		507, 419	1024	71, 59		548	1259, 1201	510	63, 53	681, 626	166, 141	101	357	368, 413	121, 82	56, 52	
		82.6		83.1			95.4		84.1	91.9	84.9			89.1	67.8	92.9	
T7						x											
						76											
T8						x											
						54											
T9	0																
T10	0																

Table 3a.3 – NGM™ allele call, RFU value, and profile percentage for two telogen hair samples and one anagen hair shaft sample for IND 3.

	D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl	13, 16	14, 19	9, 14	17, 24	x	13, 15	28, 32.2	12, 15	11, 16	13, 14	6, 8	22, 24	11, 14	15, 16	12, 15	18.3, 24
Code Profile %																
JT1 100	13, 16	14, 19	9, 14	17, 24	x	13, 15	28, 32.2	12, 15	11, 16	13, 14	6, 8	22, 24	11, 14	15, 16	12, 15	18.3, 24
	2917, 2573	6771, 4837	2665, 2039	1524, 1018	10313	9237, 9206	9005, 8232	3305, 2897	6255, 6267	2805, 2440	898, 875	2359, 2257	3743, 9889	1308, 1493	894, 804	687, 520
JT2 21.875		14			x	15			11	13, 14						
		50			265	69			57	82, 104						
										78.8						
JS1 100	13, 16	14, 19	9, 14	17, 24	x	13, 15	28, 32.2	12, 15	11, 16	13, 14	6, 8	22, 24	11, 14	15, 16	12, 15	18.3, 24
	391, 424	256, 212	129, 194	147, 159	1547	559, 536	55, 51	124, 194	255, 310	268, 140	288, 282	170, 175	317, 185	200, 121	132, 163	142, 163

Chromatogram examples

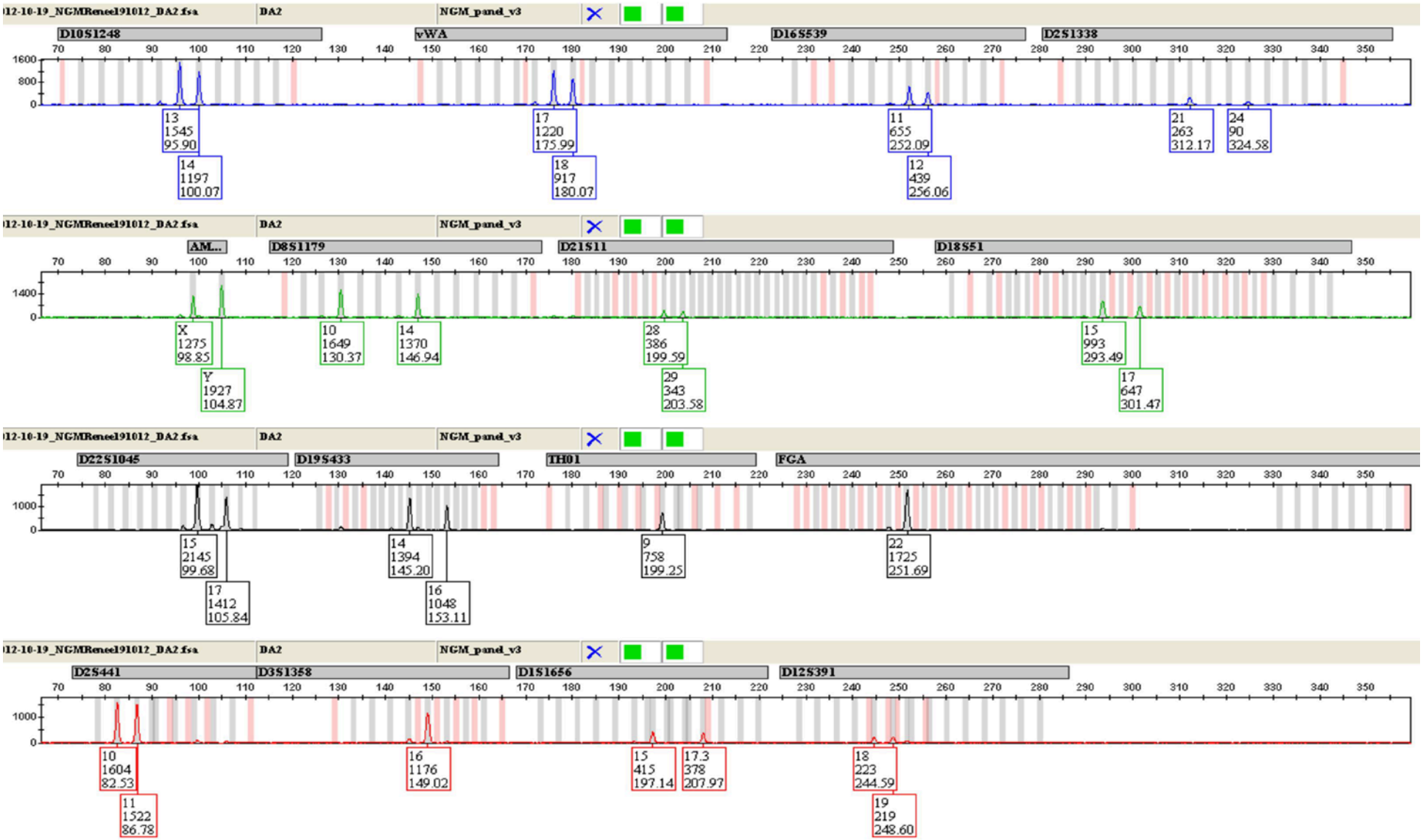

Figure 3a.1 – NGM™ kit DNA profile obtained from a single anagen hair sample for IND 2, amplified using direct PCR at 29 cycles.

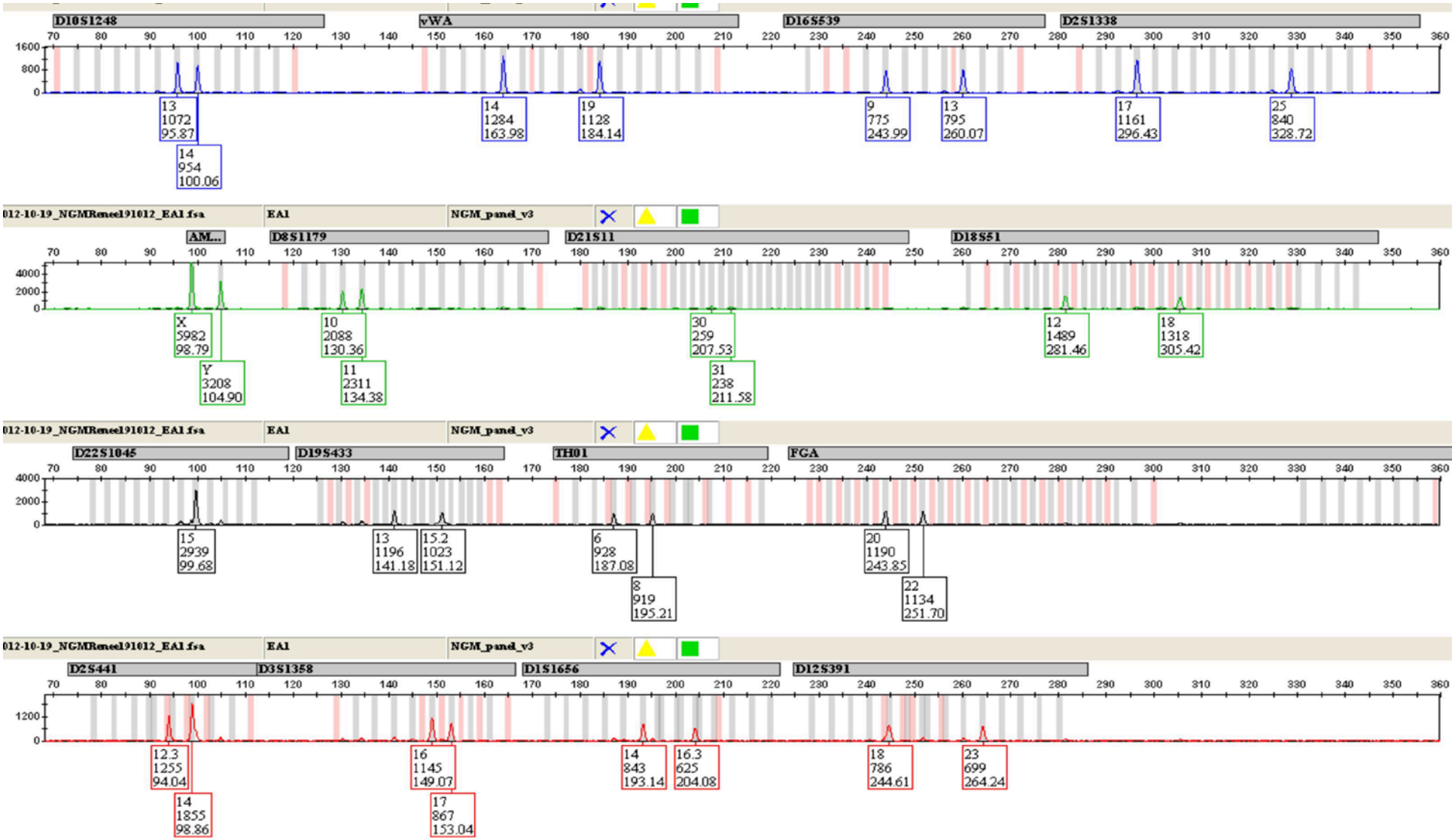

Figure 3a.2 – NGM™ kit DNA profile obtained from a single anagen hair sample for IND 4, amplified using direct PCR at 29 cycles.

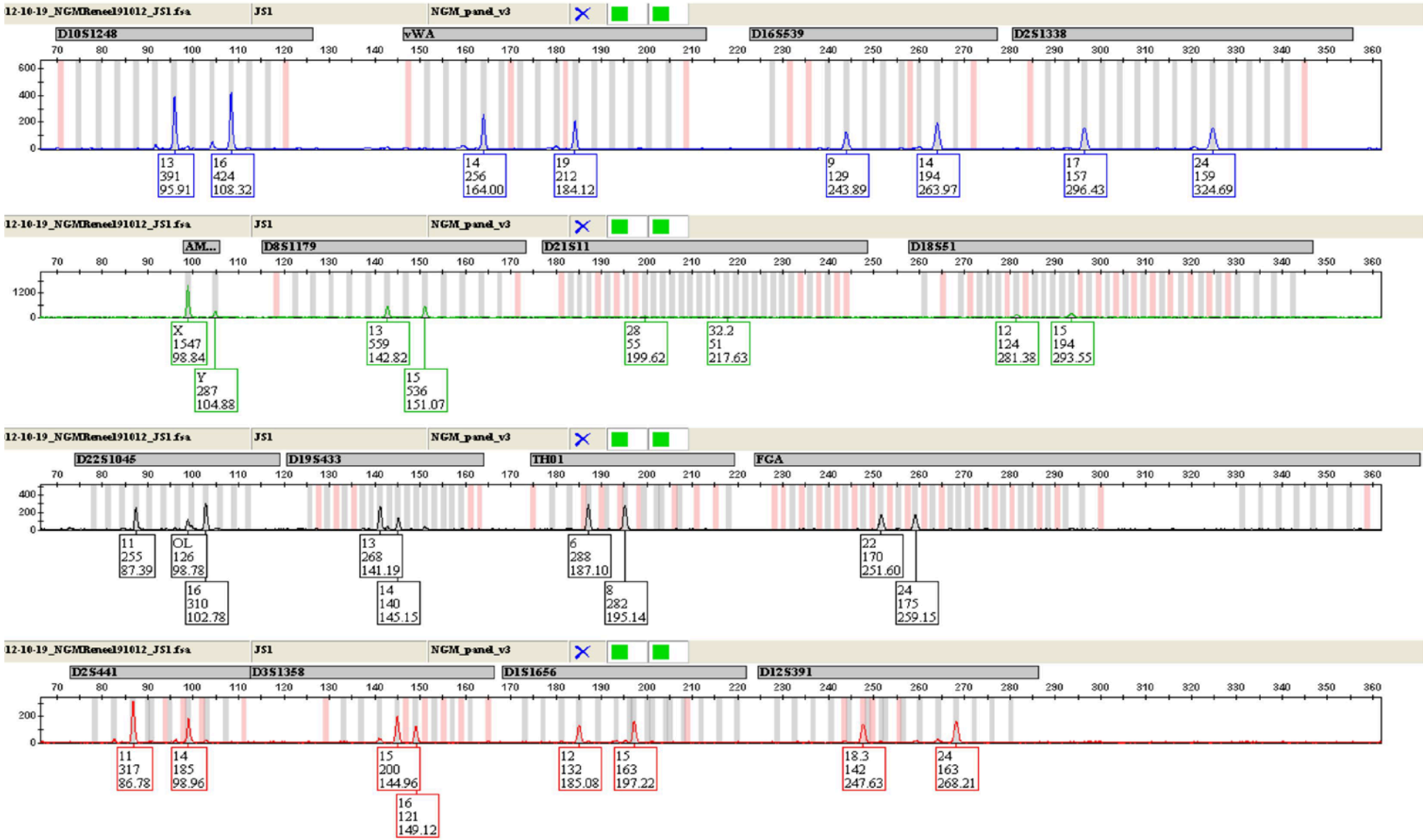

Figure 3a.3 – NGM™ kit DNA profile obtained from a single anagen hair shaft sample for IND 3, amplified using direct PCR at 29 cycles.

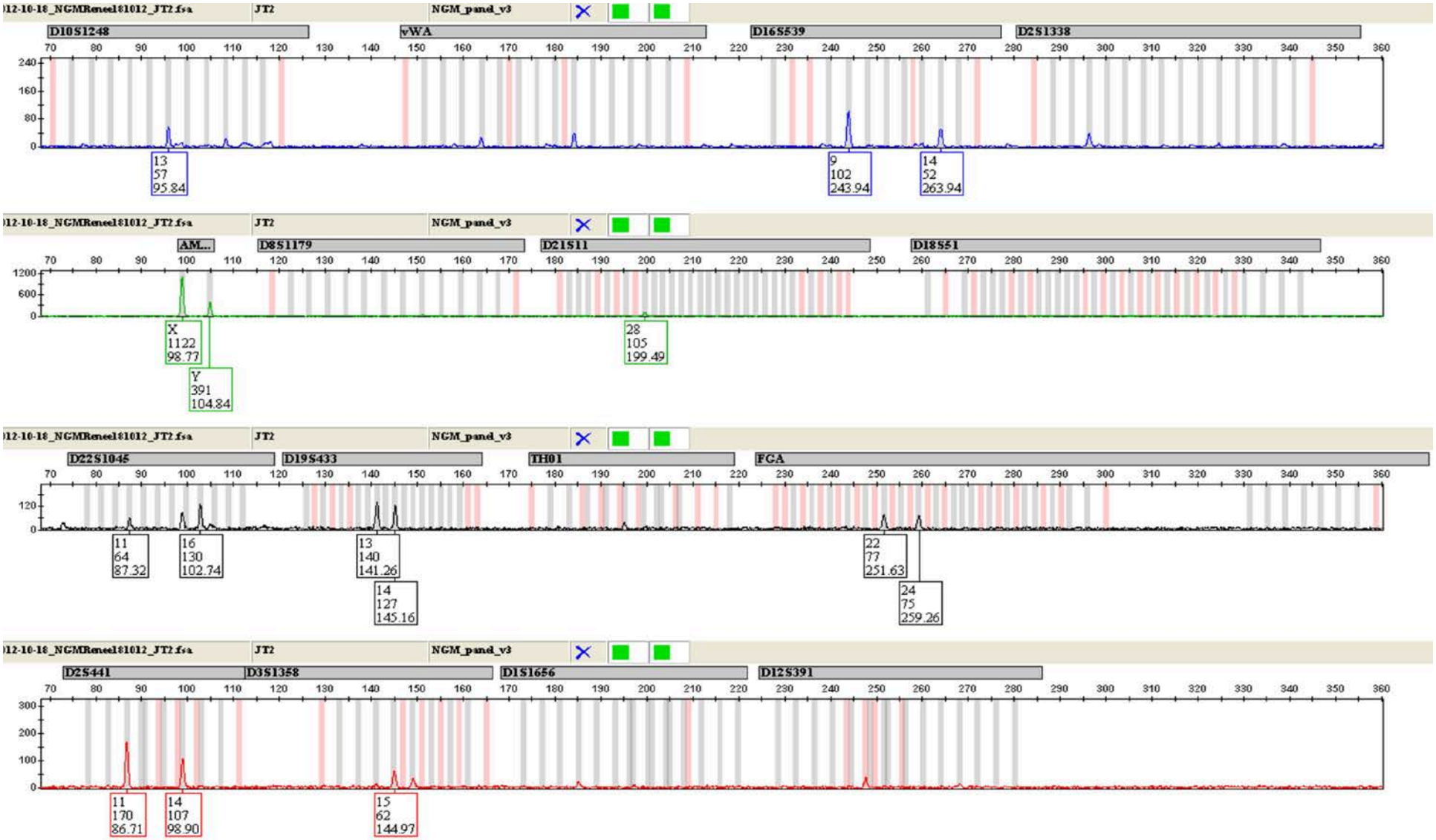

Figure 3a.4 – NGM™ kit DNA profile obtained from a single telogen hair sample for IND 3, amplified using direct PCR at 29 cycles.

b. Optimising direct PCR from anagen hair samples

Raw data examples

Table 3b.1 – NGM™ allele call, RFU value, profile percentage and heterozygous peak balance for two anagen hair samples and their corresponding dilution results for IND 2.

	Code	Profile %	D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl			13, 14	17, 18	11, 12	21, 24	X, Y	10, 14	28, 29	15, 17	15, 17	14, 16	9	22	10, 11	16	15, 17.3	18, 19
DA1		100	13, 14	17, 18	11, 12	21, 24	X, Y	10, 14	28, 29	15, 17	15, 17	14, 16	9	22	10, 11	16	15, 17.3	18, 19
			4596, 4011	4629, 4477	3607, 3720	2670, 2285	8761, 9217	8141, 8503	9080, 8232	5923, 5684	3143, 4287	4058, 4432	3083	5599	2800, 2665	4262	1977, 2223	1996, 1808
			87.3	96.7	97	85.6	95.1	95.7	90.7	96	73.3	91.6			95.2		88.9	90.6
DA2		100	13, 14	17, 18	11, 12	21, 24	X, Y	10, 14	28, 29	15, 17	15, 17	14, 16	9	22	10, 11	16	15, 17.3	18, 19
			2801, 2233	6035, 5371	899, 824	808, 737	6102, 9543	9023, 9257	9364, 8985	1279, 1198	3415, 6685	1596, 1303	3745	3755	4968, 4514	1347	441, 476	391, 368
			79.7	89	91.7	91.2	63.9	97.5	96	93.7	51.1	81.6			90.9		92.6	94.1
DDA1		100	13, 14	17, 18	11, 12	21, 24	X, Y	10, 14	28, 29	15, 17	15, 17	14, 16	9	22	10, 11	16	15, 17.3	18, 19
			860, 777	805, 803	651, 599	457, 390	1755, 1766	1539, 1618	1524, 1360	961, 909	927, 896	793, 846	610	2207	548, 541	885	351, 383	343, 319
			90.3	99.8	92	85.3	99.4	95.1	89.2	94.6	96.7	93.7			98.7		91.6	93
DDA2		100	13, 14	17, 18	11, 12	21, 24	X, Y	10, 14	28, 29	15, 17	15, 17	14, 16	9	22	10, 11	16	15, 17.3	18, 19
			398, 357	841, 771	121, 108	111, 101	894, 1448	2058, 1592	1207, 1142	149, 134	1058, 982	190, 159	191	416	712, 642	189	75, 56	63, 58
			89.7	91.7	89.3	90.9	61.7	77.4	94.6	89.9	92.8	83.7			90.2		74.7	92

Chromatogram examples

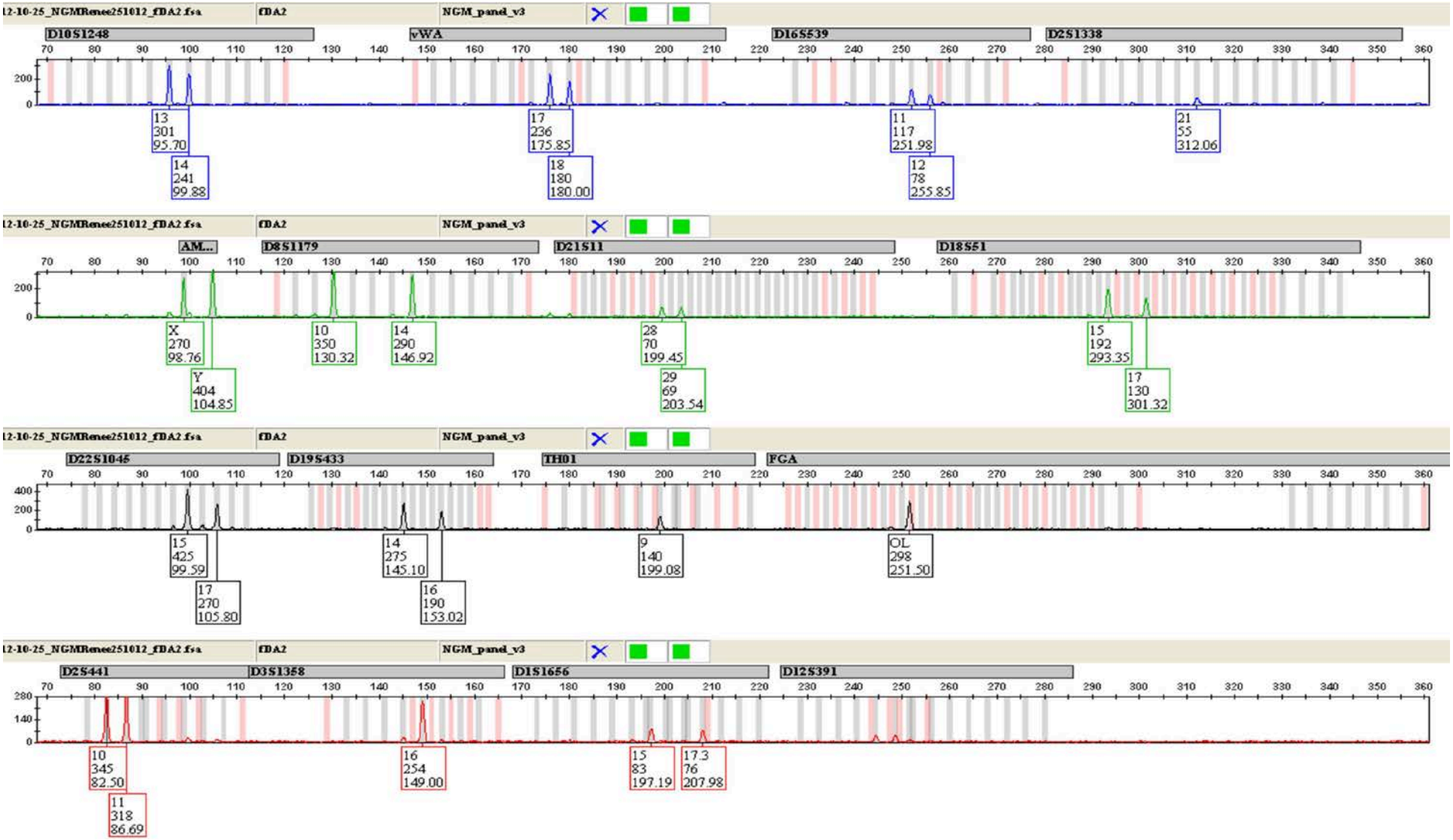

Figure 3b.1 – NGM™ kit DNA profile obtained from a single anagen hair sample for IND 2, amplified using direct PCR at 29 cycles, diluted for capillary electrophoresis.

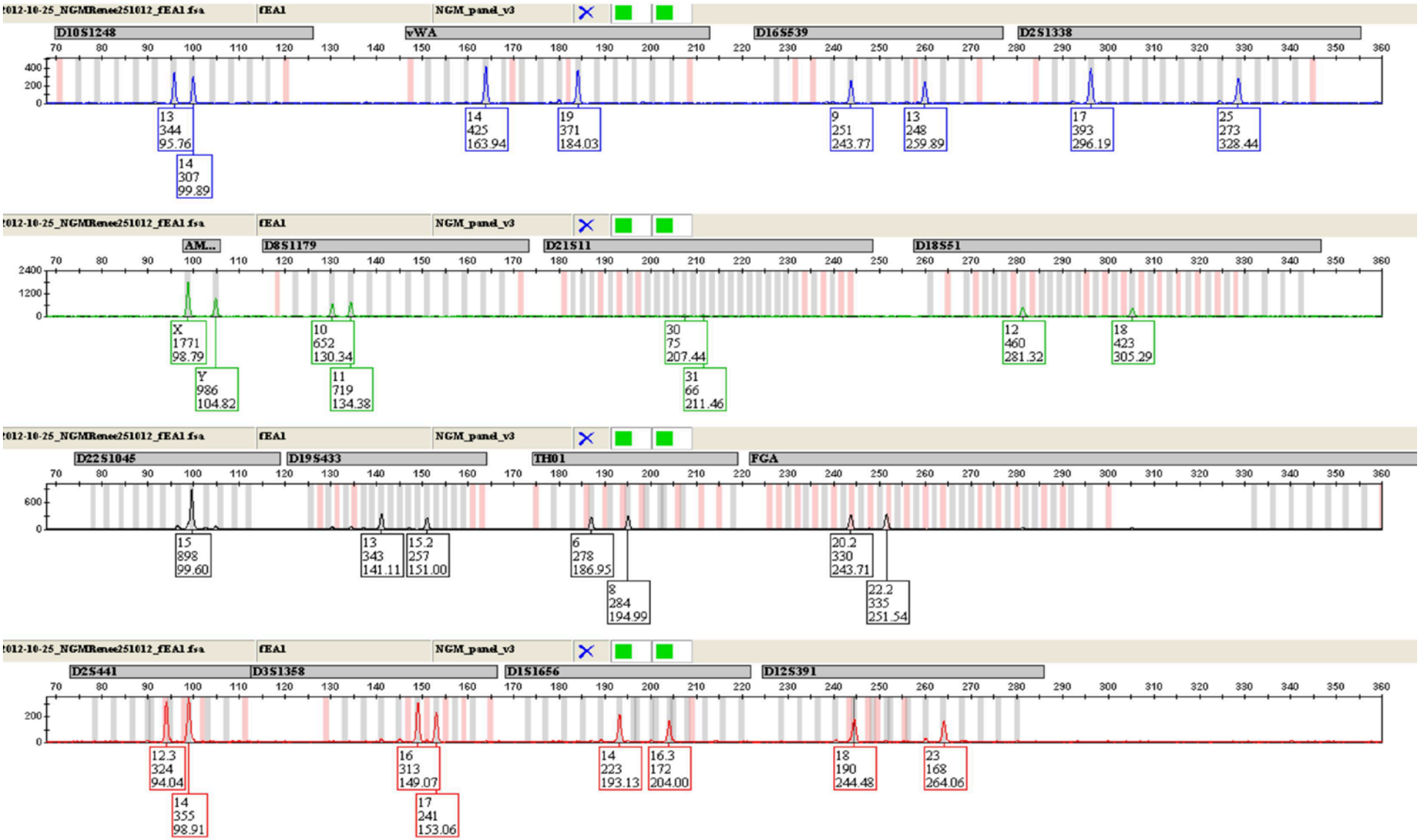

Figure 3b.2 – NGM™ kit DNA profile obtained from a single anagen hair sample for IND 4, amplified using direct PCR at 29 cycles, diluted for capillary electrophoresis.

Appendix

i. **Poster Presentation** 25th World Congress of the International Society for Forensic Genetics (ISFG), Melbourne, Australia, 2013. *Successful direct amplification of nuclear markers from a single hair follicle.*

Successful direct amplification of nuclear markers from a single hair follicle

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Flinders University
South Australia

Presented at the 2013 ISFG Conference Melbourne, Australia

Funding was provided by the Department of Justice, South Australia

Introduction

The direct PCR of samples has value in a forensic context due to the potential time saving [1–3], increase in sensitivity, and minimising of steps where potential error or contamination can occur [4]. Previous research has demonstrated the benefit of direct amplification from fibres [4] and touch DNA [5]. To date there has been no testing of human hairs with direct PCR despite the practise of hair sampling during forensic examination [6]. In many forensic laboratories a single hair may not routinely be DNA tested due to the low success rate of obtaining profiles from this medium. We report on the first use of direct amplification of DNA from a single hair in the anagen and telogen growth phases.

Method

- **Samples** A total of 30 anagen and 30 telogen head hairs from male and female donors were analysed. An additional six hairs, (two from each body limb, pubic and eyebrow), that had been stored for 5 years (aged hairs) were also analysed. Growth phase was determined using microscopy.
- **Direct PCR** was conducted by removing approximately 5 mm of the proximal tip using sterile scissors and tweezers. The hair fragment was placed into a 0.2 mL thin walled tube in a volume of 25 µL of PCR mix using either the AmpF*t*STR® NGM™ or NGM Select™ kit (Life Technologies, Victoria, Australia).
- **Amplification** was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the manufacturer's recommended conditions. The standard 29 cycles was used for all reactions.
- **Analysis:** Samples were run on an ABI 3130*xl* Genetic Analyser (Life Technologies) following standard procedures. The data were analysed using GeneMapper® v3.2. The detection threshold was set at 50 relative fluorescence units (RFU).

Results

- A full profile was obtained from 100% of anagen hair samples and aged hair samples (5 years old). Profiles 'up-loadable' (≥ 12 alleles) to the Australian National DNA Database were obtained for 33.3 % of telogen hair samples. By comparison, when incorporating an extraction step, Forensic Science SA are only able to achieve complete profiles in 53 % of samples for anagen hairs and 10 % for telogen hairs (Table1).

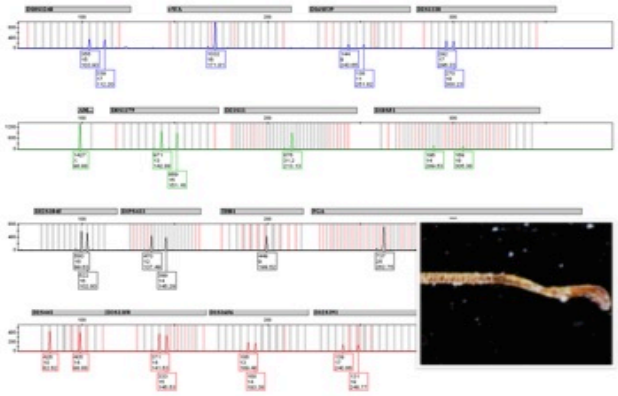

Figure 1. Chromatogram of nuclear DNA from a single **anagen** hair root, amplified using AmpF*t*STR® NGM™ PCR kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Insert: anagen hair.

Results

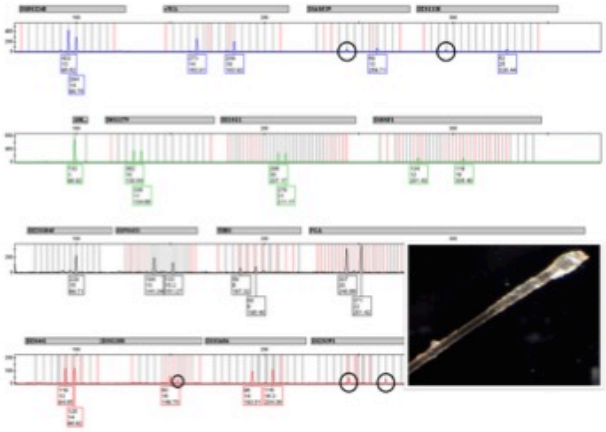

Figure 2. Chromatogram of nuclear DNA from a single **telogen** hair root amplified using AmpF*t*STR® NGM™ PCR kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Allelic drop out can be observed at the *circled* loci D16S539, D2S1338, D3S1358, and D12S391. Insert: telogen hair.

Table 1. Success rates of DNA profiles obtained from direct PCR of hair at different growth phases. An incomplete and up-loadable profile at FSSA comprises a minimum of 12 alleles including amelogenin. The average RFU values for hairs tested using direct PCR are also shown.

Hair Type	NGM™ and NGM SElect™ profile %			Average RFU
	Complete	Incomplete and up-loadable	Incomplete and not up-loadable	
Anagen	100	0	0	4217
Telogen	20	13.3	66.7	1202
Aged	100	0	0	2929

Concluding Remarks

- DNA extraction was not required to obtain DNA profiles to a forensic standard.
- Complete DNA profiles were obtained from 100 % of anagen hair samples.
- Currently, it is forensic practice not to subject telogen hair for DNA profiling. This study obtained profiles 'up-loadable' to the Australian National DNA Database from 33.3 % of telogen hairs.
- Allelic drop-in or contamination was not observed in any of the 66 DNA profiles obtained from any of the hair samples.
- The standard protocol for DNA amplification from the NGM™ and NGM SElect™ STR typing kits is suitable for direct amplification, allowing for quick and easy implementation into forensic laboratories.
- This method is cost-effective and time saving in forensic casework.
- Full profiles were obtained from aged hairs, making the technique applicable for cold case samples.

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Full Article Ottens R, Taylor D, Abarno D, Linacre A. Successful direct amplification of nuclear markers from a single hair follicle. Forensic Sci Med Pathol. 2013;9:238-43.

Corresponding abstract for poster presentation:

Title: Successful direct amplification of nuclear markers from a single hair follicle

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We report on successful amplification of DNA profiles from single hairs. Direct amplification was used on the root tip of both anagen and telogen hairs using a standard commercial forensic PCR kit to amplify 15 STR loci. All 30 anagen hairs tested from five different people gave full DNA profiles after 29 cycles with no allelic drop-in or increase in stochastic events. Six of the 30 telogen hairs tested resulted in a full DNA profile, and a further four telogen hair samples tested produced a DNA profile of five or more complete loci that could be uploaded to the National Criminal Investigation DNA Database (NCIDD, Australia). A full DNA profile was also obtained from the shaft of single anagen hairs. Current practice for many laboratories is that single telogen hairs are not subjected to DNA testing and anagen hairs are seldom tested as there is little chance of generating a meaningful DNA profile; hence this 100 % success rate in generating a DNA profile from anagen hairs is a significant advancement. A full DNA profile was obtained from six 5 year-old single hairs illustrating the power of this technique, even on aged or historic samples. The process described was trialled on current DNA profiling kits, using the manufacturer's recommended methods and no increase in cycle number, such that the methodology can be incorporated readily into a practicing forensic laboratory. For the first time in the field of human identification, single hairs can be analysed with confidence that a meaningful DNA profile will be generated and the data accepted by the criminal justice system.

Funding for Renée Ottens and Adrian Linacre was provided by the Attorney General's Department of South Australia.

Title: Genetic profiling from challenging samples

J. Templeton[1], R. Ottens[1], Viviana Paradiso[1], Damien Abarno[2], O. Handt[2], D. Taylor[2], A. Linacre[1]

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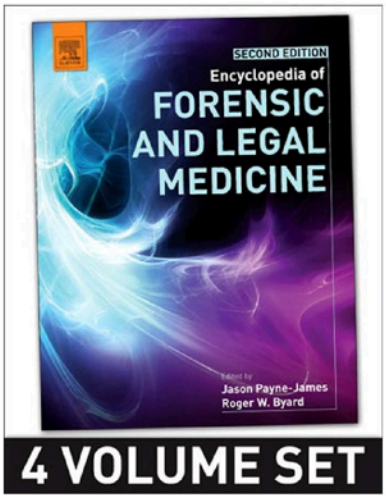
The generation of a DNA profile from trace DNA has become the focus of much interest. The current methodology subjects the sample to a process to isolate the DNA, typically using a solid phase substrate. The aim of this study is to use direct PCR to generate both mitochondrial and STR profiles from latent DNA deposited by touch and also from samples considered to be highly degraded and unlikely to generate a profile if subjected to a DNA extraction process first. Direct PCR has the potential to: minimize loss of target DNA in a critical sample; omit steps involved in standard practice - which will significantly reduce labour time and cost and; increase the likelihood of obtaining a meaningful DNA profile for interpretation. Comparison between the results obtained by direct PCR and from extracts after standard extraction processes indicate the real potential use of the method described in this paper. The technique will have niche future applications in analysing degraded samples that cannot be typed successfully using mainstream, STR-based, kits and protocols.

iii. Hair Analysis: Encyclopedia of Forensic and Legal Medicine, 2nd Edition

a. Contributed the creation of all figures and text editing.



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DNA: Hair Analysis

A Linacre and R Ottens, Flinders University, Adelaide, SA, Australia

This article is a revision of the previous edition article by E.A. Graffy, D.R. Foran, volume 2, pp 213–220, © 2005, Elsevier Ltd.

Abstract

Hairs are encountered commonly in a forensic investigation and can be a source of DNA. The amount of DNA within a single hair is often less than the optimal amount required for nuclear DNA markers. Most of the DNA is within a root sheath with trace amounts of mitochondrial DNA in the shaft. Nuclear DNA testing targets highly repetitive DNA regions called microsatellites. The quantity of starting template required for nuclear DNA profiling is 1 ng, which may be present in the root sheath but not within the hair shaft. Mitochondrial loci are targeted on hair shafts due to their greater copy number.

Glossary

Allele Alternative form of a DNA locus. For example, different lengths of DNA at a short tandem repeat.
Cortex Main body of a hair composed primarily of keratin and containing the pigment granules.
Dithiothreitol A reducing reagent used to break the di-sulfide bridges between the cysteine residues in keratin.
Ethylenediaminetetraacetic acid It chelates positive ions such as calcium and magnesium. It is used commonly in DNA extraction buffers to inhibit naturally occurring DNases (enzymes that destroy DNA).
Microsatellites Sections of highly repetitive DNA and a source of DNA polymorphisms. These are also called short tandem repeats or STRs.
Mitochondrial DNA A circular DNA molecule found in the mitochondria. The molecule is 16 569 bases in circumference in humans and there are 1000s of mitochondria per cell each containing many copies of DNA.
Polymerase chain reaction The enzymatic amplification of DNA.
Sheath Follicular tissue found surrounding a root structure, typically in anagen hairs.
Short tandem repeats (STRs) STRs is another name used for microsatellites.
Sodium dodecyl-sulphate A detergent that is used in DNA extractions as it dissolves the lipid membrane that surrounds many cells.

Background

Hairs are encountered frequently in forensic investigations. As it is estimated that we lose 100 hairs on average each day, these hairs are shed to the surroundings. In a home, shed hair might be noticeable on hairbrushes and combs, but also found on clothing and the fabrics of furniture such as chairs and sofas or automobiles. In sexual assaults pubic hair can be transferred between the assailant and the victim in potentially a two-way transfer. Physical assaults may also result in forcefully removed body or head hair (see Chapter 1 in Butler, 2012 and Chapter 4 in Goodwin *et al.*, 2011).

Prior to the advent of DNA typing, the standard forensic technique for analyzing hairs was by visual examination and then by light microscopy. Clearly if the suspect has long black hair but the hairs under examination from a potential crime scene are very short blonde hairs, then there is no reason to continue with any scientific testing. But if both the reference hairs from the suspect and those from scene are visually similar, then microscopy is a standard technique to allow comparison. A problem is that the hairs of one individual may exhibit much variation within the scalp; the fine hairs at the temple can be different morphologically to those at the nape or at the crown. Even if every morphological characteristic was the same between hairs from a suspect and reference material it is only possible to state that this is not an exclusion. There have been too many false inclusions with opinions provided on matching hairs that in hindsight should not have been given. The examiner might have said "all the morphological characteristics in hairs from the scene are consistent with those from the suspect and therefore they may have a common origin." Post-conviction exonerations using DNA typing, where hair evidence erroneously implicated an accused, has led recently to the focus of DNA typing on hair samples. Opinions

provided in hair comparisons by microscopy were an area of criticism in the report on the state of forensic science in the United States (National Academy of Science, 2009).

Microscopy does still have a role in determining the potential somatic origin (e.g., scalp, pubic, and eyebrow), species (cat, dog, or wildlife), and whether there is cellular material associated with the root (see section below). It is a nondestructive test that remains the standard screening tool to determine if DNA typing is necessary. DNA typing provides an objective scientific test to determine whether an unknown hair and reference sample from an individual match. If they match then a statistical test can be performed to determine if the DNA profiles match by chance. If they do not match then there is a definitive exclusion that the hair did not come from this individual.

Hair Structure

Hair is essentially composed of three structures. The outer surface of the hair is called the cuticle and is typically a thin layer of cells that overlap. In human hairs this looks like slates on a roof but there is a very wide variation in cuticle patterns between different mammalian species. The cortex is the main component of the hair and is primarily composed of a protein called keratin. Keratin is the same protein found in nails, hoofs, antlers, and horns. Keratin proteins tend to be very long chains of amino acids but multiple chains can bond together (analogous to multiple pieces of string wound around each other to make rope). Keratin has a high content of the amino acid cysteine. Cysteine is the only one of the 20 naturally occurring amino acids that has a sulfur residue and when a sulfur on one keratin chain lies next to a sulfur on another, the two sulfurs can bind together making a strong disulfide bridge. Within the cortex are the pigment granules that are responsible for giving the hair its color. In humans, the pigment granules are evenly spaced giving the same color throughout the hair whereas many animal hairs have a banded coloration. At the center of the hair is the medulla; this is a thin air-filled shaft that gives the hair extra strength. Under light microscopy the medulla appears as a dark core.

Of relevance to DNA typing, DNA can be found in the cortex due to residual cells that are responsible for the growth and development of the hair. DNA has not been reported to be found in the air-filled medulla although cells are regularly found adhering to the outer surface of the cuticle (see for instance Chapter 8 of Saferstein, 2007).

Hairs may also be used for forensic toxicology analysis due to their unique matrix. As hair has no active metabolism or method for secretion, unlike blood or urine, drugs will remain in the hair for longer periods once deposited and can help in investigations such as suspected poisoning or accidental drug exposure.

Hair Growth Phases

Hairs have a finite lifespan starting with active growth, then senescence, and ultimately release from the skin. The active growing phase is called anagen, with hairs in this phase called anagenic. Typically hairs grow for about 1000 days and approximately 90% of scalp hairs are anagenic. Hairs grow from their base within the scalp, called the follicle, due to the rapid replication of the germinal cells and melanocytes. As the cells replicate and divide at the base of the hair, the old cells are pushed upwards into the growing structure of the hair and are ultimately keratinized leading to the disruption of the cell and cell death. The melanocytes and germinal cells are the source of DNA later found in the cortex of the hair, as the cellular DNA from these two cell types remains associated within the keratin or cortex. When hairs reach the end of their growth phase the follicle starts to shrink and lose contact with the surrounding epidermal cells. There is a very short period lasting only a few days, called catagen (catagenic hairs), where the hairs are in the intermediate state before the last phase called telogen. Telogenic hairs account for the majority of the remaining 10% of hairs, with less than 1% being catagenic as this phase may only be a few days in duration. During the telogen phase a new follicle starts to develop below the older one leading the shrinking of the old follicle, detachment from the dermis, and then to the hair being shed. Typically it is the telogenic hairs that are encountered at crime scenes, as these are the most likely ones to be shed naturally.

A telogenic hair has a distinctive follicular tag at the root end. This is in contrast to the root end of an anagenic hair where a visible cellular sheath may be present. This simple differentiation, which is determined by microscopy, can be valuable in a forensic investigation as the presence of an anagenic hair indicates it was most likely forcibly removed. An example of hair roots from these three growth phases is shown in Figure 1.

DNA within Hair

The hair shaft is known to have very little DNA as this is only the residual DNA from the melanocytes and germinal cells. This is in contrast to the root which, if present, is potentially a rich source of DNA.

DNA typing is predominantly based on analyzing genetic markers in the nucleus. Nuclear DNA is rarely found associated with the hair shaft in sufficient amounts to generate a DNA profile. The alternative DNA typing targets the mitochondrial DNA. As explained below, mitochondrial DNA is found in larger

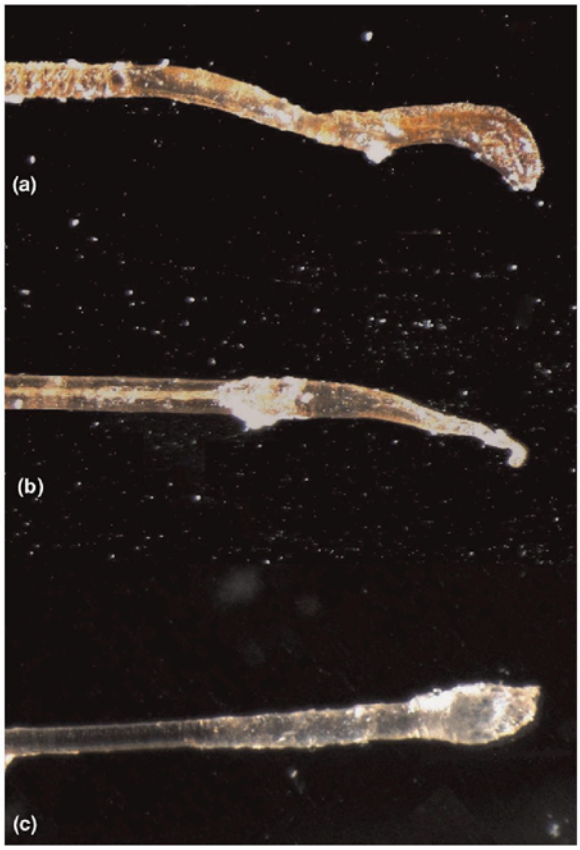

Figure 1 The three stages of hair growth: (a) anagenic hair with sheathing present, (b) catagenic hair with thin tapered root, and (c) telogenic hair with club or bulb shaped root. Images captured by Renée Ottens.

amounts than DNA from the nucleus and also is found in the shaft of telogenic hairs.

Nuclear DNA

DNA profiling methods and DNA databases are based on microsatellites (otherwise termed short tandem repeats (STRs)) on the autosomal chromosomes. Each person has two copies of their nuclear DNA, with one copy from the mother and the other from the father. Typically, most commercial STR testing kits require 1 ng of starting template. This mass of DNA equates to approximately 170 human cells. It is unlikely that this amount of DNA will be found in hair shafts and hence nuclear STR testing is unlikely to generate a DNA profile from a single anagenic or telogenic hair shaft. A single fresh anagenic hair, where a clear root sheath is visible, has the potential to generate a DNA profile. A single telogenic hair root is, however, very unlikely to have sufficient DNA to generate a profile. It is not good practice to group together numerous telogenic hairs to obtain sufficient DNA as the hairs may have come from more than one person.

Analysis of Nuclear DNA

A decision needs to be made as to whether there is any chance of generating a DNA profile using nuclear markers. If there is a visible root then this terminal section of the hair (approximately 3 mm) should be cut into a sterile tube. The cells in the root sheath should be treated like any skin cells. Typically a detergent such as sodium dodecyl-sulphate (SDS) is used to break open cell membranes and a chemical called ethylenediaminetetraacetic acid is used to remove magnesium and calcium ions (the removal of magnesium ions stops naturally occurring DNases from breaking down the DNA). Typically to ensure that all of the DNA is released from the hair section, the enzyme proteinase K (so called as it digests keratin) and a reagent such as dithiothreitol (DTT) that breaks the disulfide bridges are added. The hair shaft is incubated in this extraction buffer until the hair shaft is no longer visible. The released DNA can then be isolated using one of the commercially available kits which use a positively charged solid phase to bind DNA; the DNA can later be released into a new solution by altering the charge of the solid phase to neutral.

The isolated DNA can then be quantified during a standard process and then subjected to DNA profiling using a commercially available kit. All the kits for DNA profiling analyze STR loci. These are regions on a chromosome where a sequence of DNA bases is repeated multiple times; the number of times the repeat is found can be variable within the population. For instance there is a tiny section of chromosome 16 that has the sequence GATA where some people carry 4 GATAs (GATAGATAGATAGATA), some 5, some 6, and indeed all the possible types (alleles) to 17 repeats. As there are a total of 14 alleles (4–17) then there is in fact 105 potential genotypes. This is an example of one STR locus, however, there are commercial kits that analyze 21 STRs leading to astronomical powers of discrimination (the chance that someone else shares the same genotype at all 21 STR loci). All of the DNA databases constructed from persons who have committed a crime are based on nuclear STR typing; hence there is a significant reason to employ this type of genetic marker if possible. If there is less than 170 cells worth of DNA, and the DNA quantification results indicate that there is 200 picograms of DNA present, then is can still be tested using these STR markers. In such a case it is highly likely that not all the STR loci will generate a result leading to what is termed a partial DNA profile. If only five of the loci tested generate a result, and the others generate no data as is typical with less than optimal DNA being isolated, then this can still have a power of discrimination of more than 1 in 10 million.

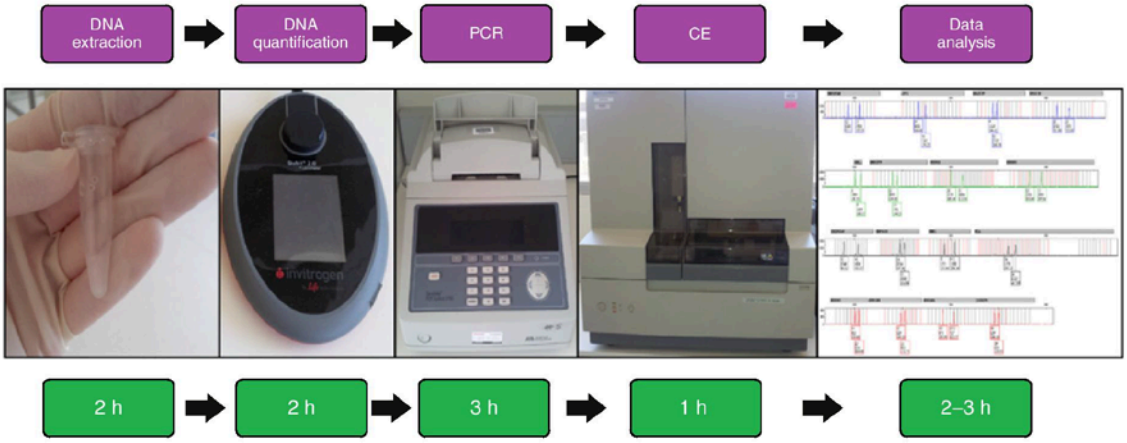

Figure 2 The standard process for DNA analysis showing the time taken for each step from DNA extraction through to data analysis. The process takes a minimum of 10 h and includes 5 steps along the process. Images captured and created by Renée Ottens.

A standard timeline for the STR typing of hair fragments is shown in Figure 2 (for more information see Chapter 6 of Goodwin *et al.*, 2011).

Mitochondrial DNA

If nuclear DNA testing is not an option, such as when there is no root sheath and the hair appears to be telogenic, then mitochondrial DNA testing is an option. Mitochondrial DNA is present in much higher copy number than nuclear DNA; typically there can be over 10 000 copies of mitochondrial DNA in one cell compared to two copies of nuclear DNA. Additionally mitochondria, the cellular organelle responsible for respiration, have a strong protein coat that protects the internal DNA from bacterial degradation.

Mitochondrial DNA testing is used less than nuclear STRs due to the mode of inheritance and resulting power of discrimination. Mitochondria and their DNA are present in the cytoplasm of the egg cell but spermatozoa do not contribute mitochondrial DNA to any fertilized embryo; hence all mitochondrial DNA comes from the mother and not the father. This can be a real advantage as everyone who shares a maternal relative will have the same mitochondrial DNA leading to linkage between a hair sample and a great granddaughter for instance. This sharing of mitochondrial DNA sequences is also the disadvantage as the power of discrimination (i.e., how many other people share the same DNA type) is poor as mitochondrial DNA cannot differentiate brothers and sisters, mother, and daughters, or even more distant genetic relatives if they share a maternal relative.

Mitochondrial DNA has been used in historic cases such as the identification of the Russian royal family who were murdered in 1918 and their skeletal remains not recovered until 1989. Here mitochondrial DNA from living known genetic relatives provided mitochondrial DNA profiles to allow matching to confirm the identity of the Tsar and his family (discussed in Chapter 13 of Goodwin *et al.*, 2011). The high copy number is also an advantage when the DNA is highly degraded such as in the body parts retrieved from the World Trade Center victims in 2001 and maternal relatives.

As mitochondrial DNA is inherited from the mother only and there is no recombination of the DNA (as happens with nuclear DNA), then all cells of an individual will have the same DNA type. This single version is called a haplotype and many of the haplotypes are known and mapped for populations around the world. This has the potential for an unknown mitochondrial DNA type to be placed within a geographical location based on common maternal ancestors.

Analysis of Mitochondrial DNA

Mitochondrial DNA typing is more likely to be performed on telogenic hairs or if there is no obvious root sheath to a hair. The extraction process is typically the same method as for nuclear DNA typing. The analysis of mitochondrial DNA is very different as it is based on variation of the DNA sequence rather than the length (as in the number of STR repeats in nuclear DNA typing). Mitochondrial DNA is a circular molecule unlike chromosomal DNA. The human mitochondrial DNA is around 16 569 bases in circumference and almost all of it is very similar between every human. Much of the DNA sequence of the mitochondrial genome encodes for genes and is under very strong selection pressure to stay the same. Any change due to a mutation in the coding region has the potential to alter the encoded amino acid which can in turn lead to an alteration in the performance or function of the protein. There are two short

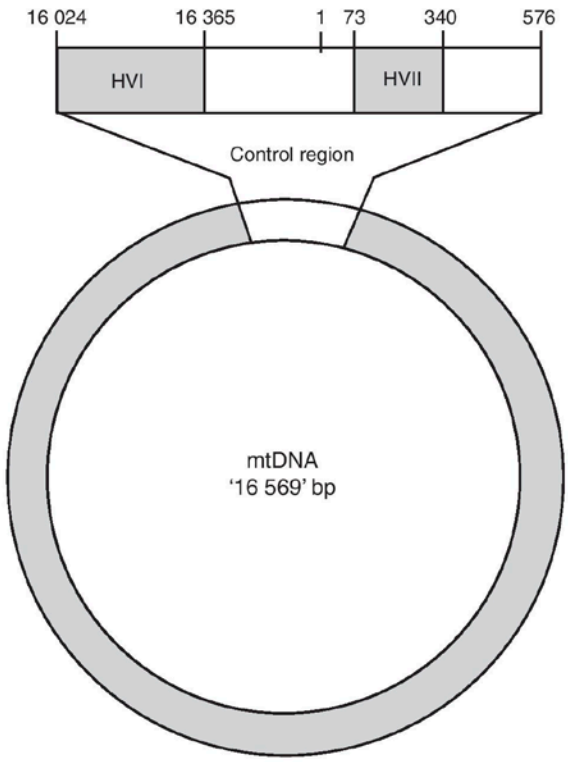

Figure 3 The hypervariable regions of the mitochondrial DNA genome showing the locations of the hypervariable regions 1 and (HVI and HVII). The entire circular mitochondrial genome is 16 569 bases in circumference and all the bases have been mapped and numbered from position 1 to base 16 569. Image created by Renée Ottens.

stretches of DNA bases within the mitochondrial DNA that do not encode a protein and these regions show sequence variation between people (who do not share a recent maternal relative). These are called the hypervariable region 1 (HVI) and hypervariable region 2 (HVII). See Figure 3 for a diagram of HVI and HVII.

In the process of DNA typing it is only these short sequences that are determined by a routine process called DNA sequencing. If the DNA sequences differ then this is an exclusion; i.e., the hair could not come from the same source as the reference material. If the HVI and/or HVII DNA sequences of a hair sample have the same DNA sequences as a reference sample then this is an inclusion. In the event of an inclusion there are three possibilities to consider: either the hair comes from the same person as the reference sample, or it comes from another person with a recent maternal relative, or it comes from someone else and matches by chance. The only difficulty is if there are one or two bases different over the 610 bases that constitute HVI and HVII. The possibility that these differences are due a mutation in the DNA sequence (changed at this one base) when passed from the mother to the offspring needs to be considered.

Mitochondrial DNA also suffers from a problem of sequence variation within the same person. This is a somatic mutation where two or more sequences exist and it is called heteroplasmy. When cells are dividing rapidly such as in melanocytes then during cell replication it is possible that a mutation occurs at a single base leading to a new bases in this mitochondrial DNA. If this mutation happens in one new cell but not in another then the result can be heteroplasmy.

Animal Testing

Dog and cat hairs are found frequently on clothing or at crime scenes and there can be associative evidence if linked to an individual animal. Microscopy can determine if a hair is from a particular species using the overall shape (most animal hairs taper at the tip whereas only human eye bow hairs show this tapering), the cuticle pattern, and the size and pattern of the medulla (in humans it is less than a third of the total diameter of the hair but in cats, rabbits, deer it can be large and lattice shaped).

Animal hairs can be subjected to the same DNA tests as human with the same choices of either nuclear DNA typing or sequencing part of the mitochondrial genome. A set of STR loci are in place for both dogs and cats and there are databases of the allele frequencies and occurrence of haplotype for both these species being developed.

New Technologies

DNA isolation methods described above are designed to extract DNA from the cortex and then purify the nucleic acid to remove any inhibitors of the amplification process. The procedures are very inefficient with up to an 85% loss of the DNA. The result is that there may be 150 cells for instance, equating to just less than 1 ng of DNA, in the starting sample but after DNA extraction and losing 75% of sample, the final extract only has 0.25 ng of DNA (or 250 pg). This is unlikely to generate a full DNA profile.

Many forensic science laboratories will not subject a single anagen hair to nuclear DNA testing as it is unlikely to generate a result. Such a sample may contain pertinent information for the investigation.

A novel approach in such cases is termed direct polymerase chain reaction (PCR) where a section of hair is placed directly into the PCR with no extraction process. The first step in using hot-start DNA polymerases is to heat the solution for 10 min at 95 °C to activate the enzyme. The heating process also denatures the proteins in the hair resulting in the loss of DNA into the solution. The benefit is that there is no loss of DNA prior to this step, and all the material is added to the reaction.

The disadvantages are that the test cannot be reproduced as all the sample is used, and if there are inhibitors present then no PCR products may be generated.

The process of direct PCR has been shown to work on single hairs over 5 years old and can be used in cold cases where no other method is available.

Molecular biology has witnessed tremendous advances in DNA sequence technology in the last few years with the advent of mass parallel DNA sequencing (often called high through-put sequencing or next-generation sequencing). Whole genomes can now be decoded in a matter of hours rather than the 3 years it took for the first human genome to be sequenced. Single cells can be used in such applications leading to open the opportunity to sequence the entire DNA content rather than target only mitochondrial DNA or the microsatellites. At the time of writing, such technologies are still too expensive to be performed regularly but there is every possibility that they will become a routine tool once the costs are reduced.

See also: Disaster Victim Management: DNA Identification. DNA: Basic Principles. DNA: Mitochondrial DNA. Substance Misuse: Hair Analysis

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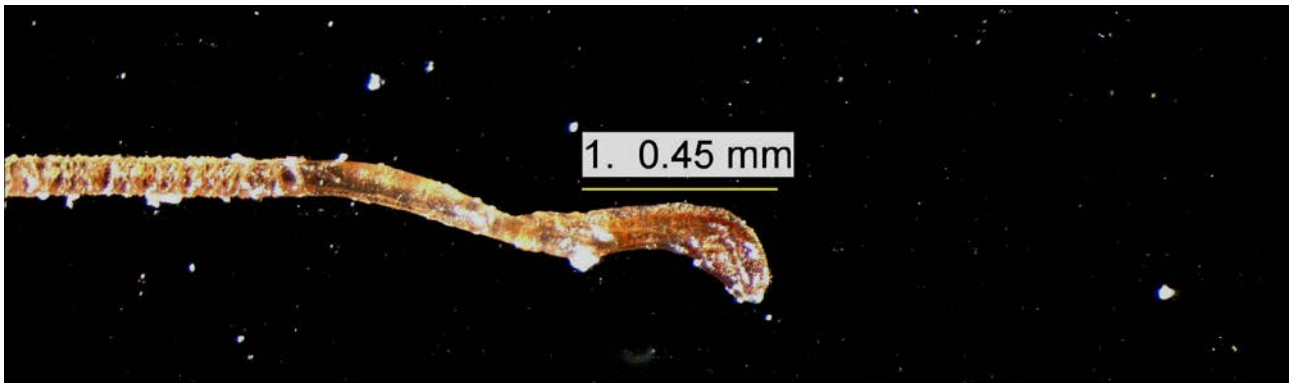
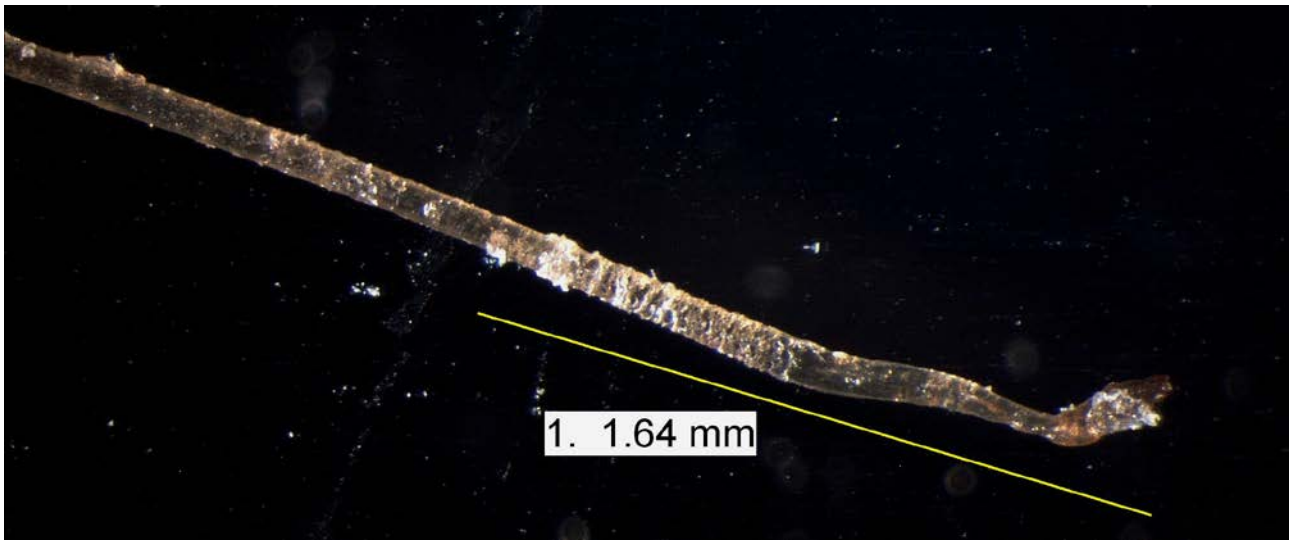
iv. Microscopy of human hair: examples.

a. Anagen





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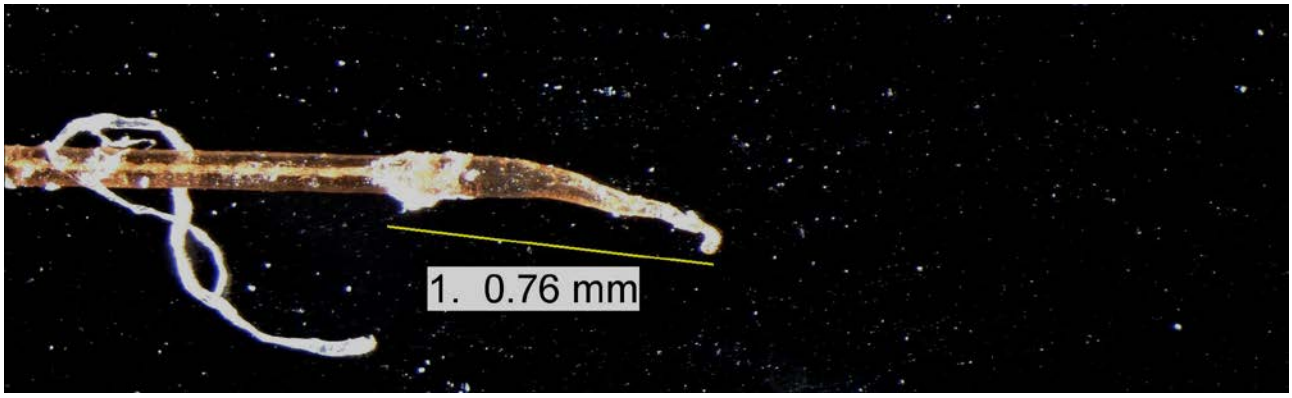
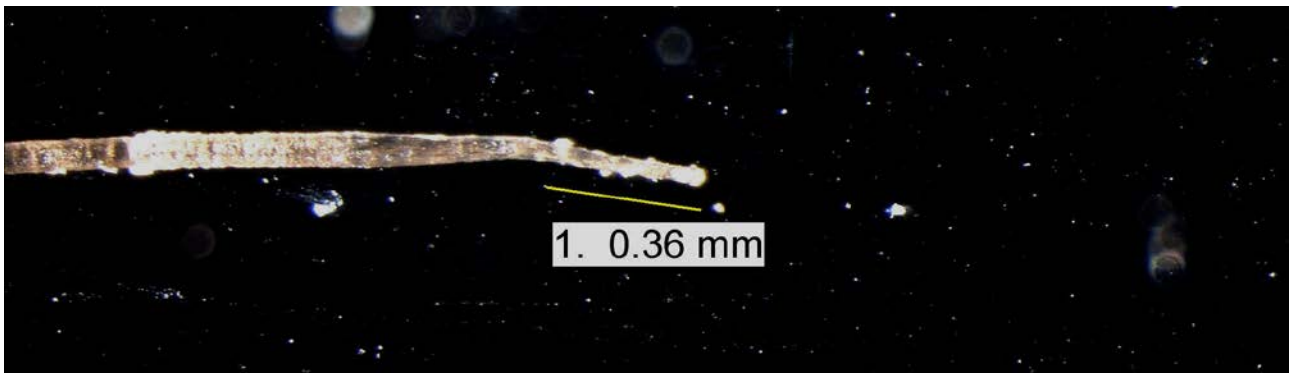
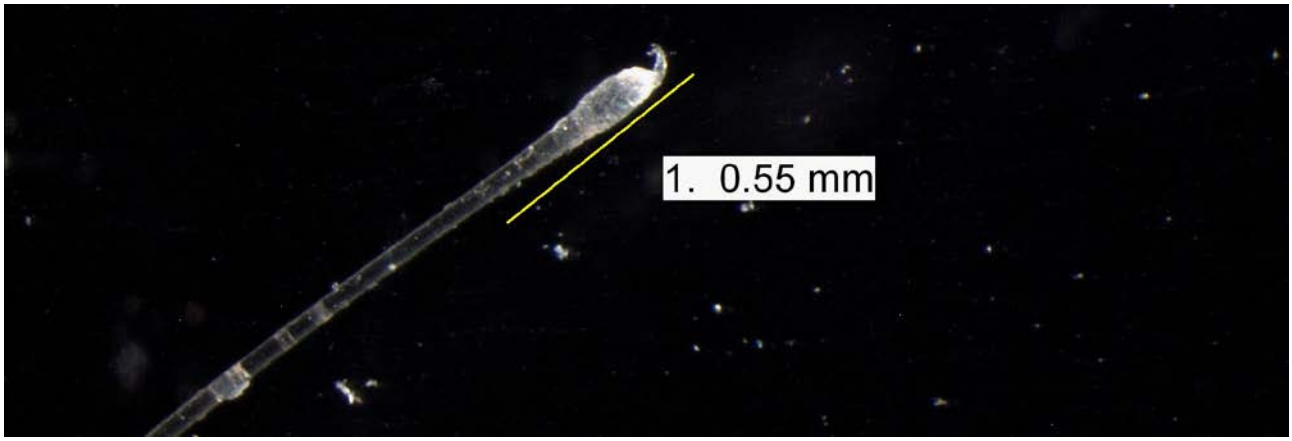
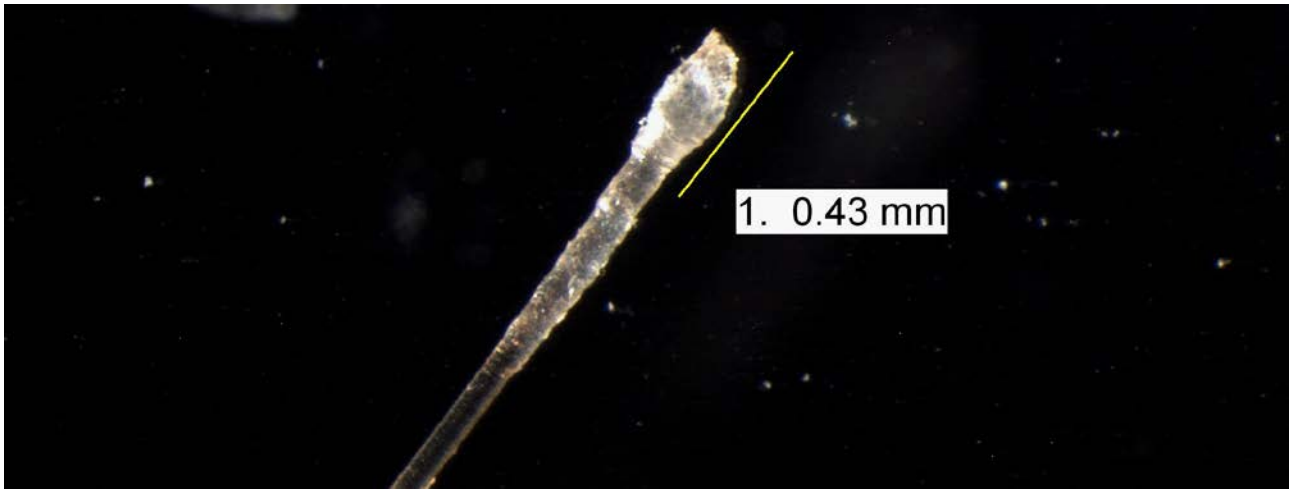




c. Telogen





CHAPTER 4

Direct PCR from Canine Hair

Blackie, R., Taylor, D., and Linacre, A., *Successful direct amplification of nuclear markers from single dog hairs using DogFiler multiplex.* Electrophoresis, 2015. http://dx.doi.org/10.1002/elps.201400560

Canine DNA Detection:

Case 2 – Determining the presence of canine DNA in an extraction obtained from a glove

Case 3 – Determining if the canine DNA from Case 2 matches bone DNA from exhumed pet

Case 4 – Determining the presence of canine DNA in a sexual assault allegation

Direct PCR from Canine Hair

4.1 Preface

Human hairs are not the only hair types found at crime scenes, it is also common to encounter animal hairs from cats, dogs or rabbits within a home, vehicle or on clothing [1-3]. Domestic animal hair can be found in abundance around the home and is easily transferred to other surfaces and areas via direct contact or secondary transfer, as most domestic pets can shed their coats several times a year with season changes [3]. Microscopy is often used to determine the type of animal hair found based on its morphology, as each species possess hair with characteristic length, colour, shape, root appearance and internal distinguishable microscopic features [1, 2]. Microscopic comparisons can be a great tool in potentially linking crime scenes, suspects and victims, but do not have the same level of discrimination power of DNA analysis. Autosomal STR markers have been researched and identified for canine and feline DNA, however the multiplex assays are not commercially available to the same extent as those for human identification. The demand and need for human DNA analysis is obviously much greater than that of animal DNA analysis, however crime scenes may arise where single animal hairs are the only physical evidence available for analysis. Like human hairs, animal hairs can be considered trace evidence and again are very difficult from which to obtain meaningful DNA information [4]. To provide DNA analysis from single animal hairs would offer investigations another powerful tool in solving cases or generating leads. The following sections investigate the optimisation of the canine STR markers within the Flinders University DNA laboratory (*4.2*), the application of canine hairs as a substrate for direct PCR (*4.3*), as well as the application of canine STR markers in casework and future implementation of the technique (*4.4*).

4.2 Multiplex setup

DogFiler is a multiplex assay developed for canine DNA profiling in forensic casework, encompassing 15 loci and one sex-determining marker [5]. To determine whether canine hairs are a suitable substrate for direct PCR methods, eight loci (including the sex-determining marker) were chosen for preliminary testing. Information regarding the optimal canine DNA concentration for use with DogFiler, or the primer concentrations of the multiplex has not been published and needed to be optimised before amplifying substrates directly. The optimal DNA concentration for human identification kits is on average 1 ng. Humans have 23 chromosomal pairs whereas canines have 39 chromosomal pairs, therefore it can be expected that the optimal DNA input for DogFiler will differ because of this difference.

Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Victoria, Australia) and QIAGEN® multiplex PCR kit (Qiagen, Victoria, Australia) were compared to determine which DNA polymerase is most suitable for canine DNA amplification. Following manufacturer's instructions for a final PCR volume of 26 µL, 2 ng of control canine DNA was amplified using Phusion® and QIAGEN® multiplex assays (Figures 4.1 and 4.2, respectively). QIAGEN® overall performed better than Phusion®. Profiles obtained using the Phusion® assay often displayed noisy baselines, split-peaks, and greater loci and allele imbalance than profiles obtained using the QIAGEN® assay. Using positive control DNA, primer concentrations were adjusted until a balance between the amplicons of the eight STR loci was observed. All final primer concentrations, primer information, PCR set-up and PCR conditions are outlined in *section 4.3*.

Using the QIAGEN® multiplex assay, canine DNA was amplified at 4, 3, 2, 1 and 0.5 ng to determine the optimal input concentration (Figure 4.3). At 2 ng and 3 ng of input DNA, balanced heterozygous allele peaks with strong RFU values averaging 6000 – 7000 were consistently obtained. At 4 ng of input DNA, baseline noise and the effects of pull-up were more noticeable, with the peaks occasionally presenting as split. At 0.5 ng of input DNA, full profiles could still be obtained, resulting in average RFU values of 2000. Peak imbalance was more common at 0.5 ng of input DNA. All concentrations resulted in full profiles, with alleles clearly defined; even at higher concentrations the baseline noise and pull-up were minimal. There was little difference overall with profile quality between 1 – 3 ng of DNA, therefore the optimal input using the eight loci selected with the QIAGEN® multiplex assay would fall within this range.

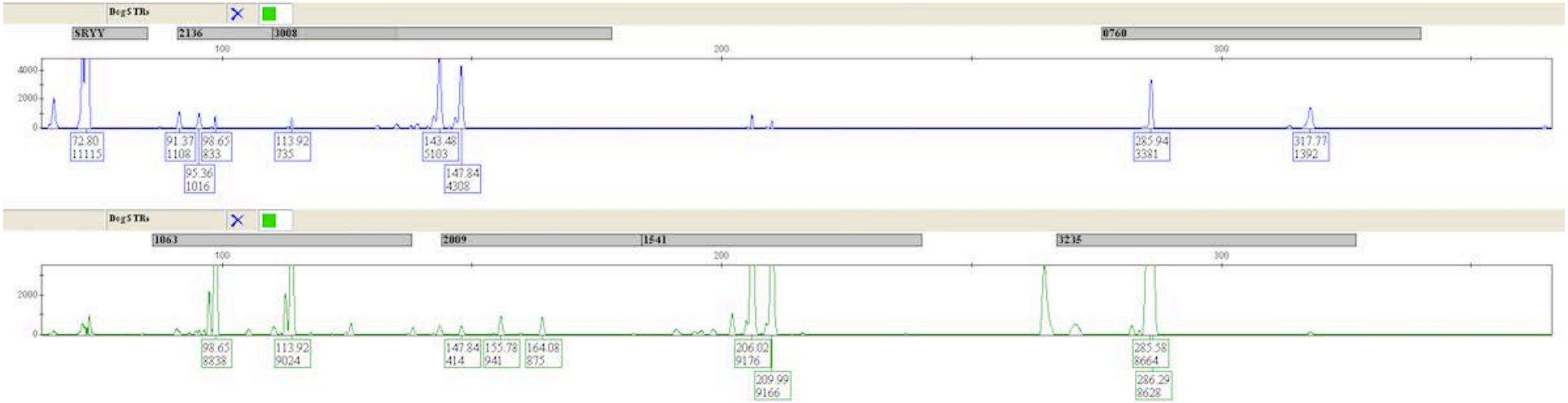


Figure 4.1 – Chromatogram of nuclear canine DNA (2 ng) amplified using Phusion® High-Fidelity DNA Polymerase and DogFiler loci at 31 cycles on a GeneAmp® System 9600 thermal cycler.

Figure 4.2 – Chromatogram of nuclear canine DNA (2 ng) amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9600 thermal cycler.

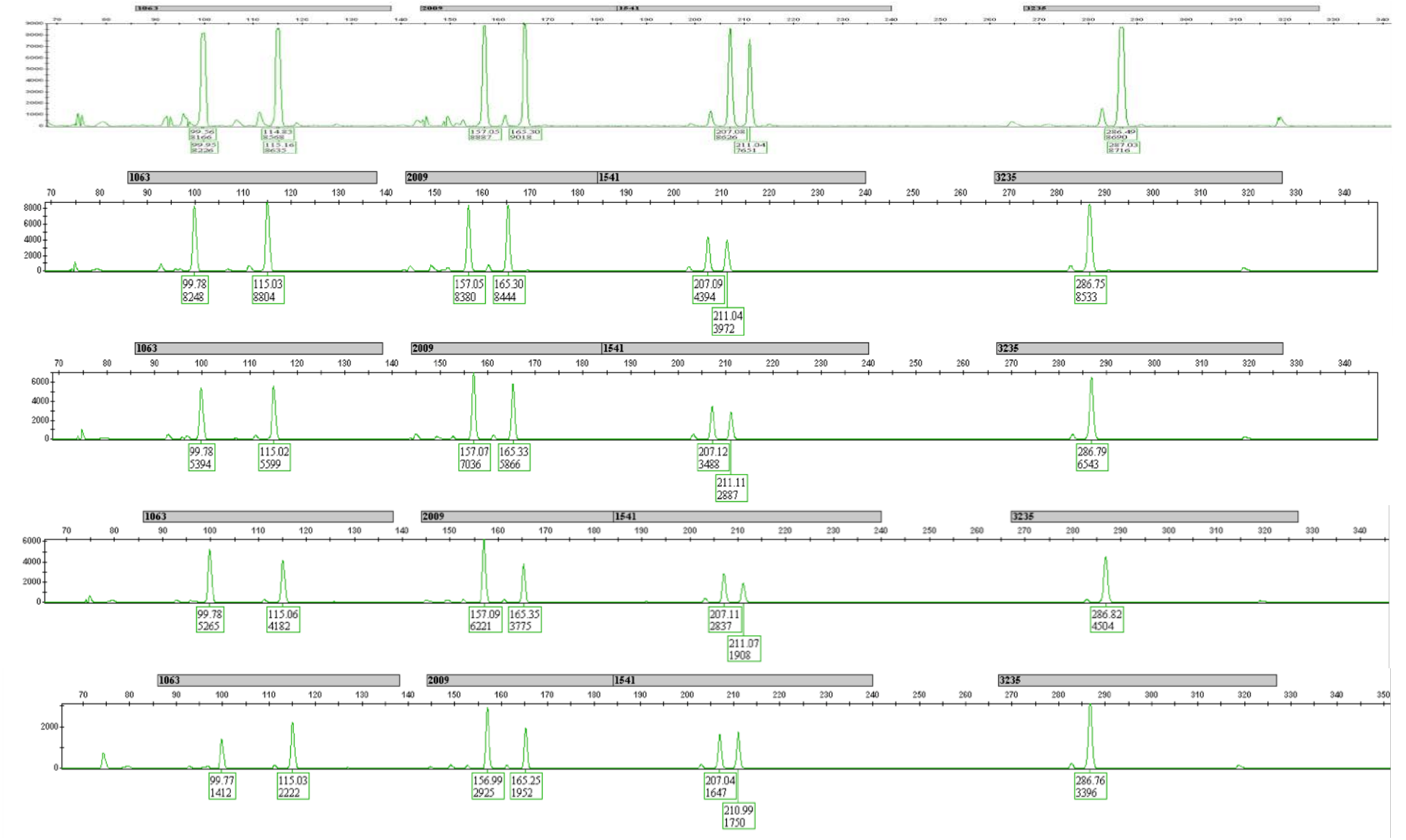


Figure 4.3 – Five chromatograms of nuclear canine DNA at concentrations: 4 ng, 3 ng, 2 ng, 1 ng and 0.5 ng (top to bottom), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9600 thermal cycler.

Shed telogen animal hairs would represent the majority of animal hair types assessed in casework and are therefore the focus of this preliminary study. To determine if there was any substantial difference in obtaining DNA profiles between canine telogen and anagen hairs, both types were analysed using microscopy and amplified directly. Animals do not have their coats washed as regularly as humans wash their hair, with animal hair more likely to retain nuclear DNA and cell-free DNA along the shaft due to skin secretions, and saliva from grooming. To increase the likelihood of obtaining DNA from telogen canine hairs, an entire hair was cut into ~ 5 mm sections and amplified in one 0.2 mL tubes (Figure 4.4)

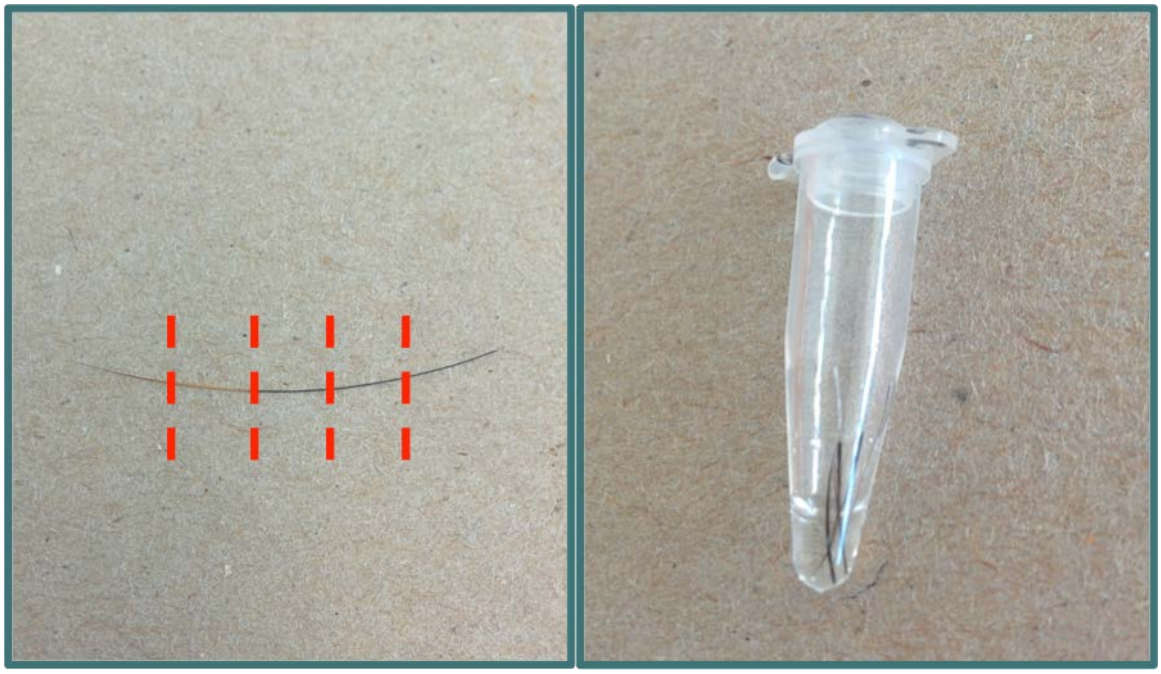


Figure 4.4 – A single canine hair (telogen) showing the approximate cut sections, and the resulting hair fragments within a 0.2 mL PCR tube containing 26 µL of QIAGEN® multiplex assay master mix and DogFiler primers.

Anagen and telogen canine hairs resulted in full profiles, displaying clear baselines and alleles (Figure 4.5). There was no significant difference in profile success rate between the two hair types during this preliminary study, indicating that there is more DNA found on the shaft of an animal hair than a human hair (again, possibly due to the frequency of washing, or animal grooming habits). As telogen hairs are more common at crime scenes, with the preliminary data showing that this hair type produces similar results to anagen hairs, *section 4.3* focuses on shed hairs only.

Canines may have several different coats of hair that are shed differently depending on the weather, mainly comprised of an undercoat and a topcoat. The undercoat is generally soft, thin and fluffy, whilst the topcoat (also referred to as guard hairs) is thicker and coarser (Figure 4.6).

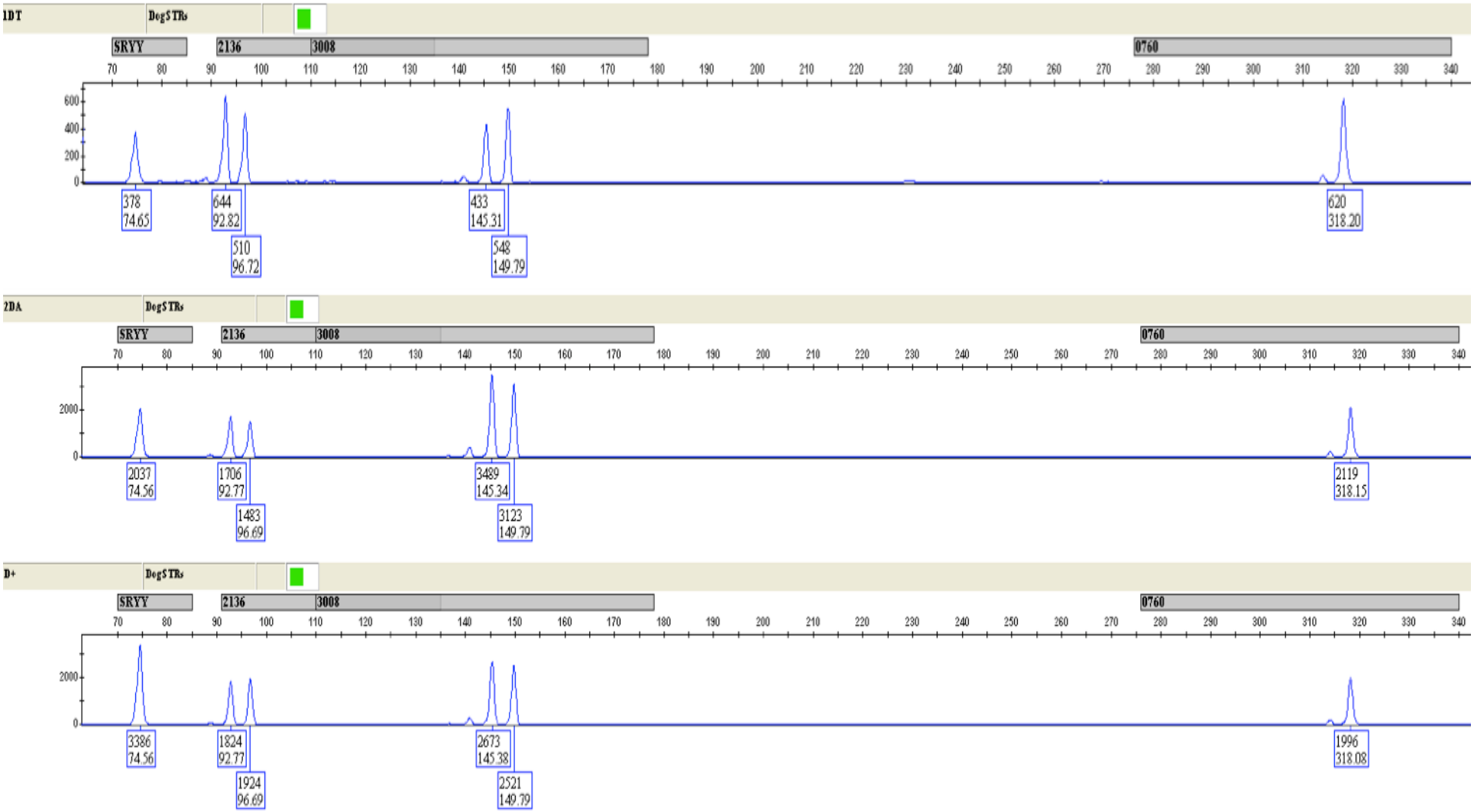


Figure 4.5 – Three chromatograms of nuclear canine DNA samples: telogen hair, anagen hair and positive control (top to bottom), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9600 thermal cycler.

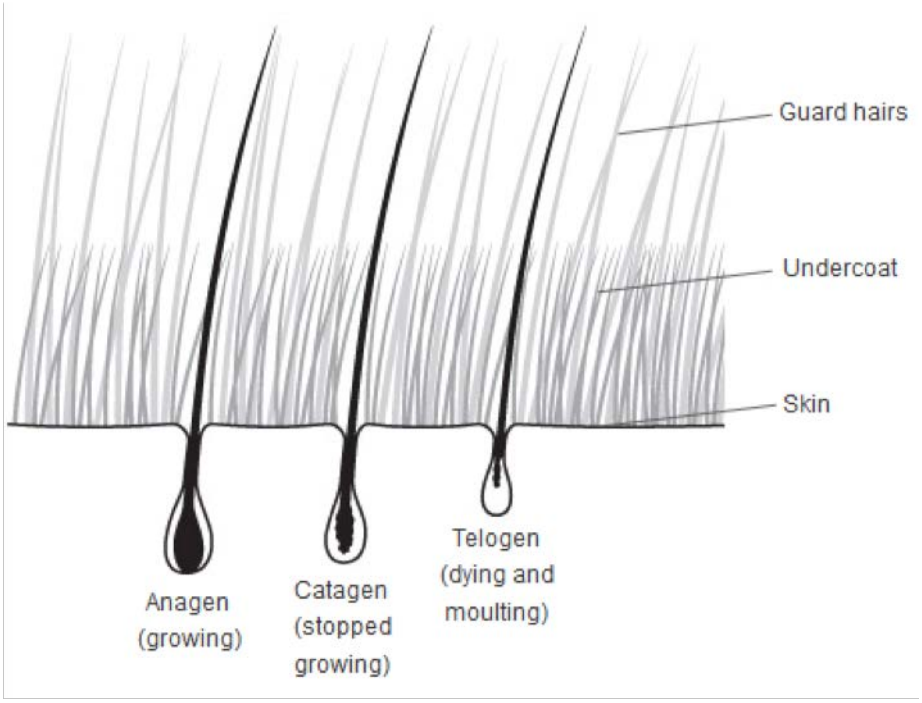


Figure 4.6 – Three stages of hair growth (anagen, catagen and telogen), showing the height and density difference of a canine's undercoat and topcoat (guard hairs). *Figure source: https://www.dyson.com.au/pets/dog-grooming.aspx*

Preliminary canine hair testing concluded with the amplification of undercoat and topcoat hair samples. Single topcoat hairs were prepared as per Figure 4.4, however due to the thin and static nature of undercoat hairs, more were added to the 0.2 mL PCR tube. Small tufts of approximately ten hairs were used instead of a single hair in order to increase the amount DNA available for amplification. Undercoat hairs showed higher signs of peak imbalance and allelic dropout than topcoat hairs, yet still resulted in full profiles for most samples tested (Figure 4.7).

With the DogFiler multiplex optimised for use, and the knowledge of what hair types are successful, a more in-depth study was conducted (*section 4.3*) to discover the full potential of using canine hairs with direct PCR.

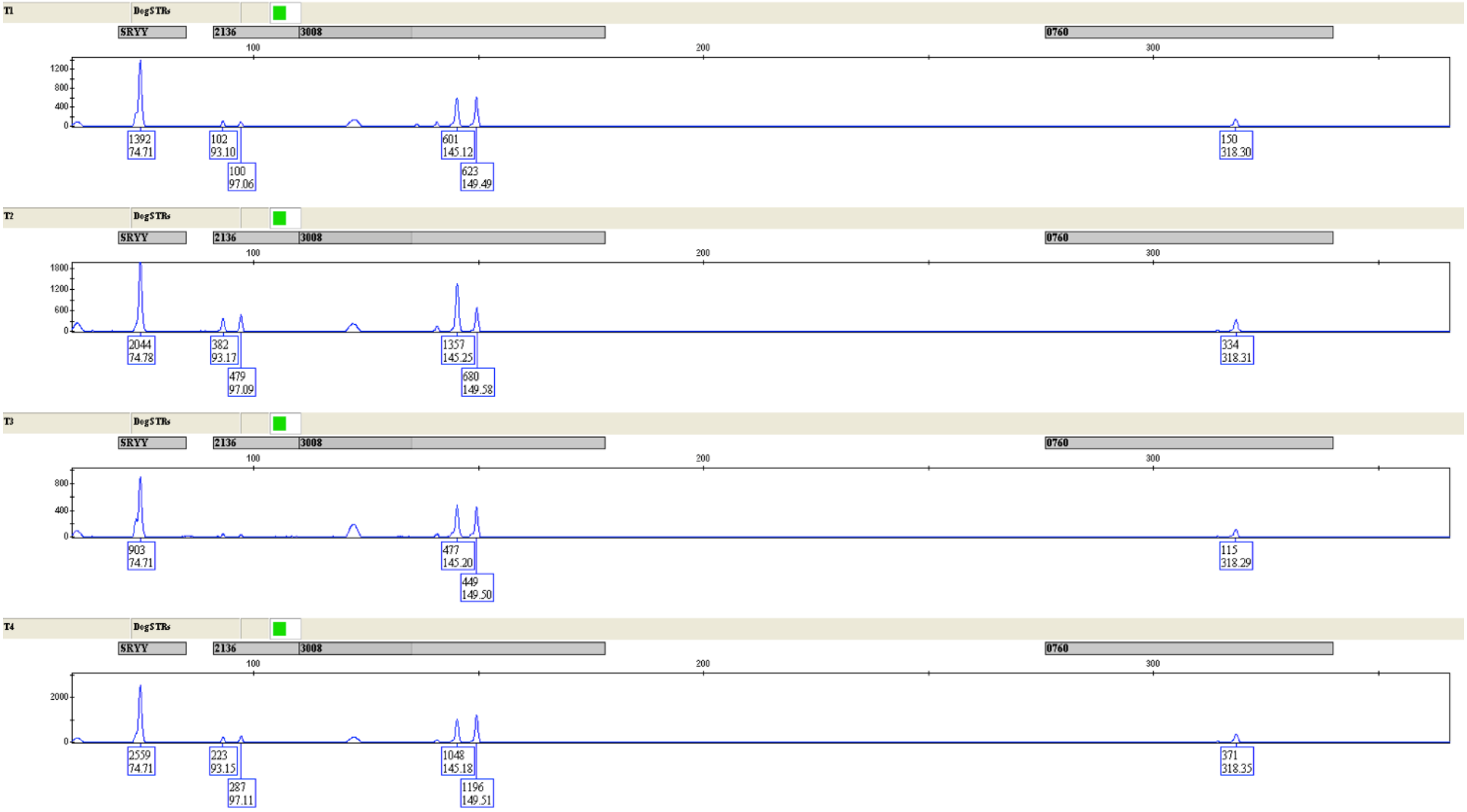


Figure 4.7 – Four chromatograms of nuclear canine DNA samples: shed undercoat hairs, amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9600 thermal cycler. Some allelic dropout and peak imbalance can be observed.

4.3 Successful direct amplification of nuclear markers from single dog hairs using DogFiler multiplex

Statement of authorship

Successful direct amplification of nuclear markers from single dog hairs using DogFiler multiplex

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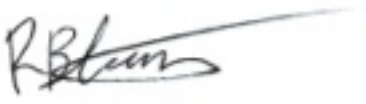
Date: April 2015

Renée Blackie (Candidate)

Performed all laboratory work (primer selection, multiplex set-up, sample collection, DNA extractions and quantifications, PCR amplification, capillary electrophoresis), data analysis and interpretation, created data tables, and wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

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Short Communication

Successful direct amplification of nuclear markers from single dog hairs using DogFiler multiplex

We report on successful amplification of canine STR DNA profiles from single dog hairs. Dog hairs are commonly found on clothing or items of interest in forensic casework and may be crucial associative evidence if linked to an individual dog. We used direct amplification from these hairs to increase the DNA yield of the sample, as well as greatly reducing analysis time. Hairs from different somatic regions were used from several different dog breeds to amplify a selection of eight loci from the validated DogFiler multiplex. Naturally shed canine hairs were processed, with a mix of coarse topcoat (guard) hairs and thinner soft undercoat hairs. Multiple sections of single hairs were amplified in 5 mm segments to determine the viability of DNA recovery from the shaft of the hair. Single guard hairs were cut into 5 mm sections and added directly into a PCR tube. Undercoat hairs, which are very fine, were amplified together in a single tube (approximately ten small hairs). Coarse hairs were found to be the most successful in producing full DNA profiles at all eight loci, matching the corresponding reference profile for that dog.

Keywords:
Direct PCR / Dog hair / Forensic identification / Nuclear DNA / Short tandem repeat
DOI 10.1002/elps.201400560

It is estimated that 36–40% of Australian and American households own a domestic dog (*Canis lupus familiaris*) (http://www.acac.org.au/ACAC_Report_2010.html) [1]. This leads inevitably to a high level of contact between owners and their pets with the result that biological samples such as hair from dogs readily transferred from the dog to the owner or any person making contact with the dog. Shed dog hairs can therefore be an important piece of forensic evidence in investigations due to these transfer events.

Naturally shed hairs are the most commonly found hair types at crimes scenes. These telogen hairs have finished the growth cycle and lack the sheathing and root bulb, which typically contain high amounts of nuclear DNA required for successful profiling [2–4]. Mitochondrial DNA (mtDNA) has a much higher copy number within cells, which has made it the primary focus of DNA analysis involving the domestic dog [5–10]. Additionally animal DNA found at crime scenes can present degradation challenges caused by environmental exposure [11].

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Abbreviations: BH, brushed hair; **CH**, hair collected from clothing; **mtDNA**, mitochondrial DNA; **RFU**, relative fluorescence unit; **SH**, hair collected from surfaces

The use of mtDNA on dog hairs in casework can be associative evidence in linking a person to a place or item, however this can be complicated due to heteroplasmy within the mitochondrial DNA [7]. STR analysis of nuclear DNA has a higher discriminating power when it comes to cases with inbred or small populations [1, 12], and the introduction of miniSTRs has helped cope with issues surrounding degraded DNA [11].

The successful analysis of mtDNA from dog hairs has been reported [13], however only limited data [11, 14] or anagen hair data from one canine individual [15] exists for nuclear DNA STR analysis from dog hairs. Previous studies conducted on human hairs and human fingernails indicate the most likely source of directly amplified DNA is from nucleated cells or cell-free DNA on the surface of the hair or nail [3, 16, 17]. Studies implementing direct PCR methods on single fibres and touch DNA samples also indicate that the likely source of DNA, nucleated, or cell-free, is present on the surface of the sample [18–20]. Studies into direct PCR of human hairs showed an increased success rate compared to methods that use a DNA extraction step [3]. The aim of this study was to demonstrate the success rate of nuclear DNA recovery from single dog hairs that represent forensic casework scenarios thus allowing routine STR typing of a single shed hair. By implementing a direct PCR approach to processing dog hairs, the DNA yield can be greatly increased as DNA is

Colour Online: See the article online to view Fig. 1 in colour.

Table 1. Sample breakdown of the type of hair used per sample collection for each breed of canine

Dog breed	Sample collection BH ($n = 4$)	SH ($n = 4$)	CH ($n = 2$)
Golden Retriever 1	4 × U	3 × G 1 × M	2 × M
Golden Retriever 2	4 × U	4 × G	2 × M
German Shepherd	4 × U	3 × U 1 × M	2 × M
Kelpie x Staffy	4 × G	4 × G	2 × G
Bull Terrier	4 × G	4 × M	2 × G

Four brushed hair (BH) samples, four surface hair (SH) samples, and two clothing (CH) samples were collected from each canine. These are categorized as guard hair (G), undercoat hair (U), or a mixture of both (M).

not lost to the extraction process, as well as reducing the time and costs involved in processing evidence.

Preliminary studies conducted on anagen and telogen canine hairs, identified by microscopy, indicated that there was no observable difference in success rates between the two growth phases for obtaining DNA profiles using direct PCR (data not shown). Plucked hairs and shed hairs were provided with informed consent from one owner. All plucked hairs were identified to be anagen, and all shed hairs collected were identified to be telogen. Since casework samples are more likely to be shed hairs (telogen), the decision was made to only use this sample type in further testing. DAPI (4',6-diamidino-2-phenylindole) fluorescent staining was not considered for this study, as part of the aims is to minimize time and resources spent on processing the samples, as well as avoid the loss of DNA through washing or staining techniques.

Hair samples were collected from randomly selected domestic dogs (*Canis lupus familiaris*), comprising of three male and two female individuals. The breeds included two Golden Retrievers, a German Shepherd, Bull Terrier, and an Australian Kelpie × Staffy (Staffordshire). The owners of the canines provided informed consent in all cases. A buccal swab was collected from each of the dogs to act as reference and three hair collections: freshly brushed hair (BH) to represent freshly shed hairs; hair collected from surfaces such as couch or floor (SH) that represent hairs of unknown age; and hair collected from the owners' clothing (CH) to mimic typical case scenarios. Hairs ranged in length from approximately 2–7 cm. A total of 50 samples were processed, ten from each of the five dogs. The ten samples included four BH, four SH, and two CH from each dog, with hair types within these samples ranging from thick guard hairs to thin fluffy undercoat hairs, or a mixture of both, as outlined in Table 1. To maximize results, hairs collected within a single area or source were used in one reaction, with no discrimination of the type of hairs found within a collection; therefore some samples processed resulted in a mixture of undercoat and guard hairs. Hair samples were not treated in any manner prior to amplification in order to minimize the loss of DNA that may be present on the surface of the hair. Previous studies have demonstrated poor success rates in yielding sufficient autosomal DNA from human and canine hairs using standard extraction methods [2, 3, 11], and therefore were not attempted in this study.

As this is a proof-of-concept study, only eight of the 16 DogFiler loci were chosen, covering the complete range of fragment sizes from ~70 to 350 bp. This range and number of loci represent the size and ranges similar to that of human STR kits, such as Profiler Plus®, with sufficient loci to provide high powers of discrimination. The DogFiler assay [1] was favored over the Mini-DogFiler assay [11] for several reasons. The DogFiler multiplex was selected due to its similar design to human identification kits in regards to the range and size of loci included, as successful sampling of human hairs using direct PCR methods with standard human identification kits has been documented [3, 21]. Preliminary testing of DogFiler loci indicated no issues with amplification from single dog hairs. The DogFiler panel has also been integrated into forensic casework and accepted in courts across the United States [1]. Half-volume reactions were also utilized in the Mini-DogFiler study, but were not considered in this study due to the nature of direct PCR (sample placed directly into PCR fluid). The interest and focus was to determine the success of canine hairs with direct PCR methods by implementing standard procedures with validated protocols with little to no changes.

The eight chosen loci for this study included: VGL0760, VGL2136, VGL3008, SRY (Y), VGL1063, VGL2009, VGL1541, and VGL3235. The final primer concentrations in a 26 μL reaction was 0.19 μM for all primers, except for primer sets VGL2009 and VGL2136 where it was increased to 0.38 μM to help balance the multiplex. Primer sets were tagged with either VIC® or 6-FAM dye (Applied Biosystems).

Direct PCR was conducted by placing one or more hairs cut into 5 mm segments, prepared using sterile scissors and tweezers, into a 0.2 mL thin walled tube. Additionally, 13 μL of PCR master mix from the QIAGEN® multiplex PCR kit (Qiagen, Victoria, Australia) along with 10 μL of the primer mix was added to the PCR tube. A further 3 μL of sterile H_2O were added to make the final volume 26 μL. The amplification was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies). The PCR began with a 15 min activation step at 95°C, followed by 31 cycles of 30 s at 94°C, 90 s at 61°C, 1 min at 72°C, and a final extension for 30 min at 60°C. The PCR conditions follow the recommended conditions for the primers [1] and enzyme used by QIAGEN. There was no alteration to the methodology of amplification deliberately to ensure that the forensic science community could adopt the process readily.

DNA was isolated from buccal swabs using a QIAGEN® Mini kit (Qiagen, Victoria, Australia) following the manufacturer's protocol. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies). From these extracts 1 ng was used in the PCR using the same conditions as for the hair samples.

Separation of the PCR products was performed on an ABI 3130*xl* Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies). An aliquot of either 1 μL or 1 μL of a 1 in 30 dilution into H_2O of the PCR sample was added to

Table 2. Summary of DNA profiles obtained using eight loci from the DogFiler STR multiplex, using canine hair from five donors, total samples $n = 50$

Hair type	Number of samples			
	0 alleles	≤ 4 loci	≥ 5 loci	Full profile
Guard			7	16
Undercoat	8	11		
Both	1	2	4	1
Total comparable profiles			28	

a solution of 0.5 μL of ABI GeneScan-600 LIZ® Size Standard and 9.5 μL of Hi-Di™ Formamide (Life Technologies). Samples were then denatured at 95°C for 3 min. Electrophoresis was conducted at 3 kV with a 10 s injection. The data were analyzed using Genemapper® v3.2. (Life Technologies). The detection threshold was set at 50 relative fluorescence units (RFU).

A total of 50 dog hair samples from five individuals were amplified directly using eight loci from the validated DogFiler multiplex system (see Table 2). A full profile, comprising of all eight STR loci, was obtained from 17 samples (an example of which is seen in Fig. 1).

In human identification, a DNA profile consisting of five or more complete STR loci can be uploaded to the Australian National Criminal Investigation DNA Database (NCIDD), and is considered discriminating enough to minimize occurrences of adventitious matches. For this study, we have also considered all profiles of five or more complete STR loci to be "comparable" using population frequencies calculated from published data [1]. Comparable but not full profiles were obtained in an additional 11 samples.

There was an assumption that BH, being the most recently shed of the hair collections, would have the least degraded DNA and therefore give better profiles overall. However, there was no distinguishable pattern of success rate when comparing samples between the three different collection types (BH, SH, and CH). When comparing hair type, guard, and undercoat, there was a significantly higher success rate with guard hairs. When the 50 samples were categorized by the hair type, a link between success rates could be established. Guard hairs were present in 23 samples and all produced five or more complete loci, whereas all 19 undercoat hair samples failed to produce meaningful data. The thicker structure and nature of the guard hairs may allow for DNA to adhere easier to the surface than the thinner undercoat hairs. Eight samples contained a mixture of guard and undercoat hairs with 62.5% ($n = 5$) producing comparable profiles. It is not clear as to why only five of the eight mixed samples gave a comparable profile considering the 100% success rate of the guard hairs on their own. The hairs do not appear to dissolve or break down during the PCR process, however the thinner nature of the undercoat hairs may leave them more susceptible to breaking down during the PCR process, releasing some melanin, a known PCR inhibitor [22–24]. A second suggestion is that there simply

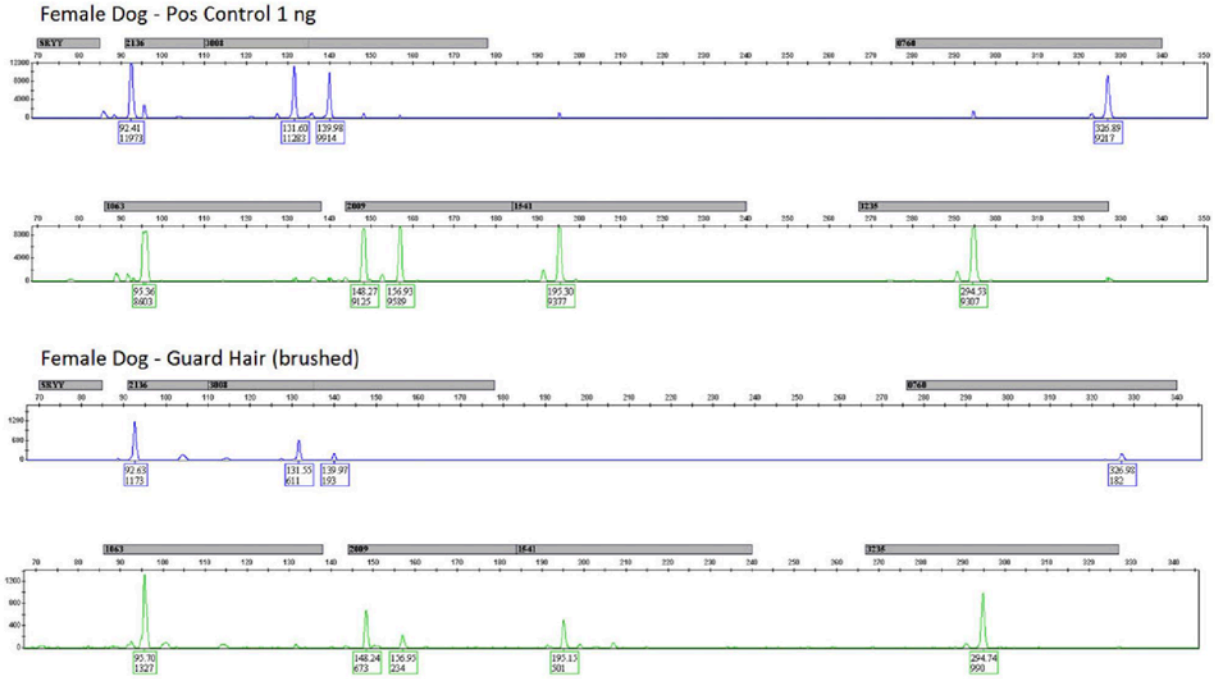

Figure 1. Chromatograms showing a positive nuclear DNA control sample compared to a single guard hair (brushed sample) from the same individual, exhibiting a matching full DNA profile.

may not be enough DNA present on the hairs of those particular samples processed. There was no discernible pattern to show that one breed of dog gave better profiles than another (data not shown). The proportion of hair types used in samples (guard, undercoat, or mixed), Table 1, were randomly selected and do not allow for equal comparison between dog breeds. In total, 56% of samples tested resulted in comparable profiles.

All DNA profiles obtained from hairs matched the DNA profile of the reference data of the donor dog. Allele sizes in this study were calculated using the fragment size obtained and published data [1] (http://www.cstl.nist.gov/strbase/dogSTRs.htm), as an allelic ladder was not available, and was not necessary to determine if the hair profiles and buccal swab profiles matched. Some profiles exhibited split allele peaks, typical of over amplification or inhibition during amplification. A dilution of the final PCR product reduced these affects as shown in previous studies [3] without causing allelic dropout. The average heterozygous peak balance (defined as the lower intensity peak divided by the higher intensity peak) of all direct amplification samples was observed to be 73.4%. Allelic dropout occurred in the expected range (larger loci) and drop-in was not observed in any sample. The average RFU value was 1819 for all samples that generated data.

By implementing a direct PCR approach to canine hair samples, we have demonstrated that nuclear STR profiles can be generated routinely from a single guard hair even if the time since being shed is unknown. There is a reduction in time and costs by omitting the extraction step and there is no loss of DNA during the extraction process allowing single hairs to be processed successfully. We make the recommendation of only processing guard hairs for direct PCR due to the higher success rate of obtaining comparable data for statistical analysis.

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The authors have declared no conflict of interest.

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4.4 Applications

4.4.1 Casework

Flinders University DNA Laboratory was asked by FSSA and SAPOL to analyse DNA samples obtained from a latex glove and determine if canine DNA was present and if that canine DNA profile matched a DNA extraction from exhumed canine bones (Case 2 and Case 3). The DNA results were presented as evidence at *R v Lowe*, a decade old attempted murder case. This section contains copies of: the joint statement of Renée Blackie and Adrian Linacre; the PCR set-up conditions; the DNA profiles obtained from the case evidence and five positive control samples (Figures 4.8 – 4.12); the calculated allele frequencies; and a news report of the final case ruling.

In a separate case, Flinders University DNA Laboratory was asked by FSSA and SAPOL to analyse a DNA extract and determine if canine DNA was present (Case 4).

Case 2

Determining the presence of canine DNA in an extraction obtained from a glove

STATEMENT OF WITNESS

Statement of:	Renée Ottens	Adrian Linacre
Occupation:	Forensic Scientist	SA Justice Chair in Forensic Science
Address:	Flinders University Adelaide, SA	Flinders University Adelaide, SA

Date:

This statement (consisting of 3 pages) each signed by us, is true to the best of our knowledge and belief and we make it knowing that, if it is tendered in evidence, we shall be liable to prosecution if we have wilfully stated anything that we know to be false or do not believe to be true.

Instruction

We were instructed by Dr Andrew Donnelly to determine if DNA from a dog was present within two samples.

Items Received

Samples 173-53.A and 173-53 were received from Dr Donnelly on 3 April at Forensic Science South Australia. The samples were transferred to the Forensic DNA Laboratory at Flinders University where the analyses were performed.

Results

A full canine specific DNA profile (8 loci from the DogFiler multiplex) was generated from sample 173.53.A and 173.53. The DNA profiles were the same. The DNA data indicate that the DNA came from a male dog.

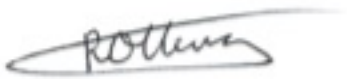
Conclusion

It is our conclusion that:

DNA from a dog (*Canis lupus familiaris*), or another member of the Canis genus, was present with both samples provided.

The data are as expected if DNA from the same male dog was present in both samples. If the DNA came from two different dogs then the DNA profiles must match by chance. It is not possible to state this chance event without access to a relevant database of DNA types within the local dog population.

Signed



Renée Ottens, BSc. (Hons)

Signed

Adrian Linacre, B.Sc. (Hons), D.Phil.

APPENDIX: CANINE SHORT TANDEM REPEAT TYPING

The DNA profiling performed in this case is similar to that used in human identification and examines short tandem repeat markers (STRs). The STR markers used in case were published by Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework (*Wictum et al Forensic Science International: Genetics, 2013, 7:1 82-91*)

The DNA markers chosen have been shown to be the same for if the DNA test is performed on saliva, hair or blood.

The test used is designed to work on the domestic dog (*Canis lupus familiaris*). The markers will generate a DNA profile from DNA originated from other members of the dog genus including the grey wolf (*Canis lupus*) and the dingo (*Canis lupus dingo*).

As well as STR markers, a gender test is included in the test to indicate if the DNA came from a male or female dog.

The DNA markers are known to be highly variable even within breeds. When two different samples give the same DNA profile then these are the data expected if the DNA came from the same dog. It is possible to estimate the probability that the two DNA samples come from the same dog if there is a suitable reference database of the frequencies of the DNA types in the local dog population.

End of statement

Case 3

Determining if the canine DNA from Case 2 matches bone DNA from exhumed pet

STATEMENT OF WITNESS

Statement of:	Renée Blackie	Adrian Linacre
Occupation:	Forensic Scientist	SA Justice Chair in Forensic Science
Address:	Flinders University	Flinders University
	Adelaide, SA	Adelaide, SA

Date: 20th May, 2015

This statement (consisting of 4 pages) each signed by us, is true to the best of our knowledge and belief and we make it knowing that, if it is tendered in evidence, we shall be liable to prosecution if we have wilfully stated anything that we know to be false or do not believe to be true.

Statement

This statement is supplemental to or statement dated 2nd May 2014.

Instruction

We were instructed by Dr Andrew Donnelly of Forensic Science SA to generate a canine DNA profile from sample taken that we understand was taken from the bone of a deceased dog. If a DNA profile can be generated from this sample we were instructed to compare this DNA profile to that obtained from a sample taken from a glove (as detailed in our previous statement dated 2nd May 2014). If the profiles matched then an opinion should be provided as to the probability that the two samples come from the same dog compared to coming from two different and unrelated dogs.

It is assumed that the information provided is accurate. If new information is provided we are willing to alter our opinions.

Items Received

Samples 173_89.1A and 173_89.2A were received at the Forensic DNA Laboratory at Flinders University on 9th July from Sherryn Ciavaglia of FSSA. It is understood that these two DNA extracts had been obtained from a bone sample taken from a deceased dog. Extract 173_89.1A was given the code bone_1 and extract 173_89.2A given the code bone_2.

Results

Canine DNA testing was performed in the forensic DNA laboratory at Flinders University. Canine DNA profiles were generated from bone_1 and bone_2 (using the test described in the Appendix). Both canine DNA profiles from the bone sample were incomplete but come from a male dog. An incomplete DNA profile is typical of insufficient DNA present within the bone samples to that is required to generate a complete profile.

The canine DNA profiles obtained from bone extracts bone_1 and bone_2 were found to match each other as would be expected. The canine DNA profiles from bone_1 and bone_2 were found to match the canine DNA profile obtained from the sample taken from a glove (sample 173-53).

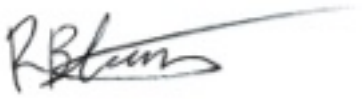
These are the DNA data expected if bone_1 and the DNA extract from the glove sample 173-53 came from the same male dog. If these two samples come from different dogs then they must match by chance. It is estimated that the chance of obtaining matching canine DNA profiles if the DNA from sample 173-53 came from a different and unrelated male dog as the bone extract bone_1 is in the order of 1 in 500 thousand.

These are the DNA data expected if bone sample bone_2 and glove sample 173-53 came from the same dog. If these two samples come from a different dog then they must match by chance. It is estimated that the chance of obtaining matching canine DNA profiles if the DNA from sample 173-53 came from a different and unrelated male dog as the DNA sample bone_2 is in the order of 1 in 500 thousand.

Conclusion

It is our conclusion that there is very strong support for the premise that the DNA taken from the glove came from the same dog as the DNA taken from a bone sample.

Signed



Renée Blackie, BSc. (Hons)

Signed

Adrian Linacre, B.Sc. (Hons), D.Phil.

APPENDIX: CANINE SHORT TANDEM REPEAT TYPING

The DNA profiling performed in this case is similar to that used in human identification and examines hypervariable regions in the dog genome called short tandem repeat markers (STRs). The STR markers used in case were published in the scientific paper 'Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework' (*Wictum et al Forensic Science International: Genetics, 2013, 7:1 82-91*).

The test used examines 8 hypervariable STR loci. As well as STR markers, a gender test is included in the test to indicate if the DNA came from a male or female dog.

The DNA markers chosen have been shown to be the same if the DNA test is performed on saliva, hair bone or blood.

The test used is designed to work on the domestic dog (*Canis lupus familiaris*). The markers will generate a DNA profile from DNA originated from other members of the Canus genus including the grey wolf (*Canis lupus*) and the dingo (*Canis lupus dingo*).

The DNA markers are known to be highly variable even within breeds. When two different samples give the same DNA profile then these are the data expected if the DNA came from the same dog. If the DNA samples come from two different dogs then the DNA profiles must match by chance. It is possible to estimate the probability that the two DNA samples come from the same dog compared to two different dogs if there is a suitable reference database of the frequencies of the DNA types in the local dog population. Allele frequencies were obtained from Wictum et al Forensic Science International: Genetics, 2013, 7:1 82-91 and a kinship factor of 0.05 was applied. In the case of an absent allele the 2p rule was applied.

End of statement

FUSA – DNA Profiling Laboratory PCR Set-Up Record

Case Number: 020/14 Operator: Renee Blackie Date: 04/04/14

DNA quant using Qubit:

Sample 53 – 0.05 ng/µL, 5 µL used in sample = 0.2 ng
Sample 53A – 0.037 ng/µL, 5 µL used in sample = 0.185 ng

PCR Tube ID	Sample used in PCR	Case ID
53	DNA extract from glove	173-53
53A	DNA extract from glove	173-53.A
NEG	Negative control, No DNA	n/a
POS	Positive control, DNA GM2 (2 ng total)	n/a

Operator Signature: _____ Witness: _____

Multi Mix Tables

	DogFiler	
# samples	**1**	2
2 x QIAGEN Buffer µL	**13**	26
Primer mix µL	**10**	20
H_2O	**-**	-
DNA	**5**	10
Final Volume µL	**28**	

Positive Control	
1	1
13	13
10	10
2	2
3	3
28	28

Negative Control	
1	1
13	13
10	5
5	5
0	0
28	28

Cycle conditions as per Wictum et al 2012, run on a GeneAmp PCR 9700 System.

	31 cycles			
95°C	95°C	62°C	72°C	72°C
1 min	30 sec	30 sec	1 min	30 min

FUSA – DNA Profiling Laboratory PCR Set-Up Record

Case Number: 020/14 Operator: Renee Blackie Date: 11/07/14

DNA quant using Qubit:

Sample 16450.1 – 0.075 ng/µL, not used, low DNA
Sample 16450.2 – 0.275 ng/µL, 5 µL used in sample Bone_2a = 1.375 ng

PCR Tube ID	Sample used in PCR	Case ID
Bone_2a	DNA extract from bone	173-89.2A
NEG	Negative control, No DNA	n/a
POS	Positive control, DNA	n/a

Operator Signature: _____ Witness: _____

Multi Mix Tables

	DogFiler	
# samples	**1**	1
2 x QIAGEN Buffer µL	**13**	13
Primer mix µL	**10**	10
H_2O	**-**	-
DNA	**5**	5
Final Volume µL	**28**	28

Positive Control	
1	1
13	13
10	10
2	2
3	3
28	28

Negative Control	
1	1
13	13
10	5
5	5
0	0
28	28

Cycle conditions as per Wictum et al 2012, run on a GeneAmp PCR 9700 System.

	31 cycles			
95°C	95°C	62°C	72°C	72°C
1 min	30 sec	30 sec	1 min	30 min

FUSA – DNA Profiling Laboratory PCR Set-Up Record

Case Number: 020/14 Operator: Renee Blackie Date: 04/08/14

DNA quant using Qubit:

Sample 16450.2 – 0.275 ng/µL, 10 µL used in sample Bone_2b = 2.75 ng

PCR Tube ID	Sample used in PCR	Case ID
Bone_2b	DNA extract from bone	173-89.2A
NEG	Negative control, No DNA	n/a
POS	Positive control, DNA	n/a

Operator Signature: _____ Witness: _____

Multi Mix Tables

	DogFiler	
# samples	**1**	1
2 x QIAGEN Buffer µL	**20**	20
Primer mix µL	**10**	10
H_2O	**-**	-
DNA	**10**	10
Final Volume µL	**40**	40

Positive Control	
1	1
20	20
10	10
2	2
8	8
40	40

Negative Control	
1	1
20	20
10	10
10	10
0	0
40	40

Cycle conditions as per Wictum et al 2012, run on a GeneAmp PCR 9700 System.

	31 cycles			
95°C	95°C	62°C	72°C	72°C
1 min	30 sec	30 sec	1 min	30 min

Canine DNA Profiles

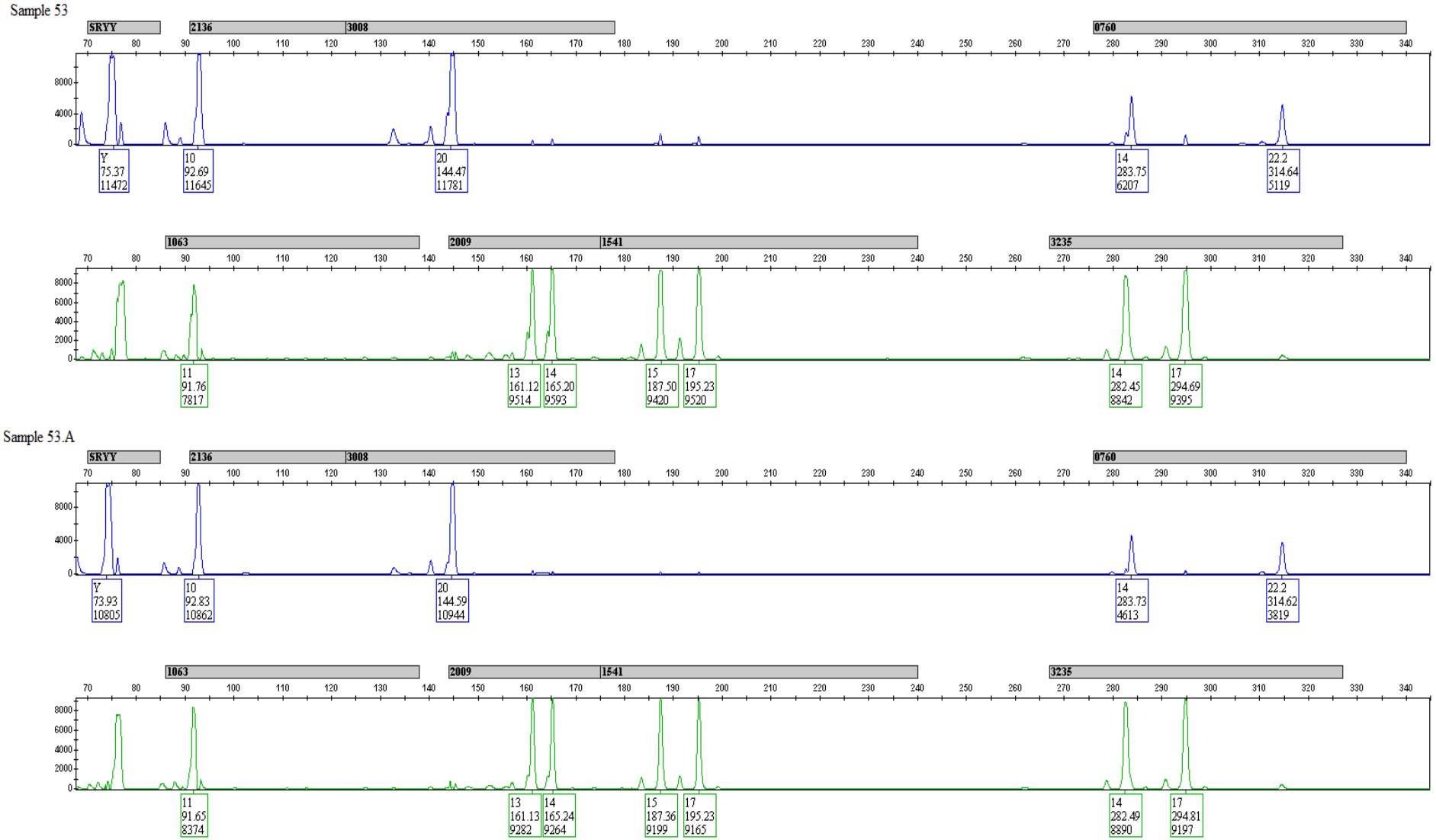


Figure 4.8 – Two chromatograms of nuclear canine DNA from glove samples 53 and 53.A, amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

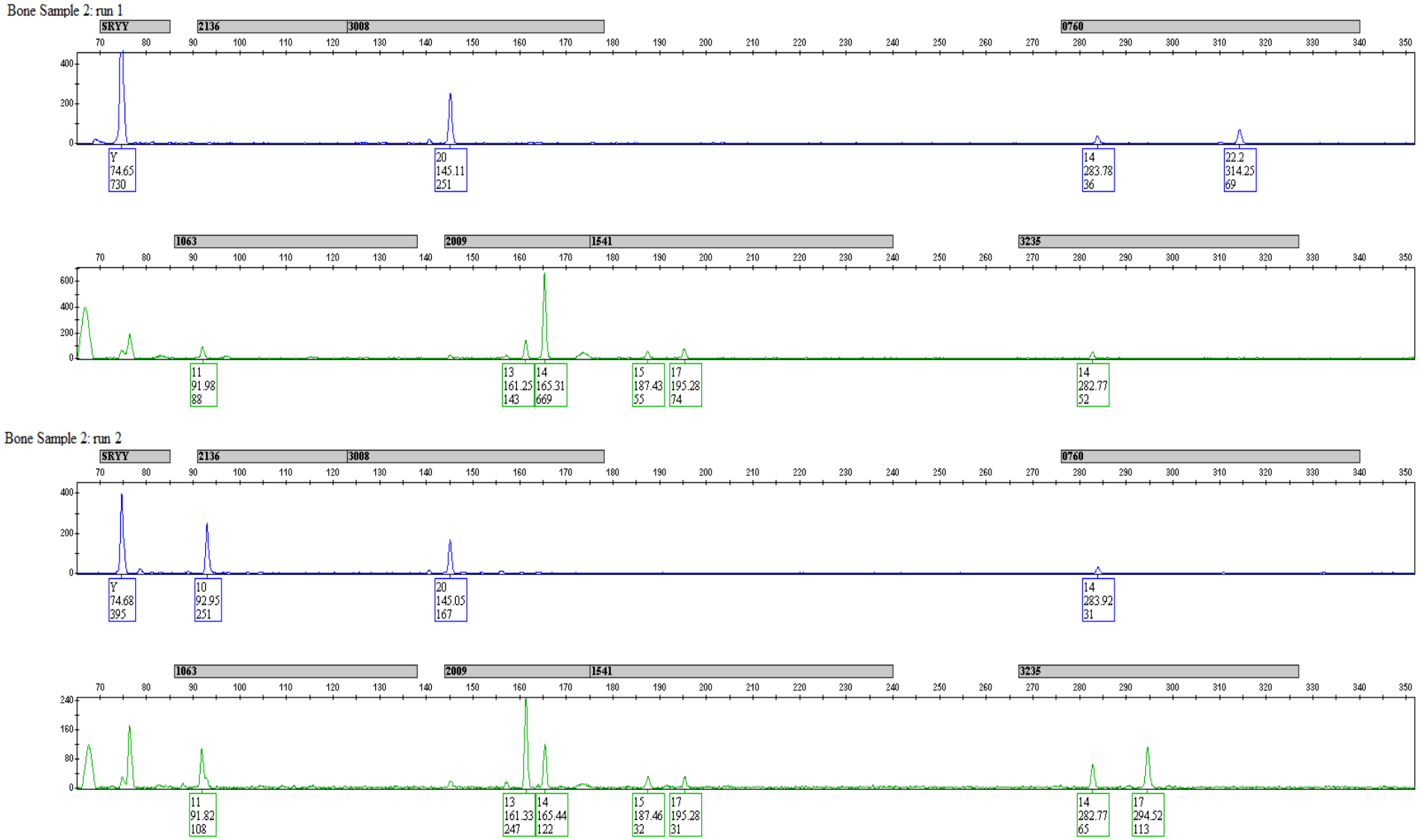


Figure 4.9 – Two chromatograms of nuclear canine DNA from Bone Sample 2 (run twice), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

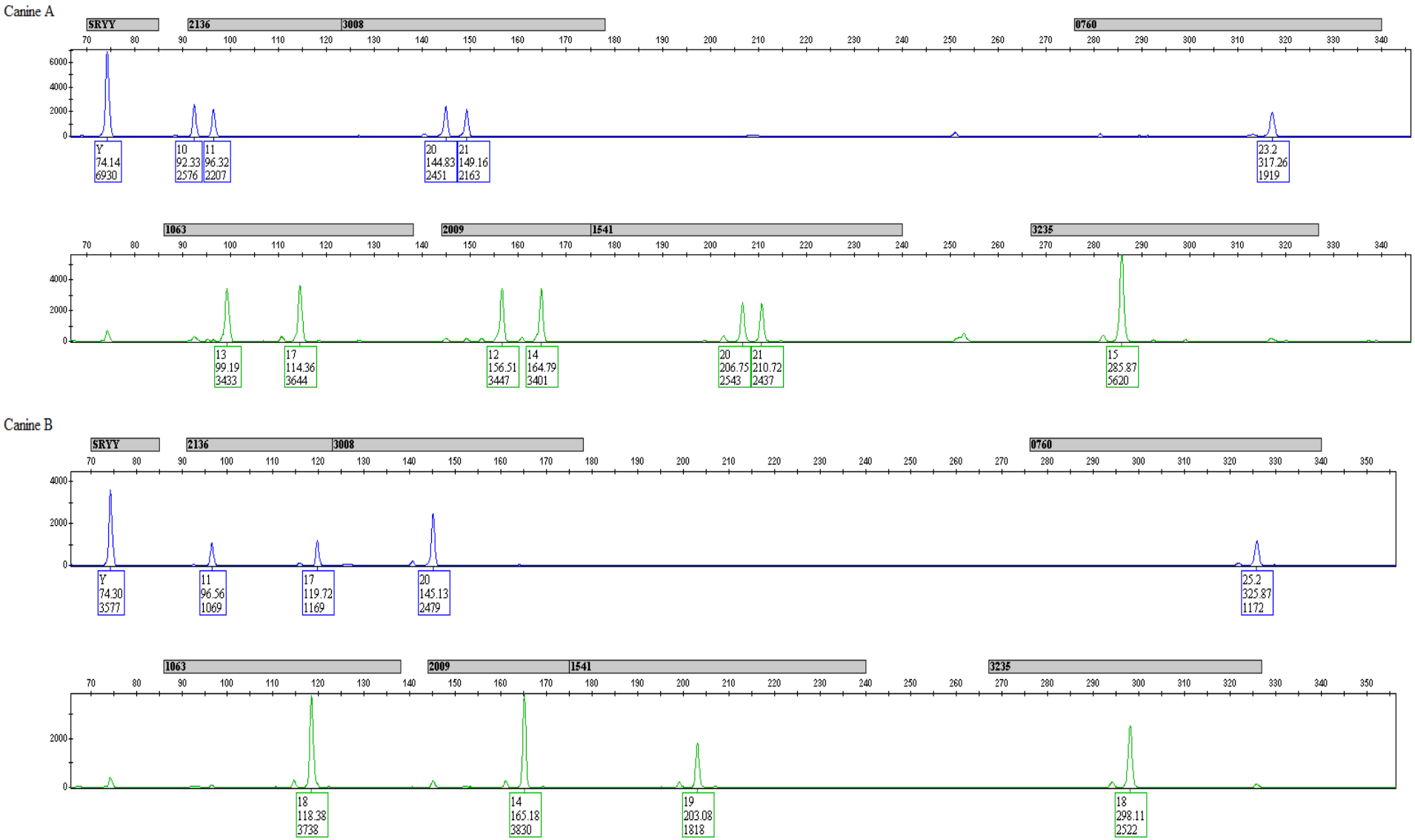


Figure 4.10 – Two chromatograms of nuclear canine DNA from positive control canines A and B (both male), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

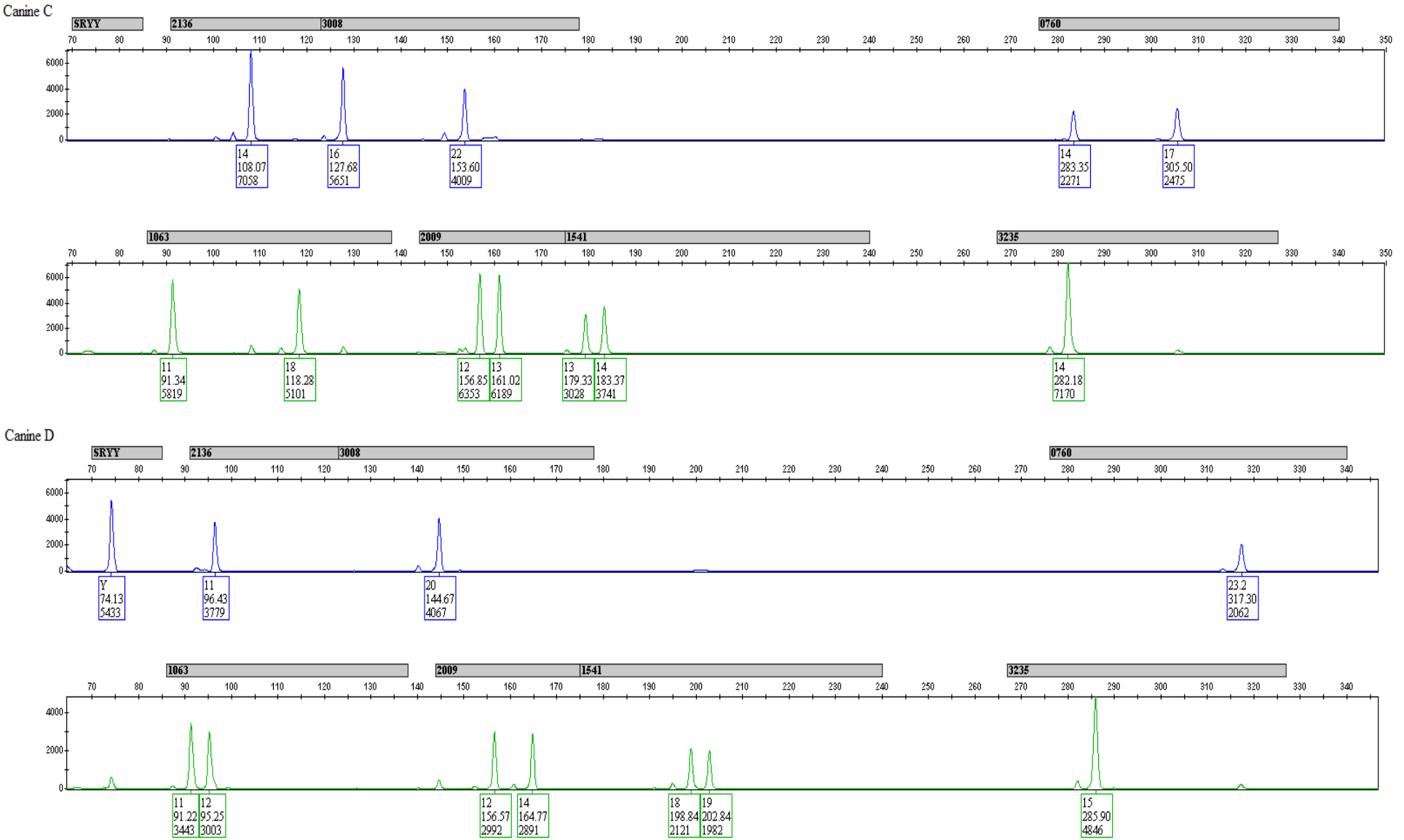


Figure 4.11 – Two chromatograms of nuclear canine DNA from positive control canines C and D (female and male, respectively), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

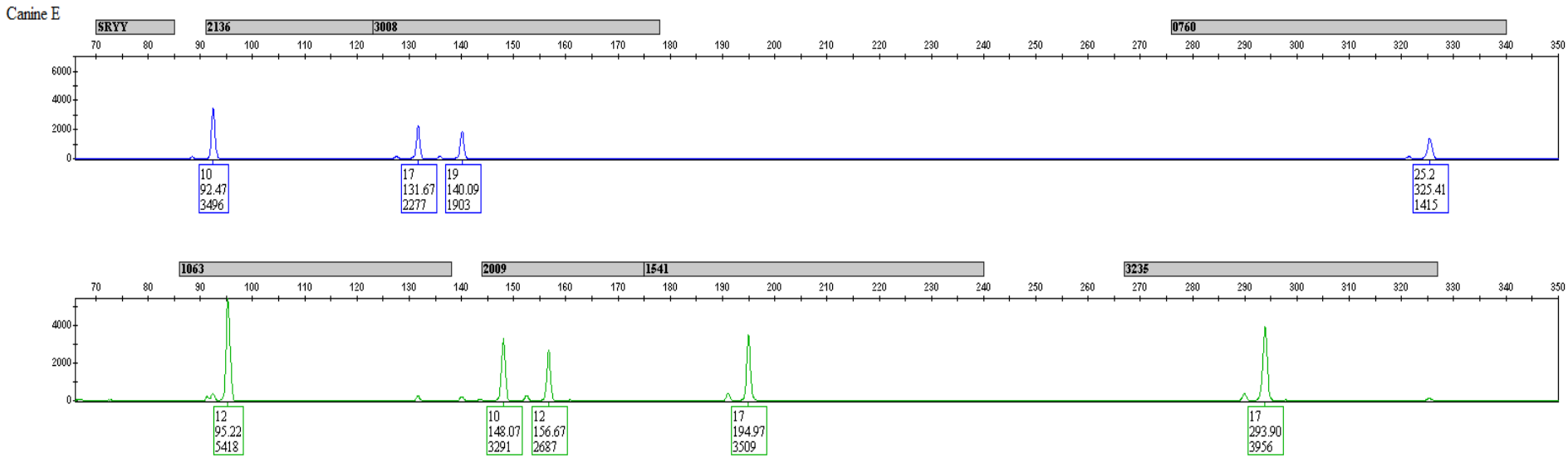


Figure 4.12 – Chromatogram of nuclear canine DNA from positive control canine E (female), amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

Allele Frequencies

Table of Canine STR Testing

Locus	SYR	2136	3008	0760	1063	2009	1541	3235
53 Glove	Y	10, 10	20, 20	14, 22.2	11, 11	13, 14	15, 17	14, 17
53.A Glove	Y	10, 10	20, 20	14, 22.2	11, 11	13, 14	15, 17	14, 17
Bone_2a	Y	NR	20, 20	14, 22.2	11, F	13, 14	15, 17	14, F
Bone_2b	Y	10, 10	20, 20	14, F	11, 11	13, 14	15, 17	14, 17

Allele Frequencies

Allele	Frequency	Gentotype Frequency
2136 10	0.12	10, 10 = 0.0144
3008 20	0.07	20, 20 = 0.0048
0760 14	0.08	14, 22.2 = 0.038
0760 22.2	0.11	
1063 11	0.02	11, 11 = 0.00039
2009 13	0.24	13, 14 = 0.146
2009 14	0.26	
1541 15	0.07	15, 17 = 0.049
1541 17	0.18	
3235 14	0.24	14, 17 = 0.066
3235 17	0.08	

⊙ = 0.0625 used. 2p rule used

Example Calculations

$\emptyset = \mathbf{0.0625}$

Heterozygous = $\frac{2[\emptyset + (1-\emptyset)Pi][\emptyset + (1-\emptyset)Pi]}{(1+\emptyset)(1+2\emptyset)}$

0760 14, 22.2

$= \frac{2[0.0625+0.075][0.0625+0.103]}{1.195}$

$= \frac{2(0.1375 \times 0.1655)}{1.195}$

$= \frac{0.0455}{1.195}$

$= 0.038$

Homozygous = $\frac{[2\emptyset + (1-\emptyset)Pi][3\emptyset + (1-\emptyset)Pi]}{(1+\emptyset)(1+2\emptyset)}$

2316 10, 10

$= \frac{[1.0625 \times 0.12][1.125 \times 0.12]}{1.195}$

$= \frac{(0.1275 \times 0.135)}{1.195}$

$= \frac{0.0172}{1.195}$

$= 0.0144$

News Report: Final Ruling

NEWS

Jury finds Nicholas Wayne Lowe guilty of attempted murder after attack with metal pole

By court reporter James Hancock

Updated Thu 30 Jul 2015, 5:25pm

A man jailed for stealing more than $1 million from the Victims of Crime Compensation Fund has been convicted of attempted murder.

The Supreme Court heard Nicholas Wayne Lowe, 33, attacked Nanette Clarke with a metal pole in her Port Lincoln home in August 2003.

A publication ban on Lowe's identity was automatically lifted after the jury delivered its unanimous guilty verdict.

It is Lowe's second trial on the current matter after a jury was unable to reach a verdict in June last year.

Lowe is already serving 10 years in jail for stealing from the compensation fund when he was a clerk in the Crown Solicitors office.

He made bogus compensation claims for family and friends and kept nearly half of the money for himself over a four-year period until his arrest in September 2012.

During the trial, Prosecutor Peter Longson told the jury that Lowe attacked Ms Clarke with a metal pole, causing serious brain damage.

He said she spent five weeks in hospital and still suffers memory problems.

"It was a year before she returned to full-time work or could drive a car," he said.

Mr Longson said Lowe's DNA profile was found on several objects at the crime scene, including a balaclava.

He said Ms Clarke's partner and son returned home to find her lying in her car.

"[She was found] unconscious, lying face first into the passenger foot well with the rest of her body and legs draped over and between the front seats," Mr Longson said.

"She was exposed from the waist down, she was just wearing a shirt on top, which is what she wore to bed."

He said there was a very large amount of blood in the house, with two significant areas around the dining table.

"There was a drag mark of blood that ran for three-and-a-half metres ... that led from the second area towards the garage door."

Lowe had been going out with the daughter of Ms Clarke's partner.

The case returns to court next week to address sentencing matters.

Topics: law-crime-and-justice, courts-and-trials, port-lincoln-5606, adelaide-5000

First posted Thu 30 Jul 2015, 4:18pm

Accessed: http://www.abc.net.au/news/2015-07-30/jury-finds-nicholas-wayne-lowe-guilty-of-attempted-murder/6660944

News Report: Final Sentencing

NEWS

Nicholas Wayne Lowe jailed for 20 years for attempted murder after attack with metal pole

By court reporter James Hancock

Updated Mon 14 Sep 2015, 12:20pm

An Adelaide man jailed for 10 years for stealing from a victims of crime fund has had 20 years added to his sentence for attempting to murder a Port Lincoln woman.

Nicholas Wayne Lowe attacked Nanette Clarke with a metal pole in her home on South Australia's Eyre Peninsula in August 2003.

She almost died from the attack.

The Supreme Court heard her partner and son returned home early from a fishing trip because of bad weather to find her lying in the foot well of her car.

Ms Clarke suffered a brain injury in the attack and still has problems with her memory, hearing loss and sense of taste and smell.

Lowe, 33, was not arrested for the crime until 2012, after DNA provided following his arrest on the victims of crime case was matched to the scene of the attack.

A jury unanimously found him guilty of attempted murder in July.

The judge set a new head sentence of 30 years for both crimes, with a non-parole period of 20 years.

Outside court, Ms Clarke welcomed the jail term.

"We are really very happy with the sentencing and look very much forward to just moving on and having some normality in our lives now," she said.

Justice Malcolm Blue described the attack as a "particularly serious version of attempted murder".

He said the motive for the "premeditated offence" was unknown.

Victim was shocked by Lowe's arrest

Lowe was dating the daughter of Ms Clarke's partner at the time of the attack.

The court was told Lowe was not considered a suspect by police.

Ms Clarke spoke in court of her shock at learning in September 2012 of Lowe's arrest for her bashing.

He had also been arrested for stealing more than $1 million from the Victims of Crime Compensation Fund.

Lowe stole the victims of crime money while working as a clerk in the Crown Solicitors Office.

Last December, Lowe was jailed for 10 years with a non-parole period of seven years for 26 counts of dishonestly dealing with documents.

Lowe will now also serve 20 years for Clarke's attempted murder, cumulative on the 10 years he is currently doing for the prior offending.

The judge said Lowe, who is married with a two-year-old son, is receiving support from family whilst in jail.

Accessed: http://www.abc.net.au/news/2015-09-14/nicholas-lowe-jailed-for-20-years-for-attempted-murder/6773244

Case 4

Determining the presence of canine DNA in a sexual assault allegation

STATEMENT OF WITNESS

Statement of:	Renée Ottens	Adrian Linacre
Occupation:	Forensic Scientist	SA Justice Chair in Forensic Science
Address:	Flinders University Adelaide, SA	Flinders University Adelaide, SA

Date: 3rd July 2014

This statement (consisting of 3 pages) each signed by us, is true to the best of our knowledge and belief and we make it knowing that, if it is tendered in evidence, we shall be liable to prosecution if we have wilfully stated anything that we know to be false or do not believe to be true.

Instruction

We were instructed by Mark Webster of Forensic Science South Australia (FSSA) to determine if DNA from a dog was present within a sample provided.

Items Received

Sample 565-2B was received into the Forensic DNA Laboratory at Flinders University from Sherryn Ciavaglia of FSSA on 4th June 2014. The sample consisted on one screw top tube containing a small amount of colourless fluid. The sample was stored securely at the Forensic DNA Laboratory prior to analysis.

Results

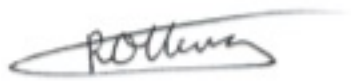
A full canine specific DNA profile was generated from sample 565-2.B. The DNA data indicate that the DNA came from a male dog.

Conclusion

It is our conclusion that:

DNA from a male dog (*Canis lupus familiaris*), or another member of the Canis genus, was present in the sample provided.

Signed



Renée Ottens, BSc. (Hons)

Signed

Adrian Linacre, B.Sc. (Hons), D.Phil.

APPENDIX: CANINE SHORT TANDEM REPEAT TYPING

The DNA profiling performed in this case is similar to that used in human identification and examines short tandem repeat markers (STRs). The STR markers used in case were published by Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework (*Wictum et al Forensic Science International: Genetics, 2013, 7:1 82-91*)

The DNA markers chosen have been shown to be the same for if the DNA test is performed on saliva, hair or blood.

The test used is designed to work on the domestic dog (*Canis lupus familiaris*). The markers will generate a DNA profile from DNA originated from other members of the dog genus including the grey wolf (*Canis lupus*) and the dingo (*Canis lupus dingo*).

As well as STR markers, a gender test is included in the test to indicates with the DNA came from a male or female dog.

The DNA markers are known to be highly variable even within breeds. When two different samples give the same DNA profile then these are the data expected if the DNA came from the same dog. It is possible to estimate the probability that the two DNA samples come from the same dog if there is a suitable reference database of the frequencies of the DNA types in the local dog population.

End of statement

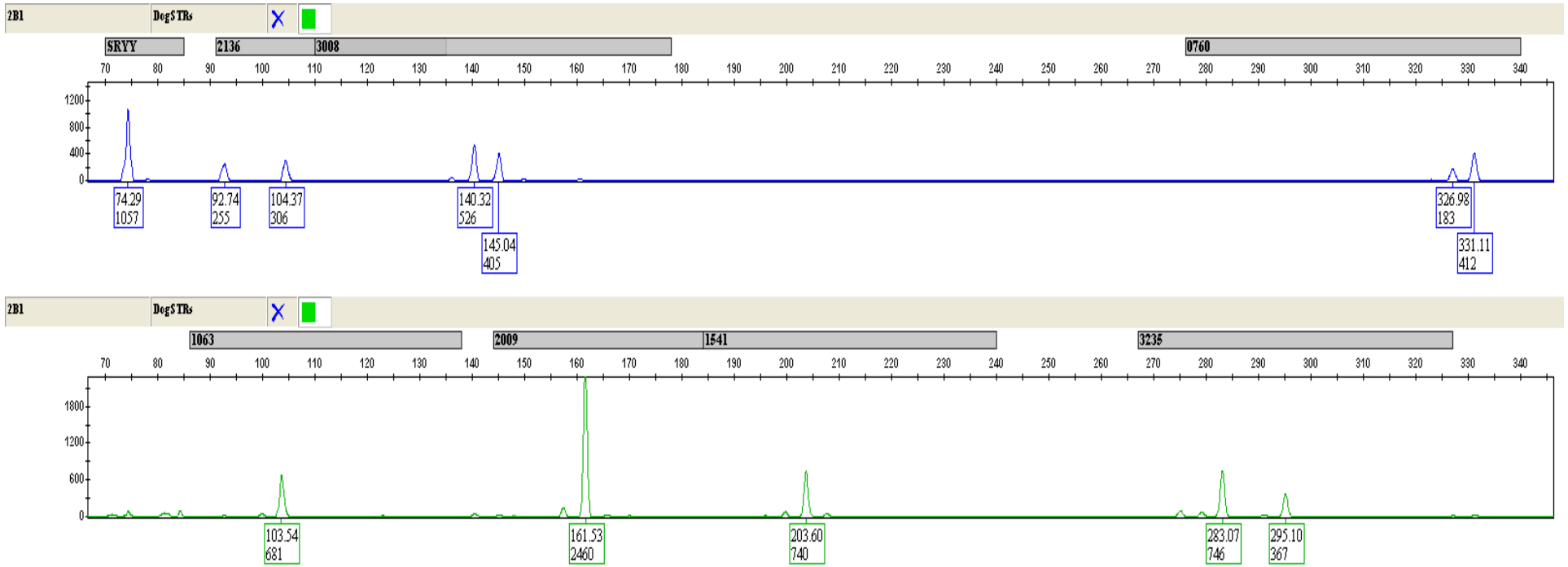


Figure 4.13 – Chromatogram of nuclear canine DNA from sample 565-2.B, amplified using QIAGEN® multiplex PCR kit and DogFiler loci at 31 cycles on a GeneAmp® System 9700 thermal cycler.

4.4.2 Cold Case Potential

The first time animal DNA was accepted as evidence in court was for the 1994 murder of Shirley Duguay, where the now infamous cat Snowball's hair implicated the murder suspect [6]. A man's leather jacket was found shortly after the discovery of the victim's bloodied abandoned vehicle. The jacket was also stained with the victim's blood and had many white domestic cat hairs inside the lining. The suspect, the victim's estranged common-law husband, lived with his parents and a white shorthaired pet cat named Snowball. One of the 27 hairs from the jacket contained enough root material for extraction and amplification of ten feline dinucleotide STR loci [7]. The results concluded that the hairs found on the coat came from Snowball, and were presented and admitted to the Supreme Court of Prince Edward Island. The suspect was found guilty of second-degree murder in July of 1996.

Since this landmark case, animal DNA amplified from hairs, faeces, urine stains, tissue samples or blood has been submitted as evidence in cases ranging from burglary, to animal abuse, sexual assaults and murder [8-16]. Although the use of animal DNA in criminal casework has continued since 1996, it is still relatively new to many countries. The first time feline DNA was submitted as evidence in a UK criminal trial was in 2012, where David Hilder was convicted for manslaughter after hairs from his pet cat were discovered on the dismembered torso of David Guy. Many cases, such as the one just mentioned, have focused on the use of mitochondrial DNA due to their high copy number and higher chance of profiling success. A shift to nuclear DNA genotyping has only emerged more recently as techniques become more sensitive [5, 17].

Direct PCR using human (*section 3.2*) and canine (*section 4.3*) hairs has shown a tremendous improvement on current methods in obtaining valuable nuclear DNA information. Both studies tested hairs of unknown age [18, 19], providing hope for cold cases where single hairs may not have been processed due to low success rates. Criminal investigations from the past, prior to routine DNA sampling, have a higher chance of human DNA contamination due to a lack of knowledge about DNA transference and DNA collection methods. Animal DNA contamination is a lot less likely to occur, making it an ideal target source for direct PCR. An unsolved Australian murder case mimics this scenario; Gerard Ross, aged 11, was abducted in October of 1997 and his body was found at a nearby pine plantation two weeks later [20]. Throughout the investigation, those collecting evidence had unfortunately contaminated it, thus making resampling and analysing with new techniques impossible. The discovery of dog hairs on his body and clothing gave hope to the investigation, as the family did not own a dog. It is unclear to what extent the dog hairs were tested and analysed, if at all.

4.5 Concluding Remarks

Similarly to human hairs, canine hairs can be successfully amplified using direct PCR, with both sets of data supporting the theory of nuclear and cell-free DNA being present on the surface of the hair, as well as in the root material. It can therefore be assumed that similar results would be achievable using hair from other animal types, such as the domestic cat. Criminal investigators all over the world are recognising the power of animal DNA in criminal casework, particularly where there may be no other linking or DNA evidence. Forensic scientists are now pooling their research data on animal genotypes, creating worldwide databases for domestic dogs and cats, ensuring that population frequency statistics can be applied in the same way human DNA is analysed [21-27]. Direct PCR of animal hairs can be a powerful tool in future criminal investigations, especially those of a time sensitive nature, as the analysis process is shortened without compromising the quality of the results.

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Supplementary Material

a. Canine allele frequencies

Raw data

Table 4a.1 – Calculated allele frequencies of the eight DogFiler loci.

Allele	VGL0760	VGL1063	VGL1541	VGL2009	VGL2136	VGL3008	VGL3235	VGL3438
7	0	0	0	0.0002238	0	0	0	0
8	0	0.0653536	0	0	0.1244405	0	0	0
9	0	0.0337959	0	0.0868397	0.2372426	0	0.0006714	0
10	0	0.0212623	0	0.0534915	0.1286929	0.0152193	0	0.0042525
11	0.0002238	0.0232766	0	0.0870636	0.0286482	0.0013429	0.0069382	0.0154432
12	0.0852731	0.0821397	0	0.032453	0.0579678	0.0217099	0.0660251	0.1931513
13	0.0747538	0.1483885	0.0013429	0.2497762	0.1284691	0.0445389	0.1651746	0.0931065
13.2	0.0006714	0	0	0	0	0	0	0
14	0.0875112	0.1998657	0.0078335	0.2670098	0.1347359	0.0447628	0.2482095	0.2238138
15	0.0080573	0.0890779	0.0700537	0.1935989	0.0980304	0.1662936	0.1421218	0.1264548
15.2	0.0004476	0	0	0	0	0	0	0
16	0.0017905	0.0250671	0.0989257	0.028872	0.0423008	0.1018353	0.1633841	0.1188451
16.2	0.0002238	0	0	0	0	0	0	0
17	0.0006714	0.0539391	0.1859893	0.0004476	0.0134288	0.1439123	0.0850492	0.0635631
17.2	0.0008953	0	0	0	0	0	0	0
18	0.0004476	0.1181737	0.2182184	0.0002238	0.0040286	0.171889	0.0897493	0.0523724
18.2	0.0465533	0	0	0	0	0.0002238	0	0
19	0.0015667	0.0868397	0.1611459	0	0.0020143	0.1573411	0.0183527	0.0485676
19.2	0.0476723	0	0	0	0	0	0	0
20	0.0011191	0.0407341	0.1101164	0	0	0.0749776	0.0091764	0.029991
20.1	0	0	0	0	0	0	0	0.0002238
20.2	0.1286929	0	0	0	0	0.0017905	0	0
21	0.0020143	0.0102954	0.0478962	0	0	0.0308863	0.0038048	0.0257386
21.2	0.1700985	0	0	0	0	0	0	0
22	0	0.0017905	0.0503581	0	0	0.0149955	0.0013429	0.0042525
22.2	0.1152641	0	0	0	0	0	0	0
23	0.0004476	0	0.0277529	0	0	0.0055953	0	0.0002238
23.2	0.1436885	0	0	0	0	0.0004476	0	0
24	0	0	0.0163384	0	0	0.0017905	0	0
24.2	0.061325	0	0	0	0	0	0	0
25	0.0008953	0	0.002462	0	0	0.0002238	0	0
25.2	0.0158908	0	0	0	0	0	0	0
26	0	0	0.0011191	0	0	0.0002238	0	0
26.2	0.003581	0	0	0	0	0	0	0
27	0	0	0.0004476	0	0	0	0	0
27.2	0.0002238	0	0	0	0	0	0	0
TOTAL	4468	4468	4468	4468	4468	4468	4468	4468
SUM	1	1	1	1	1	1	1	1

Appendix

i. **Oral Presentation** 22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Successful direct amplification of nuclear markers using single dog hairs with eight DogFiler loci*.

Presented by Renée Blackie.

Society for Wildlife Forensic Science (SWFS) meeting, Missoula, Montana, USA, 2015. *Successful direct amplification of nuclear markers using single dog hairs with eight DogFiler loci.*

Presented by Adrian Linacre.

Flinders UNIVERSITY

Government of South Australia
Forensic Science SA

Successful direct amplification of nuclear markers

using single Dog hairs

Renée Blackie (nee Ottens)
Supervisors – Adrian Linacre & Duncan Taylor

Direct PCR

Hair

Fibre

Swab

- Benefits:
  - Saves time
  - Saves money
  - Increases DNA yield
  - Can improve STR results
  - Works on challenging samples

Direct PCR Background

Sample Collection

2 hrs — DNA Extraction from Sample → Large DNA loss!!

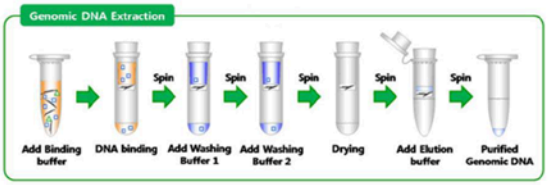
2 hrs — DNA Quantification → Needs ~0.5 - 2ng of DNA for PCR to work

2 - 3 hrs — PCR

2 - 3 hrs — Profile Analysis

1. Sample Collection

~~2. DNA Extraction~~



~~3. Quantification~~

4. PCR

Image from Bioneer http://eng.bioneer.com

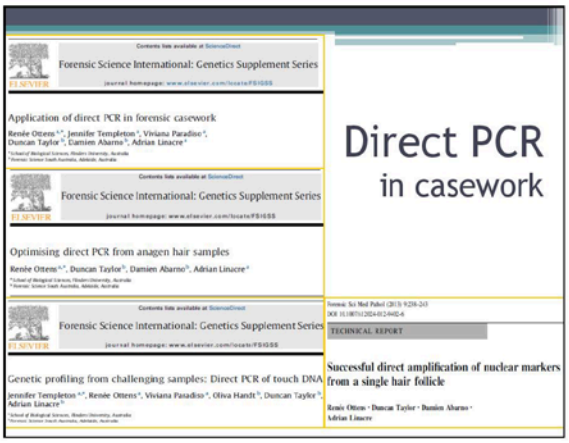
Flinders In-House Study

Extraction = up to 84 % of DNA lost

- Ultimately effects quality of STR profile

Starting DNA Concentration = 20 ng

Extraction Kit	Av Final Conc (ng/30 µL)	Av % Lost
Promega IQ	3.3	84
QIAGEN Micro	5.7	72



Canine STRs from single hairs

- Proof of concept study

Contents lists available at SciVerse ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework☆

Elizabeth Wictum [a,*], Teri Kun [a], Christina Lindquist [a], Julia Malvick [a], Dianne Vankan [b], Benjamin Sacks [c,d]

Primers

- DogFiler multiplex contains 15 loci + male sex determining marker
  - Eight loci ordered: 6-FAM → VIC →

Table 1
Locus information for DogFiler multiplex.

Locus name	Repeat location	Fluorophore or PIGtail	Primer sequence	Size range, bp	Tasha's profile repeat no.	Mutation rate
VGL0760	7: 60065445	6-FAM	F: gcagattccaggacaaagacca	276–340	13	0.0029
		Unstained	R: ggcccagaaaaggaaggagg			
VGL0910	9: 10224058	NED	F: acactttgctccacgttcct	282–350	13	0.0054
		GTTTCTT	R: acctttatgccaaaagtgtgt			
VGL1063	10: 63191724	PET	F: agccacagagcctgagagtg	86–138	11/12	None found
		GTTTCTT	R: caatcaccacctttcctcct			
VGL1103	11: 60306234	VIC	F: atctccccctggccaccacct	191–271	15/27	0.0027
		GTTTCTT	R: ggccctaaatccatgagctg			
VGL1541	15: 41210435	6-FAM	F: gagctctgtgtggaagagctta	184–240	17/19	0.0054
		GTTTCTT	R: catcctgtcctggtactctaa			
VGL1606	16: 6468079	PET	F: agcctttcgggggtcagatgt	272–340	20	0.0054
		GTTTCTT	R: ccacactgagctaaaatgc			
VGL1828	18: 28419883	NED	F: agatttgccctttggaaagt	220–284	19	None found
		GTTTCTT	R: cttttggctcctgctctgt			
VGL2009	20: 9290711	PET	F: ccatttaccagaatttgaagctg	144–184	12/13	0.0027
		GTTTCTT	R: cccgggaaaatttttctgaat			
VGL2136	21: 36673167	VIC	F: tgccaactgtttttaaagtaaca	91–135	14	None found
		Unstained	R: gcatggagaaaaagcaggtg			
VGL2409	24: 9197210	NED	F: aagcaggtgctcaacctctg	108–156	16/21	0.0027
		GTTTCTT	R: aggatagacctccatcaactgacca			
VGL2918	29: 18216971	PET	F: gatttctttcgtggatatgctgttt	188–260	15	None found
		Unstained	R: ggaaaatgtgtttttccctcca			
VGL3008	30: 8845920	6-FAM	F: agaacacggttatttgctaggc	110–178	18/19	0.0027
		Unstained	R: aagaggccaacgcagcagtgt			
VGL3112	31: 12944088	NED	F: agccaatagagcattaagtagagctg	185–217	16/17	0.0027
		gtttctt	R: ttgtgtaatgtgtgaatttaaggaaat			
VGL3235	32: 35527890	VIC	F: ggcgactcttcccctttctt	267–327	15	None found
		GTTTCTT	R: tctggaactgagcaatgtcgaaaat			
VGL3438	34: 38458581	VIC	F: acgcttgtgggtgctacact	136–188	14/21*	0.0027
		GTTTCTT	R: agcagtgatggcagagatgg			
SRY	Y	NED	F: gaacgcattcttggtgtggt	80	Neg.	None found
			R: tgatctctgagtttgcattttgg			

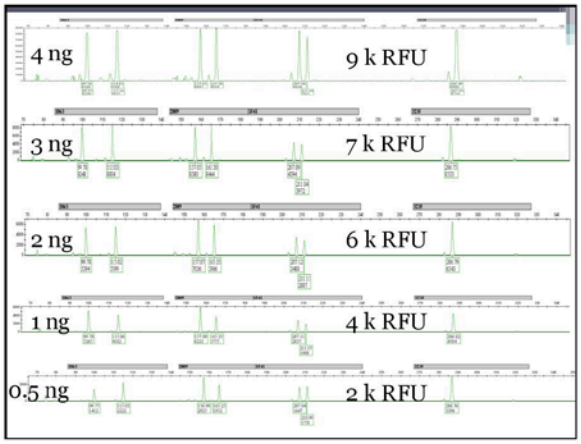
DogFiler

- No Ladder
  - Must run positive control + hair sample each time
- Create Panel + Bin Sets
- Optimal DNA Concentrations unknown
  - Human DNA 23 chromosomal pairs = 1 ng
  - Canine DNA 39 chromosomal pairs = ?
- Primer Concentrations unknown
  - Not listed in papers
- PCR:

	31 cycles			
1 min	30 s	30 s	1min	30 min
95°	95°	62°	72°	72°

Limit of Detection

- What can be detected by DogFiler?
- Two dogs – 1 male, 1 female
  - 4, 3, 2, 1, 0.5 ng of DNA
  - Repeated twice each
  - 2 - 3 ng best results – balanced peaks
  - Still strong detection at 0.5 ng



Canine Hair Types

- Telogen & Anagen
- Top Coat/ Guard hairs – thick, coarse
- Under Coat – thin, fluffy
- What is more likely to be seen in a casework scenario?

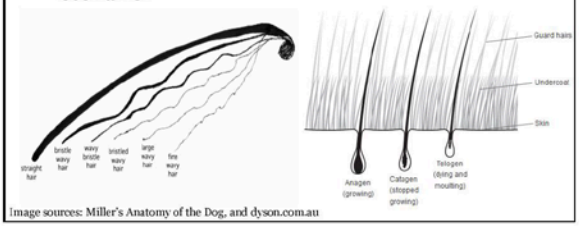
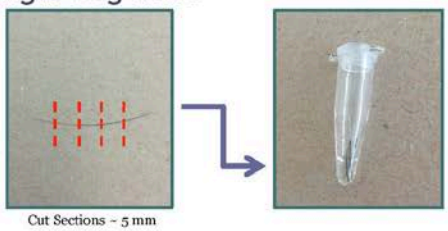


Image sources: Miller's Anatomy of the Dog, and dyson.com.au

Single Dog Hairs

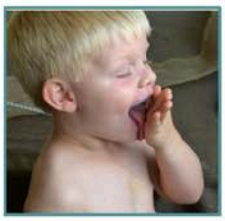


Cut Sections ~ 5 mm

Theory: Nuclear DNA present on surface of hair
- Nucleated cells or cell free DNA

Source: Skin secretion

Canine Vs Human Hygiene



Canine Results

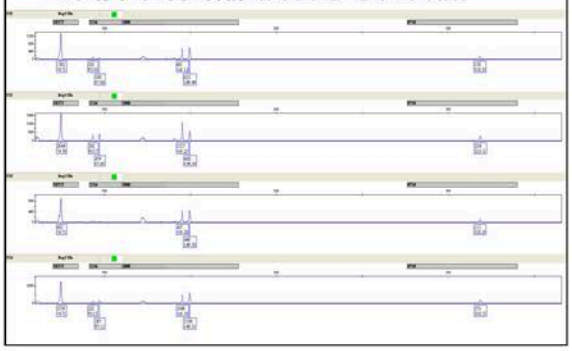
- Telogen (guard hair)

- Anagen (guard hair)

- Pos

Canine Results

- Tufts of under coat - several small hairs in one tube



Prelim Experiment

- Results showed no difference between anagen and telogen hairs
- Telogen (shed) hairs would best represent casework scenario
- Not necessary to forcefully take anagen hairs from dogs

- Note: Canine telogen hair performed better than human telogen hair

Experiment

- 5 dogs of random breeds
  - 3 male, 2 female
- Hair samples and buccal swabs collected (ref)
  - Hair freshly brushed **x 4**
  - Hair from surfaces in the house (floor, couch) **x 4**
  - Hair from clothing of owner **x 2**

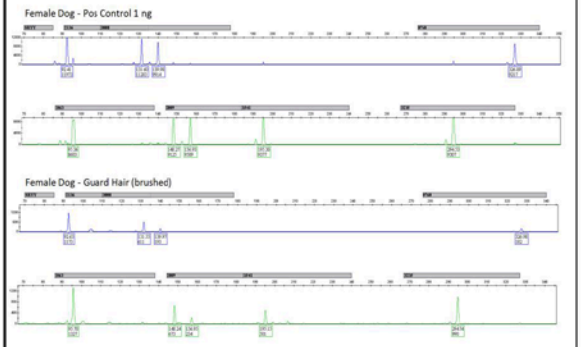
  Range of guard and undercoat hairs
- 50 samples in total

Summary of Results

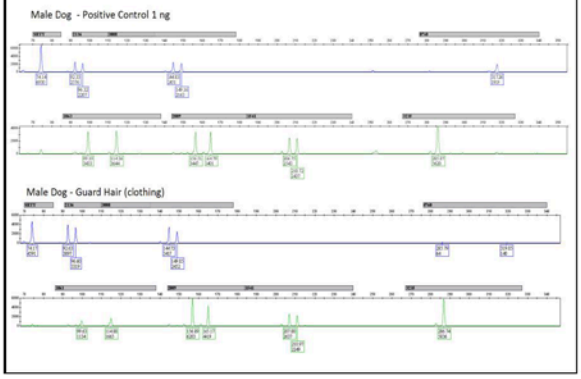
Arranged by hair type instead of location collected

Direct Sample Type	DNA Recovered/ Sample Number	Percentage Recovered
Undercoat Hairs	2/18	11 %
Guard Hairs	23/23	100 %
Mixed	6/9	67 %
Total Recovery	31/50	62%

Direct & Pos Control



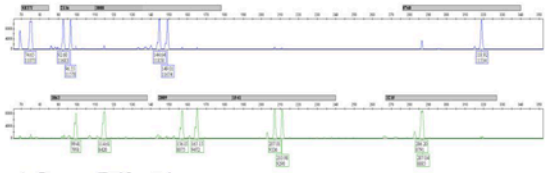
Direct & Pos Control



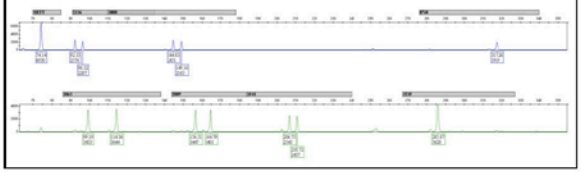
Inhibition

- Too much DNA can cause:
  - Overloaded Samples
    - Split peaks, noisy baseline, pull-up
- Overcome with:
  - PCR product dilution on gel electrophoresis
  - Additional enzyme in PCR set up

Before Dilution



After Dilution



Summary

- Extraction process circumvented
  - DNA not lost via wash steps or tube changes
  - Greater DNA yield
- Dog hairs are currently not processed for DNA due to low success rate
  - Guard hairs most successful in obtaining DNA
- Cost effective and time saving
- Already validated Canine STRs for identification
  - Easy & quick implementation in forensic labs

Corresponding abstract for oral presentation:

Successful direct amplification of nuclear markers using single dog hairs with eight DogFiler loci

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We report on successful amplification of canine STR DNA profiles from single dog hairs. Dog hairs are commonly found on clothing or items of interest in forensic casework and may be crucial associative evidence if linked to an individual dog. We used direct amplification from these hairs to increase the sensitivity and DNA yield of the sample, as well as reducing analysis time. Hairs from different somatic regions were used from several different dog breeds to amplify a selection of eight loci from the validated DogFiler multiplex. Both anagen and telogen hairs were processed, with a mix of coarse topcoat hairs and thinner soft undercoat hairs. For single coarse hairs, approximately 5 mm from the proximal tip was added directly into a PCR tube. Multiple sections of a single hair were amplified in 5 mm segments to determine the viability of DNA recovery from the shaft of the hair. Undercoat hairs, which are very fine, were amplified together in a single tube (approximately 10 small hairs). Each hair type and fragment consistently produced a full DNA profile using all eight loci that matched the corresponding reference profile for that dog.

CHAPTER 5

Direct PCR from Human Fingernails

Ottens, R., Taylor, D., and Linacre, A., *DNA profiles from fingernails using direct PCR.* Forensic Science, Medicine, and Pathology, 2015. **11**(1): p. 99-103.

http://dx.doi.org/10.1007/s12024-014-9626-8

Citations: 4

Direct PCR from Human Fingernails

5.1 Preface

From the previous direct PCR studies on canine and human hair, it can be inferred that the presence of nucleated cells or cell-free DNA would also be available on the surface of fingernails. Like hair, nails are made from keratin and have the propensity to maintain DNA on the surface, from skin secretion or sebum as the nail grows, even when exposed to extreme environmental conditions [1, 2]. Unlike hair however, fingernails are more likely to pick up PCR inhibitors such as dirt, nail polish, or cleaning chemicals through regular daily activities and exposure to the environment.

Fingernails can be used to target different sources of DNA; a victim may have collected foreign DNA under their fingernails during an attack or struggle with a perpetrator, in which case the victim's nails may either be collected or scraped underneath to collect any tissue or DNA material present [3-6]. Alternatively, fingernails have been used in victim identification [7, 8], where whole nails are collected and processed as a non-invasive method to obtain DNA information about the victim. Similarly, many difficulties arise during mass disaster victim identification [9, 10], particularly with sample collection and storage [11], making fingernails an ideal source of DNA in these scenarios.

The method for analysing DNA from fingernails varies greatly between laboratories; fingernails may be cut and pooled into one extraction, swabbed or scraped [1, 4-6, 12]. The collection method would then affect the extraction method chosen, and thus the success rate of obtaining important DNA information. Direct PCR has the potential to greatly decrease the time taken to process fingernail samples, as well as increasing the likelihood of consistently obtaining important DNA information. The following sections investigate the application of human fingernails substrate for direct PCR (*5.2*), and the future implementation and use of direct PCR in mass disaster investigations (*5.3*).

Statement of authorship

DNA profiles from fingernails using direct PCR

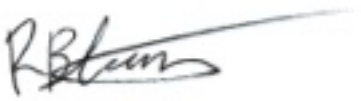
Published in Forensic Science Medicine, and Pathology

Date: October 2014

Renée Blackie (Candidate)

Performed all laboratory work (sample collection, DNA extractions and quantifications, PCR amplification, capillary electrophoresis), data analysis and interpretation, created data table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

5.2.1 *Published manuscript, a technical report, Forensic Science Medicine, and Pathology*

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TECHNICAL REPORT

DNA profiles from fingernails using direct PCR

Renée Ottens · Duncan Taylor · Adrian Linacre

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Abstract We report on the successful routine amplification of DNA profiles from small sections of fingernails using direct PCR. The data are from 40 nail clippings from eight donors where approximately 4 mm^2 of nail is added directly to the PCR. The NGM^{TM} kit was used that amplifies 15 STR loci plus amelogenin. No increase in cycle number was used and no enrichment of the PCR products was performed. Full DNA profiles were observed in 17 of the 40 profiles with 21 generating partial DNA profiles. The process omits the DNA extraction process, and hence there is no opportunity to quantify the DNA prior to amplifying the STRs, but by not performing a DNA extraction step, the amount of DNA available for PCR is maximized. Single source DNA profiles were observed in 29 of the 38 profiles obtained. The source of the DNA is assumed to be adhering to the underside of the nail. This simple method offers a significant reduction in time to generate DNA profiles from nail clippings, such as those taken from victims of mass disasters, and should be included into a forensic process relatively easily as it requires no change to manufacturer's instructions for amplification.

Keywords Direct PCR · Fingernails · Forensic science · NGM STR loci

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Introduction

We report on a highly successful method of direct amplification of DNA from human fingernail clippings. Fingernail clippings can be a source of human identification with the DNA preserved within the keratin structure of the nail. Isolation of DNA from the fingernail normally requires incubation of 24 h or more, until the nail has completely digested, in a buffer containing proteinase K and dithiothreitol (DTT). Direct PCR of samples has gained much interest in a forensic context due to the potential saving in time [1–4], increase in sensitivity and minimizing of steps open to error or contamination [5]. Much of the focus has been on direct amplification from a body fluid stain [6] with an emphasis also on the speed of the DNA typing [7] and the types of DNA polymerase used. Previous reports have illustrated the potential benefit of direct amplification from fibers [5], hair [8, 9], and touch DNA [10, 11]. The potential to generate a profile from fingernail clippings is dependent on the presence of nucleated cells or cell free DNA present on the surface of the nail from skin secretion or sebum. The aim of this study was to develop a simple, operational method that could be used routinely in forensic science casework with no special laboratory requirements, reducing the time taken to process evidence that provides a greater DNA profiling success rate than standard extraction method [12].

Materials and methods

Samples

Fingernail clippings were collected from both male and female donors working in the forensic DNA laboratory

at Flinders University. Confirmed consent was provided in all cases and clearance was provided by the Southern Adelaide Clinical Human Research Ethics Committee. Each donor was asked to provide a clipping from each finger after showering or washing their hands. A total of 40 fingernail clippings from eight donors were analyzed. DNA extracts from buccal swabs were also provided as references of donors. Fingernail clippings were not treated in any manner prior to amplification.

Direct PCR amplification and conditions

Direct PCR was conducted by using a nail cutting approximately 2 mm × 2 mm in size, prepared using sterile scissors and tweezers. A single fragment was placed into a 0.2 mL thin walled tube containing 10 µL of PCR master mix from the AmpFℓSTR® NGM™ kit (Life Technologies, VIC, Australia) along with 5 µL of the primer mix and 1 µL of AmpliTaq Gold® DNA polymerase. A further 9 µL of sterile H_2O were added to make the final volume 25 µL. The amplification was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the manufacturer's recommended conditions. The standard 29 cycles was used for all reactions. There was no alteration to the methodology of amplification deliberately to ensure that the process could be adopted readily by the forensic science community. The NGM™ kit amplifies 15 STR loci plus the amelogenin locus.

Analysis of buccal swab

DNA was isolated from buccal swabs using a Qiagen Mini kit (Qiagen, VIC, Australia) following the manufacturer's protocol. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies). From these extracts 1 ng of DNA was used in the PCR using the same conditions as for the direct fingernails samples.

DNA extraction comparison

Two nail fragments from each donor, measuring approximately the same size as was used in the direct PCR method (2 mm × 2 mm), were extracted using a Qiagen Mini Kit. A total of 16 extractions were performed following the manufacturer's protocol. The fragments were incubated for 24 h and eluted in a final volume of 50 µL. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies). From these extracts 1 ng, where possible, or 10 µL of DNA extract if the DNA was at a sub-optimal concentration, was used in the PCR using the same conditions as for the direct fingernail samples.

Capillary electrophoresis

Capillary electrophoresis was performed on an ABI 3130*xl* Genetic Analyser (Life Technologies) using POP-4™ polymer (Life Technologies). An aliquot of either 1 µL, or 1 µL of a 1 in 30 dilution into H_2O, of the PCR sample was added to a solution of 0.5 µL of ABI GeneScan-600 LIZ® Size Standard and 9.5 µL of Hi-Di™ Formamide (Life Technologies). Samples were then denatured at 95 °C for 3 min. Electrophoresis was conducted at 3 kV with a 10 s injection. The data were analyzed using Genemapper® v3.2 (Life Technologies). The detection threshold was set at 50 relative fluorescence units (RFU).

Results and discussion

A total of 40 fingernail clippings from eight individuals were amplified directly using the NGM™ kit and the data analyzed (see Table 1). A full profile, comprising all 15 STR loci and the amelogenin locus, was obtained from 17 samples (an example of which is seen in Fig. 1). Profiles resulting in allelic drop-out, from the major component, of two alleles or less (almost complete NGM™ DNA profile) occurred in ten samples. Eleven samples produced a DNA profile of five or more complete STR loci (partial profile) that could be uploaded to the Australian National Criminal DNA Database (NCIDD). Only two samples from the 40 tested generated profiles of seven alleles or less.

Seven profiles exhibited split allele peaks, typical of over amplification or inhibition during amplification. A dilution of the final PCR product reduced these affects as shown in previous studies [8] without causing allelic dropout. The average heterozygous peak balance (defined as the lower intensity peak divided by the higher intensity peak) of all direct amplification samples was observed to be 79.9 %. Dropout occurred in the expected range (larger loci). The heterozygous peak balance for the extracted samples was overall lower at 68.9 %, with 75 % of samples exhibiting split peaks and pull-up that could not be reduced without losing alleles at the larger loci.

Additional alleles from a minor contributor were observed in nine (22.5 %) samples (an example of which is seen in Fig. 2). Six samples contained a single minor allele, and the remaining three samples contained between three and six minor alleles. The minor alleles were all consistent with a cohabiting partner of the donor. These figures are consistent with previous control sample studies, showing foreign DNA contribution to fingernail samples to be between 13 and 23 % [12]. By contrast, the extracted samples in this study showed a much larger foreign DNA contribution at 81.2 %, where the major or minor component could not be distinguished in 61.5 % of those samples

Table 1 Summary of DNA profiles obtained using the NGM ™ STR kit from fingernail clippings 2 mm × 2 mm in size from eight donors, total samples $n = 40$

Full profile	Almost full profile	Partial profile (uploadable)	Incomplete profile	Foreign DNA detected	
15 complete loci	Drop-out of ≤ 2 alleles	≥5 complete loci	≤4 complete loci	Single allele	Multiple alleles (≤6 alleles)
42.5 % ($n = 17$)	25 % ($n = 10$)	27.5 % ($n = 11$)	5 % ($n = 2$)	15 % ($n = 6$)	7.5 % ($n = 3$)
Profiles uploadable to NCIDD (%)			Total percentage of profiles containing foreign DNA		
95			22.5		

Number of samples where foreign DNA was detected is also shown

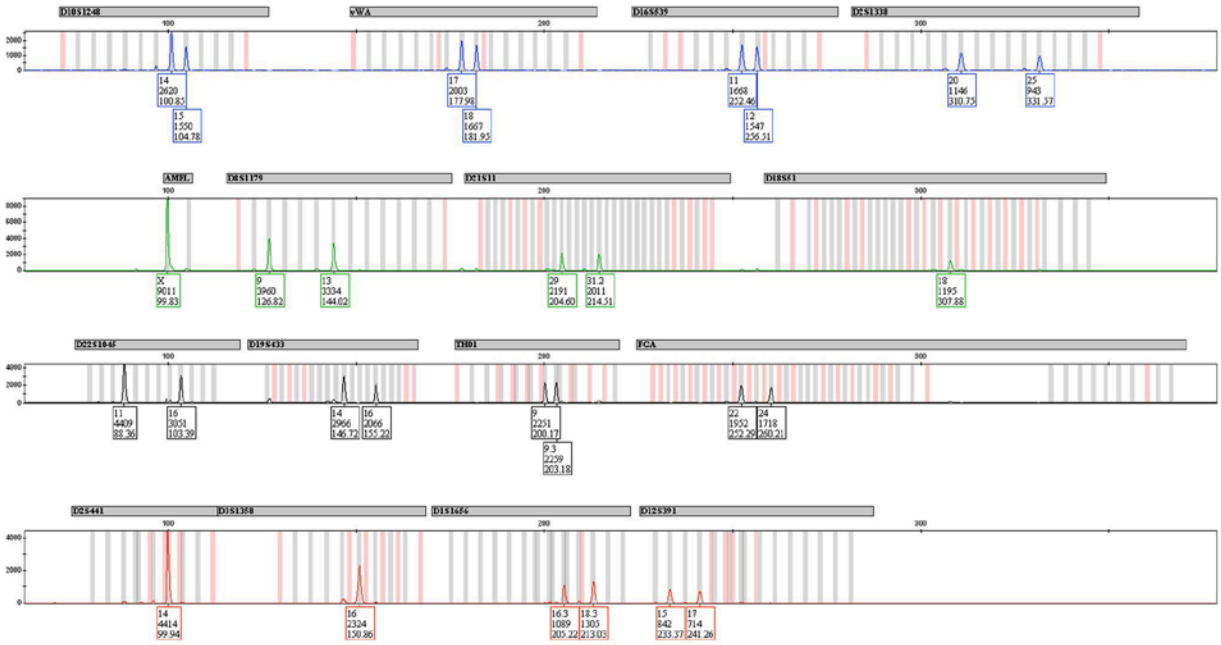

Fig. 1 Chromatogram of nuclear DNA from a fingernail clipping exhibiting a full DNA profile

(eight profiles in total). A presumption in sampling DNA directly is that nuclear material is present on the surface of the sample and is released and amplified during PCR. Fingernails would come into contact with foreign DNA during daily activity, and it would not be unexpected to see this additional DNA when sampling directly or via extraction methods [13].

A DNA profile containing five or more complete STR loci was obtained from 95 % of the samples tested using direct PCR. The profiles obtained from extracted DNA displayed similar success rates; however this can vary greatly between different extraction methods. Matte et al. [12] used wooden scrapers to obtain DNA from fingernails and found that between 36 and 87 % of their control samples (washed nails) yielded sufficient DNA for amplification using a 9 STR loci kit, depending on the extraction method used, but make no comment on the quality of the profiles obtained.

The DNA profiles obtained from clean washed fingernails in this study generated mostly single source profiles, or displayed a clear major profile if additional minor alleles were present; making them ideal for fast human identification, such as mass disaster victim identification where time and resources are limited. The successful recovery of DNA from fingernails that have been subjected to harsh conditions that may replicate mass disaster scenarios, such as water submersion, has been reported previously [14], indicating the robust nature of fingernails and their ability to hold DNA. Fingernail clippings do not need any special storage (such as refrigeration) and due to the small amount of sample needed, additional testing can be carried out using direct and standard extraction methods if a repeat test is required.

Unwashed nail clippings could also be processed directly for the purpose of obtaining foreign DNA as a victim may have come in close contact with a

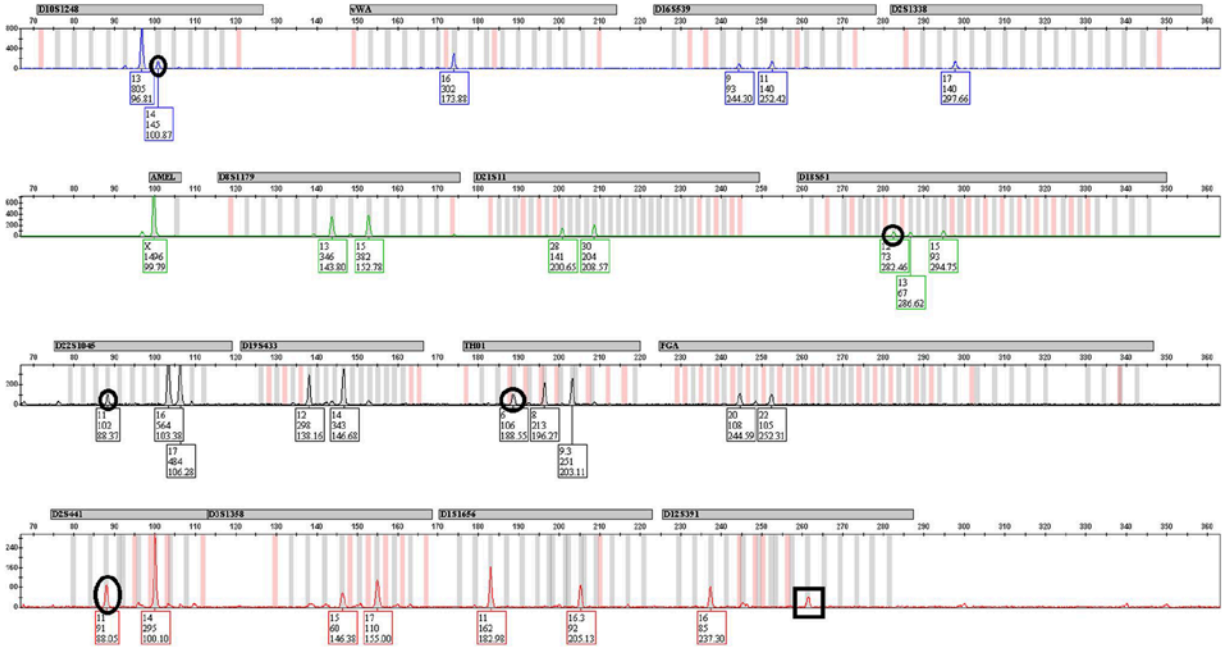

Fig. 2 Chromatogram of nuclear DNA from a fingernail clipping exhibiting a mixed DNA profile. Minor allelic contributions can be observed at the *circled* loci. Drop-out of the major component observed at locus D12S391 in the *square*

perpetrator. Foreign DNA recovered from fingernails can be significant evidence in a forensic investigation [15–17]. Direct PCR maximized the amount of DNA available for PCR as it is not being lost to the extraction process [5, 8, 18].

Conclusion

Direct PCR produced DNA profiles from a small fingernail clipping that could be uploaded to the NCIDD in 95 % of samples tested. This is a substantial increase compared to the process of performing standard DNA extraction then amplification of the extract [12] and greatly reduces the time and money spent processing samples. The profiles obtained were of higher quality than the extracted samples in this study, showing a higher heterozygous peak balance and limited effects from overloading. It was found that direct PCR of some fingernail clippings produced over-loaded DNA profiles after standard 29 amplification cycles and we recommend a dilution of the product prior to electrophoresis of the sample.

Only two samples failed to produce up-loadable profiles. One of these samples produced no alleles, whereas the other produced seven alleles (including amelogenin) across four loci. As each sample only required a 4 mm^2 section of a single fingernail clipping, additional testing either by direct PCR or standard extraction methods can be carried out if required. This simple process of direct PCR from fingernail clippings can be readily adopted for use into forensic DNA practice as standard validated methods were used and we demonstrate that the process has the capability of generating high quality full DNA profiles in reduced time.

Key points

1. We report on a successful and novel method for obtaining DNA profiles from fingernail clippings using direct PCR.
2. Profiles that were up-loadable to the Australian National DNA Database were obtained from 95 % of the samples tested.
3. The standard protocol for DNA amplification from the NGM™ STR typing kit was not modified, allowing for fast implementation into forensic laboratories.
4. Only 4 mm^2 of fingernail sample is required for testing, allowing for retesting or standard extraction testing if required. Standard extraction generally involves the "pooling" of all fingernail clippings taken.
5. Direct PCR provides more DNA to PCR than standard extraction methods, resulting in more useful DNA profiles therefore saving time and money in forensic casework.

6. Fingernail clippings could be used for fast human identification in disaster situations.

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5.3 Applications

The challenges faced with mass disaster investigations (DVI) and the subsequent victim identification has been well documented [10, 11, 13-17]. An extensive amount of evidence and scene management is required immediately after a mass disaster and can be hindered by the type of disaster, the terrain, the overall environmental conditions, the number of staff and organisations available for assistance, and the coordination of those teams, just to name a few. DNA evidence must also be documented, collected and stored appropriately and as fast as possible to reduce further contamination and degradation. Often due to the sheer size of a mass disaster operation, DNA degradation is very difficult to avoid thus creating delays in identification, and increasing the costs of the downstream processes. DNA analysis has often required the use of shorter primer sets, or mini-STRs, to help combat the DNA breakdown and degradation often associated with DVI cases [18-22]. The nature of the DVI can mean DNA degradation is unavoidable, as in the case of bombing or plane crashes. Some studies have even focused on the use of SNPs or mitochondrial DNA to overcome these same issues [23-27].

As mentioned in *sections 5.1* and *5.2*, the human fingernail has the propensity to hold DNA within its keratin structure for long periods of time, even through extreme environmental conditions. This sample type therefore has great benefits for DVI situations, particularly when it comes to storage of the sample. Fingernails do not require refrigeration, and can be collected and stored very quickly leading for the next stages of processing. Refrigeration of samples is a huge hurdle when it comes to mass numbers of victims, especially in remote or humid locations. When sampling fingernails, only a small section is required for direct PCR, allowing for resampling or further testing if required. Fingernails and human hair, where available, would be an effective additional (or alternative) source for DNA sampling in DVI cases to traditional blood or tissue. Both sample types have shown to work well with standard STR typing, without the need to use modified or additional methods for processing [28-30]. The speed of processing of these sample types also decreases the overall cost of processing – both are huge benefits in DVI when pressure surrounds every aspect of mass disaster investigations.

5.4 Concluding Remarks

Based on the results of this study, the use of direct PCR with human fingernails has improved the probability of obtaining meaningful profiles compared to processing the same samples using standard extraction methods, as well as reducing the time and costs involved with the analysis process. Without the need to modify or adjust validated methods, direct PCR of human fingernails can be applied in investigations quickly. The robustness of fingernails and their propensity to hold DNA through extreme environmental conditions is greatly beneficial to DVI and missing person investigations that are time sensitive. The data continues to support the theory that nuclear and cell-free DNA are present on the surface of the substrate, and is easily made available to the PCR matrix during the direct amplification process.

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Supplementary Material

a. DNA profiles from fingernails using direct PCR

Chromatogram examples

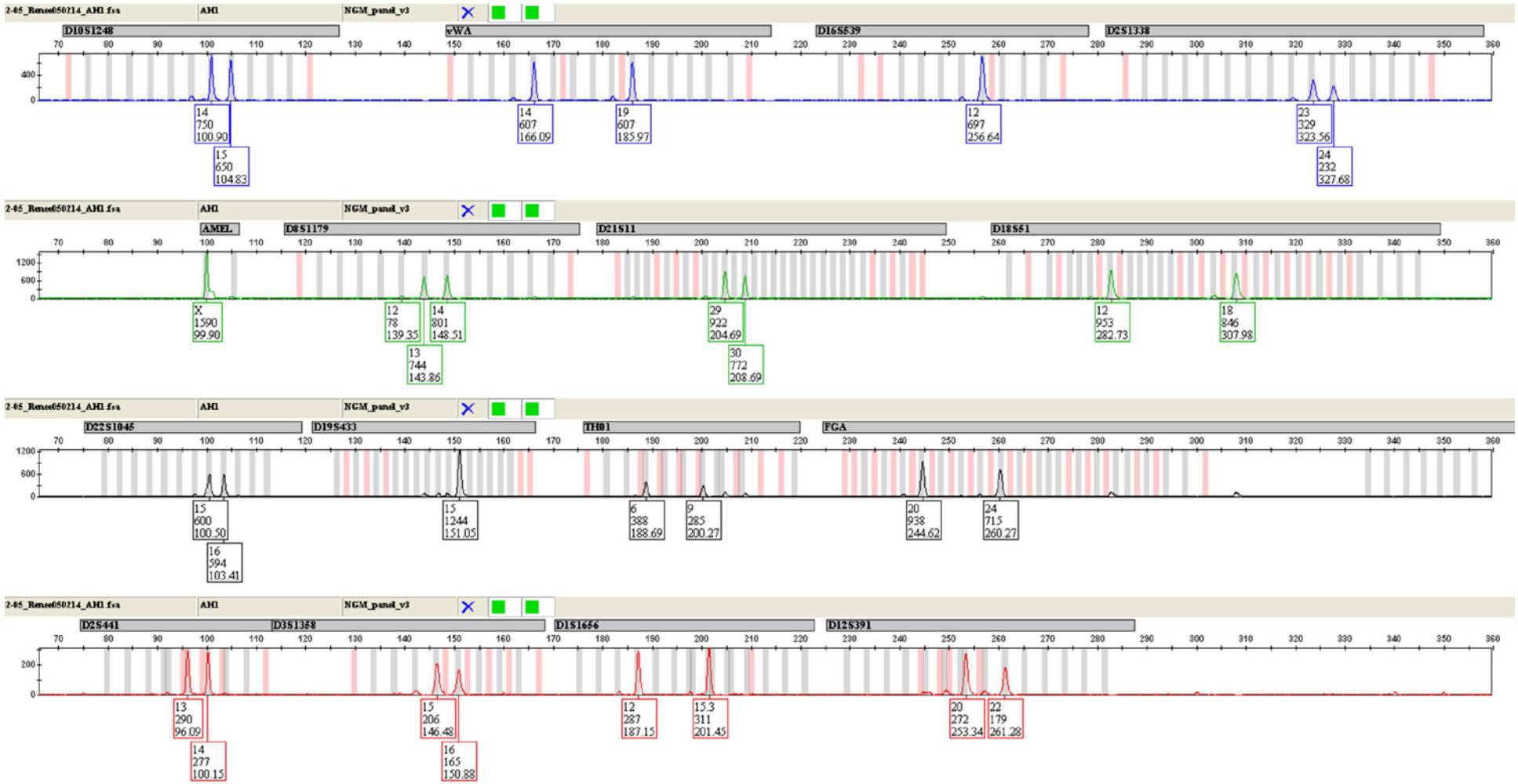


Figure 5a.1 – NGM SElect™ kit full DNA profile obtained from a human fingernail sample for IND 1, amplified using direct PCR at 29 cycles.

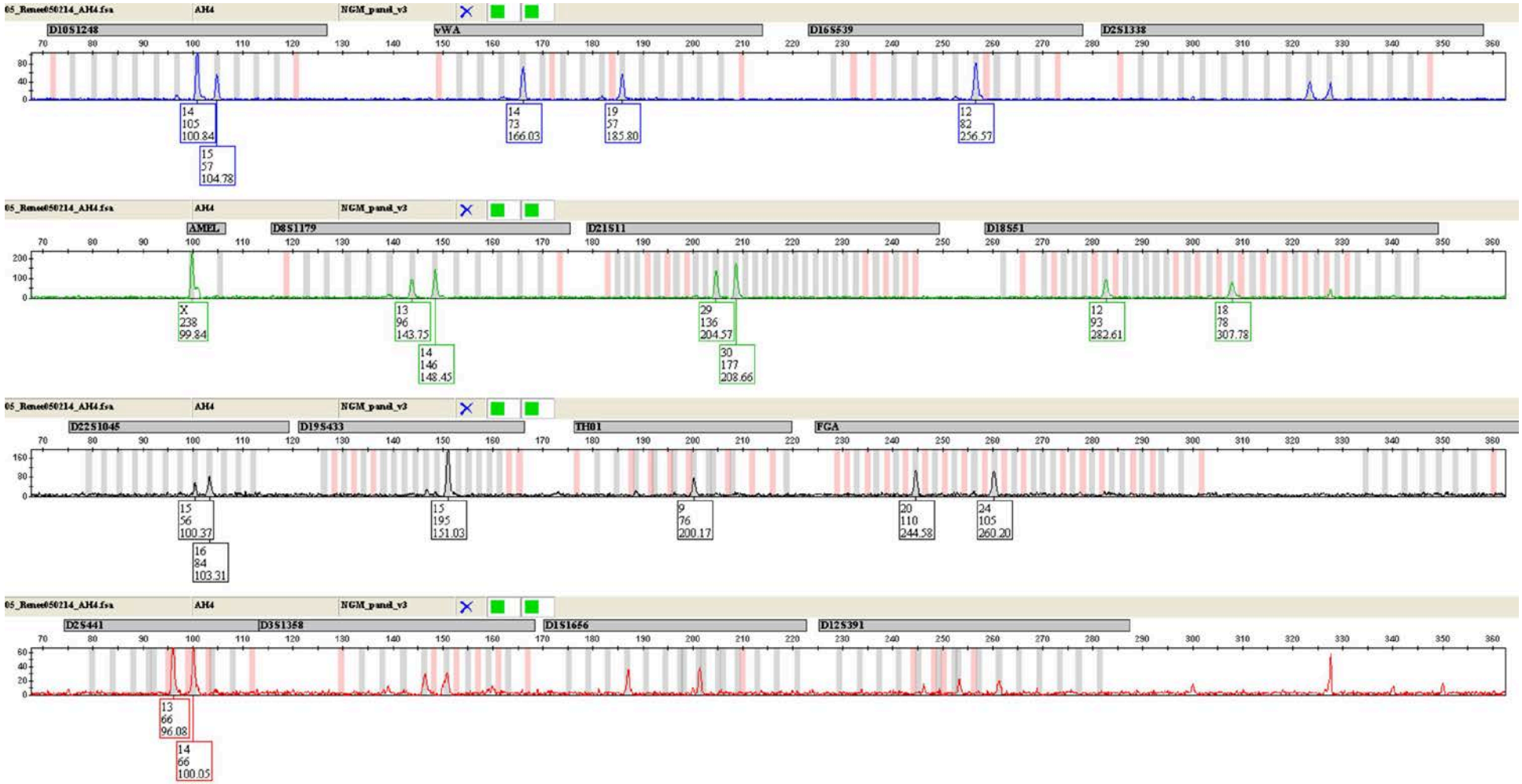


Figure 5a.2 – NGM SElect™ kit partial DNA profile obtained from a human fingernail sample for IND 1, amplified using direct PCR at 29 cycles.

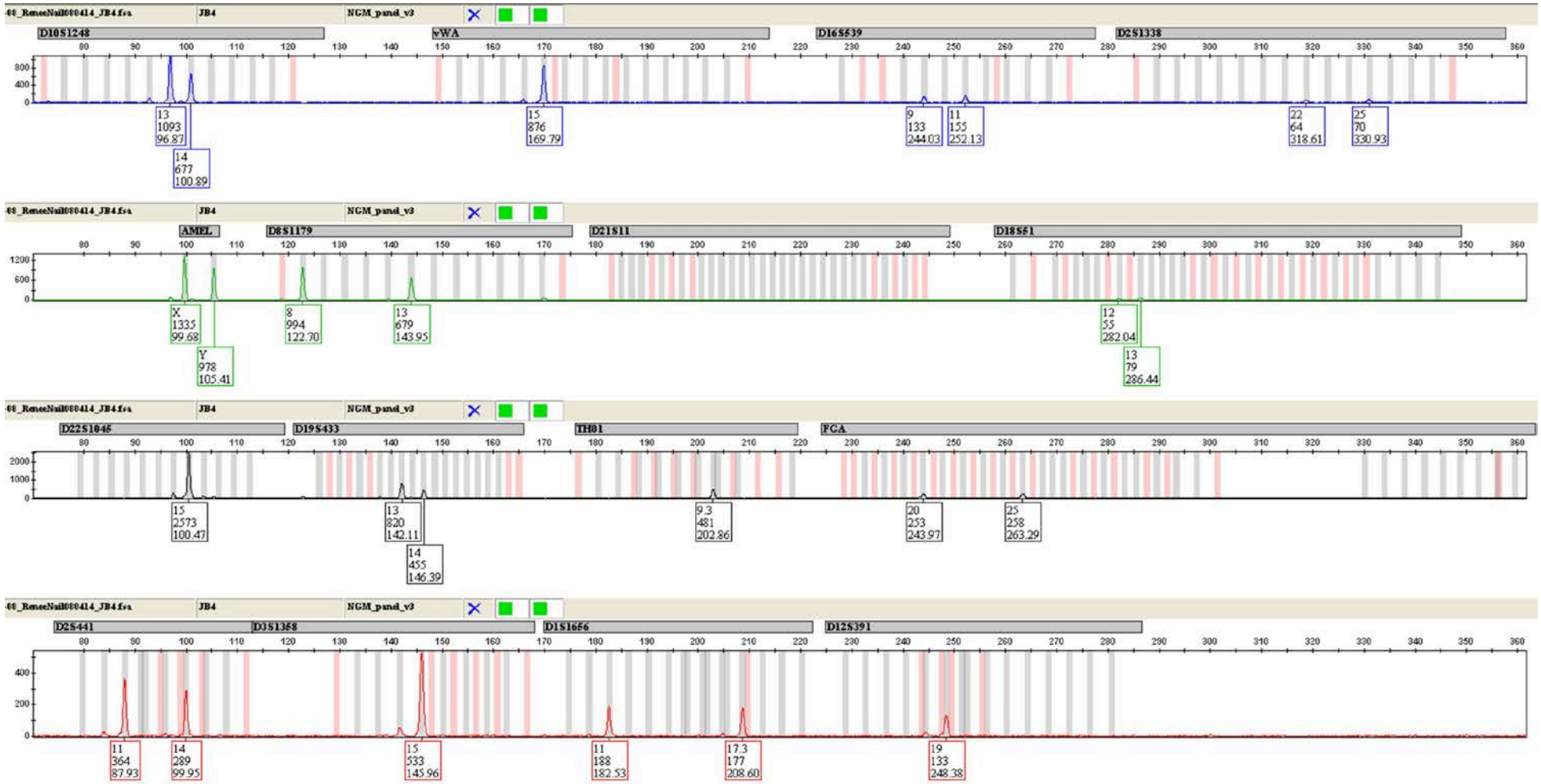


Figure 5a.3 – NGM SElect™ kit DNA profile obtained from a human fingernail sample for IND 4, amplified using direct PCR at 29 cycles.

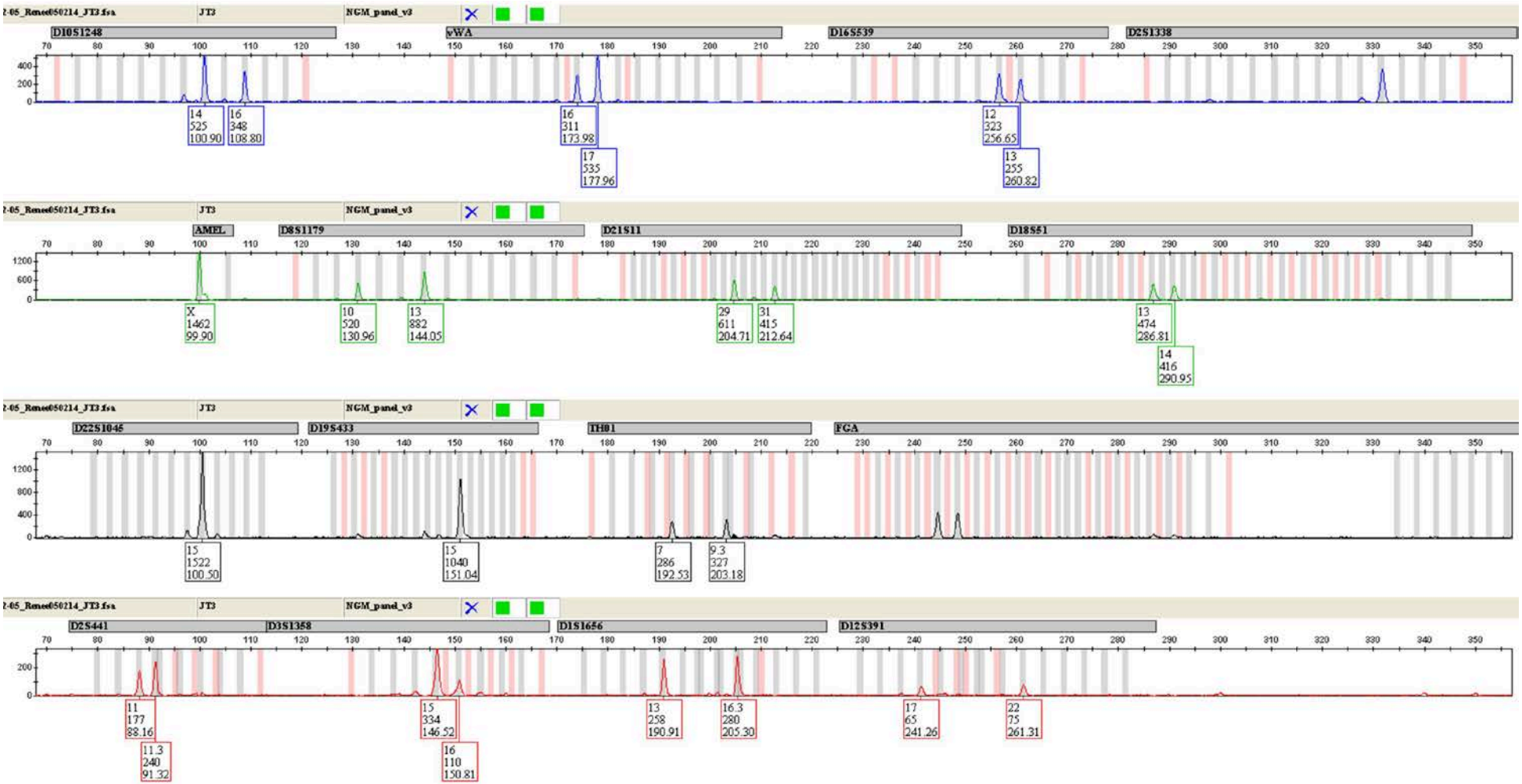


Figure 5a.4 – NGM SElect™ kit DNA profile obtained from a human fingernail sample for IND 5, amplified using direct PCR at 29 cycles.

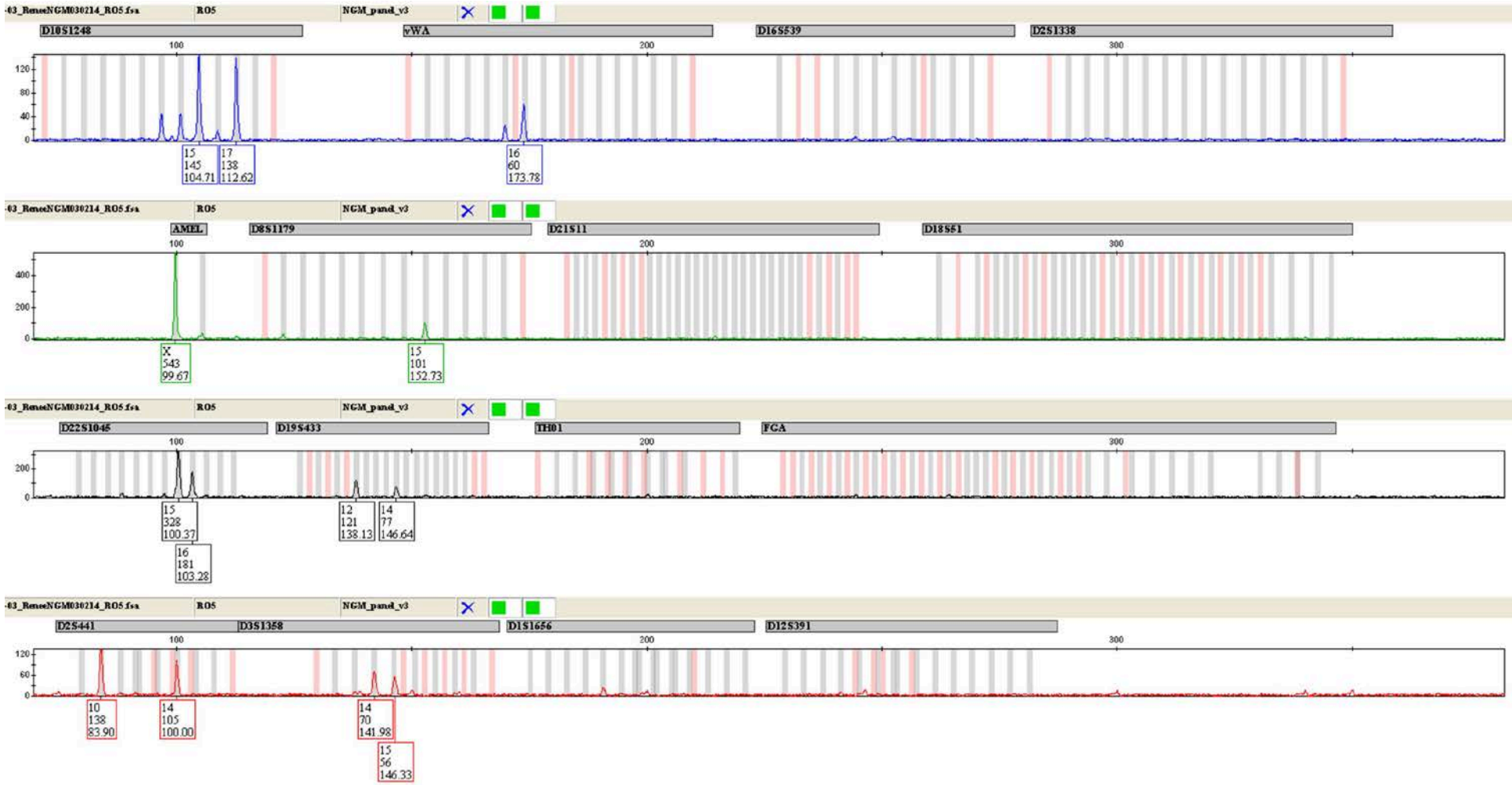


Figure 5a.5 – NGM SElect™ kit partial DNA profile obtained from a human fingernail sample for IND 6, amplified using direct PCR at 29 cycles.

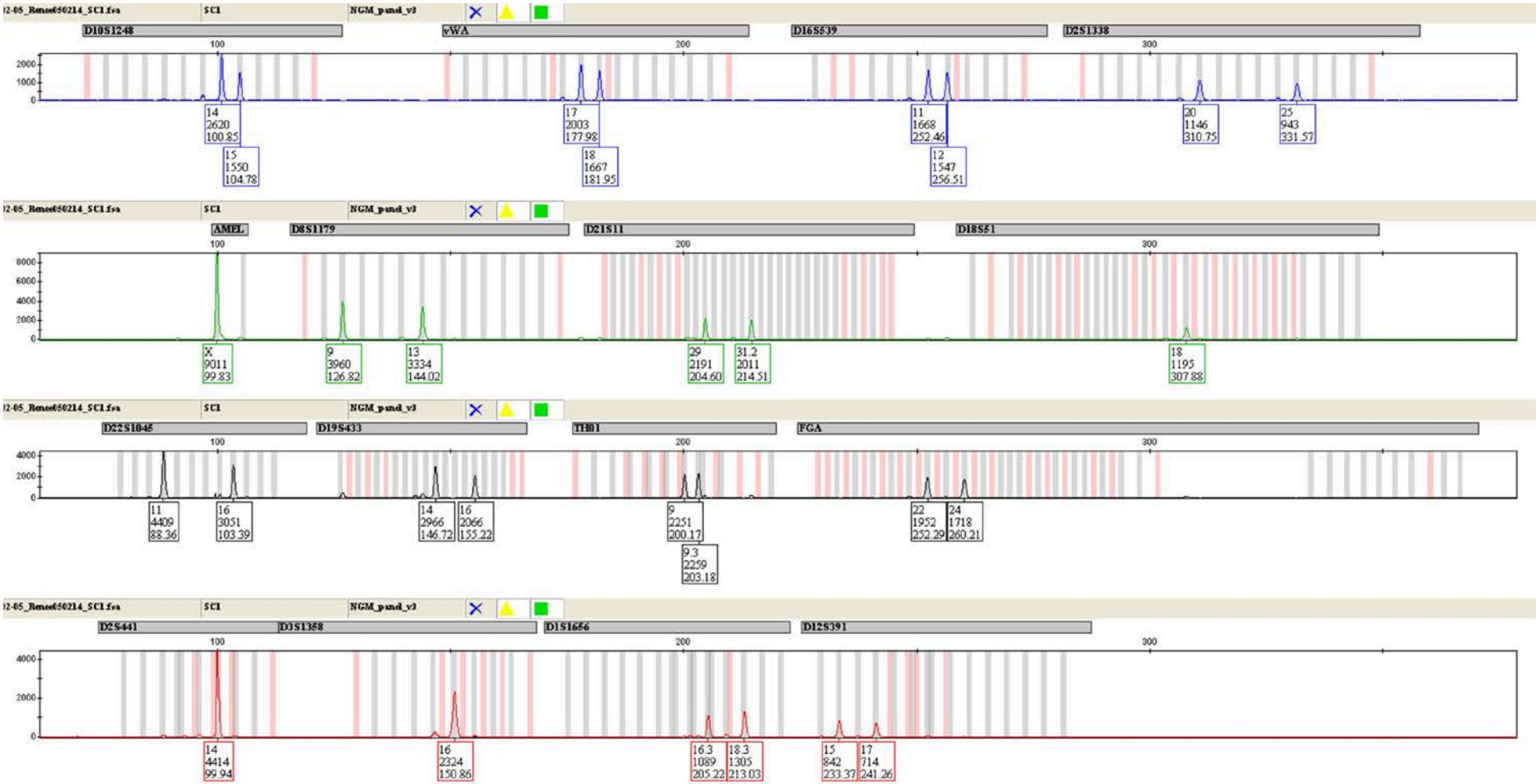


Figure 5a.6 – NGM SElect™ kit DNA profile obtained from a human fingernail sample for IND 7, amplified using direct PCR at 29 cycles.

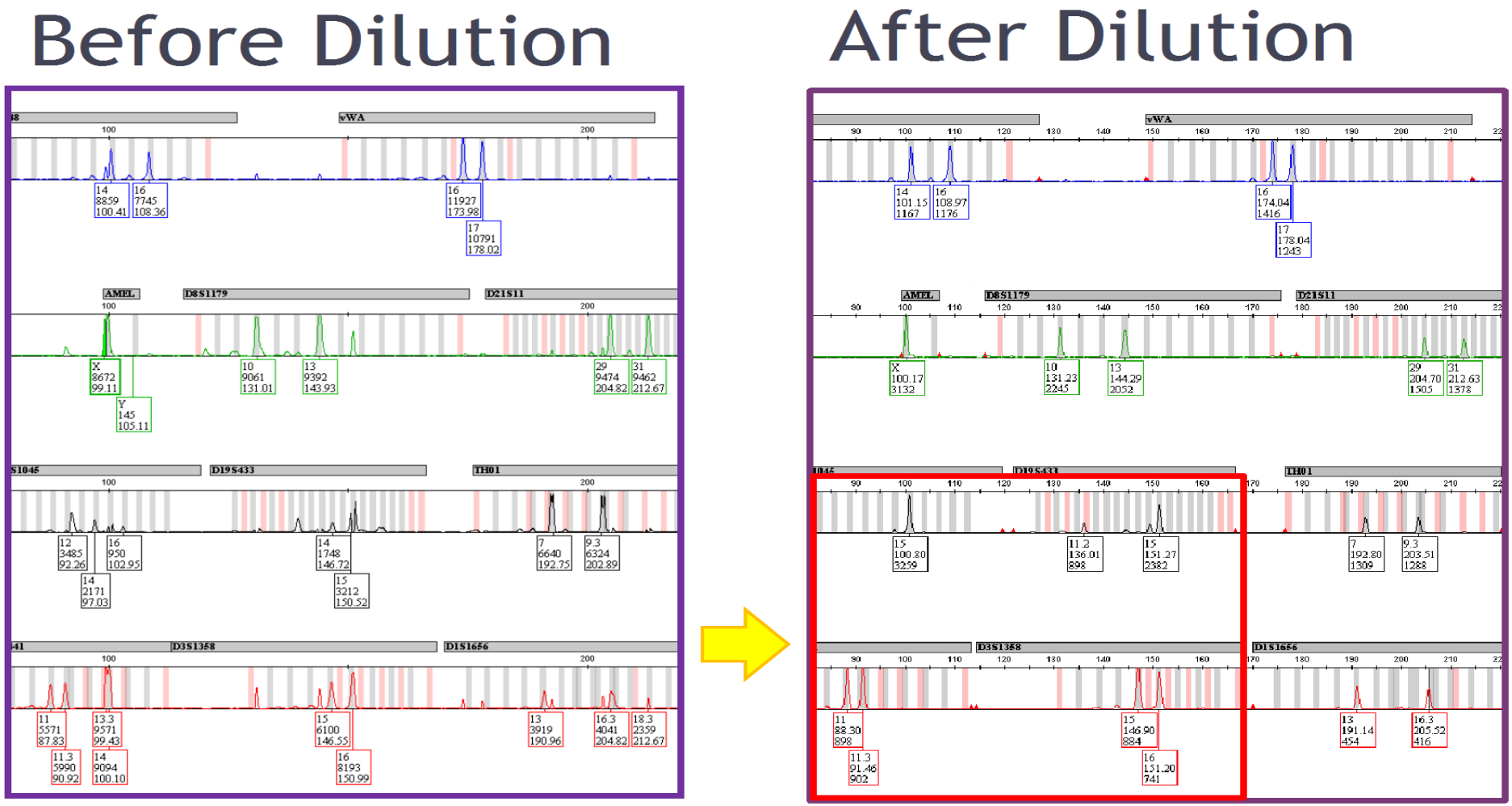
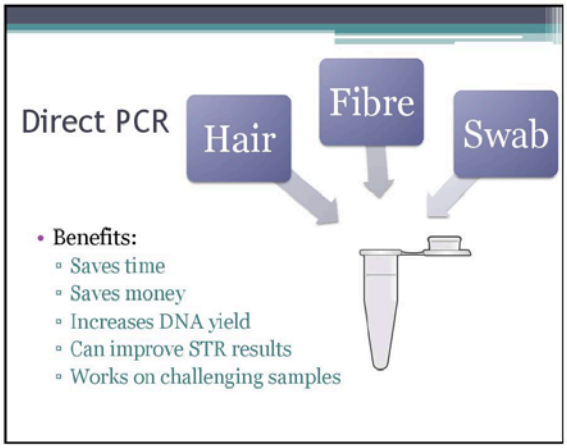
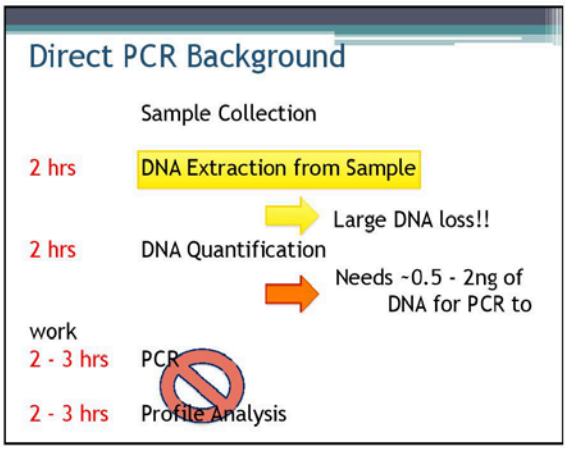


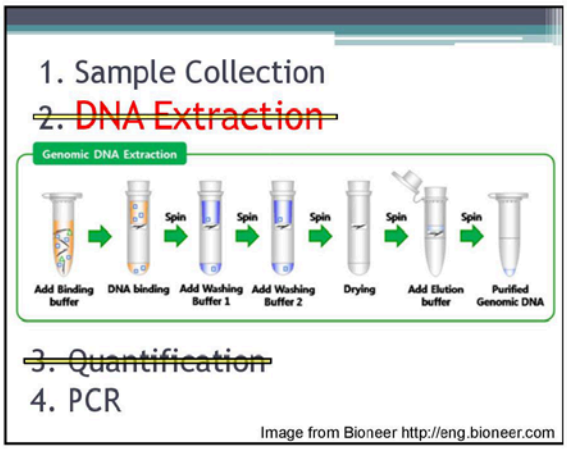
Figure 5a.7 – NGM SElect™ kit DNA profile obtained from a human fingernail sample, amplified using direct PCR at 29 cycles. Side by side comparison of the same sample before and after PCR product dilution for capillary electrophoresis. Pull-up effects are greatly reduced.

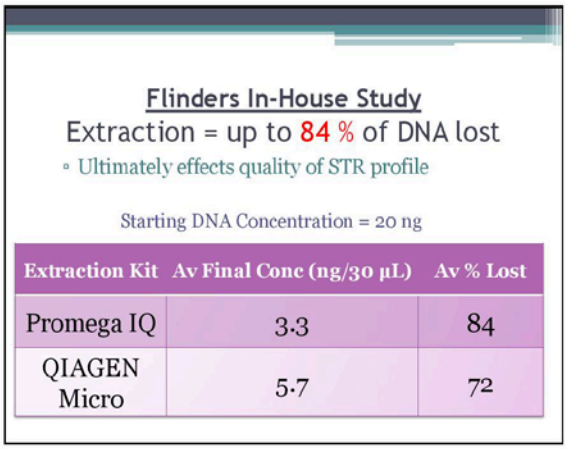
Appendix

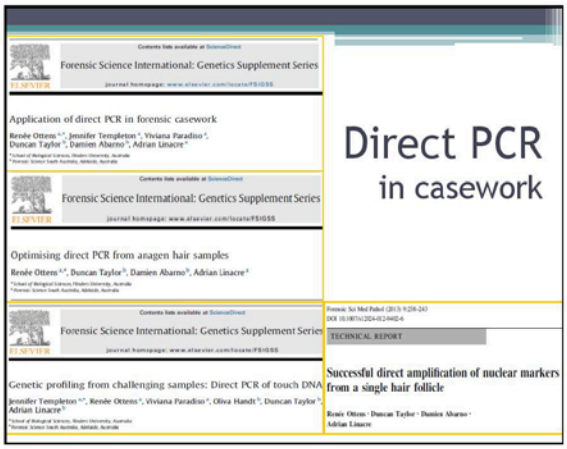
i. **Oral Presentation** 22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Successful direct amplification of nuclear markers from fingernail clippings.*











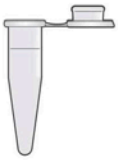
Nail Study

- 40 fingernail clippings from 8 donors
  - Collected post hand-washing/ shower
- ~2 mm² of fingernail used
- Placed directly into PCR tube
- NGM™ human identification kit used
  - 15 STR loci + amelogenin
- Standard 29 cycles (NO increase or adjustment!)
  - Typically increased for low copy DNA

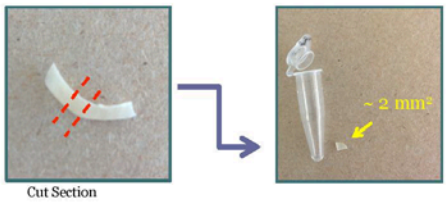
Typical Process

- No Standard method
- Clippings from one or both hands pooled for single extraction
- Scrapings under nails pooled
- Swabbing nails

One extraction



Fingernail Clipping



Cut Section

Theory: Nuclear DNA present on underside of fingernail
- Nucleated cells or cell free DNA

Source: Skin secretion or sebum

DNA - single source or mixed?

- Previous study (Extraction method)[1]
  - Foreign DNA contribution between 13 – 23 %
  - Mostly linked to cohabiting partner
- Presence of foreign DNA not unexpected
  - Daily activities

[1] Matte M, Williams L, Frappier R, Newman J. Prevalence and persistence of foreign DNA beneath fingernails. Forensic Sci Int Genet. 2012;6(2):236-43.

Results n = 40

Full Profile	Almost Full	Partial Profile	In-complete	Foreign DNA Detected	
15 complete loci	Drop out of ≤2 alleles	≥5 complete loci	≤4 complete loci	Single Allele	Multiple Allele
42.5%	25%	27.5%	5%	15%	7.5%
n = 17	n = 10	n = 11	n = 2	n = 6	n = 3
Profiles up-loadable to the National DNA database (Australia)				Total observed foreign DNA	
95%				22.5%	

Results

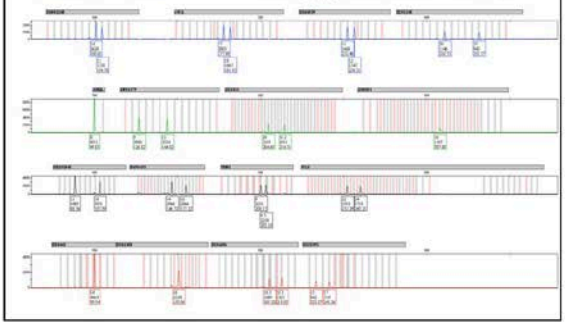
DNA obtained: 38 Samples
29 Single Source
9 Mixed Source*

* mixed profiles had clear Major and Minor Components

All minor alleles attributed to cohabiter of donor

Full Profile

average peak balance = 79.9 %



Additional Alleles + Drop out

Optimisation

- STR kits are optimised for 1 ng input of DNA
- Input DNA unknown for Direct PCR samples
- Too much DNA can lead to:
  - Peak Imbalance
  - Noisy Baseline
  - Pull-up
  - Split peaks

Diluting PCR products
can reduce these affects

Before Dilution / After Dilution

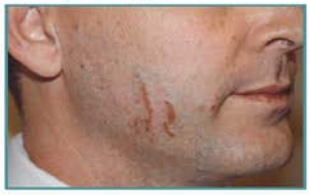
Application

- Fast Human Identification
  - Clean nails mostly single sourced/ clear major
  - Nails can be washed pre or post collection
  - Special storage not required
  - DVI identification
  - Robust material
    - Study shows DNA can withstand harsh conditions on fingernails and can still be retrieved [2]

[2] Harbison SA, Petricevic SF, Vintiner SK. The persistence of DNA under fingernails following submersion in water. In: Brinkman B, Carracedo A, editors. Progress in Forensic Genetics 9. Int Congress Ser. Amsterdam: Elsevier Science Bv; 2003. p. 809-13.

Application

- Casework
  - Focuses on mixed profiles (assaults, close contact)
  - Direct PCR yields higher DNA



Summary

- Extraction process circumvented
  - DNA not lost via wash steps or tube changes
  - Greater DNA yield
- Cost effective and time saving
- Small sample required
  - Allows for retesting via Direct or Extraction
- Minimal to no changes made to **protocols** of already validated STR human identification kits
  - Easy & quick implementation in forensic labs

Flinders UNIVERSITY

Government of South Australia
Forensic Science SA

Thank You

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Department of Justice SA

Corresponding abstract for oral presentation

Successful direct amplification of nuclear markers from fingernail clippings

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We report on successful direct amplification of DNA profiles from fingernail clippings. The collection of fingernail clippings for DNA analysis is fast and non-invasive. Fingernails will not degrade in the same manner as other biological materials and do not need special storage. This makes fingernails an ideal sample type in mass disaster circumstances where storage and conditions may not be optimal. Direct amplification has the advantages of increasing PCR sensitivity and DNA yield, allowing for human identification to be made in a significantly reduced time. Using the NGM™ 15 loci STR kit, a small section (~ 2 mm^2) of clipped nail was placed directly into a 0.2 mL thin walled tube for amplification. No adjustments to the manufacturer's protocol were made except the addition of 5 units of AmpliTaq Gold® DNA polymerase. Ten nail clippings (one from each finger) were analysed from seven individuals. Full profiles were obtained routinely after 29 cycles with no inherent stochastic effects thus reducing time, cost and increasing the opportunity to obtain an STR profile.

CHAPTER 6

Direct PCR from Fibres

Blackie, R., D. Taylor, and A. Linacre, *DNA profiles from clothing fibers using direct PCR.* Forensic Science, Medicine, and Pathology, 2016. **12**(3): p. 331-335.
http://dx.doi.oirg/10.1007/s12024-016-9784-y

Direct PCR from Fibres

6.1 Preface

Similarly to human and animal hairs, single fibres or items of clothing may often be present at crime scenes, potentially offering valuable forensic information. Single fibres may come loose from their original source and be transferred to other surfaces or areas during direct or indirect physical contact, such as a struggle during a physical assault or in the attempt to remove evidence of a crime [1]. Single fibres are typically analysed microscopically to identify morphological features such as weave pattern, colour, or whether the fibre is natural, man-made or both [2, 3]. The morphology of a fibre can provide information to assist in linking it to its original source or location. The addition of autosomal DNA information from a single fibre could provide new avenues for investigation, with the ability to link a person, as well as a garment, to a crime. This chapter explores the use of direct PCR with fibres from clothing, to offer an additional analysis tool in criminal investigations. The following sections explore the testing of fibres, using direct PCR, within the Flinders University DNA laboratory (*6.2*), the application of single fibres as a substrate for direct PCR (*6.3*), as well as the application of single fibres in casework and future implementation of the technique (*6.4*).

6.2 Preliminary data

To investigate the suitability of single fibres as a substrate for direct PCR methods, several preliminary experiments were conducted. Firstly, to ascertain if inhibition or over-amplification would occur, the following conditions were set up: using sterilised white cotton, fabric squares measuring approximately 2 mm x 2 mm were held for 15 seconds (Figure 6.1); single fibres measuring approximately 5 mm were held for 15 seconds; 10 µL of saliva was pipetted onto fabric squares and left to dry; and 1 ng of control DNA (extracted from individual's buccal swab) was pipetted onto fabric squares and left to dry. All fabric squares and single fibres came from the same source of white cotton, which had been sterilised under ultra violet light for 10 minutes. Hands were washed with soap and water 30 minutes prior to holding fabric squares and single fibres. Ten of each sample type was processed.

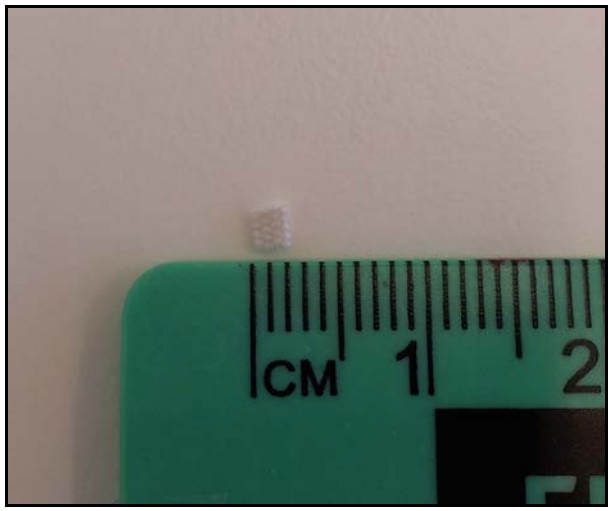

Figure 6.1 – Example of size of white cotton fabric cut for direct PCR used in control and preliminary experiments.

All samples were amplified using the NGM™ kit (Life Technologies, Victoria, Australia). Each sample (i.e. either the entire material square, or the entire fibre) was prepared in a 0.2 mL thin walled tube containing 25 µL of reagents following the manufacturer's instructions. A total of 29 PCR cycles was performed on a GeneAmp® 9700 96-well thermal cycler (Life Technologies) following manufacturer's instructions. PCR products (1 µL) were combined with 0.5 µL Liz 500™ (Life Technologies) separated using a 3130*xl* Genetic Analyser (Life Technologies). The data were analysed using GeneMapper® ID v3.2 (Life Technologies) with a threshold of 50 RFU for allele assignment.

Touched fabric square samples all produced profiles of six alleles or less (counting homozygous loci as a single allele to represent an unknown reference profile). However, 50 % of these samples showed a greater number of alleles between 40 – 49 RFU (represented by dots in Table 6.1), just below the 50 RFU threshold, indicating the possibility for protocol enhancement (such as cycle number increase) or analysis using a continuous software programme such as STRmix™ or TrueAllele® where peaks at lower analytical thresholds can be considered by modelling signal and noise [4-7]. Utilising the information down to 40 RFU would mean three of the ten touched fabric square samples would be considered partial profiles (five or more complete STR loci), and therefore up-loadable to the Australian NCIDD.

Nine of ten single fibre samples produced profiles of five or more complete STR loci, with seven of these profiles containing between 1-3 non-donor alleles (Table 6.2). Although the fabric squares have a higher surface area than the single fibres during contact, the single fibres performed better. It is possible that the larger physical space of the fabric squares within the PCR tube, and therefore PCR matrix, somehow prevents all available surface DNA from being released. DNA retention to the fabric squares is explored later in this section. As a single fibre is used in the analysis it is assumed that any resulting DNA profile will be mostly attributed to the individual touching the fibre. The individual touched the fibres and fabrics 30 minutes after washing their hands. During the 30-minute interval, the individual commenced regular activities. With this in mind, secondary transfer of DNA needs to be considered when analysing profiles obtained [1, 8-10]. It cannot be assumed that a DNA profile from a single fibre is wholly from the individual touching the item.

All fabric square samples with saliva returned full profiles of the individual (Table 6.3). DNA was not extracted from the saliva prior to placing on the fabric; it is assumed that cell-free DNA would be present in the saliva [11, 12], and that cell membranes would also be broken down during the initial heating stage of the PCR process releasing DNA into the PCR matrix.

Table 6.1 – NGM™ profiles showing allele call, RFU value and profile percentage obtained from ten fabric square (white cotton) samples that were held for 15 seconds and amplified using direct PCR methods at 29 cycles. Dots indicate alleles present but less than 50 RFU threshold.

		D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl		15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
Code	Profile %																
TF1	2					x 317											
TF2	8	..	16 69	..	.	x 326	13 95			15, 16 124, 118	.			. 14 50	..	.	
TF3	4					x 88	13 68				14 55						
TF4	3	15 . 52	.	.	.	x 64		.				.					
TF5	5	..	16 62	..	..	x 238				..	12, 14 61, 60		.	.	..		
TF6	2	.	.			x 218											
TF7	4		.			x 361	13 60			15 56							
TF8	9	..	16 59	..	..	x 145	13, 15 70, 77	31.2 71		..		.	25 53	..			
TF9	3					x 2445				16 67							
TF10	3					x 1639	15 53										

Table 6.2 – NGM™ profiles showing allele call, RFU value and profile percentage obtained from ten single fibre strand (white cotton) samples that were held for 15 seconds and amplified using direct PCR methods at 29 cycles. Dots indicate alleles present but less than 50 RFU threshold. Alleles highlighted in red indicate non-donor alleles.

		D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl		15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
Code	Profile %																
TS1	96.8	15, 17 253, 191	16 359	9, 11 156, 141	17 221	x, y 6617, 130	8, 13, 15 1049, 383, 346	26.2, 31.2 72, 259	14, 18 69, 117	15, 16 541, 328	12, 14 378, 349	9 243	25 317	10, 14 200, 344	14, 15 201, 158	13, 14 78, 106	17, 19 51, 52
TS2	87.5	15, 17 202, 213	16 193	9, 11 132, 111	17 76	x, y 9532, 380	8, 13, 15 834, 295, 311	31.2 231	14 59	15, 16 351, 340	12, 14 522, 255	9 303	25 185	10, 14 202, 3684	14, 15 137, 149	13 . 57	..
TS3	78.1	15, 17 88, 167	16 78	9, 11 61, 101	18 63	x, y 8996, 403	8, 13, 15 361, 206, 140	31.2 119		15, 16 191, 196	12, 14 88, 176	9 153	25 95	10, 14 51, 2121	14, 15 63, 87		
TS4	68.7	15, 17 77, 79	16 62			x, y 5280, 130	8, 13, 15 118, 58, 94	31.2 56		15, 16 114, 54	12, 14 91, 77	9 120	25 68	10, 14 69, 124	14, 15 57, 63		
TS5	100	15, 17 301, 207	16 605	9, 11 186, 141	17, 18 136, 121	x 1966	13, 15 322, 312	31.2 274	14, 18 110, 98	15, 16 512, 529	12, 14 338, 416	9 462	25 601	10, 14 162, 259	14, 15 177, 121	13, 14 155, 113	17, 19 70, 52
TS6	96.8	15, 17 231, 284	16 305	9, 11 78, 68	17, 18 126, 115	x 3292	13, 15 291, 264	31.2 176	14 112	15, 16 281, 436	12, 14 392, 261	9 235	25 163	10, 14 166, 87	14, 15 91, 131	13, 14 93, 74	17, 19 45, 37
TS7	62.5	15, 17 152, 105	16 147			x, y 3547, 114	8, 13, 15 63, 78, 131	31.2 175		15, 16 176, 128	12, 14 152, 177	9 131	25 139	10, 14 79, 113			
TS8	65.6	15, 17 89, 87	16 83	9 56		x, y 3693, 94	8, 13, 15 57, 155, 88	31.2 158		15, 16 100, 196	12, 14 298, 129	9 138	25 65	14 76	14 52		
TS9	50	13, 15, 17 62, 54, 64	16 79			x 2817	13, 15 177, 78			15, 16 245, 158	12, 14 88, 51	9 56		14 77	14 55		
TS10	9.3					x 1639	15 53										

Table 6.3 – NGM™ profiles showing allele call, RFU value and profile percentage obtained from ten fabric square (white cotton) samples that had 10 µL of saliva pipetted onto the centre and amplified using direct PCR methods at 29 cycles.

		D10S1248	vWA	D16S539	D2S1338	AM	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
Positive Ctl		15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
Code	Profile %																
SF1	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		4478, 4372	11349	3302, 3032	2131, 1988	9909	9138, 9191	9392	2945, 2645	4614, 6728	6488, 5293	5854	5982	4698, 10657	3198, 3029	2584, 2291	1967, 1784
SF2	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3164, 3103	8341	2470, 2254	1618, 1465	10237	7902, 7705	9163	2926, 2531	5477, 4974	4940, 4771	4816	6049	3555, 9230	2417, 2235	1958, 1911	1676, 1422
SF3	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		2957, 2975	7917	2676, 2438	1954, 1840	10189	6180, 5837	8100	3043, 2976	4182, 4090	4414, 3953	4372	6606	2685, 7535	2046, 1921	1643, 1573	1399, 1340
SF4	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		2648, 2430	7436	2163, 2090	1497, 1359	10343	5522, 5529	6613	2417, 2122	4000, 3703	3434, 3171	3252	6940	2687, 6905	1893, 1759	1387, 1322	1185, 1074
SF5	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3339, 2924	9757	2307, 2034	1500, 1244	11009	6495, 5834	8512	2063, 1724	5659, 4952	4182, 3423	3613	6725	4096, 9078	2623, 2278	1656, 1525	1225, 1106
SF6	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3149, 3037	8500	268, 2521	1951, 1620	10565	6719, 6937	9220	3890, 3464	4804, 4347	4910, 4347	4583	6171	3004, 8667	2282, 2057	1971, 1872	1553, 1487
SF7	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		5041, 4468	11112	4919, 4212	3417, 3298	9615	8993, 9047	9148	4783, 4036	4402, 6050	5971, 4634	6208	5857	5847, 10926	4681, 4906	3105, 2931	2461, 2244
SF8	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		2255, 2250	5614	1715, 1668	1196, 1143	9599	4753, 4517	7342	3721, 3146	3115, 2605	3263, 3416	2860	6844	1966, 4711	1394, 1426	1373, 1305	1170, 1076
SF9	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3755, 3444	9880	2594, 2345	1654, 1456	10490	8456, 7940	8819	2462, 2194	5192, 5622	5530, 5048	5260	7017	4430, 9923	3169, 2736	2158, 1907	1495, 1375
SF10	100	15, 17	16	9, 11	17, 18	x	13, 15	31.2	14, 18	15, 16	12, 14	9	25	10, 14	14, 15	13, 14	17, 19
		3552, 3265	7732	1490, 1240	954, 809	10581	7163, 6822	6331	2307, 1890	4997, 5156	4945, 4133	3766	5921	3831, 9760	2113, 2126	1808, 1518	904, 894

As mentioned previously in *section 2.5*, the level of fluorescence in a treated DNA sample can be used to indicate how much DNA is present [13, 14]. The data from *section 2.5* supported a linear correlation between DNA mass and the RFU value, therefore a decrease in RFU value would be indicative of a loss of DNA mass. This same principle is applied to determine if DNA is retained on fabric or released entirely during direct PCR. The average RFU values of positive control samples of known DNA masses (2800M, Promega, Victoria, Australia) at 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ng were compared to the average RFU values of the same DNA concentrations amplified directly from fabric squares. Fabric samples were prepared by pipetting the series of control DNA (2800M) onto sterilised fabric squares measuring 2 mm x 2 mm and allowed to dry. This was prepared three times for each mass of DNA (0 – 1 ng) for a total of 33 fabric samples. Each sample was placed directly into a 0.2 mL thin walled tube containing 25 µL of reagents from the NGM^{TM} kit, following the manufacturer's instructions. A total of 29 PCR cycles was performed on a GeneAmp® 9700 96-well thermal cycler following manufacturer's instructions. PCR products (1 µL) were combined with 0.5 µL Liz 500™ separated using a 3130*xl* Genetic Analyser. The data were analysed using GeneMapper® ID v3.2 with a threshold of 50 RFU for allele assignment. A single set of the positive control samples were amplified in the same way. All samples were run in triplicate on the 3130*xl* for a total data set of 33 positive control profiles and 99 fabric profiles (see supplementary material: *raw data examples*). The linear correlation between DNA mass and RFU value was confirmed for both sets of data, with R^2 values above 0.9. The data indicated that overall there was an average RFU value decrease of 21.7 % between the control and fabric samples, suggesting that approximately one fifth of DNA present on this fabric type is not released into the PCR matrix during amplification.

There is obviously a wide range of variables that could contribute to the availability of DNA on the fabric and how much is released to the PCR matrix. This could include the composition of the fabric: if it is synthetic, natural or a combination of both; any dyes present that may inhibit the direct PCR process; exposure of the DNA to environmental pressures; the type of DNA left behind; how much pressure is applied to the fabric through touch; an individual's 'shedder' status; as well as transference factors (direct, or indirect) to name a few. It would be unrealistic to explore all of these factors, and many are constantly changing due to a range of other variable conditions. With these issues in mind, *section 6.3* explores the potential of using single fibres from worn clothing items with direct PCR. The clothing items were selected randomly and consist of various fabric types and colours, as best to mimic a casework scenario.

Statement of authorship

DNA profiles from fibres using direct PCR

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Renée Blackie (Candidate)

Performed all laboratory work (sample collection, PCR amplification, capillary electrophoresis), data analysis and interpretation, created data table, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

6.3.1 *Published manuscript, a technical report, Forensic Science Medicine, and Pathology.*

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TECHNICAL REPORT

DNA profiles from clothing fibers using direct PCR

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Abstract We report on the successful use of direct PCR amplification of single fibers from items of worn clothing. Items of clothing were worn throughout the course of a day, with the individual commencing regular activities. Single fibers were taken from the cuff of the clothing at regular intervals and amplified directly. The same areas were subjected to tape-lifting, and also amplified directly for comparison. The NGM™ kit that amplifies 15 STR loci plus amelogenin was used. A total of 35 single fiber samples were processed and analyzed from five items of clothing, with 81 % of samples returning a profile of 14 alleles or more. All tape-lift samples amplified directly produced DNA profiles of 15 alleles or more. The aim was to develop a simple, operational method that could be used routinely in forensic science casework and that has the potential to generate more complete profiles, which would not be detected using standard extraction methods on this type of sample. For ease of implementation, the process also adheres to standard methods with no increase in the cycle number.

Keywords Direct PCR · Single fibers · Forensic science · NGM STR loci

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Introduction

Direct PCR was first used on forensic relevant samples in 2010, illustrating the potential benefit of using this technique with fibers [1]. Fibers have not been explored in any further detail using this technique since then, with much of the research focusing on other sample types such as hair, fingernails, blood, and trace DNA on various substrates [2–11]. The presence of PCR inhibitors in fabrics, such as dyes, can represent a challenge when directly amplifying single fibers. The previous two decades have seen a vast improvement in all aspects of the DNA profiling process, providing highly robust human identification kits that are more capable of dealing with PCR inhibition than ever before [12, 13]. Even with the increase in sensitivity of modern STR typing kits, trace amounts of nuclear DNA still limits the possibility of generating meaningful DNA profiles using current standard operating procedures. Direct PCR therefore has the potential to increase the number of alleles generated from trace DNA samples. By circumventing the extraction process, time and costs are also reduced, as well as reducing laboratory error or exogenous DNA contamination.

Clothing found at crime scenes can be analyzed for DNA using a variety of techniques such as swabbing, tape-lifting, or extracting directly from cut sections of the fabric [1]. Single fibers, however, are less likely to be processed for DNA and instead are analyzed microscopically [14–16]. The ability to obtain nuclear DNA profiles from single fibers would be of great benefit to investigations, especially where microscopy may fail to provide substantial information. We report on the successful direct amplification of single fibers from worn items using the NGM™ human identification kit.

Materials and methods

Samples

A female participant wore five different long-sleeved clothing items of varying fabric compositions and colors, for 12 h each. The five items were: A1—a maroon cardigan, cotton; A2—a cream with light gray print sweater, cotton; A3—a light gray with dark gray print sweater, cotton; A4—a dark green cardigan, cotton/acrylic; and A5—a light gray cardigan, 80 % acrylic/20 % nylon. The individual washed each item prior to wearing, and was the only individual in direct contact with them between washing and wearing the items. The items were not removed from the individual during the time period. A single fiber, approximately 5 mm in length, was cut from the inside seam of the right cuff of each item at hours 0, 2, 4, 6.5, 9, and 11 of wear, with a tape-lift conducted after 12 h of wear. Samples collected at time points 0, 2, 4, 6.5, and 9 h were amplified using direct PCR methods, with the 11-h fiber samples undergoing standard extraction methods. All clothing items were worn during the course of a regular workday where normal office-based activity was conducted for that individual.

Tape-lifts were conducted on each worn item on the inside of the right cuff using Sellotape® brand adhesive tape. Tape measuring approximately 5 cm × 2.5 cm in size was used for each tape-lift. The tape was pressed over and removed from the targeted area 20 times. A 1 cm × 1 cm section was removed from the center of the tape and placed into a 0.2 mL thin walled tube for direct PCR amplification.

A total of 35 samples were processed and analyzed from the five items of clothing. DNA extracts from buccal swabs were also provided as references of the participant and their cohabiting partner. The fibers were not treated in any manner prior to amplification.

Direct PCR amplification and conditions

Direct PCR was conducted by placing the relevant sample, either a single fiber or section of adhesive tape, into a 0.2 mL thin walled tube containing 10 μL of PCR master mix from the AmpFℓSTR® NGM™ kit (Life Technologies, Victoria, Australia) along with 5 μL of the primer mix and 1 μL (5 U) of additional AmpliTaq Gold® DNA polymerase (Life Technologies). The addition of the AmpliTaq Gold® DNA polymerase is to increase the overall units of enzyme in the reaction to assist in overcoming inhibitors that may be present on the fiber. A further 9 μL of sterile H_2O were added to make the final volume 25 μL. The amplification was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the manufacturer's recommended conditions. During PCR, 29 cycles was used for all reactions. The NGM™ kit amplifies 15 STR loci plus the amelogenin locus.

DNA extraction from single fibers

A single fiber from each item, measuring approximately 5 mm, was extracted using aQIAamp® DNA Mini Kit. A total of five extractions were performed following the manufacturer's protocol for DNA purification from buccal swabs (as swabs are made from fibers). The fibers were incubated for 1 h and eluted in a final volume of 30 μL. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies). From these extracts, 10 μL of DNA was used in the PCR setup using the same conditions as for the direct fiber samples.

Capillary electrophoresis

Capillary electrophoresis was performed on an ABI 3130*xl* Genetic Analyser (Life Technologies) using POP-4™ polymer (Applied Biosystems). An aliquot of 1 μL of the PCR sample was added to a solution of 0.5 μL of ABI GeneScan-600 LIZ® Size Standard and 9.5 μL of Hi-Di™ Formamide. Samples were then denatured at 95 °C for 3 min. Electrophoresis was conducted at 3 kV with a 10 s injection. The data were analyzed using GeneMapper® v3.2. The detection threshold was set at 50 relative fluorescence units (RFU).

Results

Two of the five samples tested at time 0 h, directly after washing, produced just one allele each—the X-chromosome amelogenin marker. All samples from item A4 produced no alleles, and upon further testing it was concluded that an inhibitor, most likely the dye (a dark green), had interfered with the direct PCR process. No alleles were observed for the five 11-h fiber samples that were processed using standard extraction methods. The remaining 16 timed fiber samples (2, 4, 6.5, and 9-h wear) amplified directly using the NGM™ kit were analyzed (see Table 1).

All fiber samples, except for two, produced mixed DNA profiles displaying major and minor contributors. A single minor allele was observed in seven of these samples, with the remaining samples containing between two and 15 minor alleles. All alleles obtained could be attributed to the donor or their cohabiting partner. Only one profile contained a single allele that could not be attributed to either. Item A3 at two time points (4 and 9 h) produced profiles

Table 1 Summary of alleles obtained from single fibers amplified directly from items worn over a 9-h period

Item Hours item worn for	A1 Total number of alleles obtained	A2	A3	A4	A5
0	1	0	0	0	1
2	15 (0)	29 (1)	3 (1)	0	30 (6)
4	16 (1)	21 (0)	14 (14)	0	33 (7)
6.5	25 (9)	5 (1)	2 (1)	0	24 (1)
9	42 (15)	17 (2)	21 (21)	0	14 (1)
11[a]	0	0	0	0	0
12[b]	39 (15)	43 (16)	15 (6)	0	22 (8)

Data includes 11-h time point of fibers processed with standard extraction methods, and 12-h time point of tape-lifts amplified directly. Number of foreign/non-donor alleles in brackets

[a] 11-h time point samples are single fibers processed with standard extraction methods

[b] 12-h time point samples are from directly amplifying a section of adhesive tape after a tape-lift

comprising of alleles *only* from the cohabiting partner, and none from the participant wearing the item. Time points 2 and 6.5 h produced insignificant data of three alleles or less. There is no distinguishable pattern of allelic increase when items are worn for longer periods of time (Table 1).

A full DNA profile of the participant, comprising all 15 STR loci and the amelogenin locus, was obtained from two samples (example Fig. 1). A further nine samples produced a DNA profile of five or more complete STR loci (partial profile) that could be uploaded to the Australian National Criminal DNA Database (NCIDD). Three samples in total produced five alleles or less, with two samples producing 14 and 21 alleles of the cohabiter only. An additional four profiles, ranging between 15 and 43 alleles, were obtained using direct amplification of the adhesive tape, with profiles typically showing a large number of non-donor alleles (example Fig. 2).

Discussion

A presumption in sampling DNA directly is that nuclear material is present on the surface of the sample and is released and amplified during PCR. The human body is constantly shedding dead skin, perspiring, and coming into contact with foreign DNA during daily activity. To see non-donor DNA appearing on the fibers of worn items, via secondary and/or subsequent DNA transfer, would not be unexpected when sampling directly [17–19]. The participant of this study was asked if there was any change to their routine on the day they wore item A3, as it produced two profiles comprising of alleles only from their cohabiting partner. They noted the only significant difference was that they did not shower in the morning before work, but at night instead. As the participant and their partner

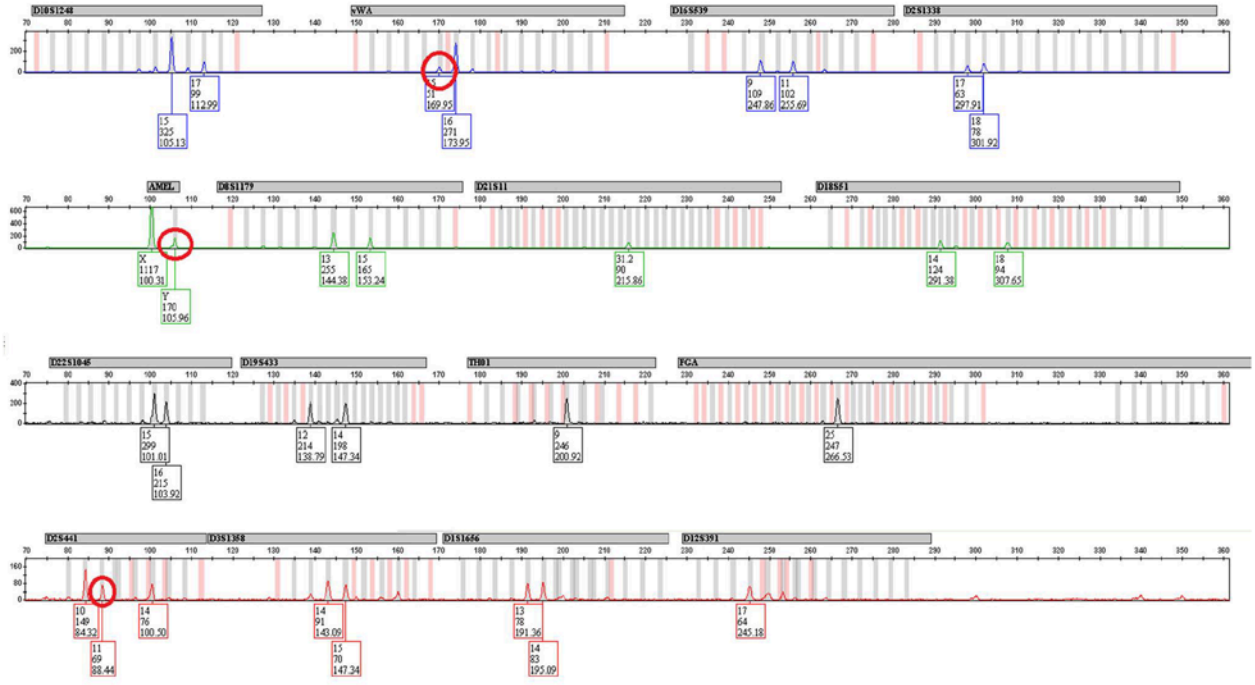

Fig. 1 Chromatogram of nuclear DNA from a single clothing fiber after 2 h of wear. A fiber was amplified directly using AmpF*ℓ*STR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Sample was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. Non-donor allelic contributions are highlighted with *circles*

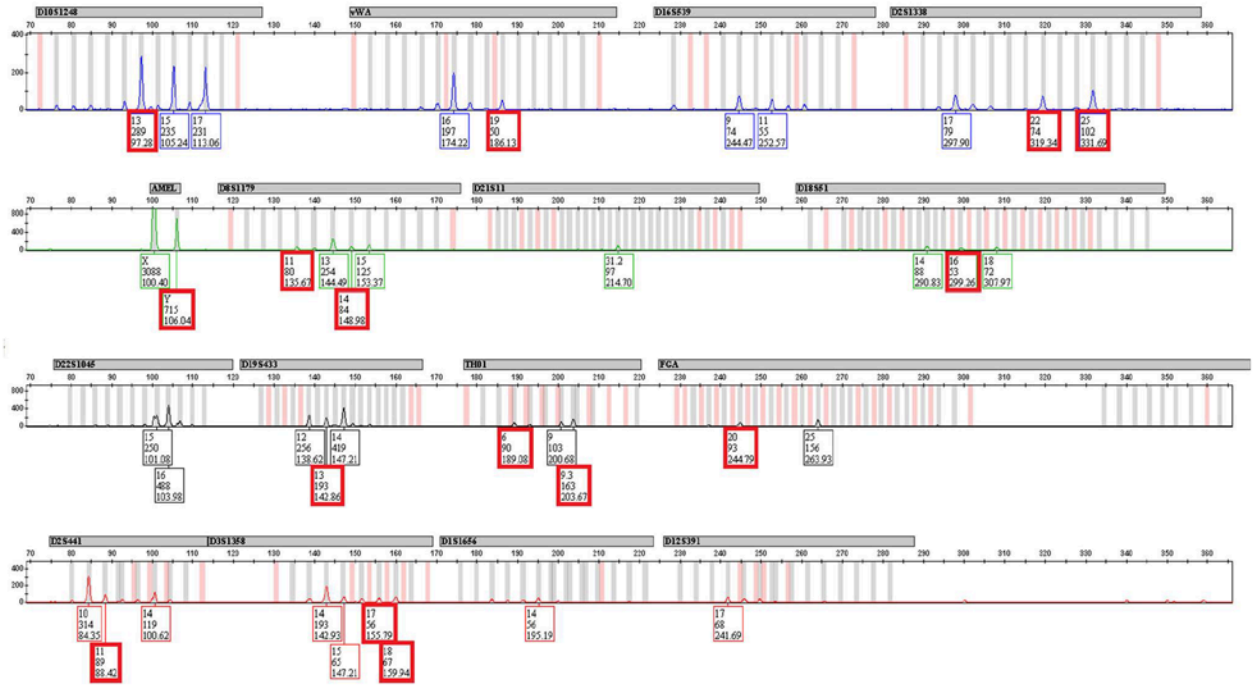

Fig. 2 Chromatogram of nuclear DNA from a tape-lift of clothing item after 12 h of wear. A 1 cm^2 section of tape was amplified directly using AmpF*ℓ*STR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Sample was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s. Non-donor allelic contributions are highlighted with *boxes*

share a bed, we propose that there was opportunity for the participant to accumulate shed cells and free DNA from their partner onto their own body during the night. Thus allowing any foreign DNA collected on the skin, in this case that of their partner, to be readily transferable to worn items of clothing.

The presence of DNA on clothing directly after washing has also been previously reported [20]; indicating DNA can be transferred between items during the wash cycle. This could possibly explain alleles present at the 0 h time point. However, improved multiplexes and instrumentation also greatly increases the sensitivity of detection, allowing DNA profiles to be obtained from just a few cells, or cell-free DNA [21–24]. By amplifying the samples directly, the amount of DNA available to the PCR matrix is maximized, as the nuclear DNA is not lost to the extraction process [1, 4, 5]. Therefore, any DNA or cell-free DNA that is not destroyed during the washing process, and any contact made with foreign DNA, has a higher chance of being detected during direct amplification.

The profiles obtained from direct PCR of tape-lifts are consistent with previous studies, showing a higher number of alleles obtained using tape-lift extraction compared to other extraction methods such as swabbing [25–27]. These results are also consistent with previous research indicating that DNA from the non-wearer is often found on items of clothing, mostly detected as a minor profile [18, 28, 29]. The direct PCR technique combined with the tape-lift extraction is a much faster process than previously described, and does not require substantial adaption to current amplification methods.

Conclusion

Direct PCR of single fibers produced DNA profiles of at least 14 alleles or more, which could be uploaded to the Australian NCIDD in 81 % of samples tested. Similarly, all tape-lift samples amplified directly produced DNA profiles of 15 alleles or more. Only three single fiber samples failed to produce up-loadable profiles. The results are a significant increase compared to the process of performing standard DNA extraction prior to amplification, where single fibers failed to produce any alleles. Only one item tested contained direct PCR inhibitors within the fiber—most likely the composition of dyes.

Each sample only required a single fiber, or a small cut-out of adhesive tape, allowing for additional testing to occur, as well as targeting several areas of the clothing item if required. This simple process of direct PCR from single fibers and adhesive tape can be readily adopted for use into forensic DNA practice as standard validated methods were used. DNA profiles can be generated at a reduced cost and

faster than when standard extraction methods are implemented, producing higher quality results.

Key points

1. We describe a successful method for obtaining DNA profiles from single clothing fibers and adhesive tape using direct PCR.
2. Profiles that were suitable for up-loading to the Australian National Criminal DNA Database were obtained from 81 % of the single fiber samples tested.
3. The standard protocol for DNA amplification from the NGM™ STR typing kit was not modified, allowing for fast implementation into forensic laboratories.
4. Only a single fiber is required for testing, allowing for testing of multiple sections of clothing.
5. Direct PCR provides more DNA to the PCR matrix than standard extraction methods, resulting in more useful DNA profiles, therefore saving time and money in forensic casework.

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6.4 Applications

Section 6.3 highlights the potential of using single fibres from clothing in conjunction with direct PCR. Amplifying sections of tape-lifts directly also provided a higher number of alleles than standard extraction methods. Using multiple tape-lifts and single fibres from one item of clothing could possibly provide a series of DNA profiles to assist in an investigation where DNA information may not be generated otherwise. Consensus and composite profiling can also be implemented using data generated from the direct amplification of these samples. Composite profiling combines all alleles observed from multiple replicates. An allele only needs to appear once to be recorded. Consensus profiling requires alleles to appear more than once and is only recorded if observed in each replicate (Figure 6.2).

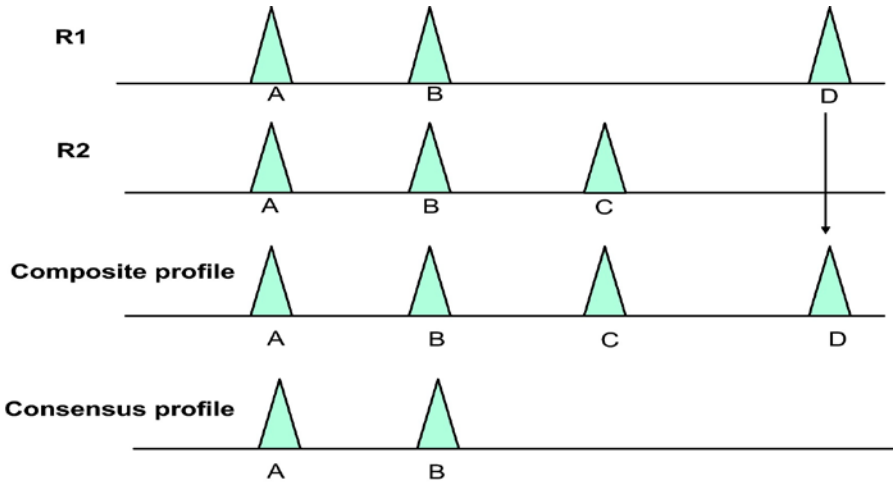

Figure 6.2 – Understanding the difference between composite and consensus profiles by comparing two replicates, R1 and R2. The consensus profile only contains alleles A and B as they appear in both replicates, whereas the composite profile contains all alleles (A, B, C and D) observed in both replicates. *Figure source: Bright [15]*.

Caution should be observed when applying these two analysis techniques. Consensus and composite profiles are created by amplifying DNA from a single extraction multiple times, or by injecting the same PCR product multiple times. Only the latter should be applied when using direct PCR methods, as per the recommendations of the SWGDAM guideline for interpreting composite and consensus profiles [16]. It should be noted that only American laboratories are required to follow SWGDAM guidelines. Further research using direct PCR and consensus profiling may need to be conducted to determine how conservative the profiles are, that alleles are associated as donor or non-donor (major or minor contributors) with a reduction or elimination of allelic drop-in [17, 18]. Semi-continuous or fully continuous methods that utilise all the information from replicates

generated from a single DNA extract [19], could also be applied to profiles run from the same direct PCR product.

Projecting forward into the use of fibres and direct PCR would be to investigate the suitability of other common fabric types with this method. The items used in the preliminary experiments (*section 6.2*) and explored further in *section 6.3* were either cotton or mostly cotton blends. Denim would be an obvious choice, however it is likely that indigo dyes commonly present in denim will inhibit the PCR process [20, 21]. As mentioned previously, it would be unrealistic to explore the suitability of all fibre types, however many common blends are yet to be investigated. Textile fibres from carpet, tents, sails, ropes and cordages are also commonly found at crime scenes [2] and are worthy of investigation using direct amplification methods.

It is also clear that directly amplifying sections of a tape-lift yields impressive data that would not be obtained by standard methods, or without an increase in cycle number. Tape-lifting is the most effective and common method to remove trace evidence from items, clothing, and corpses at a crime scene [2]. This has long been the standard procedure for fibre investigations [2, 22]. Tape-lifts are able to collect foreign hairs and fibres, as well as skin flakes, and any other DNA (cell-free) that has been transferred directly or indirectly. The identification of skin flakes on tape-lifts using microscopy has enabled the DNA to be successfully extracted and amplified [22]. The process however, is laborious and only resulted in DNA profiles from approximately 15 % of 500 individually extracted skin flakes. From the successful samples, only 5 % yielded full DNA profiles. LT-DNA typing methods using 33 cycles were implemented to obtain this data. Using standard methods, only 0.5 – 1 % of selected samples yielded full profiles. Although these success rates can be considered low, it has opened up a pathway to re-examine tape-lifts for cellular DNA where previously only fibres and hairs were collected for examination. This method was applied to cold cases and resulted in a conviction in two cases [22]. Direct amplification of tape-lifts from cold cases has the potential to improve on these results as cycle number increase is not required, and significant DNA mass is not lost to the extraction step. The process is significantly faster, and multiple samples can be amplified from a single tape-lift.

6.5 Concluding Remarks

Although this research conducted on fibres is considerably small in the vast world of fabrics, the benefits highlighted are huge. Two additional methods for acquiring DNA from fabrics include amplifying single fibres or small fragments directly, and using small sections of tape from a tape lift. Using these tools to complement current analysis techniques of fibres can offer investigators more information in a short amount of time. It is clear that there are limitations involved when using fibres as a direct PCR substrate, there are too many variables to investigate thoroughly, and inhibition from dyes will likely be an ongoing issue. The discrimination power offered by DNA however, is a valuable asset to any investigation and always worthy of pursuing. Like previous substrates, these methods do not require the enhancement of cycle number or significant protocol adjustments to those used in forensic laboratories on typical trace DNA samples.

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Supplementary Material

a. DNA profiles from clothing fibres using direct PCR

Clothing items

Figure 6a.1 – Red/maroon coloured cardigan, labelled cotton, item ID for experiment is A1.

Figure 6a.2 – Cream with grey print jumper, labelled cotton, item ID for experiment is A2.

Figure 6a.3 – Light grey with dark grey print jumper, labelled cotton, item ID for experiment is A3.

Figure 6a.4 – Dark green cardigan, labelled cotton/acrylic, item ID for experiment is A4.

Figure 6a.5 – Light grey cardigan, labelled 80 % acrylic/ 20 % nylon, item ID for experiment is A5.

Laboratory setup examples

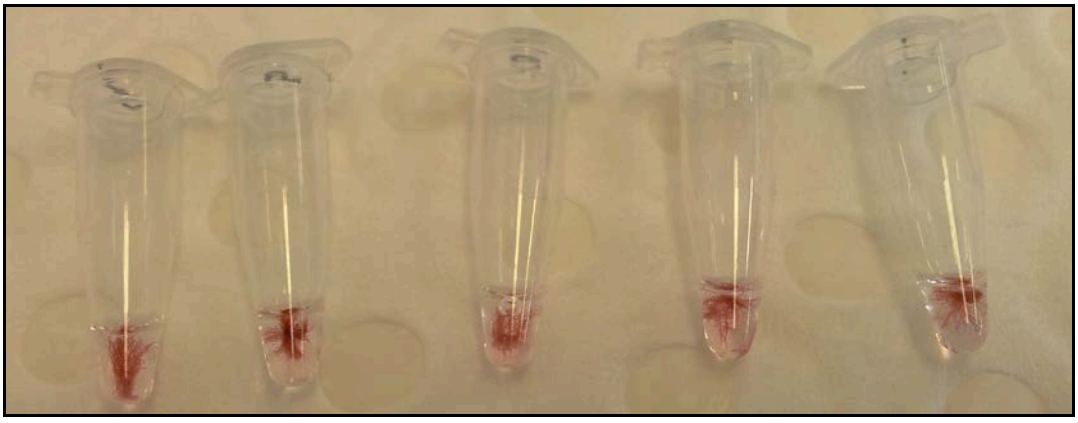

Figure 6a.6 – Example of fibres from item A1 inside 0.2 mL PCR tubes.

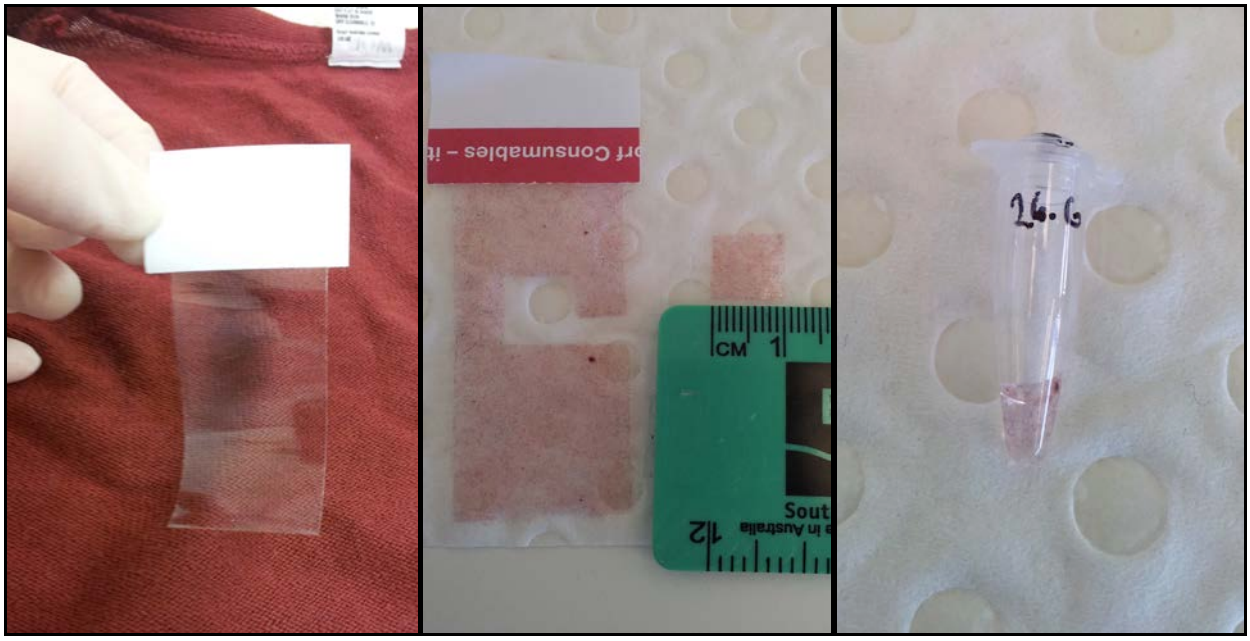

Figure 6a.7 – Tape lifting item A1, central piece of tape cut for direct PCR, and cut tape inside a 0.2 mL PCR tube.

Raw data examples

Table 6a.1 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.1 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM			D8S1179		D21S11		D18S51			D22S1045		D19S433		TH01		FGA			D2S441		D3S1358		D1S1656			D12S391		
01_n_a	13	15	16	19	9		22	25	X	Y		14	15	29	31.2	16	18		16	13	14	6	9.3	20	23		10		18			13		18		23
	251	175	164	87	158		268	93		194	225	204	226	237	158	204	213		441	208	330	398	210	259	243		105		79			81		99		96
01_n_b	13	15	16	19	9		22	25	X	Y		14	15	29	31.2	16	18		16	13	14	6	9.3	20	23											
	202	144	129	68	131		195	70		126	152	152	159	113	81	128	130		264	141	223	250	118	135	127											
01_n_c	13	15	16	19	9		22	25	X	Y		14	15	29	31.2	16	18		16	13	14	6	9.3	20	23											
	179	117	110	55	105		158	55		98	113	107	111	86	57	88	90		207	106	163	179	94	90	96											
01_1_a	13		16				22								31.2					13		6										13				
	64		98				59								61					72		139										51				
01_1_b			16																			6														
			64																			76														
01_1_c			16																			6														
			51																			57														
01_2_a			16	19	9		22	25		Y		14	15	29	31.2	16	18		16	13	14	6	9.3	20							12	13				
			98	50	111		78	86			1034	174	142	71	134	162	133		255	90	122	264	183	58							57	64				
01_2_b					9				X	Y		14	15			16	18		16		14	6	9.3													
					53					404	376	66	50			53	57		101		55	89	61													
01_2_c									X	Y		14					18		16			6	9.3													
										391	377	59					52		85			88	65													
01_3r_a							22										18		16				9.3													
							78										58		55				141													
01_3_b																							9.3													
																							57													
01_3_c																							9.3													
																							71													

Table 6a.2 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.2 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
02_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20		10	14	17	18	12		18	23
	134	86	204	147	128	174	177	109	363	398	248	117	52	268	129	216	229		507	127	388	541	276		58	156	165	78	164		80	88
02_n_b	13	15		19	9	13		X	Y		14	15	29		16	18	16		13	14	6	9.3					17	18	12	13	18	
	240	247		196	51	239			123	482	244	283	122		105	99	267		190	140	345	316					60	85	54	84	118	
02_n_c	13	15	16	19	9	13	22	25 X	Y		14	15		31.2	16	18	16		13	14	6	9.3	20			14	17		12			
	83	56	120	83	80	105	96	60	195	213	144	64		107	59	104	114		261	62	179	246	110			72	64		53			
02_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2			16		13	14	6	9.3	20	23		14	17	18	12	13	18	23
	166	52	98	156	178	163	74	201	564	289	227	80	245	258			338		179	187	395	242	173	139		184	62	127	139	128	148	78
02_1_b	13		16	19	9	13	22	25 X	Y		14	15	29	31.2			16		13	14	6	9.3	20	23		14		18	12	13	18	
	141		74	121	135	122	50	136	461	252	202	64	167	166			251		140	146	265	164	117	87		78		65	63	57	65	
02_1_c	13		16	19	9	13		25 X	Y		14		29	31.2			16		13	14	6	9.3	20	23								
	92		53	82	88	83		91	253	130	107		87	89			142		85	75	146	94	70	55								
02_2_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13 *		6	9.3	20	23	10	14	17	18	12	13	18	23
	156	128	258	213	342	65	161	96	860	516	132	222	144	116	373	155	779		312		479	118	84	107	186	193	114	142	134	134	161	99
02_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13		6	9.3	20	23	10	14	17	18	12	13	18	23
	134	111	240	198	307	64	132	78	691	409	108	179	115	93	305	125	584		226		363	104	66	88	113	97	71	92	88	85	100	58
02_2_c	13	15	16	19	9		22	25 X	Y		14	15	29	31.2	16	18	16		13		6	9.3		23	10	14	17	18	12	13	18	
	97	88	184	146	232		97	56	485	293	74	124	77	66	210	89	429		165		265	72		60	81	73	50	63	66	61	73	
02_3r_a	13	15		19	9	13	22	25 X	Y		14	15	29		16	18	16		13	14	6	9.3	20	23			17	18	12	13	18	23
	221	224		192	50	237	138	77	109	426	225	259	107		107	60	242		173	136	323	281	202	192			52	78	53	77	103	73
02_3_b	13	15		19		13	22	25 X	Y		14	15	29		16	18	16		13	14	6	9.3	20	23				18		13	18	
	215	216		178		202	117	64	106	386	216	256	96		92	81	222		154	131	281	240	157	159				69		53	78	
02_3_c	13	15		19		13	22	X	Y		14	15	29		16	18	16		13	14	6	9.3	20	23							18	
	164	157		132		160	89		74	280	149	171	69		69	56	171		102	85	200	179	119	114							52	

Table 6a.3 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.3 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
03_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18		13	18	23
	255	242	301	140	350	406	262	259	636	244	474	367	413	258	489	313	1247		733	533	475	623	331	296	196	400	83	210		199	216	226
03_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14		18		13	18	23
	174	163	202	88	227	263	151	153	320	124	270	201	174	106	240	158	643		385	279	234	292	136	128	73	138		70		63	65	63
03_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14		18		13	18	23
	214	201	233	103	258	301	172	164	382	141	297	231	197	120	270	165	742		436	324	244	317	151	137	85	165		81		62	73	72
03_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10		17	18	12	13	18	23
	358	231	244	123	280	230	151	309	529	354	351	293	302	202	242	395	902		495	273	234	1059	315	106	170		286	184	269	122	193	100
03_1_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20		10		17	18	12		18	
	223	145	154	75	170	136	84	161	263	175	196	172	133	90	121	213	428		231	138	105	462	130		63		98	64	79		57	
03_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20				17	18	12			
	170	111	116	56	121	97	58	116	194	133	145	118	93	63	79	143	306		169	91	81	325	91				75	53	60			
03_2_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16		16		13	14	6	9.3	20	23	10	14		18	12	13	18	
	291	130	285	192	127	185	282	144	1378	928	350	311	265	110	239		817		619	398	443	497	213	214	129	142		114	101	161	70	
03_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16		16		13	14	6	9.3	20	23	10			18	12	13		
	183	83	191	130	80	118	163	88	757	490	184	169	133	58	122		417		305	209	230	260	102	116	56			53	51	73		
03_2_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16		16		13	14	6	9.3	20	23	10	14		18	12	13		
	185	84	196	130	84	120	169	86	838	561	217	194	148	69	129		465		335	223	256	281	126	137	73	98		67	64	100		
03_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	338	164	155	135	143	93	154	209	273	342	676	278	178	139	131	69	694		287	338	516	437	219	161	53	134	79	53	161	87	102	
03_3_b	13	15	16	19	9		22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		14			12			
	228	100	101	88	82		81	115	202	235	505	215	108	80	85	60	474		187	229	320	268	122	88		74			95			
03_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23					12			
	177	84	80	75	83	53	79	109	129	146	319	135	88	65	69	54	313		119	152	226	210	88	72					68			

Table 6a.4 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.4 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
04_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	195	503	357	266	492	238	430	220	595	703	715	383	517	381	554	526	685		393	331	565	700	382	434	263	381	298	476	157	161	229	345
04_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	155	400	279	205	384	185	302	151	341	420	479	256	262	189	315	297	430		256	224	329	384	162	222	114	157	118	190	60	51	81	112
04_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	18	23	
	138	330	244	182	337	159	273	134	317	341	423	230	229	170	273	270	373		210	182	271	337	157	183	107	150	108	168	52		80	107
04_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	372	269	64	182	215	199	249	193	484	449	277	541	224	179	222	262	1072		410	391	540	215	309	243	113	174	229	277	62	127	76	122
04_1_b	13	15		19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18		13		23
	225	161		110	131	115	139	109	282	264	181	349	114	90	128	170	582		218	217	283	119	160	122	62	91	109	132		53		50
04_1_c	13	15		19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18				
	194	149		103	113	100	112	87	212	209	148	287	95	71	91	122	461		174	163	219	95	123	94	50	64	92	114				
04_2_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3		23	10	14	17	18	12	13		23
	182	216	129	140	79	106	104	127	445	236	100	159	51	107	108	92	625		328	324	295	335		96	127	103	62	130	199	123		56
04_2_b	13	15	16	19	9	13	22	25 X	Y		14	15		31.2	16	18	16		13	14	6	9.3		23	10	14		18	12	13		
	152	179	124	131	78	109	117	160	341	167	76	111		90	78	100	467		241	237	236	270		80	78	60		82	135	84		
04_2_c	13	15	16	19	9	13	22	25 X	Y		14	15		31.2	16	18	16		13	14	6	9.3		23	10	14		18	12	13		
	164	195	136	146	89	120	127	179	372	175	87	126		102	85	113	498		253	259	259	300		89	77	61		84	153	93		
04_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12		18	23
	110	187	442	130	140	218	194	248	430	343	193	144	174	152	261	126	625		449	63	426	304	201	233	144	199	103	86	63		143	71
04_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13		6	9.3	20	23	10	14	17	18			18	
	94	152	388	112	119	170	146	184	323	266	163	119	121	105	192	131	454		317		288	220	126	151	90	110	67	65			82	
04_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13		6	9.3	20	23	10	14					18	
	68	111	270	84	90	135	115	144	231	179	103	79	89	82	160	100	313		203		218	176	102	123	66	77					66	

Table 6a.5 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.5 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
05_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	343	245	461	337	166	336	493	413	559	620	441	314	220	544	291	365	1084		856	310	381	624	299	372	430	226	185	217	226	152	98	196
05_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13		23
	263	187	366	280	161	328	523	458	316	361	274	203	130	316	251	318	618		487	186	218	387	176	255	169	85	67	82	83	61		81
05_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	316	229	519	400	235	490	783	672	416	487	450	322	205	506	415	519	802		757	303	349	618	288	387	213	104	105	125	128	86	63	131
05_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	415	224	649	88	534	220	294	170	1707	842	347	593	316	129	246	197	1336		543	294	1302	478	408	282	133	312	269	227	143	252	313	128
05_1_b	13	15	16		9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		14	17	18		13	18	
	199	103	330		308	133	199	123	680	339	155	279	127	54	156	142	507		208	122	509	192	156	136		71	75	65		70	94	
05_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23		14	17	18	12	13	18	23
	255	133	420	55	405	177	272	172	850	404	191	331	163	69	205	189	587		247	142	624	237	200	164		64	95	82	55	91	121	51
05_2_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	338	331	451	389	216	426	164	123	571	1095	630	547	405	170	143	252	1709		746	945	627	405	129	163	349	227	293	179	275	159	140	80
05_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	212	204	299	265	168	354	178	149	317	592	358	305	231	99	148	247	916		383	493	342	246	83	118	155	92	132	83	136	78	81	
05_2_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	188	185	278	240	153	318	166	137	289	540	336	286	208	95	137	225	827		340	443	309	219	76	101	138	80	120	78	131	70	74	
05_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	365	417	424	465	440	176	418	205	1052	646	462	326	119	137	258	180	1184		603	673	525	825	111	236	349	154	123	187	179	167	145	90
05_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	182	208	233	251	228	86	199	95	545	332	273	196	62	70	133	133	594		316	342	273	405	59	116	177	96	72	116	93	90	69	
05_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14		18	12	13	18	
	155	173	179	204	222	86	195	96	432	254	184	132	56	62	124	122	432		206	228	201	359	57	98	153	69		77	74	70	64	

Table 6a.6 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.6 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
06_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	601	499	446	493	260	563	602	351	1312	692	520	556	741	567	575	644	1501		605	1002	896	1297	326	301	541	296	368	358	318	205	317	261
06_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	484	404	344	388	207	466	440	263	759	420	317	330	346	265	324	354	905		343	562	459	675	151	147	223	98	133	132	109	69	105	83
06_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	381	316	309	353	196	430	403	239	627	341	315	332	342	267	335	363	751		323	539	451	659	156	143	184	85	122	125	103	63	105	83
06_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	535	303	548	253	235	293	514	150	1090	765	740	630	363	493	357	264	1040		318	617	775	726	285	518	151	295	234	302	183	121	150	194
06_1_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	390	215	404	185	172	213	347	100	767	544	594	517	251	327	252	212	706		219	415	516	477	179	331	95	184	135	182	93	68	78	106
06_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	364	200	361	161	146	184	308	90	805	562	598	511	228	303	240	198	731		211	416	506	463	168	308	79	137	115	156	81	52	64	78
06_2_a	13	15	16	19	9	13		X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	342	160	64	131	167	80			269	478	279	196	158	122	176	118	609		219	149	214	381	80	116	117	193	78	133	82	114	87	54
06_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	395	192	98	204	269	142	141	234	271	484	323	224	196	156	234	199	655		237	157	265	502	101	172	94	148	74	113	87	114	96	56
06_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	508	249	126	254	341	175	187	308	366	667	464	319	252	208	322	268	881		343	232	359	660	126	223	126	198	102	157	117	151	122	71
06_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	461	452	398	423	190	242	179	315	487	745	396	323	259	158	285	407	1832		393	720	726	578	227	407	212	208	198	138	122	187	225	68
06_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	309	318	283	307	127	161	108	192	288	435	264	218	145	92	171	287	1093		236	433	412	326	123	215	101	93	105	73	65	99	103	
06_3_c	13	15	16	19	9	13		25 X	Y		14	15	29		16	18	16		13	14	6	9.3	20	23	10		17	18			18	
	155	149	121	131	56	62		80	147	226	127	105	67		86	137	559		109	190	177	162	55	98	52		56	51			52	

Table 6a.7 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.7 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
07_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	384	519	412	303	346	261	570	497	722	373	329	466	396	374	333	500	1675		703	476	738	839	234	288	464	464	473	236	257	227	306	343
07_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	301	413	316	232	306	239	595	542	445	232	213	311	223	212	272	416	1038		433	293	415	495	147	191	205	198	191	95	93	86	127	158
07_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	412	563	511	385	511	399	968	893	619	339	361	525	379	360	458	710	1526		750	509	728	882	218	326	235	203	254	135	138	120	178	210
07_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	300	293	168	388	280	219	177	126	1088	764	507	211	367	237	224	140	1050		699	449	422	674	191	231	431	608	184	199	297	156	114	79
07_1_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	441	441	278	664	538	430	390	281	1346	980	805	336	532	359	438	357	1442		1022	651	564	1003	251	381	342	396	158	174	254	134	104	71
07_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	297	305	187	440	363	288	265	191	870	623	513	213	334	219	280	224	917		621	400	350	605	149	240	220	251	101	114	157	83	66	
07_2_a	13	15	16	19	9	13		X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	594	335	266	317	186	191			495	609	389	493	293	255	204	228	950		459	638	640	561	211	232	299	292	282	156	203	186	180	193
07_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	456	277	267	318	203	206	222	335	338	430	301	381	243	215	187	250	713		318	443	530	491	175	209	138	117	153	86	134	113	113	127
07_2_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	340	207	205	245	154	162	176	261	261	342	249	316	186	170	147	195	546		284	393	423	376	138	157	112	94	128	77	107	94	87	95
07_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	417	279	262	452	199	277	227	217	612	645	398	463	221	265	255	301	1008		736	505	735	599	266	318	199	246	223	168	224	271	115	101
07_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	286	193	195	335	135	185	145	146	411	417	326	375	154	184	179	255	755		548	382	521	435	166	209	128	132	153	114	144	177	70	60
07_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	301	195	188	324	135	177	147	142	477	496	334	388	161	184	196	273	852		572	404	531	466	168	213	122	114	137	107	135	161	61	50

Table 6a.8 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.8 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
08_n_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	867	524	572	540	525	401	569	948	1147	1230	847	815	677	903	1171	665	2332		1252	1702	1122	1228	582	214	420	355	528	489	441	321	322	530
08_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	746	435	507	469	461	360	446	753	747	724	631	608	404	517	795	456	1593		882	1182	687	758	321	143	211	147	235	219	193	130	124	203
08_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	723	427	523	498	509	400	480	814	681	738	668	635	431	555	895	518	1490		897	1224	736	823	353	147	192	148	253	233	198	138	139	235
08_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	770	641	396	226	590	411	424	357	2151	1062	546	963	275	324	596	404	1595		1225	1323	1212	892	331	845	353	354	258	415	544	378	215	240
08_1_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	562	464	299	171	465	324	310	254	1242	621	394	675	163	202	411	315	900		700	764	702	490	169	500	156	115	115	194	242	162	88	103
08_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	335	272	173	98	266	177	172	144	790	401	244	429	96	122	254	195	577		444	475	440	298	116	298	130	138	97	153	174	121	64	76
08_2_a	13	15	16	19	9	13		X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	586	361	506	90	446	377			955	1106	631	675	320	351	346	500	1030		836	827	917	984	437	337	263	256	311	220	239	266	128	170
08_2_b	13	15	16		9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	106	119
	262	166	265		224	189	173	203	337	361	265	280	129	142	179	235	407		297	286	381	403	184	148	94	111	125	83	18	23	52	73
08_2_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	408	252	409	75	336	285	270	314	500	558	435	460	197	213	278	357	636		527	523	593	638	266	227	125	125	163	117	144	153	68	85
08_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	453	373	343	452	413	519	358	134	1641	723	734	504	175	239	537	323	1233		912	442	736	948	358	306	243	289	271	226	219	180	114	150
08_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	370	321	306	403	361	437	290	113	1143	500	602	431	139	181	452	324	899		670	300	537	698	264	214	147	147	180	155	157	125	71	89
08_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	322	265	241	323	311	371	243	95	946	415	474	333	113	148	372	274	731		484	238	414	566	196	179	127	122	145	119	126	100	56	72

Table 6a.9 – NGM™ profiles showing allele call and RFU value for triplicate samples of 0.9 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
09_n_a	13	15	16	19	9	13		X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	762	1169	427	579	388	262			1094	1253	1286	851	971	702	77	60	2536		1662	1288	1236	1463	395	225	361	640	557	432	386	373	165	131
09_n_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	591	900	328	467	615	651	504	548	740	851	991	660	653	526	743	724	1703		1177	891	847	1113	461	337	185	304	254	194	175	168	142	181
09_n_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	579	887	367	519	728	768	583	643	572	663	856	569	626	496	690	692	1381		1034	785	744	1028	444	335	142	236	225	172	158	148	127	172
09_1_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	930	735	219	510	280	290	114	85	1423	2181	923	740	345	384	165	91	2757		843	1065	1243	1371	357	307	591	655	473	433	309	260	182	
09_1_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	762	603	227	541	344	417	215	167	778	1242	766	636	300	331	223	135	1672		607	809	1015	1147	331	347	245	257	222	209	190	168	122	
09_1_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	572	466	187	449	337	440	268	215	512	838	538	449	228	260	226	137	1109		428	553	748	868	296	306	173	176	160	152	143	128	108	
09_2_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	825	486	817	528	584	433	758	638	996	1857	963	858	745	472	509	371	2231		1203	1123	630	2087	686	591	414	605	400	466	362	377	453	172
09_2_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	534	321	571	354	357	268	433	363	559	1039	546	496	396	250	257	217	1295		648	588	344	1187	345	307	182	243	184	218	178	174	210	77
09_2_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	453	271	478	297	304	222	364	309	432	808	461	426	310	190	212	165	1031		566	528	285	921	280	245	156	223	161	192	147	148	153	59
09_3r_a	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	468	511	582	825	455	177	373	491	3228	4090	606	681	193	356	468	405	1390		836	806	886	1013	337	348	328	269	172	207	319	189	189	205
09_3_b	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	339	361	506	713	351	133	292	389	1691	2150	441	516	142	249	348	331	926		564	551	573	678	218	243	186	87	101	128	218	129	115	120
09_3_c	13	15	16	19	9	13	22	25 X	Y		14	15	29	31.2	16	18	16		13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	218	248	307	436	241	94	193	259	1206	1522	290	327	97	174	247	244	645		371	349	395	497	170	173	165	166	77	103	164	102	89	98

Table 6a.10 – NGM™ profiles showing allele call and RFU value for triplicate samples of 1 ng of neat DNA (blue), and on white cotton fabric squares (green, yellow and red), amplified using direct PCR methods at 29 cycles. Results used to determine loss of DNA (or DNA retention to the fabric) based on RFU values.

	D10S1248		vWA		D16S539		D2S1338		AM		D8S1179		D21S11		D18S51		D22S1045		D19S433		TH01		FGA		D2S441		D3S1358		D1S1656		D12S391	
1_n_a	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	547	630	831	495	533	702	702	477	921	1117	986	1116	1074	662	961	1321	3121	1738	1938	1514	1421	868	541	540	668	534	609	593	634	593	253
1_n_b	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	427	492	680	409	422	563	522	366	470	589	643	715	526	325	555	762	1851	1044	1152	834	786	434	280	228	255	201	228	219	228	202	85
1_n_c	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	396	457	696	420	447	589	551	385	430	541	665	723	558	351	599	828	1704	1056	1188	856	807	455	301	201	241	208	235	225	242	219	89
1_1_a	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	653	777	614	753	581	365	959	258	3643	1843	785	1069	432	669	917	970	2404	1556	1277	963	1490	809	910	789	367	412	514	551	441	351	348
1_1_b	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	291	351	281	340	241	149	361	95	1283	651	341	458	139	220	320	361	923	564	457	327	491	243	298	223	58	108	141	141	109	79	76
1_1_c	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	318	385	303	370	272	168	415	111	1459	742	377	515	168	257	388	426	1026	657	513	378	555	303	357	299	141	146	192	181	149	116	106
1_2_a	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	866	1117	642	698	531	675	474	462	1232	741	1048	1204	707	445	656	735	2121	1501	1294	1461	1399	919	577	298	344	615	509	499	231	224	233
1_2_b	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	536	684	456	507	346	433	298	295	709	415	710	811	444	298	421	476	1418	914	783	954	922	590	363	147	158	310	266	292	136	115	119
1_2_c	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	416	540	371	405	287	357	249	243	440	268	509	592	299	199	294	334	933	694	588	674	629	402	255	99	99	228	187	205	90	74	81
1_3r_a	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	349	509	165	322	358	288	365	413	9355	6490	643	567	311	187	248	243	1252	649	873	718	847	314	344	235	8624	242	254	279	276	178	99
1_3_b	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	23
	339	513	154	291	319	242	300	339	9014	6431	627	544	262	154	225	271	1141	564	736	575	683	214	257	190	7103	177	189	201	194	116	62
1_3_c	13	15	16	19	9	13	22	25	X	Y	14	15	29	31.2	16	18	16	13	14	6	9.3	20	23	10	14	17	18	12	13	18	
	271	421	128	247	280	212	258	288	9094	4441	431	380	204	125	169	203	783	390	518	413	554	182	209	139	3849	130	135	155	153	93	

Chromatogram examples

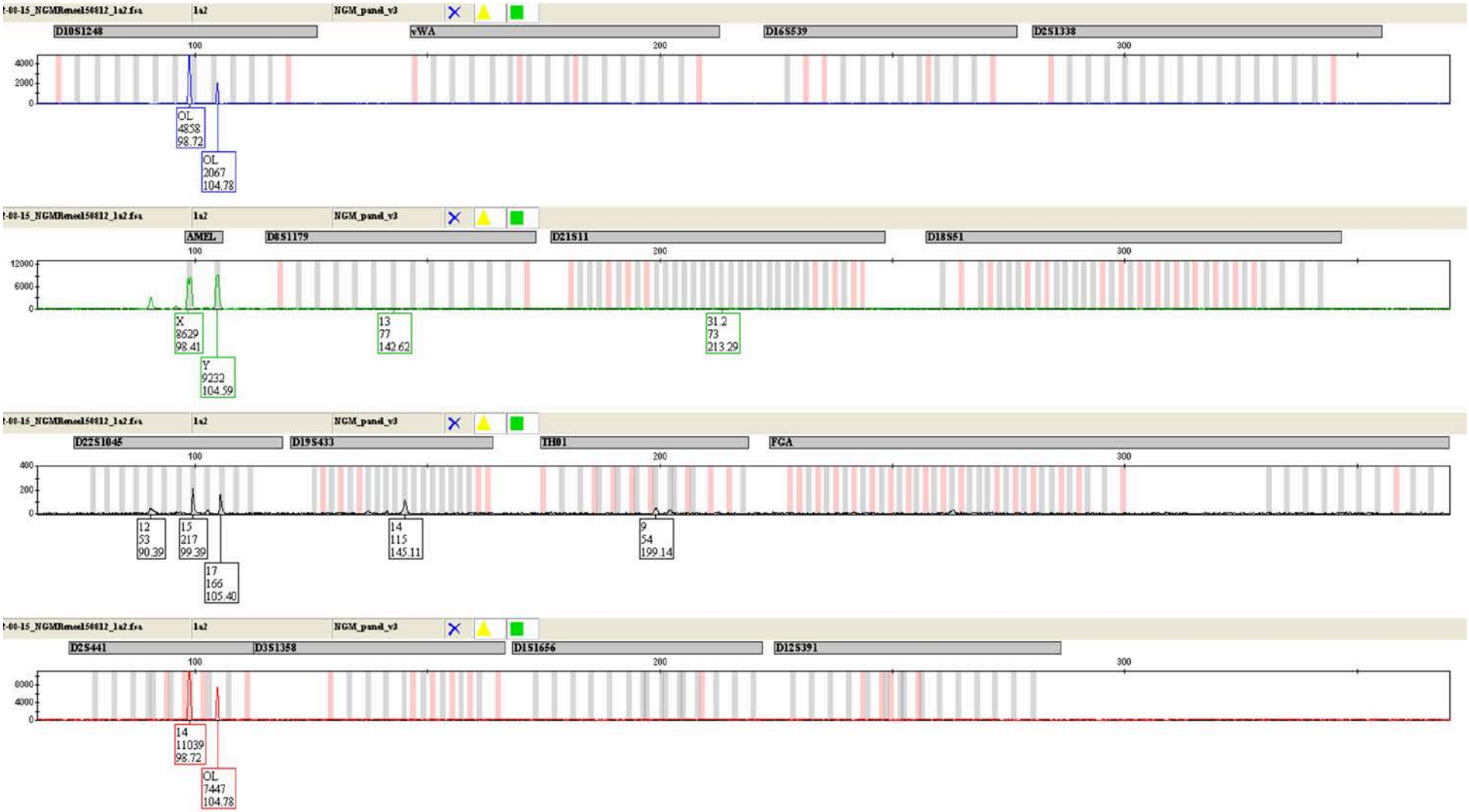

Figure 6a.8 – NGM™ DNA profile obtained from a single fibre from the cuff of item A1 after 2 hours of wear, amplified using direct PCR at 29 cycles.

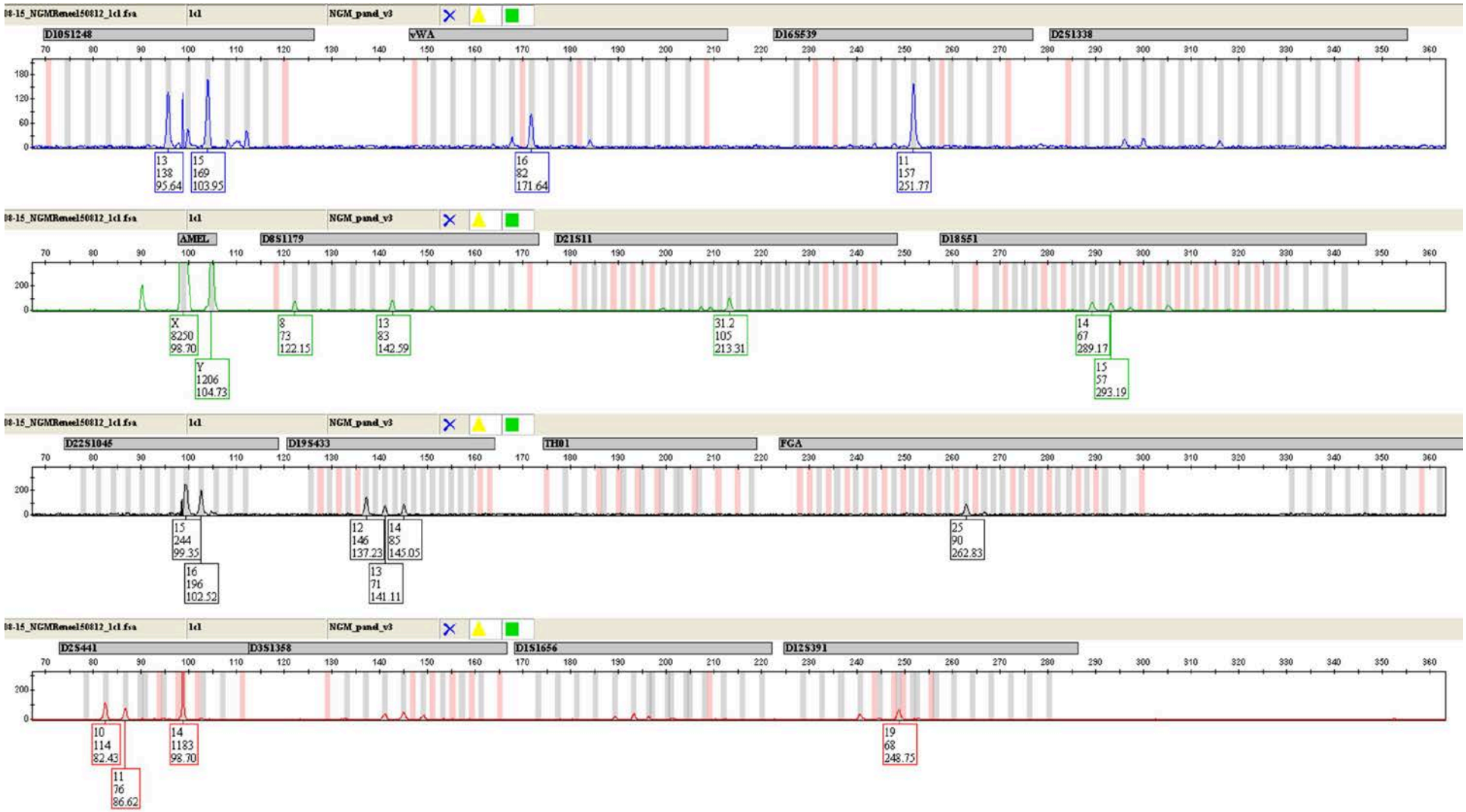

Figure 6a.9 – NGM™ DNA profile obtained from a single fibre from the cuff of item A1 after 6 hours of wear, amplified using direct PCR at 29 cycles.

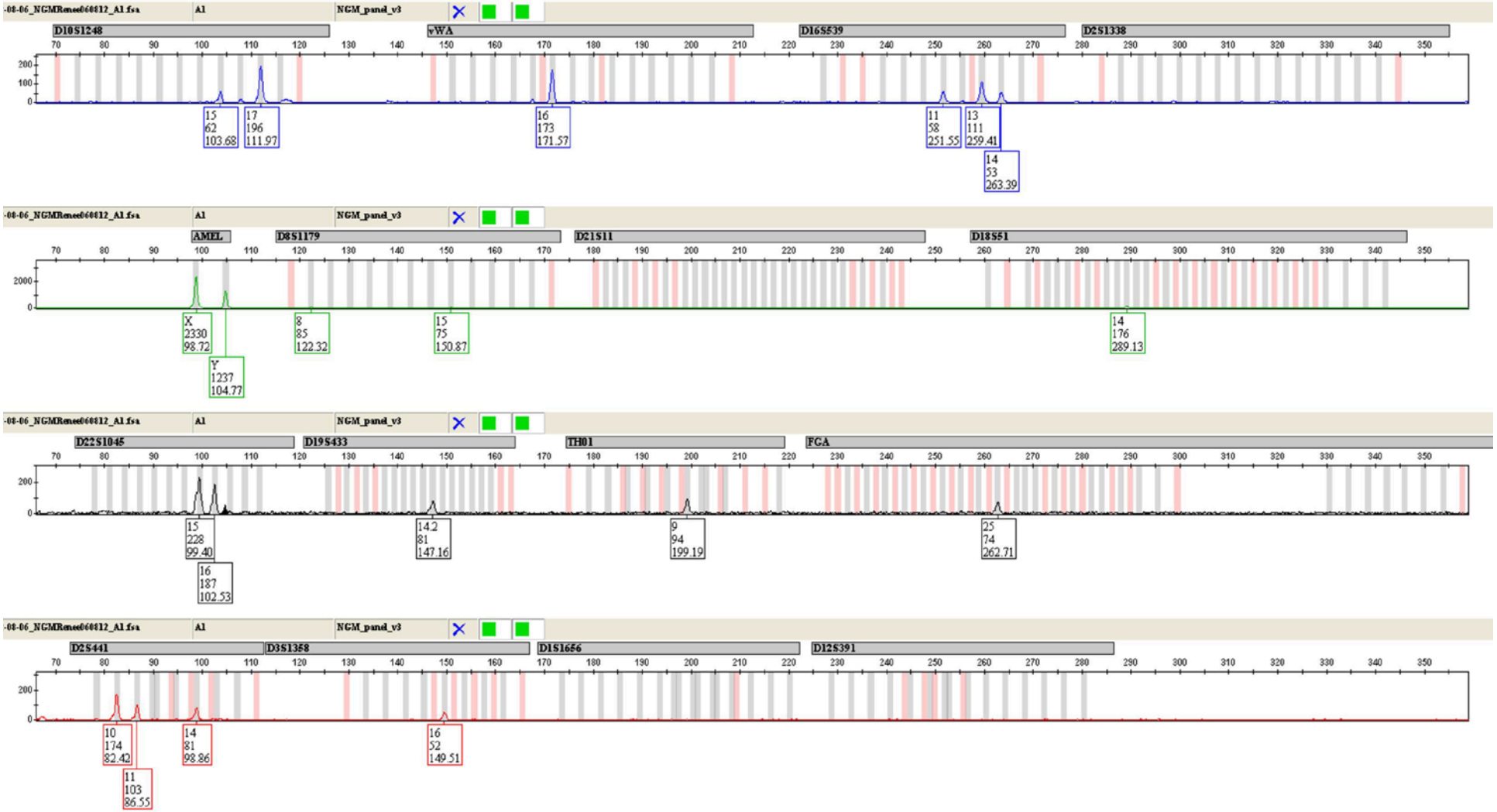

Figure 6a.10 – NGM™ DNA profile obtained from a single fibre from the cuff of item A1 after 9 hours of wear, amplified using direct PCR at 29 cycles.

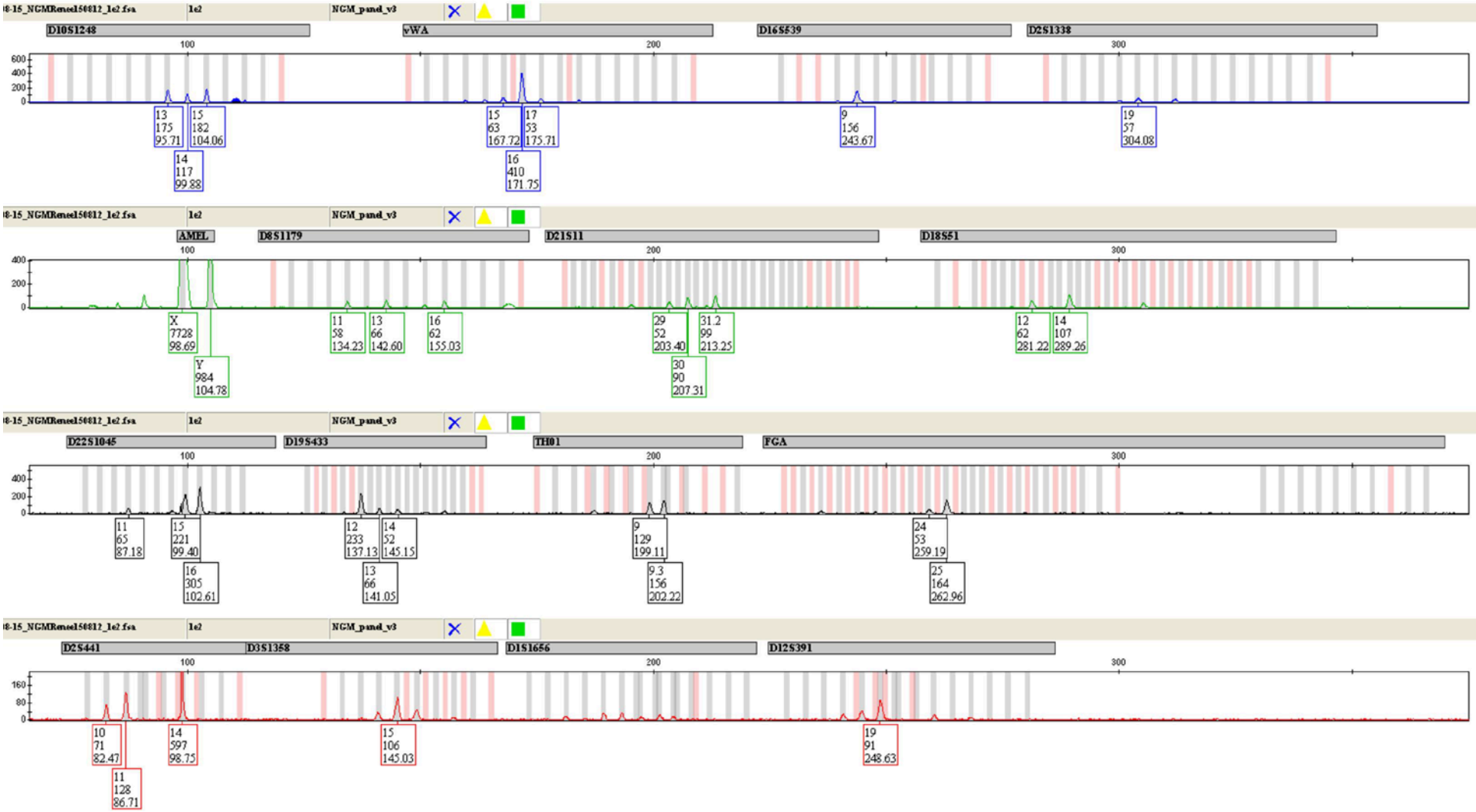

Figure 6a.11 – NGM™ DNA profile obtained from a single fibre from the cuff of item A1 after 11 hours of wear, amplified using direct PCR at 29 cycles.

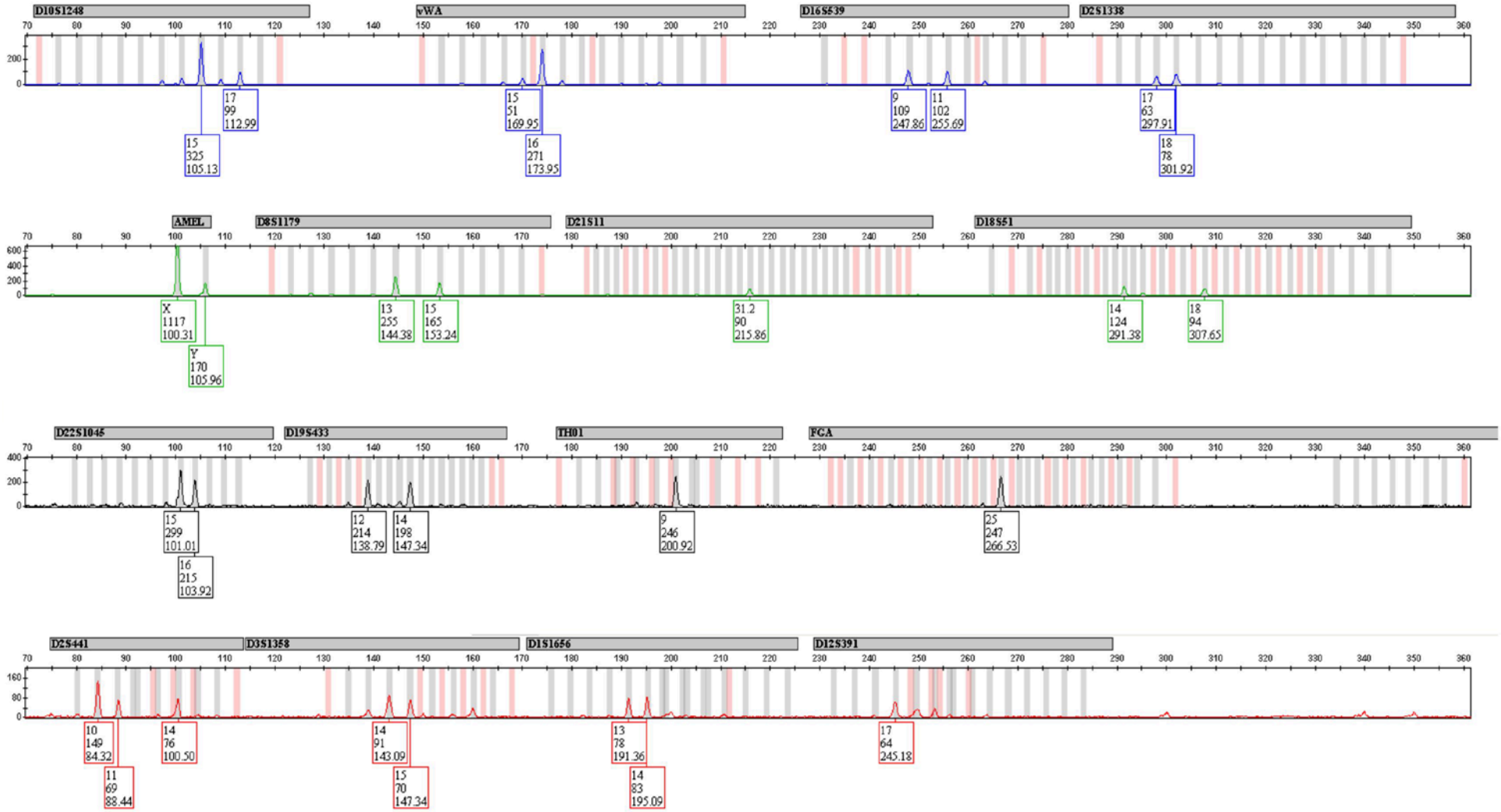

Figure 6a.12 – NGM™ DNA profile obtained from a single fibre from the cuff of item A1 after 2 hours of wear, amplified using direct PCR at 29 cycles.

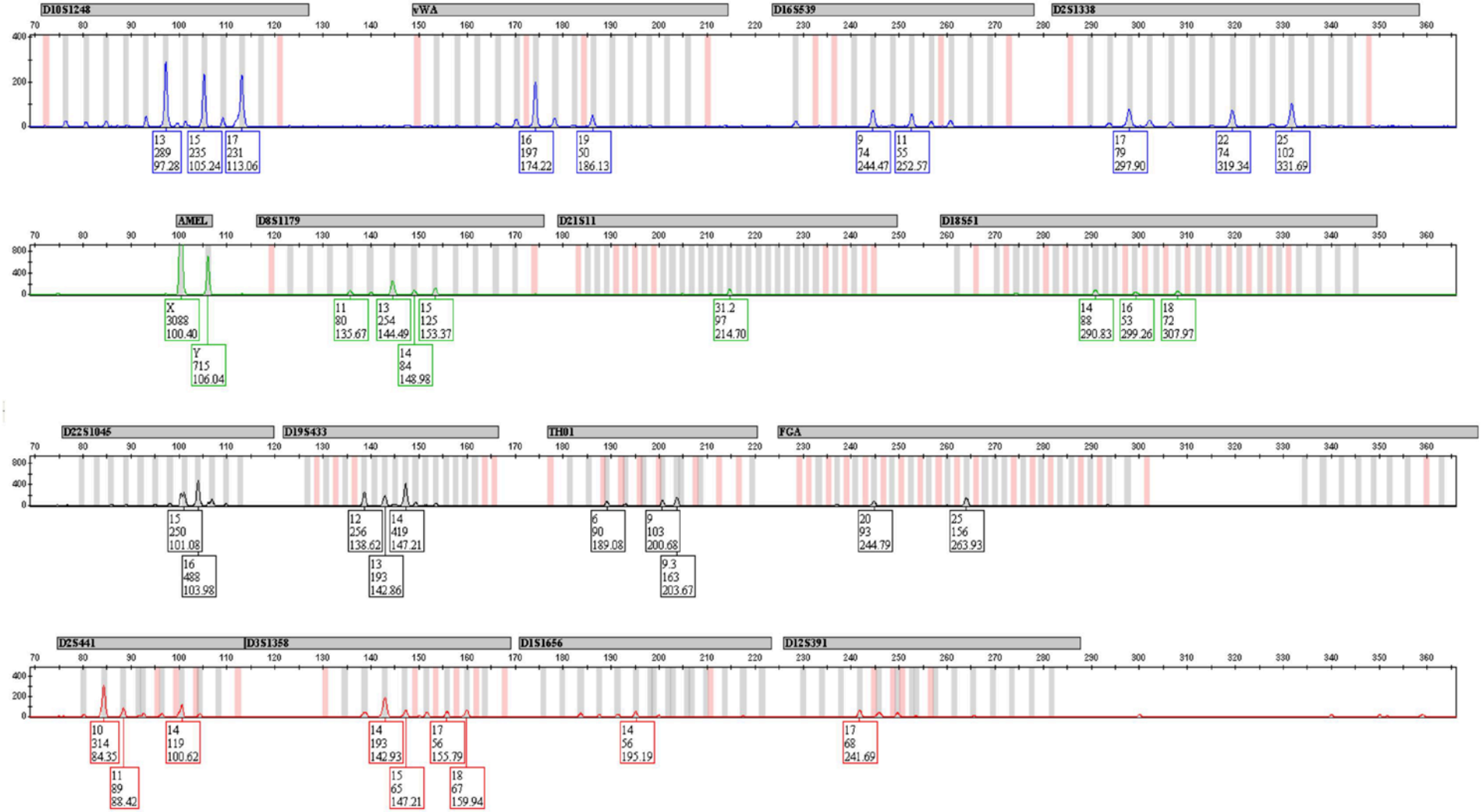

Figure 6a.13 – NGM™ DNA profile obtained from a tape lift from the cuff of item A1 after 12 hours of wear, amplified using direct PCR at 29 cycles.

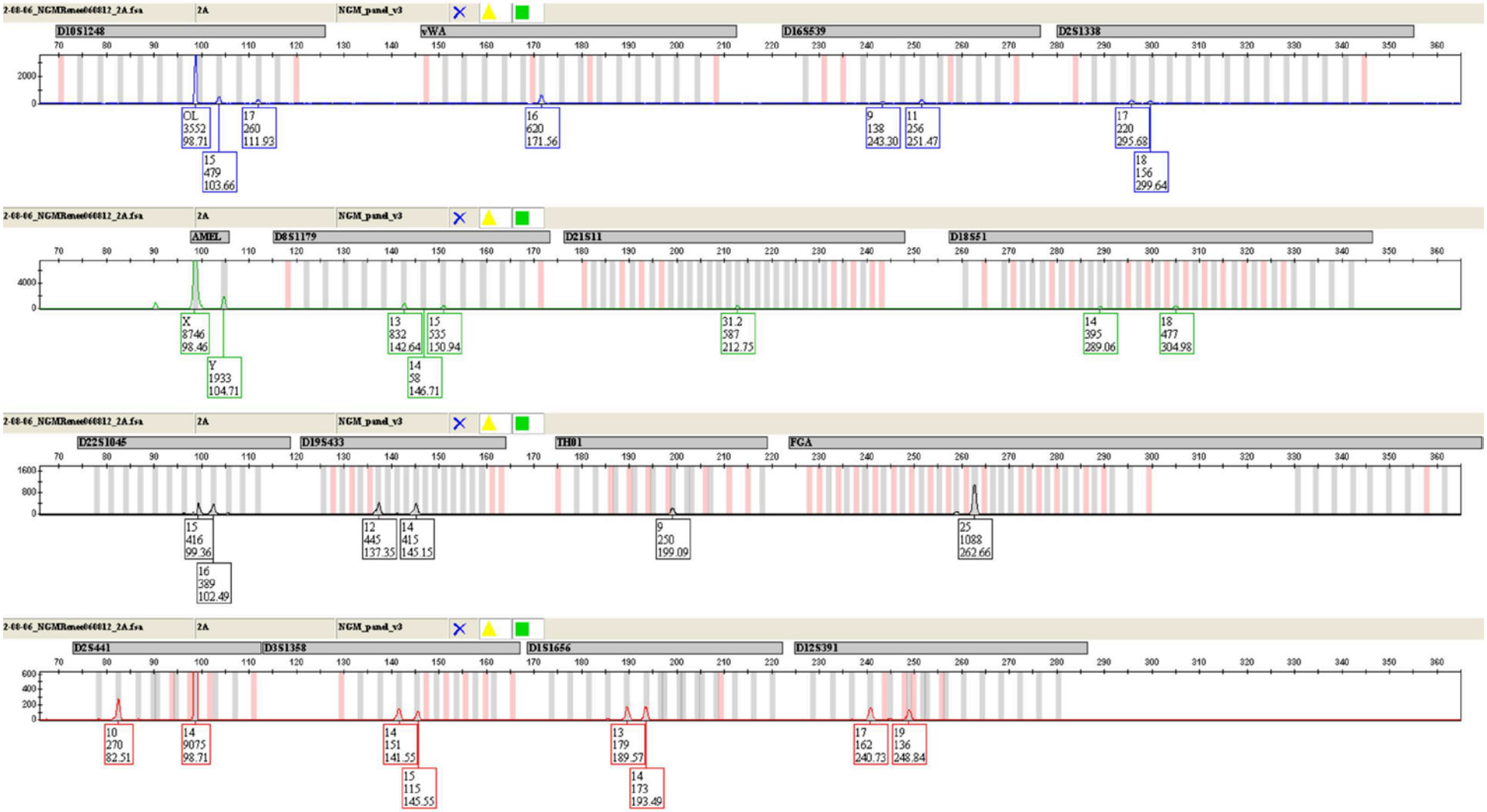

Figure 6a.14 – NGM™ DNA profile obtained from a single fibre from the cuff of item A2 after 2 hours of wear, amplified using direct PCR at 29 cycles.

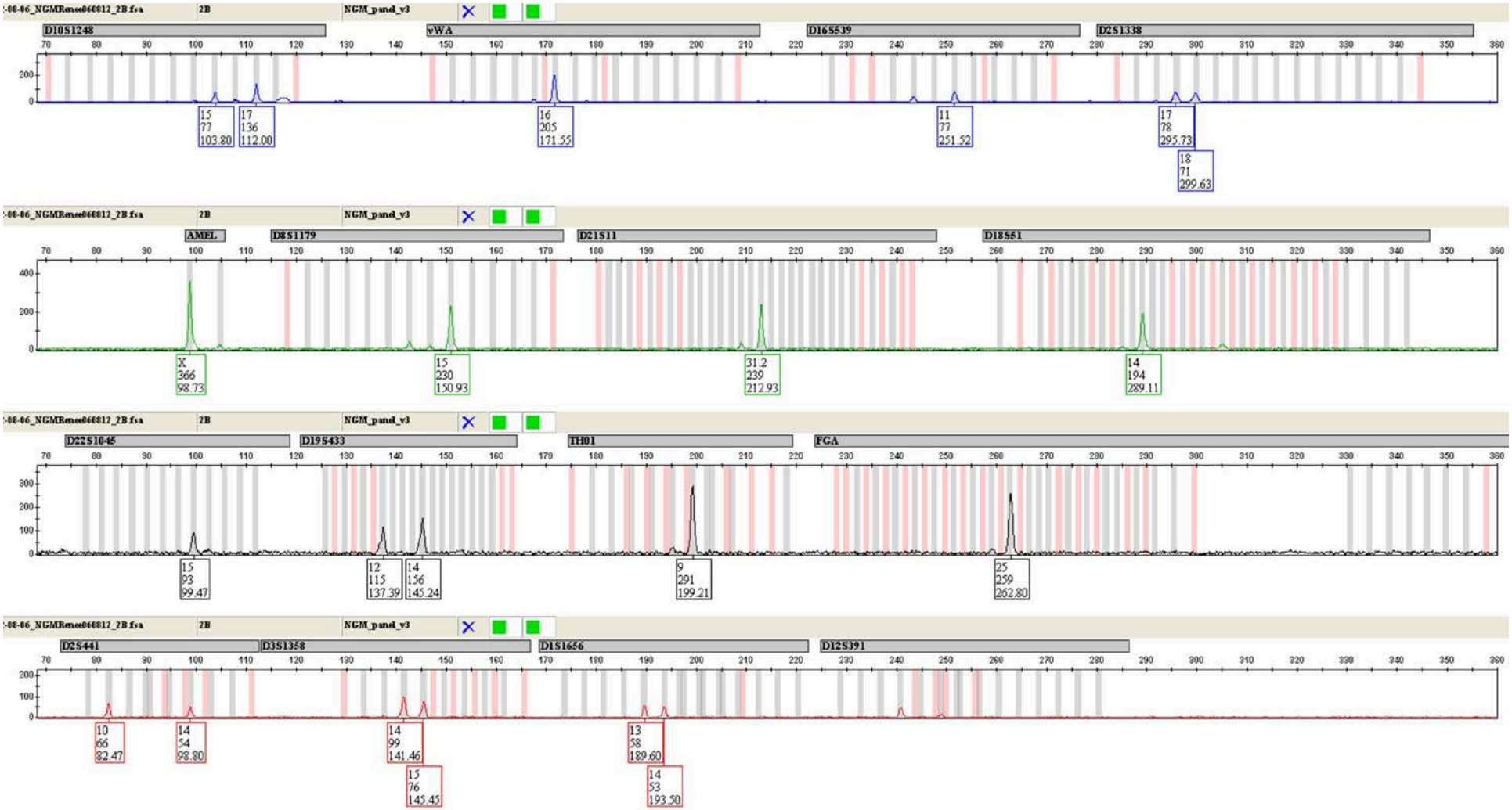

Figure 6a.15 – NGM™ DNA profile obtained from a single fibre from the cuff of item A2 after 4 hours of wear, amplified using direct PCR at 29 cycles.

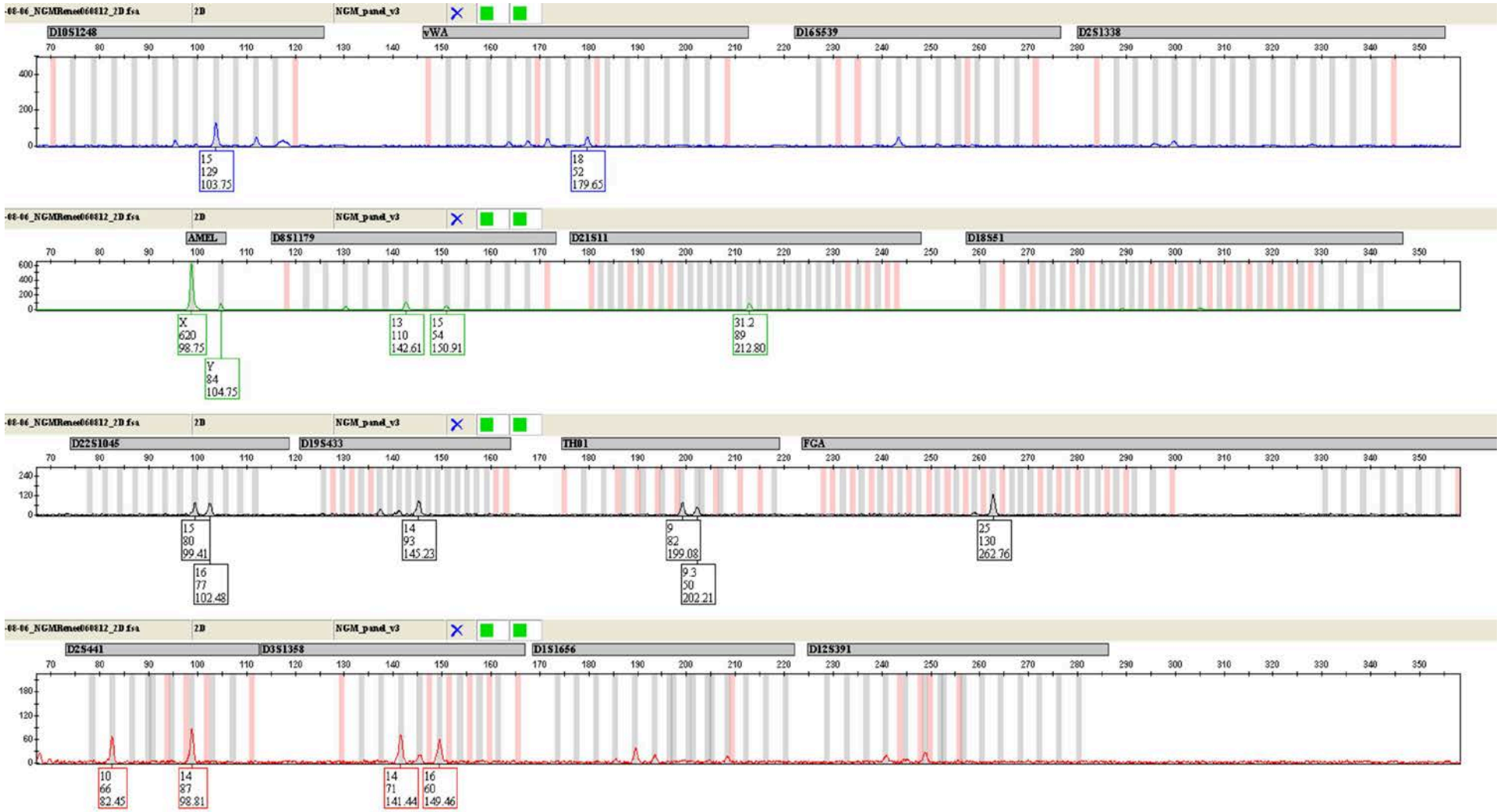

Figure 6a.16 – NGM™ DNA profile obtained from a single fibre from the cuff of item A2 after 9 hours of wear, amplified using direct PCR at 29 cycles.

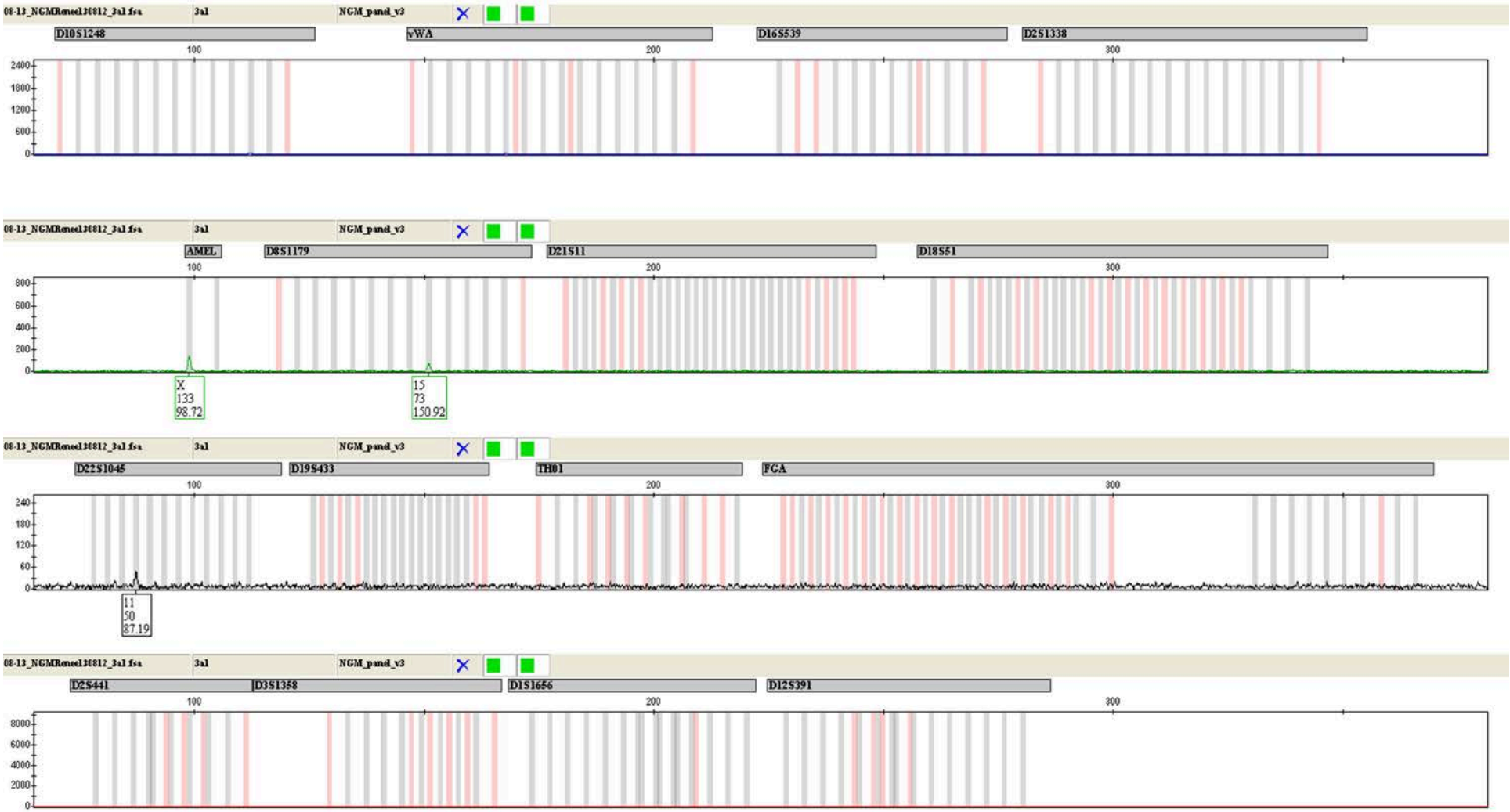

Figure 6a.17 – NGM™ DNA profile obtained from a single fibre from the cuff of item A3 after 2 hours of wear, amplified using direct PCR at 29 cycles.

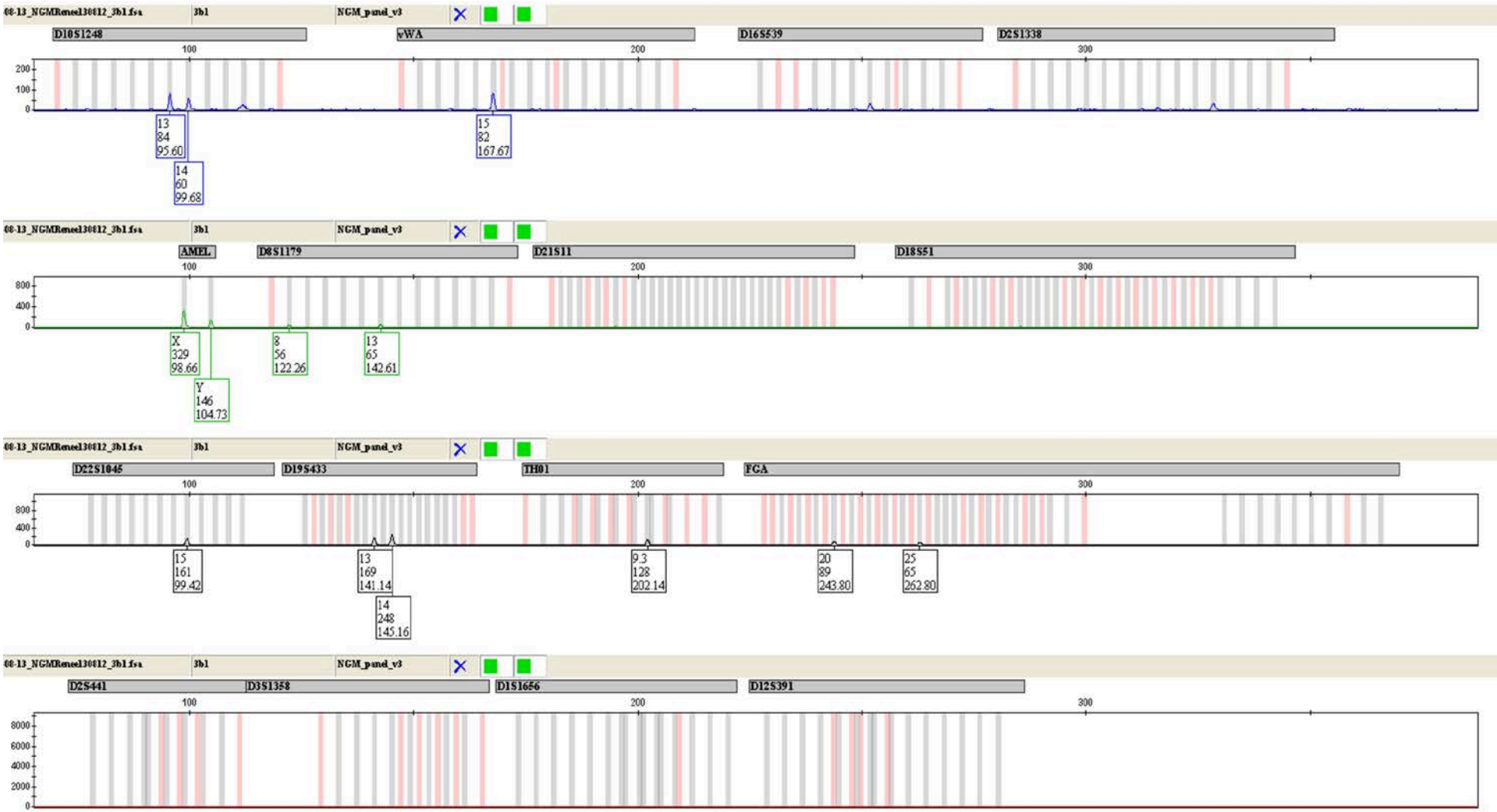

Figure 6a.18 – NGM™ DNA profile obtained from a single fibre from the cuff of item A3 after 4 hours of wear, amplified using direct PCR at 29 cycles.

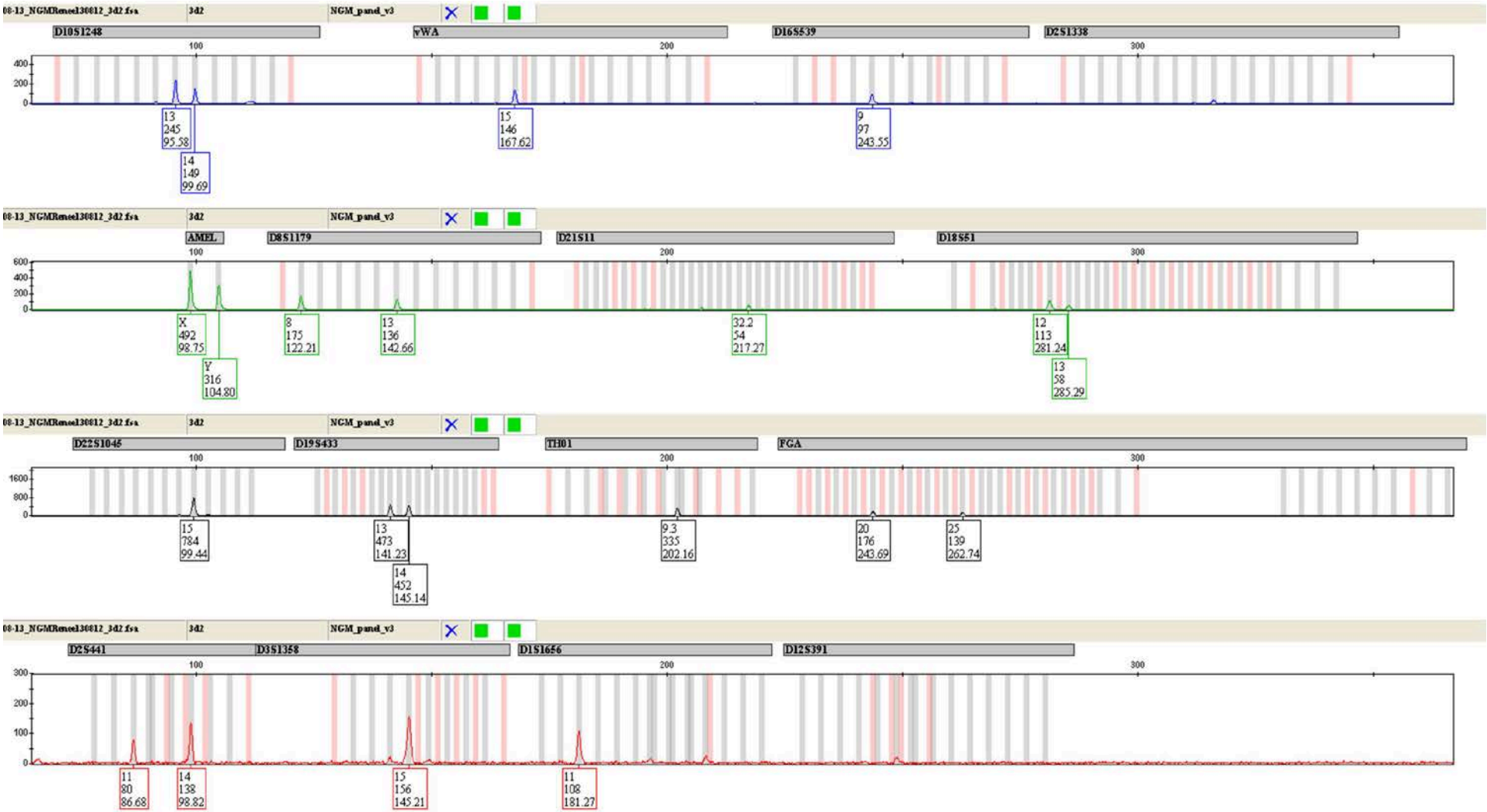

Figure 6a.19 – NGM™ DNA profile obtained from a single fibre from the cuff of item A3 after 9 hours of wear, amplified using direct PCR at 29 cycles.

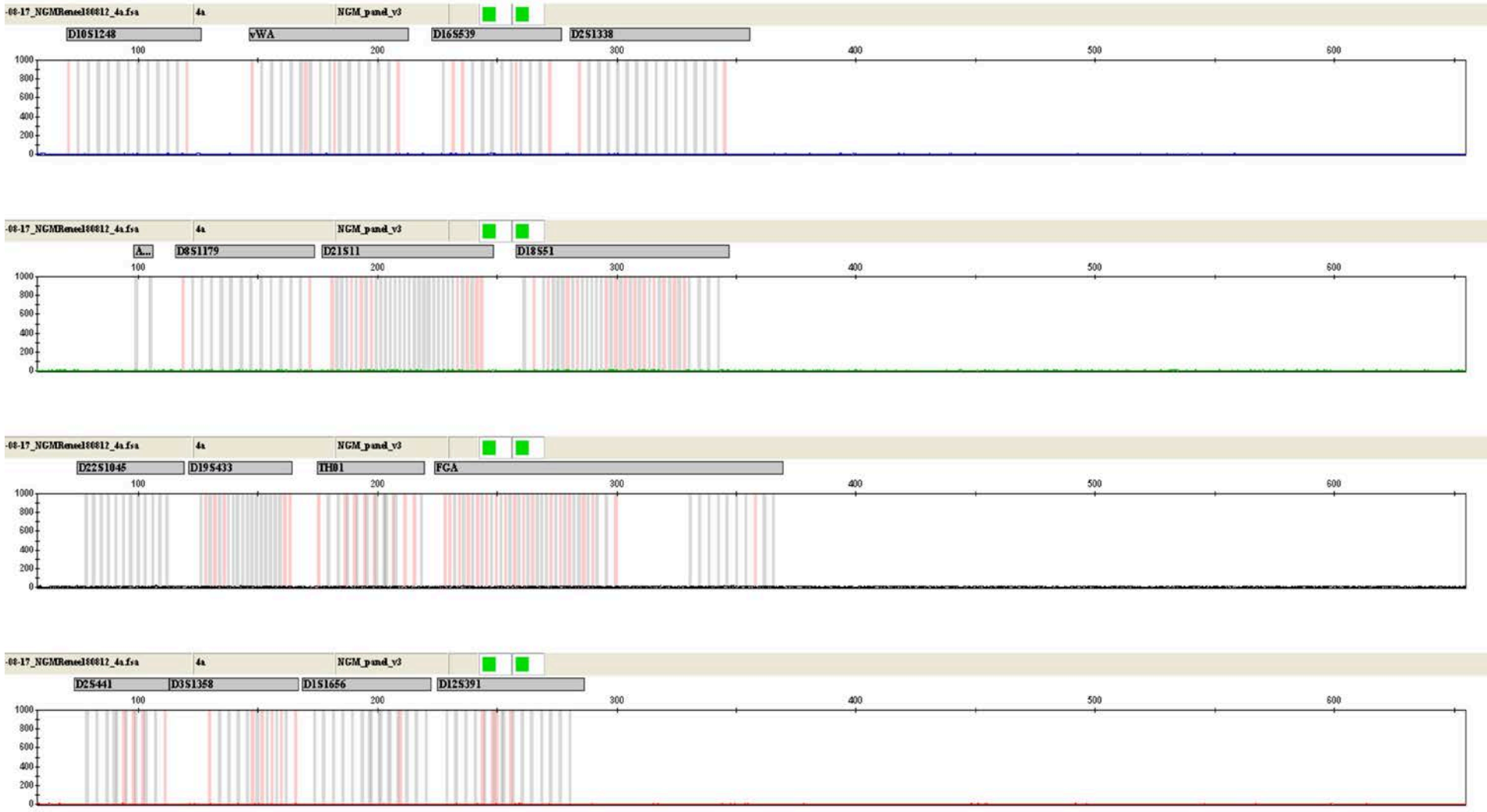

Figure 6a.20 – NGM™ DNA profile obtained from a single fibre from the cuff of item A4 after 9 hours of wear, amplified using direct PCR at 29 cycles. Fibre contains dye that causes inhibition.

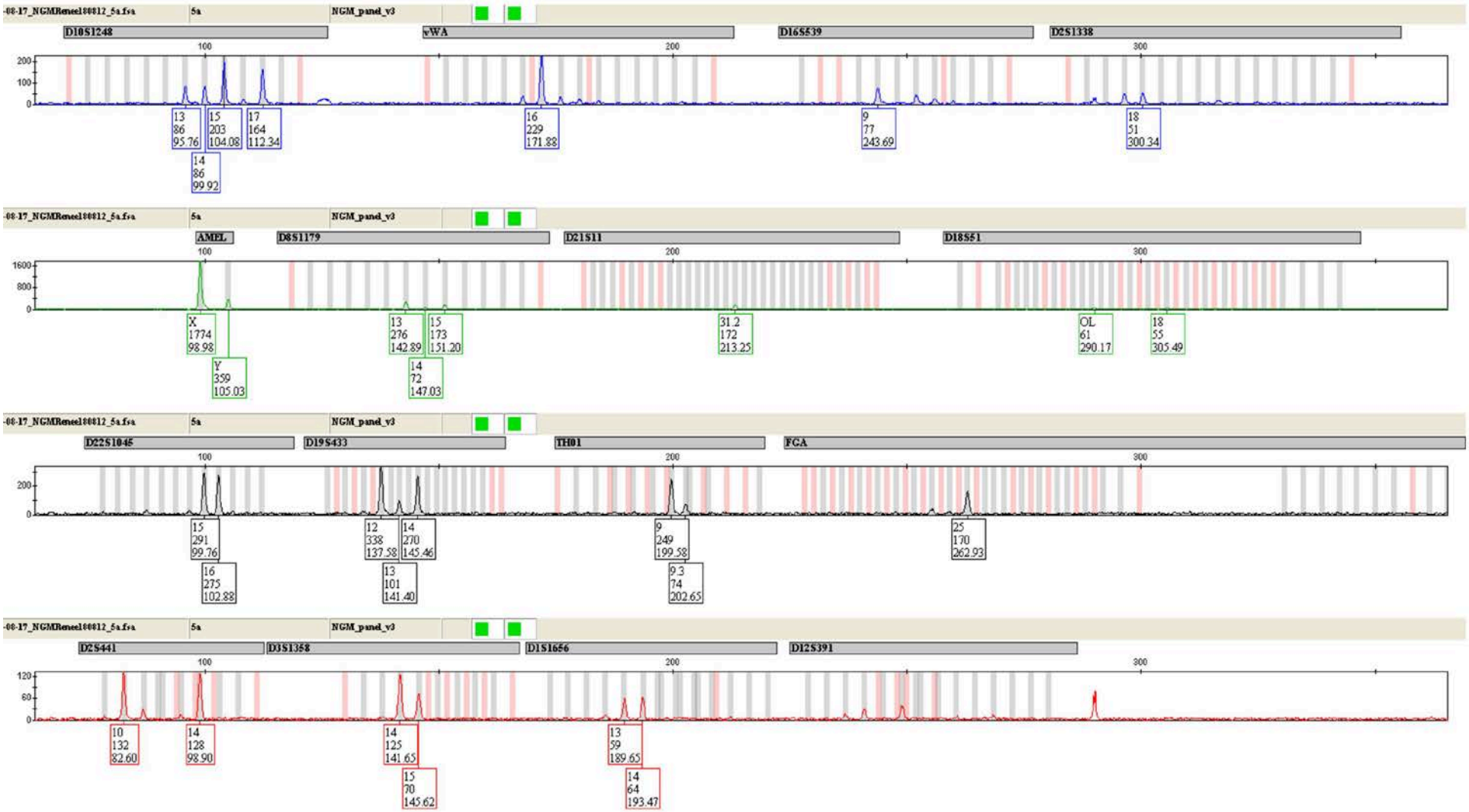

Figure 6a.21 – NGM™ DNA profile obtained from a single fibre from the cuff of item A5 after 2 hours of wear, amplified using direct PCR at 29 cycles.

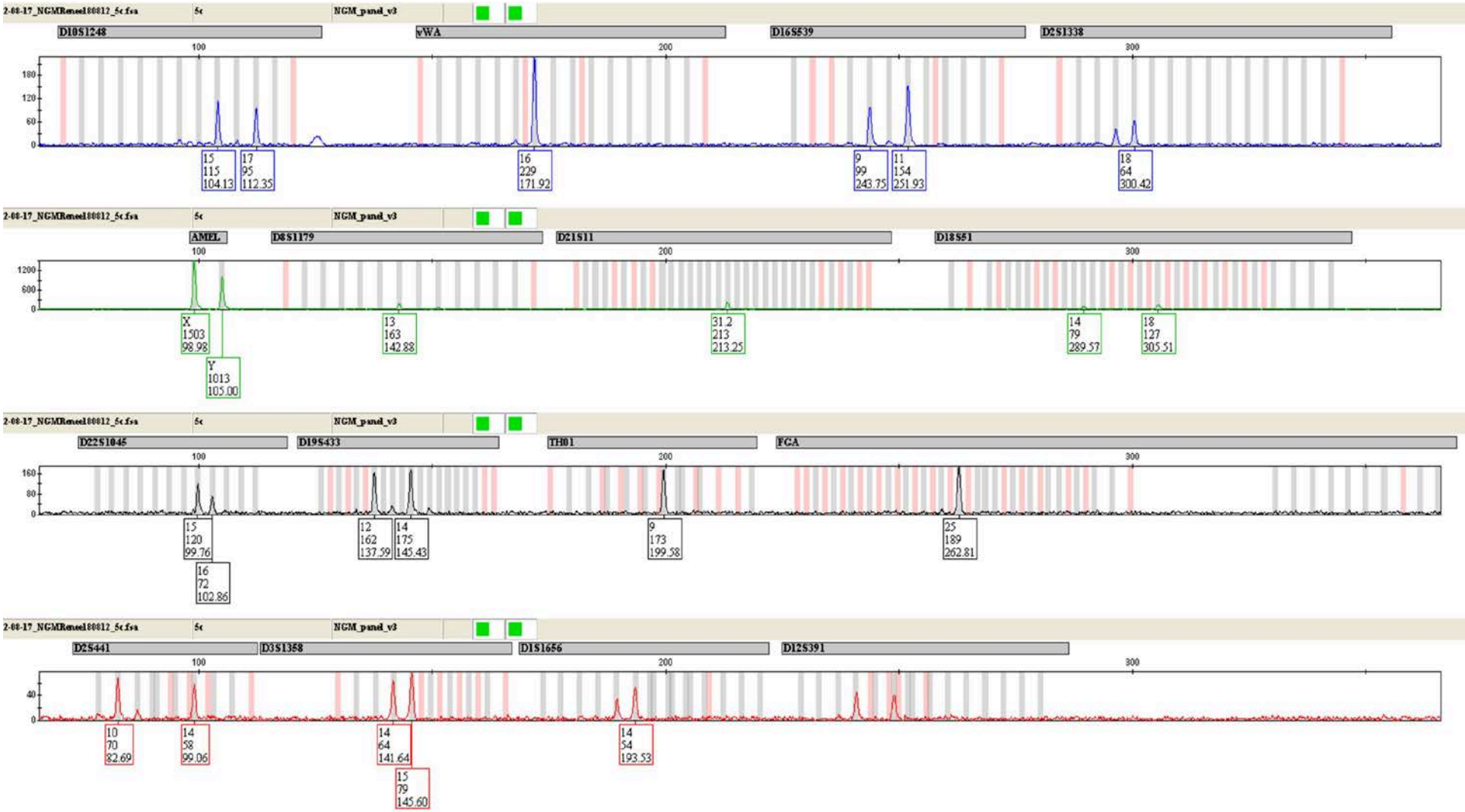

Figure 6a.22 – NGM™ DNA profile obtained from a single fibre from the cuff of item A5 after 6 hours of wear, amplified using direct PCR at 29 cycles.

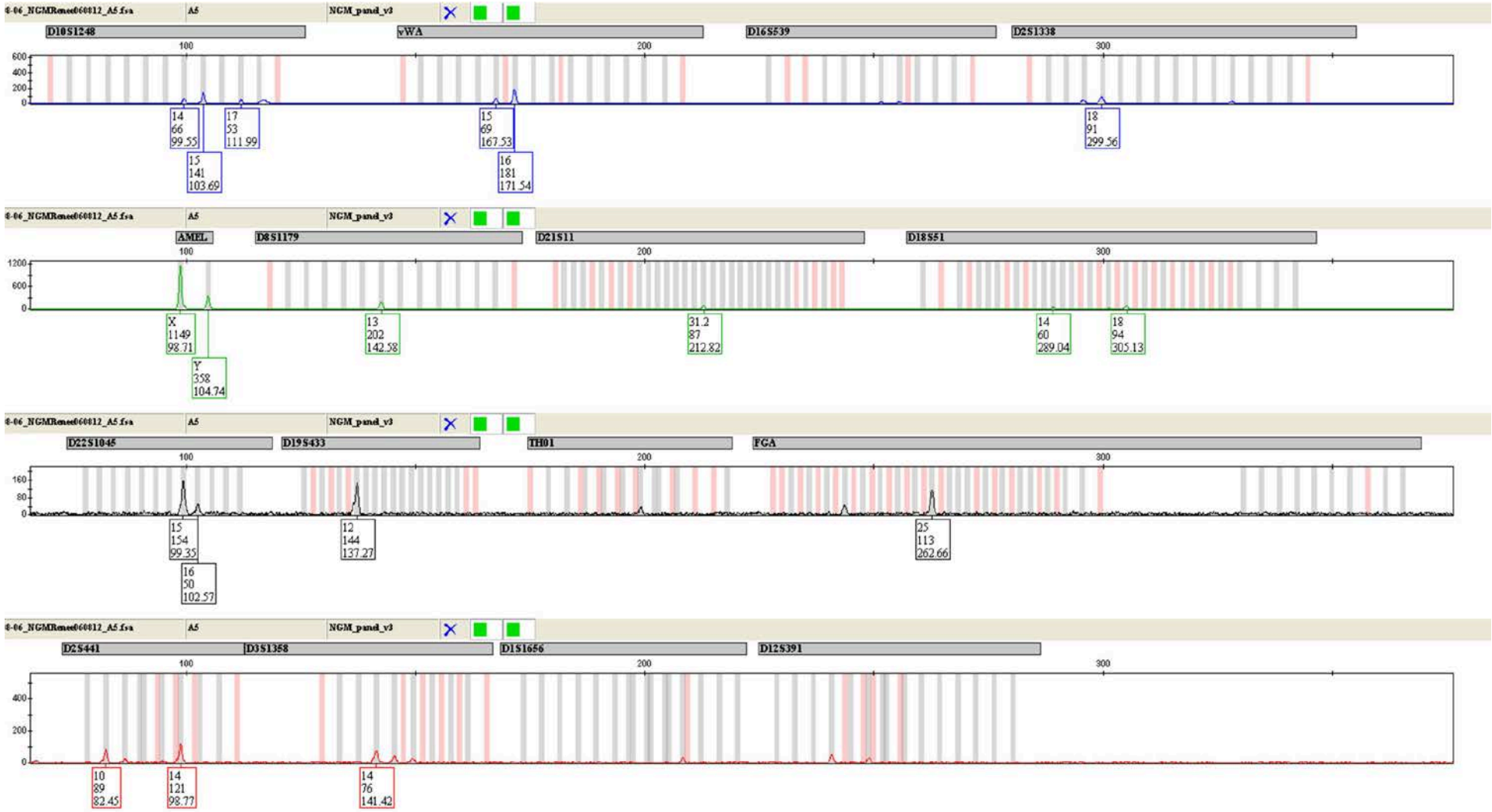

Figure 6a.23 – NGM™ DNA profile obtained from a single fibre from the cuff of item A5 after 9 hours of wear, amplified using direct PCR at 29 cycles.

CHAPTER 7

Direct Phenotypic SNPs

Manuscript prepared as: a technical note, Journal of Forensic Sciences.

Successful direct amplification of human hair and fingernails using IrisPlex SNP markers

Direct Phenotypic SNPs

7.1 Preface

SNP data have been used for over a decade to infer ancestry, lineage and human migration movement, in conjunction with Y-STR and mitochondrial DNA haplotypes [1-4]. However, using SNP data to predict phenotypes is a recent progression within the forensic science community [5-9]. As SNP amplicons are typically smaller than STR amplicons, there is a greater chance of obtaining valuable DNA information from difficult trace and degraded samples [10-13]. Although SNP data does not offer the same level of discrimination power for identification, standard STR profiles may not be of much significance if there is no reference profile or existing profile on a database for comparison. The use of phenotypic or ancestry SNPs, therefore, has the potential to aid in inclusion or exclusions of suspects or victims where no other information is available.

The first step of SNP analysis involves regular PCR techniques of small amplicons; therefore direct PCR can be applied at this stage in conjunction with samples that routinely amplify well with direct methods. The following sections investigate the application of single human hairs and fingernail clippings as substrates for direct PCR using IrisPlex SNP markers (*7.2*), and the future implementation and use of the direct PCR technique in conjunction with other SNP markers (*7.3*).

7.2 Successful direct amplification of human hair and fingernails using IrisPlex SNP markers

Statement of authorship

Successful direct amplification of human hair and fingernails using IrisPlex SNP markers

Manuscript prepared as: a technical note, Journal of Forensic Sciences.

Date: March 2016

Renée Blackie (Candidate)

Performed all laboratory work (sample collection, DNA extractions and quantifications, PCR and SNP amplification, capillary electrophoresis), data analysis and interpretation, created data table and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Duncan Taylor (Co-Supervisor)

Assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

Adrian Linacre (Supervisor)

Supervised the project, assisted with experimental design, commented on data, and edited the paper.

I hereby certify that the statement of contribution is accurate

Signed  Date March 2016

7.2.1 Manuscript prepared as: a technical note, Journal of Forensic Sciences.

Successful direct amplification of IrisPlex SNP markers from a single human hair and fingernail fragments

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ABSTRACT

We report on the successful direct amplification of IrisPlex Single Nucleotide Polymorphism markers using single hair follicles and fingernail clippings. SNPs from the validated IrisPlex system were chosen. A section (~ 5 mm) of a single hair from the proximal tip, and a section of a fingernail clipping (~ 4 mm^2) were used separately in the initial PCR amplification. These were placed directly in the PCR tube. All following steps adhered to the IrisPlex protocol. Complete SNP profiles were obtained from all 25 single hair samples and 40 % of 25 nail samples with a further 28 % of nail samples yielding 4 out of the 6 targeted SNPs. In all cases the SNPs matched the reference profiles of the individuals tested. The results from this study highlight the potential use of DNA trace samples as a source for directly amplifying SNPs.

Keywords: forensic science, direct PCR, anagen hairs, fingernail fragments, IrisPlex, SNPs

The use of Single Nucleotide Polymorphisms (SNPs) in forensic science is becoming popular due to the ability to provide additional informative data to the use of standard Short Tandem Repeats (STRs) (1-10). The STR profile from a forensic sample is only of value if it can be matched to a reference profile or a DNA profile held on a national database. SNPs however, are able to provide phenotypic information for individual identification and allow inferences to be made on hair and eye colour as well as ancestry and lineage to potentially help identify new suspects or narrow down a large list of suspects (11, 12). This study aimed to generate a set of six informative SNPs that form the IrisPlex (8) from trace material encountered in forensic science.

Single hairs and fragments of fingernails were selected for this study. Both sample types are notoriously difficult to obtain meaningful nuclear DNA due to low initial template DNA, the large variation in extraction methods used, and the poor efficiency of extraction methods (13-17). Direct PCR, where the sample of interest is placed directly into the amplification reaction, has the benefit of no loss of DNA template and therefore provides a potential increase in relative sensitivity compared to performing a DNA extraction. There have been recent reports on using direct PCR for a range of substrates using STR typing (13, 18-22) and here we report on applying direct PCR to the generation of phenotypically informative SNPs on a single hair and a fragment of fingernail.

2. Materials & Methods

2.1 Samples

Five donors (three female and two male) provided five recently plucked hairs and five fingernail clippings each. This generated a total of 50 samples comprising 25 anagen hairs and 25 nail clippings to be used in this experiment. The collection of samples was in line with approval from the Southern Adelaide Clinical Human Research Ethics Committee. The growth phase of the hairs were determined by microscopy. Fingernails were collected by the donor within 15 minutes post hand-washing or showering.

2.2 DNA Extraction from buccal swabs

Buccal swabs were provided to allow comparison to the reference SNP profile of the donors. DNA was isolated from buccal swabs using a QIAGEN® Mini kit (Qiagen) following the manufacturer's protocol and eluted into a final volume of 100 µL. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies, Victoria, Australia) following manufacturer's recommended protocol. From these extracts 1 ng was used in the PCR.

2.3 Direct PCR from hair and fingernails

Direct PCR was conducted by removing approximately 5 mm of proximal tip of the hair or approximately 4 mm^2 of each fingernail clipping, using sterile scissors and tweezers. The single hair and single nail fragments were placed into separate 0.2 mL thin walled tubes. The tubes contained 12 µL of the IrisPlex primer mix for 6 SNPs (using the same primer concentrations as published) (8) and 12 µL of PCR master mix from the QIAGEN® multiplex PCR kit (Qiagen, Victoria, Australia) making a total volume of 24 µL.

2.4 Amplification of SNPs

The amplifications were conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the recommended protocol (95 °C for 15 mins, followed by 94 °C for 30 sec, 61 °C for 90 sec, 72 °C for 60 sec, and 60 °C for 30 mins) with the exception of a decrease in cycle number. The standard 33 cycles was decreased to 31 cycles for direct samples based on previous knowledge of over-amplification of these sample types (13, 15, 23). The standard IrisPlex protocol was followed for the remaining SNP steps (8), which includes: cleaning 5 µL of PCR product with 2 µL of ExoSAP-IT and incubating at 37 °C for 45 min followed by 80 °C for 15 min; SBE reaction with 3 µL of combined SBE primers, 1 µL of SNaPshot™ multiplex reaction mix, and 1 µL of cleaned PCR product amplified at 96 °C for 2 mins, followed by 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 30 sec; SBE cleanup using 1 µL of added SAP to the SBE PCR product

and incubating at 37 °C for 45 min followed by 75 °C for 15 min; capillary electrophoresis of cleaned SBE product was performed on an ABI 3130*xl* Genetic Analyser (Life Technologies) using POP-4™ polymer (Life Technologies).

2.5 Analyses of data

The data were analyzed using GeneMapper® v3.2 (Life Technologies). The detection threshold was set at 50 relative fluorescence units (RFU).

3. Results

Full IrisPlex SNP profiles were obtained from 100 % of the 25 single hair samples and 10 of the 25 (40 %) of fingernail samples (Table 1). Additionally, 7 of the 25 (28 %) fingernail SNP profiles displayed all SNP alleles except for 1 or 2 alleles that exhibited drop-out. This is typical of insufficient template DNA and might be expected from some fingernail fragments. A full profile from a fingernail clipping and single hair matching the donor profile can be observed in Figure 1. Only eight of the fingernail samples failed to generate any SNP alleles. The eight failed profiles were all from two of the five participants, suggesting that these individuals may have a lower propensity to load their hands with DNA, or a poor 'shedder' status.

All SNP profiles obtained from hairs and fingernail clippings matched the SNP profile of each individual's reference profile. There were no observations of allelic drop-in or contamination in any of the 42 SNP profiles obtained.

4. Discussion

We have demonstrated that SNP profiles can be generated routinely from a both a single hair and from a fragment of a fingernail. Nuclear STRs are infrequently obtained from a single hair when subjected to DNA extraction prior to amplification and therefore mitochondrial DNA typing is often

used. The SNPs used in this test where all 25 hairs generated a full SNP profile are all nuclear-based indicating the possibility of gaining an informative SNP profile from such single hair.

Of the 25 nail clippings, 17 generated SNP profiles containing information at at least four loci. Given that the nails were collected from donors post hand-washing or showering, a question arises as to the source of the DNA. In all cases the SNPs from the fingernails matched that of the donor with no additional alleles indicating that the DNA came from cellular material intrinsic to the nail itself or shed from the skin of the donor.

When using direct PCR there is a reduction in time and costs by omitting the extraction step and there is no loss of DNA during the extraction process, allowing these samples to be processed successfully to obtain SNP profiles of a forensic standard. As direct PCR only requires a small sample, such as a 4 mm^2 section of fingernail, typically there will be enough material to attempt multiple, direct PCRs or a standard extraction when initial direct PCR did not yield sufficient results. This may be important in jurisdictions where there is a requirement of secondary testing. Only necessary minimal alteration at the PCR amplification stage of the validated IrisPlex methodology occurred to ensure that the forensic science community could adopt the process readily. Preliminary studies using the same sample types with the SNP*for*ID 34-plex ancestry test (24) have also shown promising results within our laboratory, indicating the great potential for the use of direct PCR in the field of forensically informative SNPs.

Acknowledgement

Funding was provided by the Attorney General's Department of South Australia.

Conflict of Interest

The authors have declared no conflict of interest.

Ethics

Donors provided signed consent under the approval of the Southern Adelaide Clinical Human Research Ethics Committee (SAC HREC), application number 502.13.

References

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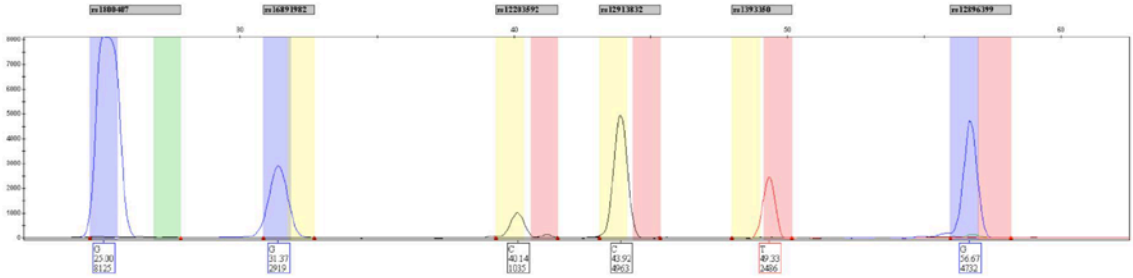
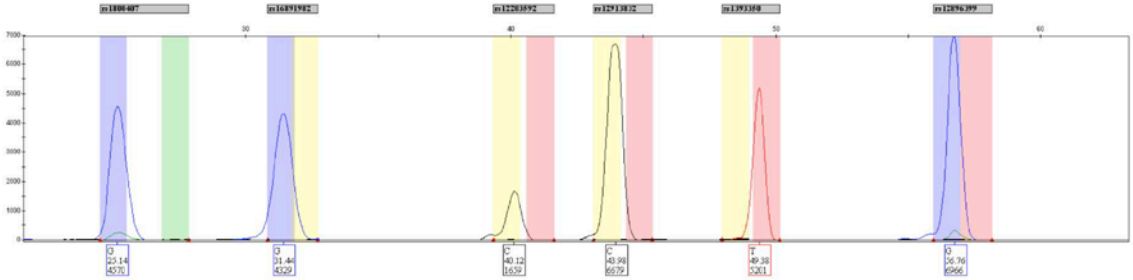
Table 1 Summary of SNP profiles obtained using direct PCR using five fingernail fragments and five hair samples from five donors (total samples n = 50).

	SNP Profile Obtained		
Sample Type	No results	Partial Profile (dropout of ≤ 2 SNPs)	Full Profile (All 6 SNPs)
Fingernail Clipping	32 % (n = 8)	28 % (n = 7)	40 % (n = 10)
Anagen Hair			100 % (n = 25)
Total	**n = 8**	**n = 7**	**n = 35**

Positive control



Hair Sample



Nail Sample

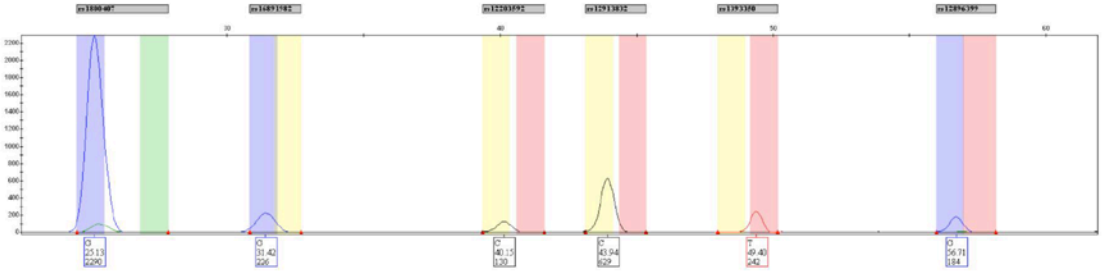


Figure 1 Chromatograms of nuclear DNA from a positive control, single anagen hair root, and a fingernail clipping from one individual. Samples amplified using the IrisPlex SNP procedure at 31 cycles on a GeneAmp® System 9600 thermal cycler and separated on a 3130*xl* Genetic Analyzer.

7.3 Applications

Section 7.2 outlines the successful direct amplification of two human substrates with IrisPlex SNPs and promising results with the SNP*for*ID 34-plex ancestry test. A natural progression of research would be to expand on the number and type of SNP panels used with a direct PCR approach, such as the more recent HIrisPlex panel [14] that determines hair colour as well as eye colour, and the vast amount of individual identification SNPs available [8, 15-17], including the SNP*for*ID 52-plex panel [13, 18, 19]. Of course, human hair and fingernails are not the only substrates that can be amplified directly: fibres from clothing, fibres from swabs used on a range of surfaces, as well as tape used in tape lifting fabrics, could also be investigated in conjunction with SNP testing.

There are an increasing number of forensic informative SNPs being discovered, and the SNaPshot® multiplex kit can only incorporate the use of 30 – 50 SNPs in a single assay [20-23]. The drive to obtain more information from a single sample is leading to the combination of ancestry and phenotypic informative SNPs through the means of next generation sequencing (NGS), where thousands of genetic markers can be genotyped simultaneously [24-27]. As NGS technology continues to develop and improve, the systems will be able to combine different forensic assays to accommodate the new SNP markers that are continually being discovered. Less input DNA is required for NGS, and if coupled with direct PCR techniques, could lead to great improvements when processing difficult trace evidence and degraded samples.

7.4 Concluding Remarks

The results from this study highlight the potential use of DNA trace samples as a source for directly amplifying forensic informative SNPs. A wide range of substrates and SNPs can be combined to further increase the likelihood of obtaining profiles from degraded and challenging samples. As the number of forensic informative SNPs continues to grow, newer technologies such as NGS are being implemented to provide incredible amounts of data from single samples. By combining individual identification SNPs, ancestry inference SNPs, and phenotypic inference SNPs into one process, the application of data can be applied to a widening range of investigations such as missing persons, DVI scenarios, and the inclusion or exclusion of suspects or victims to assist an investigation.

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Supplementary Material

a. Successful direct amplification of human hair and fingernails using IrisPlex SNP markers

Raw data

Table 7a.1 – IrisPlex SNP results for individual 1, amplifying anagen hair roots and fingernail samples using direct PCR methods.

Sample	rs1800407	rs16891982	rs12203592	rs12913832	rs1393350	rs12896399
Pos Control	**G**	**G**	**C**	**C/T**	**C**	**G/T**
Nail 1	G	G	-	C/T	C	G/T
Nail 2	G	G	-	C/T	C	G/T
Nail 3	G	G	C	C/T	C	G/T
Nail 4	G	G	C	C/T	C	G/T
Nail 5	G	G	-	C/T	C	G/T
Hair 1	G	G	C	C/T	C	G/T
Hair 2	G	G	C	C/T	C	G/T
Hair 3	G	G	C	C/T	C	G/T
Hair 4	G	G	C	C/T	C	G/T
Hair 5	G	G	C	C/T	C	G/T

- no SNP allele present

Table 7a.2 – IrisPlex SNP results for individual 2, amplifying anagen hair roots and fingernail samples using direct PCR methods.

Sample	rs1800407	rs16891982	rs12203592	rs12913832	rs1393350	rs12896399
Pos Control	**G**	**G**	**T**	**C/T**	**C**	**G**
Nail 1	G	•	-	C/T	C	G
Nail 2	G	G	T	C/T	C	G
Nail 3	G	G	T	C/T	C	G
Nail 4	G	G	T	C/T	C	G
Nail 5	G	G	T	C/T	C	G
Hair 1	G	G	T	C/T	C	G
Hair 2	G	G	T	C/T	C	G
Hair 3	G	G	T	C/T	C	G
Hair 4	G	G	T	C/T	C	G
Hair 5	G	G	T	C/T	C	G

- no SNP allele present

• allele present but below 50 RFU threshold

Table 7a.3 – IrisPlex SNP results for individual 3, amplifying anagen hair roots and fingernail samples using direct PCR methods.

Sample	rs1800407	rs16891982	rs12203592	rs12913832	rs1393350	rs12896399
Pos Control	**G**	**G**	**C/T**	**C**	**C/T**	**G/T**
Nail 1	G	G	C/T	C	C/T	G/T
Nail 2	G	G	C/T	C	C/T	G/T
Nail 3	G	G	•/T	C	C/T	G/•
Nail 4	G	G	C/T	C	C/T	G/•
Nail 5	G	G	C/T	C	C/T	G/T
Hair 1	G	G	C/T	C	C/T	G/T
Hair 2	G	G	C/T	C	C/T	G/T
Hair 3	G	G	C/T	C	C/T	G/T
Hair 4	G	G	C/T	C	C/T	G/T
Hair 5	G	G	C/T	C	C/T	G/T

• allele present but below 50 RFU threshold

Table 7a.4 – IrisPlex SNP results for individual 4, amplifying anagen hair roots and fingernail samples using direct PCR methods.

Sample	rs1800407	rs16891982	rs12203592	rs12913832	rs1393350	rs12896399
Pos Control	**G**	**G**	**C**	**C**	**T**	**G**
Nail 1	-	-	-	-	-	-
Nail 2	G	G	C	C	T	G
Nail 3	-	-	-	-	-	-
Nail 4	G	-	C	C	T	G
Nail 5	-	-	-	-	-	-
Hair 1	G	G	C	C	T	G
Hair 2	G	G	C	C	T	G
Hair 3	G	G	C	C	T	G
Hair 4	G	G	C	C	T	G
Hair 5	G	G	C	C	T	G

- no SNP allele present

Table 7a.5 – IrisPlex SNP results for individual 5, amplifying anagen hair roots and fingernail samples using direct PCR methods.

Sample	rs1800407	rs16891982	rs12203592	rs12913832	rs1393350	rs12896399
Pos Control	**G**	**G**	**C/T**	**C**	**C/T**	**G/T**
Nail 1	-	-	-	-	-	-
Nail 2	-	-	-	-	-	-
Nail 3	-	-	-	-	-	-
Nail 4	-	-	-	-	-	-
Nail 5	-	-	-	-	-	-
Hair 1	G	G	C/T	C	C/T	G/T
Hair 2	G	G	C/T	C	C/T	G/T
Hair 3	G	G	C/T	C	C/T	G/T
Hair 4	G	G	C/T	C	C/T	G/T
Hair 5	G	G	C/T	C	C/T	G/T

- no SNP allele present

Note: re-amplification of fingernail clippings where initial amplification failed to produce all SNP alleles, returned full SNP profiles.

Appendix

i. **Poster Presentation** 22nd International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Adelaide, Australia, 2014. *Successful direct amplification of SNP markers using single human hairs & fingernail clippings.*

Successful direct amplification of SNP (Single Nucleotide Polymorphism) markers using single human hairs & fingernail clippings

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Forensic Biology Lab
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Presented at the 2014 ANZFSS Conference Adelaide, Australia

Funding was provided by the Department of Justice, South Australia

Introduction

We report on the successful direct amplification of **IrisPlex** SNP markers using single hair follicles and fingernail clippings. The use of phenotypic SNPs in forensic science is fast becoming popular due to the nature of the information provided. Standard STR profiles may not be of much significance if there is no reference profile or existing profile on a database for comparison. Phenotypic SNPs have the potential ability aid in inclusion or exclusions of suspects or victims where no other information is available. In this study, we aimed to determine if SNPs could be amplified using direct PCR from single hair follicles. We chose to amplify SNPs from the validated IrisPlex system, an informative tool for determining eye colour. A section (~ 5 mm) of hair from the proximal tip, and sections of fingernail clippings (~ 2 mm^2) was used separately in the initial PCR amplification. All following steps adhered to the IrisPlex protocol [1]. Profiles obtained from the hairs and nails consistently matched the reference profiles of the individuals tested. The results from this study highlight the potential use of DNA trace samples as a source for directly amplifying informative SNPs.

Method

- **Samples** A total of 25 anagen hairs from 5 (male and female) donors were analysed. Growth phase was determined using microscopy. Additionally, 25 fingernail clippings were also processed from the same donors. DNA extracts from buccal swabs were provided as references of donors. A total of 50 samples used in this experiment.
- **Direct PCR** was conducted by removing approximately 5 mm of the hair proximal tip or ~ 2 mm^2 of each fingernail clipping, using sterile scissors and tweezers. The hair and nail fragments were placed into separate 0.2 mL thin walled tubes in a volume of 24 µL of PCR mix using IrisPlex primer sets for 6 SNPs and QIAGEN multiplex buffer. IrisPlex protocols followed for remaining SNP steps.
- **Amplification** was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the recommended protocol. The standard 33 cycles was decreased to 31 cycles for direct samples.
- **Analysis:** Samples were run on an ABI 3130*xl* Genetic Analyser (Life Technologies) following standard procedures. The data were analysed using GeneMapper® v3.2. The detection threshold was set at 50 relative fluorescence units (RFU).

Results

- A full profile was obtained from 100% of anagen hair samples and 40 % of fingernail samples. Additionally, 28 % of fingernail SNP profiles displayed all SNP alleles except for 1 or 2 (drop-out).
- As each fingernail sample only required a 2 mm^2 section, additional testing either by direct PCR or standard extraction methods can be carried out if required on samples that did not yield results.

Table 1. Summary of SNP profiles obtained using direct PCR with fingernail and hair samples. Total n = 50.

	SNP Profile Obtained		
Sample Type	**Full Profiles (All 6 SNPs)**	**Partial Profiles (drop out ≤ 2 alleles)**	**No Results (zero alleles obtained)**
Fingernail Clipping	40 % (n = 10)	28 % (n = 7)	32 % (n = 8)
Anagen Hair Fragment	100 % (n = 25)		
Total	**n = 35**	**n = 7**	**n = 8**

Results

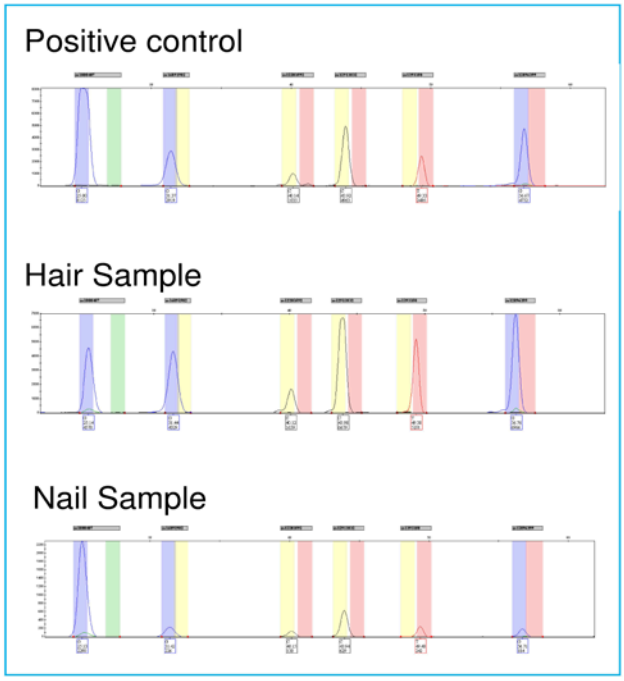

Figure 1. Chromatogram of nuclear DNA from single **anagen** hair root, and **fingernail** clipping, amplified using IrisPlex SNP procedure at 31 cycles on a GeneAmp® System 9600 thermal cycler.

Concluding Remarks

- DNA extraction was not required to obtain SNP profiles to a forensic standard.
- Complete SNP profiles were obtained from 100 % of anagen hair samples, and 40% of fingernail samples.
- Allelic drop-in or contamination was not observed in any of the 42 SNP profiles obtained from any of the hair or nail samples.
- This method is cost-effective and time saving in forensic casework.
- Additional testing either by direct PCR or standard extraction methods can be carried out if required on nail samples that did not yield results.

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Corresponding abstract for poster presentation:

Successful direct amplification of Single Nucleotide Polymorphism (SNP) markers using single human hairs with IrisPlex

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We report on the successful direct amplification of IrisPlex SNP markers using single hair follicles. The use of phenotypic SNPs in forensic science is fast becoming popular due to the nature of the information provided. Standard STR profiles may not be of much significance if there is no reference profile or existing profile on a database for comparison. Phenotypic SNPs have the potential ability aid in inclusion or exclusions of suspects or victims where no other information is available. In this study, we aimed to determine if SNPs could be amplified using direct PCR from single hair follicles. We chose to amplify SNPs from the validated IrisPlex system, an informative tool for determining eye colour. A section (~ 5 mm) of hair from the proximal tip was used in the initial PCR amplification. All following steps adhered to the IrisPlex protocol. Profiles obtained from the hairs consistently matched the reference profiles of the individuals tested. The results from this study highlight the potential use of DNA trace samples as a source for directly amplifying informative SNPs.

ii. *Collaborative EDNAP exercise on the IrisPlex system for DNA-based prediction of human eye colour*

a. *Contributed laboratory work and data analysis for the Flinders DNA Laboratory*

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Collaborative EDNAP exercise on the IrisPlex system for DNA-based prediction of human eye colour

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ABSTRACT

The IrisPlex system is a DNA-based test system for the prediction of human eye colour from biological samples and consists of a single forensically validated multiplex genotyping assay together with a statistical prediction model that is based on genotypes and phenotypes from thousands of individuals. IrisPlex predicts blue and brown human eye colour with, on average, >94% precision accuracy using six of the currently most eye colour informative single nucleotide polymorphisms (HERC2 rs12913832, OCA2 rs1800407, SLC24A4 rs12896399, SLC45A2 (MATP) rs16891982, TYR rs1393350, and IRF4 rs12203592) according to a previous study, while the accuracy in predicting non-blue and non-brown eye colours is considerably lower. In an effort to vigorously assess the IrisPlex system at the international level, testing was performed by 21 laboratories in the context of a collaborative exercise divided into three tasks and organised by the European DNA Profiling (EDNAP) Group of the International Society of Forensic Genetics (ISFG). Task 1 involved the assessment of 10 blood and saliva samples provided on FTA cards by the organising laboratory together with eye colour phenotypes; 99.4% of the genotypes were correctly reported and 99% of the eye colour phenotypes were correctly predicted. Task 2 involved the assessment of 5 DNA samples extracted by the host laboratory from simulated casework samples, artificially degraded, and provided to the participants in varying DNA concentrations. For this task, 98.7% of the genotypes were correctly determined and 96.2% of eye colour phenotypes were correctly inferred. For Tasks 1 and 2 together, 99.2% (1875) of the 1890 genotypes were correctly generated and of the 15 (0.8%) incorrect genotype calls, only 2 (0.1%) resulted in incorrect eye colour phenotypes. The voluntary Task 3 involved participants choosing their own test subjects for IrisPlex genotyping and eye colour phenotype inference, while eye photographs were provided to the organising laboratory and judged; 96% of the eye colour phenotypes were inferred correctly across 100 samples and 19 laboratories. The high success rates in genotyping and eye colour phenotyping clearly demonstrate the reproducibility and the robustness of the IrisPlex assay as well as the accuracy of the IrisPlex model to predict blue and brown eye colour from DNA. Additionally, this study demonstrates the ease with which the IrisPlex system is implementable and applicable across forensic laboratories around the world with varying pre-existing experiences.

1. Introduction

The field of forensic genetics is making great strides with the rapid scientific and technological evolution in obtaining new knowledge and creating innovative tools for solving crimes more and more effectively. Forensic DNA Phenotyping (FDP), a nascent advancement in this field, is one example of recent innovative developments in forensic genetics and involves the prediction of an individual's externally visible characteristics (EVCs) using biological samples obtained at a crime scene or from an anonymous body (parts) that may belong to a missing person [1–4]. Conventional DNA identification involves the comparison of DNA profiles derived from short tandem repeat (STR) marker genotypes obtained from evidence and reference samples, which is useful in cases when the sample donor is known from their DNA profile. In certain circumstances, sample donors may not be identified, i.e. a match (or familial match) of the DNA profiles with known suspects such as those in criminal offender DNA (profile) databases or with ante-mortem samples in cases of missing persons is not successful, or when DNA profile comparisons with putative relatives of missing persons does not reveal the degree of similarities indicating biological relationship. In these situations, FDP can be used to help investigative authorities focus their search for unknown suspects or missing persons towards individuals with particular DNA-predicted externally visible phenotypes. The DNA-based prediction of EVCs can thus aid investigations by police and other authorities by reducing the number of possible suspects or other individuals if conventional STR typing of the evidence fails to produce identification [1,2]. Furthermore, reconstructing appearance information from biological samples such as bones or teeth or other remains of deceased individuals is relevant in anthropological research disciplines including those relying on ancient DNA analysis [1,2,5].

Several model-based approaches, amongst others [4,6], have been developed for predicting a particular phenotype from DNA most notably human eye (iris) colour [7]; the IrisPlex system is one such tool [8]. IrisPlex can accurately predict blue and brown eye colour with a precision of >94%, according to a previous study [9], using six of the most informative eye colour markers: rs12913832 (HERC2), rs1800407 (OCA2), rs12896399 (SLC24A4), rs16891982 (SLC45A2 (MATP)), rs1393350 (TYR) and rs12203592 (IRF4) in a single genotyping assay and a prediction model based on thousands of individuals for which IrisPlex genotype and eye colour phenotype data are available [7,8,10]. The 94% accuracy is based on using a threshold of $p > 0.7$, however it is possible to use IrisPlex prediction with a lower $p > 0.5$ threshold. The assessment of precision accuracy is based on a broad European dataset of >3800 individuals using IrisPlex can be found in Table 3 of that publication [9]. The IrisPlex assay represents the first FDP system that successfully underwent developmental validation using the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines for use in forensic casework [10]. The IrisPlex prediction model, first established on thousands of Dutch Europeans, has been evaluated in several populations within and outside Europe and was shown to perform reliably, independent of the bio-geographic origin of the individual tested [9,11].

In an international effort to test the reliability and consistency of the IrisPlex system for eye colour prediction through an inter laboratory exercise, the European DNA Profiling (EDNAP) Group, a working group of the International Society for Forensic Genetics (ISFG), carried out a collaborative study led by the Department of Forensic Molecular Biology of the Erasmus University Medical Center Rotterdam (Netherlands), who initially developed and validated the IrisPlex system [8–10] and for this reason were chosen to conduct this further assessment on the IrisPlex tool alone. Of the 21 participating laboratories, 18 were from Europe,

2 were from Australia and 1 was from the U.S.A. The prime aim of this exercise was to implement the method and assess the performance of the system across different forensic laboratories with varying levels of experience, from complete novices with no SNP typing experience to participants with SNaPshot experience and those with specific IrisPlex experience.

Notably, some authors previously raised issues about marker content and model outcomes of the IrisPlex system [12–15]. The present collaborate EDNAP exercise, however, represents a rather technical exercise to test the performance of the IrisPlex system across laboratories with varying levels of pre-existing experience. Therefore, issues about marker and model choice for predicting eye colour from DNA may be addressed in more dedicated future studies. Here, we present the results of this collaborative EDNAP exercise, placing emphasis on the reliability and consistency in using the IrisPlex system for blue and brown eye colour prediction from DNA.

2. Materials and methods

2.1. Samples and materials provided to the participating laboratories

The organising laboratory (Department of Forensic Molecular Biology, Erasmus MC University Medical Center Rotterdam, Netherlands) divided the entire exercise into three different tasks. All participants were provided with a detailed written laboratory protocol [8] as well as the eye colour prediction model that is an interactive excel spread-sheet as published earlier [10]. Furthermore, for assay interpretation guidelines, participants were given a protocol stating a 50 relative fluorescent units (rfu) peak height threshold should be used for allele calls using the IrisPlex specific GeneMapper software (Applied Biosystems) Bin and Panel set provided. For a broader understanding of average peak heights and balance ratios, participants were asked to refer to the previous developmental validation publication of the IrisPlex system [10]. In addition to the samples and the primers provided for each task, all reagents, which include: 1× PCR buffer, 2.7 mM $MgCl_2$, 200 mM of each dNTP, 0.5 U AmpliTaq Gold Polymerase, SNaPshot™ Multiplex chemistry for the single base extension (SBE) reactions (Applied Biosystems, Foster City, CA), Exonuclease Shrimp Alkaline Phosphatase (ExoSAP-IT), and Shrimp Alkaline Phosphatase (SAP) (USB Corporation, Cleveland, OH), required for running the IrisPlex system were shipped on dry ice to each of the 21 participating laboratories. The laboratories were asked to use their own internal sizing standard (LIZ 120) and formamide for the capillary electrophoresis run.

Due to an ExoSAP-IT™ degradation issue noted during the early phase of the exercise, which subsequently was acknowledged by the producing company as a bad batch of enzyme, aliquots of a newly delivered and tested ExoSAP-IT™ were shipped again to the requesting laboratories, while the others opted to use their in-house standard cleaning protocols. As this was a clean-up procedure, it did not impede on the testing of the IrisPlex assay overall. Purified products were run by the laboratories using their in-house Genetic Analysers (for type, see Table 1) and analysed with the previously published eye colour prediction model [10] provided by the organising laboratory for predicting human eye colour from IrisPlex genotypes.

As a disclaimer for the choice of samples used in this assessment, please note that it is well established and documented [7,9,16,17], that the IrisPlex system through its use of six eye colour associated SNPs performs very well in predicting blue and brown eye colour with Area Under the receiving operator Curve (AUC) values >0.9; however its use for predicting intermediate eye colour (current AUC of ~0.7) is not at an optimum level yet. This is due to the current lack of knowledge on DNA predictors for these non-blue, non-brown eye colours i.e. green eye colour, individuals with heterochromia etc. which is not only a limitation of the IrisPlex but of all currently available DNA test systems for eye colour [4,6]. DNA variants with similarly high prediction effects on non-blue/brown eye colours as the IrisPlex SNPs have on blue and brown eye colour have yet to be identified. Therefore, the IrisPlex system was previously promoted for the prediction of blue and brown eye colours and thus the organising laboratory opted to test variations in blue and brown eye colour alone for Tasks 1 and 2 to evaluate the current IrisPlex system assay and prediction performance on these categories. Task 3 however incorporates all three categories as this task was based on samples provided by the participating laboratories who were not asked to focus on blue and brown eye colour alone when selecting their Task 3 volunteers.

Tasks 1 and 2 contain samples from individuals of European (80% per task) and non-European bio-geographic origin (20% per task) including one admixed individual in Task 1. The individuals used in Task 3, including information about their bio-geographic background, were at the discretion of the participating laboratories and were unknown to the organising lab.

2.2. Task 1 – IrisPlex eye colour prediction from biological samples with eye colour knowledge

The organising laboratory provided all participating laboratories with five blood samples (labelled Ind1–Ind5) and five saliva

Table 1
DNA extraction and quantification protocols used by the 21 laboratories for both Tasks 1 and 2. Grey boxes indicate no data received from the participating laboratory.

Lab ID	Extraction Protocol	Quantification Protocol	Polymer	Genetic Analyzer
1			POP 4	3130xl
2	EZ1 DNA Investigator Kit on an EZ1 Advanced XL (Qiagen)	Quantifiler® Duo DNA Quantification Kit (Life Technologies)	POP 6	3100
3			POP 4	3130
4	Task 1: EZ1 advanced (Qiagen); Task 3 DNA Blood Mini Kit (Qiagen)	ABI prism® 7900 (Life Technologies-LT) using Quantifiler® Human DNA Quantification Kit (LT).	POP 4	3130xl
5	Chelex + Qiaquick	Task 1: Qubit and Task 2: RT-PCR Quantifiler®	POP 4	3130xl
6	QIAamp® DNA Mini (Qiagen, Hilden, Germany)	qPCR using the 7900HT Fast Real-time PCR System (Applied Biosystems, Darmstadt, Germany) and Alu Primers	POP 4	3500xl
7	Chelex	Quantifiler® Duo	POP 7	3130xl
8	5% solution of BT Chelex 100 resin (Bio-Rad)	Quantifiler® Human DNA Quantification Kit (Life Technologies) on AB 7900 RT-PRC	POP 4	3130
9	QIAmp DNA blood Mini kit from Qiagen	Quantifiler® Human kit on Applied Biosystems 7500 Real-Time PCR System	POP 7	3500xl
10	QIAmp DNA blood Mini kit from Qiagen	Nanodrop	POP 7	3500xl
11		Quantifiler® Human	POP 4	3130xl
12	QIAamp mini columns (Qiagen)	real-time quantitative PCR assay using ALU repeats from Nicklas et el.	POP 7	3130xl
13	EZ1 robot (Qiagen)	Quantifiler®	POP 7	3130xl
14	Qiagen EZ1 Advanced XL extraction robot with the EZ1 DNA Investigator Kit	Quantifiler® Human DNA Quantification Kit (Life Technologies)	POP 4	3130xl
15	QiAmp DNA Mini kit (Qiagen)	Nanodrop	POP 4	3130xl
16	QiAmp DNA Mini kit (Qiagen)		POP 7	3500
17	Qiagen EZ1 Investigator Handbook	Thermo Scientific NanoDrop 2000/2000c spectrophotometer	POP 4	3130
18	Applied Byosistems Prepfiler Forensic DNA Extraction kit	Task 1 and Task 3 a 1% agarose gel; Task 2 - a RT PCR	POP 4	3130
19	Chelex	Quantifiler® DUO in an 7500 Real-Time PCR System	POP 7	3130
20	Phenol–Chloroform	Quantifiler® Human DNA Quantification Kit and AB 7300.	POP 4	3130
21	Qiagen M48 robotic station and MagAttract DNA Mini M48 Kit	Quantifiler kit® and ABI 7500	POP 7	3130xl

samples (labelled Ind6–Ind10) on FTA cards of 10 individuals with blue ($N = 5$) or brown ($N = 5$) eye colour. To produce these samples, fresh venous blood and saliva samples were collected from ten different individuals and 100 μL of each of the samples were pipetted on to the FTA cards. A digital eye image from each of these 10 individuals who donated blood or saliva was also provided to the participants. All the laboratories were instructed to use their own in-house DNA extraction and quantification protocols. All participating laboratories were asked to generate the IrisPlex genotype profile from each of the 10 samples and using the provided IrisPlex prediction model, to conclude the eye colour prediction of the 10 donor individuals by noting the probability and precision accuracy per each sample and individual using the guidelines implemented in a previous publication [9] as provided to the participants. An example report was also provided to each laboratory to ease the fill-out for return. As the participants were provided with eye pictures, they already knew the eye colour of the study individuals of Task 1 before analyses. However, the conclusion of the eye colour phenotypes had to be based on the genotypes determined by each individual laboratory considering the probability and precision accuracy guidelines provided by the organising laboratory. The term 'precision accuracy' relates to the previous publication [9], which undertook a study on the final prediction called by the IrisPlex model in terms of probability values on over 3800 European individuals. It assesses the highest probability value (which is defined as the eye colour of the individual) and how correct the eye colour prediction was at thresholds that increase in increments of 0.05p; from no threshold to $p > 0.95$ [9].

2.3. Task 2 – IrisPlex eye colour prediction from DNA of simulated casework samples without eye colour knowledge

The laboratories were provided with five DNA samples extracted from simulated casework samples (labelled CW1–CW5) from 5 individuals with blue ($N = 2$) or brown ($N = 3$) eye colour. DNA samples were extracted by the organising laboratory with the QIAamp DNA mini kit (Qiagen, Hagen, Germany). The following biological materials were used for DNA extractions: 2 buccal swabs (CW1 and CW2) – both samples subjected to UV radiation using the Bio-Link (Vilber Lourmat) for 1 min at a strength of 50 J/cm^2 before DNA extraction; saliva on glass slide (CW3) stored at room temperature for 1 week before DNA extraction, whole blood on glass slide (CW4) stored at room temperature for 1 week before DNA extractions, and semen DNA extracted from a frozen donated sample (CW5). DNA concentrations were measured using the nanodrop system and 3 μL of the following solutions were provided: CW1: 0.5 ng/μL, CW2: 0.1 ng/μL, CW3: 0.25 ng/μL, CW4: 2 ng/μL, and CW5: 50 ng/μL (see Table 2 for overview). The samples CW1–CW4 were freshly extracted, quantified and run using the IrisPlex system prior to DNA degradation, thereby serving as a control to their degraded counterparts. In contrast to Task 1, in Task 2 the laboratories were not provided with any eye colour phenotype information of the sample donors. Participants were also not provided with any other sample information such as DNA concentration or treatment prior to DNA extractions of the DNA extracts they received for Task 2. They were asked to generate the IrisPlex genotype profile for each individual and report back the obtained eye colour probabilities and accuracies using the model and materials provided, and to finally conclude the most likely eye colour category per individual. An example report was also provided.

Table 2
Details of the simulated casework samples provided to the participating laboratories for the Task 2.

Sample #	Sample type	Treatment	Concentration (ng/μL)
CW1	Buccal Swab	UV for 1 min	0.5
CW2	Buccal Swab	UV for 1 min	0.1
CW3	Saliva on Slide	RT for 1 week	0.25
CW4	Blood on slide	RT for 1 week	2
CW5	Semen	–	50

2.4. Task 3 – participant-driven IrisPlex eye colour prediction

This part of the study was optional. Each participating laboratory was instructed to collect and genotype samples from five different individuals of any eye colour. Selection of volunteers and biological materials was at the discretion of the participants. An important caveat in this task is that IrisPlex cannot guarantee a high prediction accuracy of the non-blue and non-brown eye colours; however, in contrast to Tasks 1 and 2 no eye colour phenotype restrictions were imposed on the participants in their choice of volunteers for Task 3. The laboratories were asked to report the DNA concentration, IrisPlex genotypes, eye colour probability outcomes and accuracy percentages, and a digital high-resolution eye image of the genotyped individuals. The organising laboratory instructed that the iris photo should be taken in natural light conditions (no fluorescent bulb light) with and without flash lens using a digital camera focusing on eyes only (no full portrait).

3. Results and discussion

3.1. Sample extractions and quantifications by each participating laboratory

As the DNA extraction and quantification method can influence genotyping outcomes due to the quality and quantity of DNA extracted and consequently input into a downstream reaction, it can thus influence phenotype inference from the genotypes produced in FDP systems. Therefore we included extraction and quantification monitoring in this exercise. As part of Task 1, the host laboratory provided the participating laboratories with biological samples (blood and saliva samples on FTA cards) from which the participants extracted and quantified DNA using their methods of choice. To note, the affiliated laboratory number in the author list does not represent the laboratory number described throughout the paper. Protocols used for DNA extraction and quantification were different and are listed in Table 1. As evident, the different extraction and quantification methods used by the participating laboratories provided varying results, as summarised in a box plot diagram (Fig. 1), even though the same volume of biological sample was provided to each of the participants on FTA cards. Labs #2, 4, 13, 14 and 17 used the Qiagen EZ1 investigator kit for extraction and reported on average higher quantification values as compared to Labs #6, 9, 10, 12 and 15 that used the Qiagen QIAamp DNA mini kit extraction protocol. Lab #20 applied a Phenol–Chloroform extraction approach, which yielded on average higher quantification values compared to all the other methods used. Lab #18 used the Prepfiler Forensic DNA extraction kit and obtained on average higher final DNA amounts than all other methods, except Phenol–Chloroform. Labs #5, 7, 8, and 19, which used the Chelex extraction protocol, reported comparatively lower quantification values than all other methods used in this exercise. Worthy to note, this figure assumes that all laboratory input DNA volumes were similar (i.e. the recommended 1 μL). The precise extraction volumes used by all labs were not available to the organising laboratory. This figure merely represents the differing extraction methods yielding varying final DNA concentrations, however, it is expected that the participants followed all recommendations provided by the organising lab which specifically states a 1 μL volume with at least a concentration of 32 pg

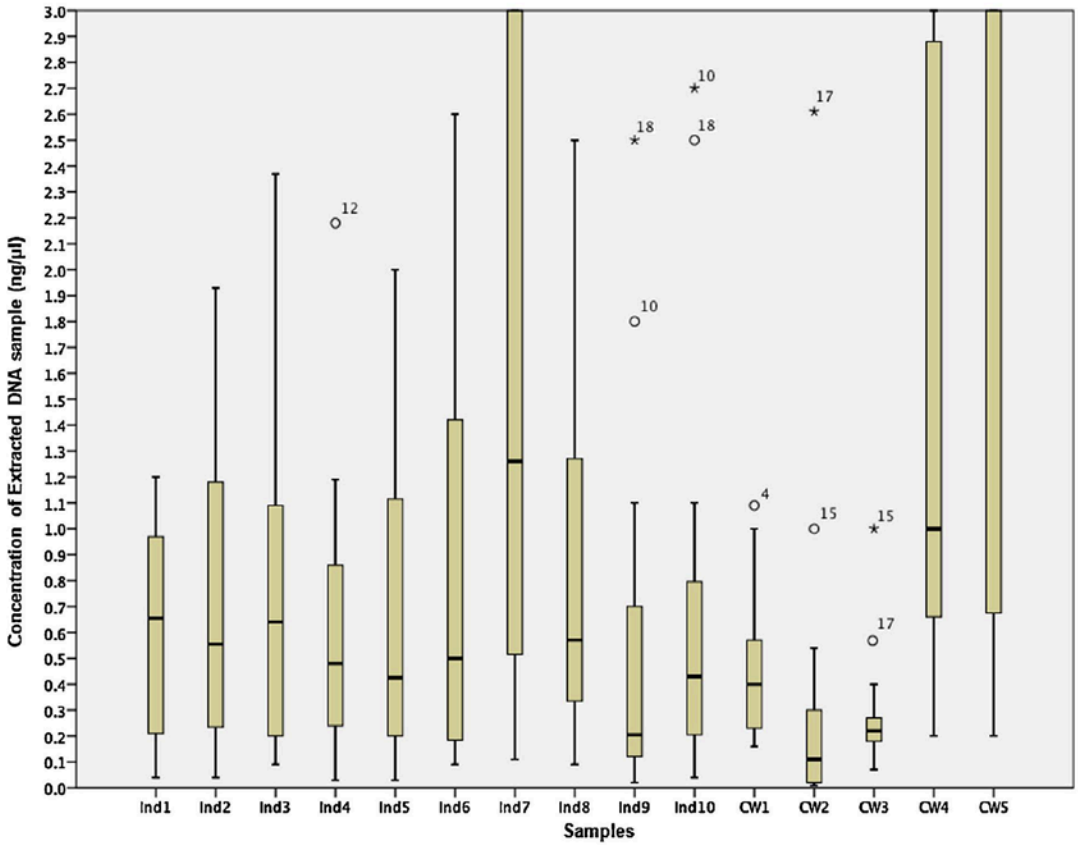


Fig. 1. Box-plot (scaled to 3 ng/µL) showing the variation in obtained DNA concentrations using different methods for extraction and quantification between the 21 laboratories for the 15 samples used in Task 1 and 2. Note that for Task 1 (samples Ind1–Ind10), biological samples were provided to the participants so that DNA extraction and DNA quantifications were carried out by the participants on the same volumes of biological materials provided on FTA cards, while for Task 2 (samples CW1–CW5) participants were provided with already extracted DNA samples that varied in treatment and DNA concentrations. Medians are represented by the horizontal lines and the boxes depict the 25–75% quartiles. The whiskers represent the minimum and the maximum values. Outliers are marked with the laboratory number where they were reported.

DNA input for IrisPlex profiling. The DNA samples provided for Task 2 were previously extracted by the host laboratory using the QIAamp DNA mini kit (Qiagen). The participating laboratories were requested to measure DNA concentrations using their method of choice and to report back the values. Because different quantification methods were used, the obtained concentration estimates differed (Fig. 1 and Supplementary Table 1), similar to Task 1, even though equal aliquots of the very same DNA solutions per each sample were provided to each of the participants. As evident, sample CW2 was recorded as the most variable (0.01–2.61 ng/µL), which contradicts recorded measurements by the organising laboratory of 100 pg (Fig. 1).

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.04.006.

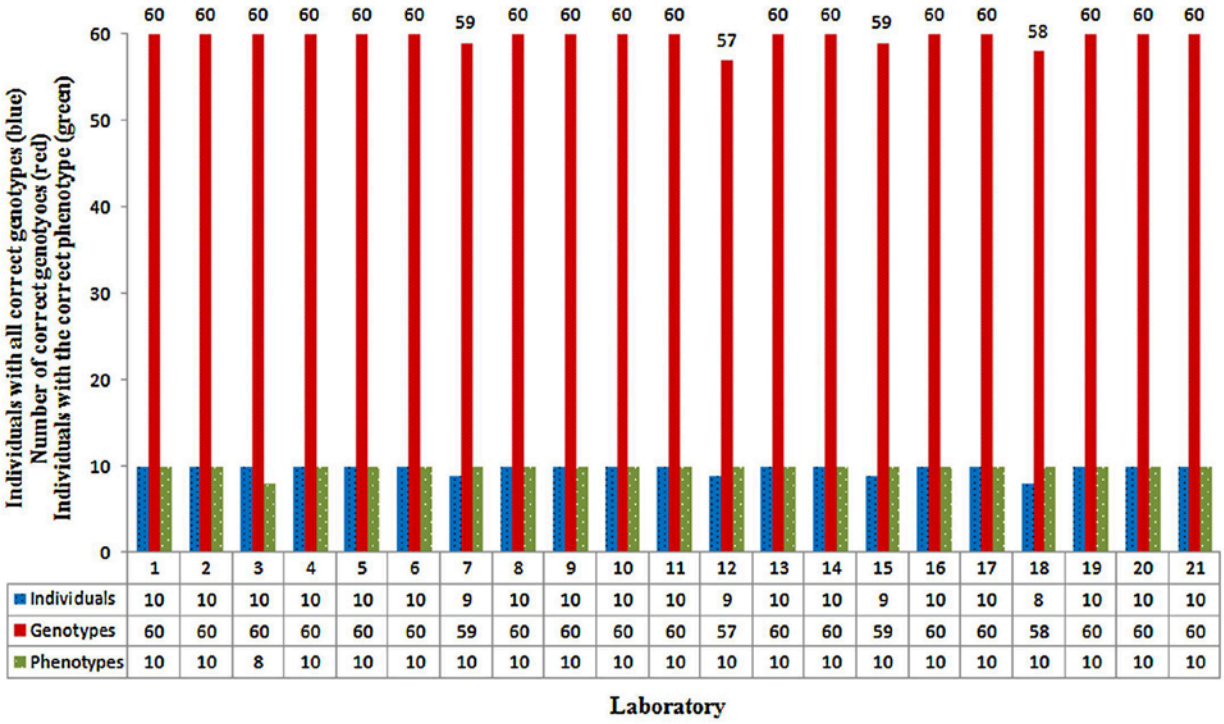
Overall, the recorded DNA quantification data indicate that all samples shipped, both the biological samples on FTA cards of Task 1 and the extracted DNA samples of Task 2, and remained rather stable during transportation and short-term storage at the participating laboratories. For the impact of the varying amounts of DNA obtained by the participants in Task 1 and the varying DNA concentration measures obtained in Task 2 on genotype and phenotype accuracy, see the specific chapters on Tasks 1 and 2 below. From the DNA quantification data reported by the participating laboratories for the samples used in Task 3 (Supplementary Table 1) it is evident that all the samples genotyped for this portion of the exercise were of reasonable quantity. When conducting genotyping analyses and calling the peaks, the 50 rfu fluorescence threshold was set for calling alleles for a locus in all tasks and samples.

3.2. Task 1 – IrisPlex eye colour prediction from biological samples with eye colour knowledge

All participating laboratories reported the predicted eye colour and their probabilities in the format as requested by the organising laboratory. Fig. 2 depicts the accurate genotype and eye colour phenotype calls for all the ten samples as obtained by the 21 participating laboratories. Supplementary Table 2 lists the genotypes of the ten individuals with their respective eye colour probability and accuracy. Fig. 3 shows the eye colour images of the 10 individuals used in this task.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.04.006.

Twenty of the 21 laboratories (95%) predicted the eye colour of all 10 individuals included in Task 1 correctly from IrisPlex (Fig. 2; green bars). Overall, 208 (99%) of the 210 samples analysed in this task by all the 21 laboratories were reported with the correct eye colour phenotype prediction. An overview of the samples with incorrect genotypes that were discordant with the organising laboratory is provided in Table 3. Only one laboratory (Lab #3) faced difficulties in concluding the correct eye colour phenotype for two samples (Individual 1 and 10). The phenotype for both



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Individuals	10	10	10	10	10	10	9	10	10	10	10	9	10	10	9	10	10	8	10	10	10
Genotypes	60	60	60	60	60	60	59	60	60	60	60	57	60	60	59	60	60	58	60	60	60
Phenotypes	10	10	8	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Fig. 2. Accuracy of the IrisPlex genotype calls (6 SNPs) and the IrisPlex-based eye colour phenotype prediction of the 10 samples provided in Task 1 as reported by each of the 21 participating laboratories. Blue indicates the number of individuals that were correctly genotyped at all 6 IrisPlex SNPs (i.e. for which a correct IrisPlex profile was reported). Red indicates the total number of genotypes across all 6 SNPs and all 10 samples that were correctly reported. Green indicates the number of individual samples for which the correct eye colour phenotype was reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

individuals was reported as inconclusive, although the correct IrisPlex genotypes were obtained and reported. These two individuals had eye colour probabilities for blue, intermediate and brown of 0.306, 0.142, 0.552 and 0.299, 0.253, 0.448 respectively (Fig. 3(a) and (k)), and did not cause a problem for the other 20 laboratories to conclude the correct brown eye colour for both samples.

Overall, 1253 (99.4%) of the 1260 genotypes generated for the 6 IrisPlex SNPs in the 210 samples analysed by all the 21 laboratories were reported correctly. The 7 (0.6%) incorrect genotypes were reported by 4 laboratories, while 17 participants reported the correct 6-SNP IrisPlex profiles for all 10 samples. Importantly, none of these incorrect genotypes led to erroneous eye colour phenotype predictions being reported. Lab #7 reported an incorrect homozygous genotype T instead of the true heterozygous CT for rs12203592 for Individual 8 due to a dropout of the C allele, since the respective peak was below the 50 rfu threshold. Lab #12 reported discordant heterozygous instead of correct homozygous genotypes for Individual 9 across three markers (rs12913832, rs16891982 and rs1393350). Drop-in of the alleles C, G and T was observed for each of the markers rs12913832, rs16891982 and rs1393350 respectively. However, the DNA concentration for this particular sample was reported by Lab #12 to be very low (0.02 ng/μL), much lower than the concentration obtained by the other

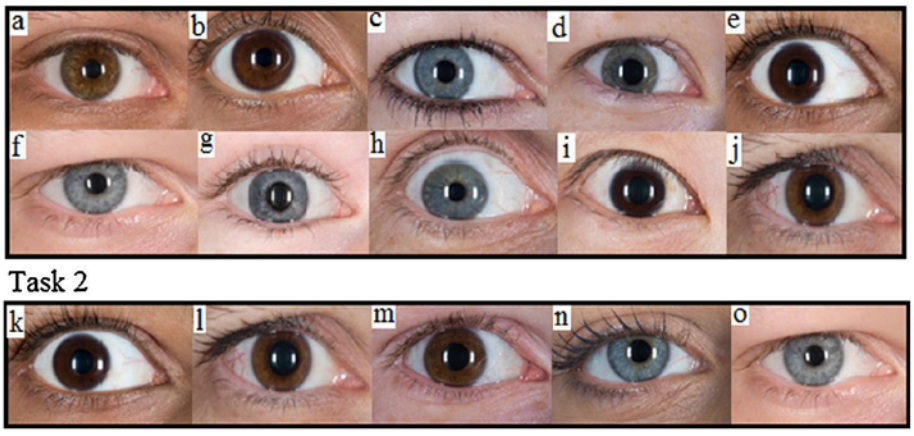


Fig. 3. Eye colour images of the 10 individuals whose samples were used in Task 1 and the 5 individuals whose samples were used in Task 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 3
Tasks 1 and 2 genotyping results that were discordant between the host and the participating laboratories.

	Sample	Lab#	Locus	Comments
Task 1	Individual 8	7	rs12203592	Drop-out of C
	Individual 9	12	rs12913832	Drop-in of C
	Individual 9	12	rs16891982	Drop-in of G
	Individual 9	12	rs1393350	Drop-in of T
	Individual 2	15	rs16891982	Drop-out of C
	Individual 3	18	rs12203592	Drop-in of T
	Individual 8	18	rs12203592	Drop-out of T
Task 2	CW2	6	rs12913832	Drop-out of T
	CW2	7	rs12913832	Drop-out of C
	CW2	15	rs1393350	Drop-in of T
	CW2, CW3	17	rs12913832	Drop-out of T, C respectively
	CW2	17	rs1800407	Drop-out of A
	CW3	18	rs1393350	Drop-out of T
	CW2	21	rs12896399	Drop-out of T

laboratories for this sample (Supplementary Table 1). In accordance with the provided protocol, 1 μL of DNA solution of this sample was used for the IrisPlex PCR. Therefore, for this sample the amount of DNA input was below the previously established sensitivity threshold of the IrisPlex assay [10], which explains the high failure rate for genotypes of this sample. Lab #15 reported incorrect homozygous genotype of the G allele (instead of the correct heterozygous GC genotype) for individual 2 at rs16891982 due to a dropout of the C allele. Lab #18 experienced at marker rs12203592 a drop-in of allele T for individual 3 and dropout of allele T in individual 8. One explanation could be primer degradation due to incorrect storage of the primer or an incorrect volume addition of this primer to the assay, but unlikely due to a sample issue as the DNA concentrations reported for the individuals 2, 3 and 8 by the Labs #15 and 18 were more than 1.1 ng/μL (provided that the correct input of 1 μL was used). For cases such as these, an erroneous result can be avoided by re-running several analyses of the sample, and is usually recommended when using all genotyping platforms, this includes the IrisPlex system.

3.3. Task 2 – IrisPlex eye colour prediction from DNA of simulated casework samples without eye colour knowledge

In Task 2, the provided five DNA extraction aliquots from simulated casework samples (Table 2) were evaluated by each participating laboratory. Notably, the samples used for DNA extractions not only came from different biological sources (saliva, blood, and semen) but also experienced different environmental conditions (UV radiation, storage at room temperature) and were provided to the participants in varying DNA concentrations (0.1–50 ng/μL), all unknown to the participants. Therefore, and due to the fact that no eye colour phenotypes were provided of the sampled individuals, this task was more challenging than Task 1. Fig. 4 depicts the accurate genotype and eye colour phenotype calls for all the 5 samples as reported by the 21 participating laboratories. Supplementary Table 2 shows the reported genotypes of the five samples with their respective eye colour prediction probabilities and precision accuracy. Fig. 3 shows the eye colour images of the 5 individuals used in this task. An overview of the samples with incorrect genotypes that were discordant with the organising and the other participating laboratories is provided in Table 3.

Eighteen (86%) of the 21 laboratories predicted the eye colour of all 5 individuals correctly from IrisPlex (Fig. 4; green bars). Overall, 101 (96.2%) of the 105 samples analysed by all the 21 laboratories together were reported with the correct eye colour phenotype. The 4 samples (3.8%) for which the eye colour phenotypes were incorrect had been reported by 3 laboratories. Lab #3 predicted the eye colour of 2 of the 5 individuals (CW2 and CW3) as inconclusive, although the genotypes for these samples were reported correctly. Both samples clearly had to be designated as brown from the obtained probabilities ($p = 0.448$ and $p = 0.552$, respectively), and the phenotypes indeed were brown (Fig. 3(l) and (m) respectively), as was correctly interpreted by 18 other laboratories. The other 2 incorrectly phenotyped samples were reported by Labs #6 and 17 due to the drop-out of the T allele at rs12913832 in sample CW2. The 2 laboratories reported an incorrect homozygous C allele instead of a heterozygous CT allele, thereby, reporting an incorrect blue eye colour instead of the correct brown eye colour phenotype (Fig. 3(l)).

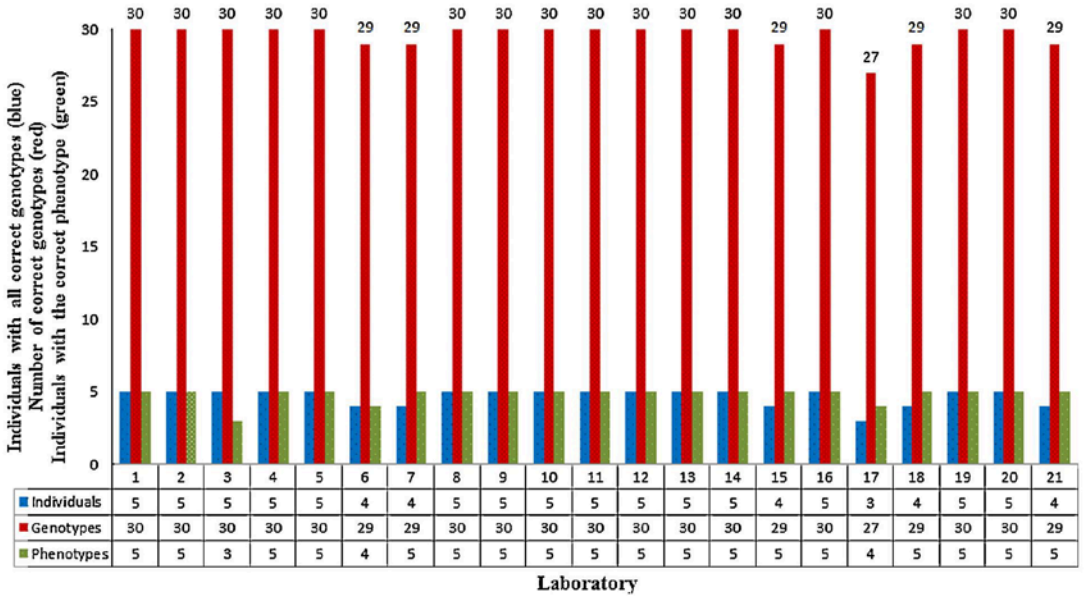


Fig. 4. Accuracy of the IrisPlex genotype calls (6 SNPs) and the IrisPlex-based eye colour phenotype prediction of the 5 samples provided in Task 2 as reported by each of the 21 participating laboratories. Blue indicates the number of individuals that were correctly genotyped at all 6 IrisPlex SNPs (i.e. for which a correct IrisPlex profile was reported). Red indicates the total number of genotypes across all 6 SNPs and all 5 samples that were correctly reported. Green indicates the number of individual samples for which the correct eye colour phenotype was reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Overall, 622 (98.7%) of the 630 genotypes generated for the 6 IrisPlex SNPs in the 105 samples analysed by all laboratories together were correctly reported in Task 2. The 8 (1.3%) incorrect genotypes were produced in 2 samples (CW2 and CW3) by 6 laboratories, while 15 of the 21 laboratories (71.4%) reported the correct 6-SNP IrisPlex profile for all 5 samples. In contrast to the 2 (25%) genotype errors in sample CW2 by Lab #6 and 17 that caused phenotype errors as mentioned in the previous paragraph, the remaining 6 incorrect genotypes (75%) did not have any impact on the eye colour phenotype accuracy. At rs12913832, Lab #7 reported an incorrect genotype due to a drop-out of the C allele for sample CW2. A drop-in of the T allele for CW2 and drop-out of the T allele for CW3 at rs1393350 resulted in incorrect genotyping by Labs #15 and 18 respectively. Furthermore, incorrect genotypes were reported by Lab #21 at rs12896399 for sample CW2 due to a drop-out of the T allele. Lab #17 experienced problems in the first typing of samples CW1, 2 and 3 and subsequently retyped these samples in different DNA dilutions. At rs12913832, drop-out of the T allele for CW2 (as mentioned above); drop-out of the C allele for CW3, and drop-out of the A allele at rs1800407 for CW2 were reported which resulted in erroneous results for this laboratory. A dilution step performed by the participating laboratory, due to a misleading quantification result, of the already low quantity degraded samples provides a likely explanation for the drop-out of the alleles in this set of samples.

Several laboratories ($n = 3$; Labs #3, 6 and 17) experienced difficulties with correct phenotyping of the simulated and treated casework samples in Task 2 for which no eye colour phenotypes were provided as opposed to the untreated biological samples provided together with eye colour phenotypes in Task 1 ($n = 1$; Lab #3). Similarly, more laboratories ($n = 6$; Labs #6, 7, 15, 17, 18 and 21) had difficulties in correct genotyping of Task 2 samples in relation to Task 1 samples ($n = 4$; Labs #7, 12, 15 and 18). Within Task 2, the most genotyping and phenotyping difficulties i.e. allelic drop-outs and drop-ins were reported for 2 particular samples (CW2 and CW3). Sample CW2 was reported with different incorrect genotypes by 5 of the laboratories (Labs #6, 7, 15, 17 and 21) and sample CW3 was reported incorrectly by 2 laboratories (Labs #17 and 18) (see Table 3 for overview). Sample CW2 must therefore be noted as being a difficult sample to genotype. From Fig. 1, it is evident that, of the laboratories that reported quantification data for Task 2, sample CW2 was recorded as the most variable (0.01–2.61 ng/μL), which strongly deviates from the recorded measurements by the organising laboratory of 100 pg. Given its unusual quantification range, severe degradation and heterozygosity at 3 (rs12913832, rs1800407 and rs12896399) of the 6 SNPs, increased incidence of allelic drop-out may be expected in sample CW2 as compared to the homozygous sample CW1 (which also experienced UV degradation) that caused no problems for genotyping. This demonstrates, as expected and as also known for any other genotyping assay, that the combination of low quality and low quantity template DNA provides challenges for correct genotyping including for the IrisPlex assay. However, it should be emphasised that 244 of the 252 (96.8%) genotypes of the most challenging samples CW2 and CW3 were generated correctly by 15 of the 21 (71.4%) participating laboratories, which demonstrates the reliability of the IrisPlex assay for difficult DNA samples. This also represents the necessity of employing duplicate analysis when genotyping samples of low DNA quantity in final case work applications.

3.4. Task 3 – participant-driven IrisPlex testing

The optional Task 3 of the exercise, where participants were asked to recruit their own volunteers for IrisPlex genotyping and eye colour prediction, was performed by 20 of the 21 laboratories. Lab #7 could not perform this task due to reported ethical issues. Supplementary Table 3 summarises the data for this task. Based on the digital eye images provided by the participants (Fig. 5), the organising laboratory judged the correct phenotypes by two independent experienced observers. Lab #12 performed this exercise and reported the genotype and phenotype, but provided no eye images to the host laboratory for inspection. As it was not possible to judge the accuracy of the results provided by this participant, they were excluded from the analyses. As can be seen in Fig. 6, 16 of the 19 laboratories (84.2%) predicted the eye colour of all analysed individual samples correctly, while 3 laboratories faced difficulties in concluding the correct eye colour from the estimated probability combinations for some samples. Overall, 96 (96%) of the 100 samples analysed by the 19 laboratories were reported with the correct eye colour prediction, as judged by the organising laboratory based on the digital eye images sent by the participants. The 4 samples reported with incorrect eye colour were from 3 different laboratories (1x#2, 2x#3, and 1x#5). Lab #2 reported blue eye colour ($p = 0.678$) for their sample 1 but the eye image showed brown colour and the estimated brown eye probability was only 0.191 (Fig. 5(a)). Lab #3 obtained the following probabilities for their sample 1: Blue – 0.207, Intermediate – 0.161 and Brown – 0.632 and reported an inconclusive result, while the probability for brown was by far higher than for the other two categories so that brown should have been concluded instead and indeed the respective eye image showed brown (Fig. 5(b)). Sample 4 of Lab #3 appeared blue from the images (Fig. 5(c)) but a high brown eye probability ($p = 0.892$) was obtained while the blue eye probability was low ($p = 0.024$). Lab #5 obtained probabilities of Blue – 0.375, Intermediate – 0.264 and Brown – 0.361 for their sample 1, and reported blue eye colour but the image indicates brown eye colour (Fig. 5(d)). In this case, however, the eye colour could have been reported inconclusive since the brown and the blue eye colour probabilities were very similar. It is therefore important to use and report the level of precision accuracy based on each probability threshold with the final prediction, i.e. $p < 0.5$ highest probability value, $p > 0.5$ highest probability value. This can be found in Table 2 of our previous publication [9]. It is noteworthy to emphasise that the IrisPlex genotypes in Task 3 were not verified independently in contrast to those in Tasks 1 and 2. Therefore we cannot know for sure if any of the 4 incorrect phenotype predictions in Task 3 may have been caused by incorrect genotypes, although the high genotyping accuracy rates achieved in Tasks 1–3 suggest this might be somewhat unlikely. Due to violation of anonymity, the geographic origin of these individuals cannot be determined.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.04.006.

Further to note, participants for Task 3 were not asked to restrict their choice of volunteers to blue and brown eye colour only. This was different from Tasks 1 and 2 where only volunteers with blue and brown eye colour were used due to known limitations of the IrisPlex system to accurately predict non-blue and non-brown eye colours [8–10]. However only Lab #1 reported 2 individuals as intermediate ($p = 0.411$ and $p = 0.405$) and from the eye images (Fig. 5(e) and (f) respectively), we can confirm that the individuals were correctly predicted as intermediate as they contain substantial pupillary rings of a different colour (i.e. majority of iris blue colour with obvious brown pupillary ring). It is also worth noting that although no restrictions were imposed on the choice of samples for Task 3, all the laboratories (except Lab #1) chose individuals with either blue or brown eyes and hence it is most likely that all the participants were guided by knowledge of (or clearly considered the) limitations of IrisPlex for accurately predicting non-blue and non-brown eye colour phenotypes. Furthermore, according to general knowledge, the frequency of

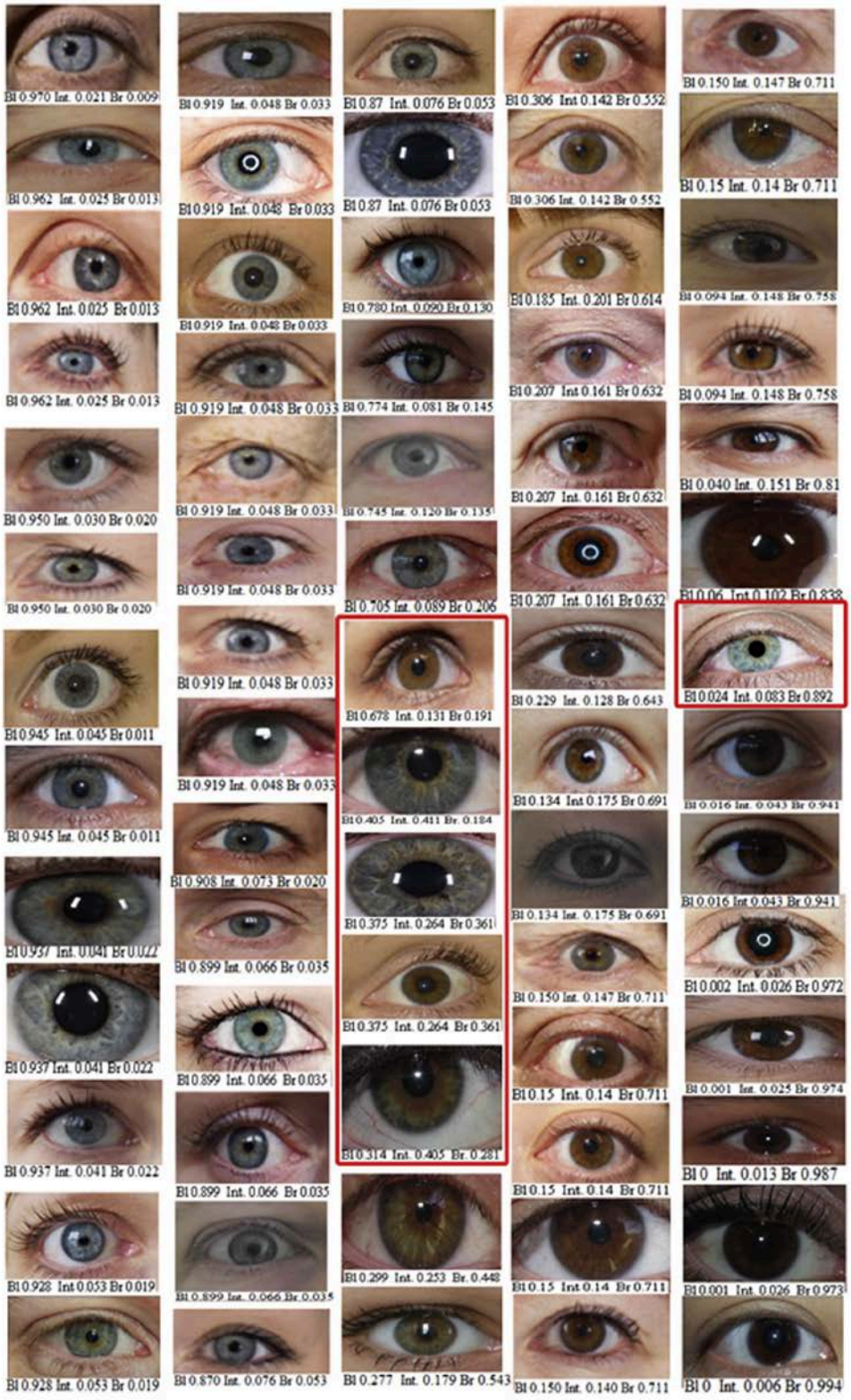


Fig. 5. Eye images sent by the participating laboratories used for the voluntary aspect of the study, Task 3. Eye images include probability values for blue, intermediate and brown eye colour provided by the participants as determined from the IrisPlex genotypes. The area surrounded by the red lines indicates the incorrect eye colour prediction as assessed by the host laboratory from inspection of the eye images provided, and compared with the eye colour phenotype reported by the participants based on IrisPlex analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

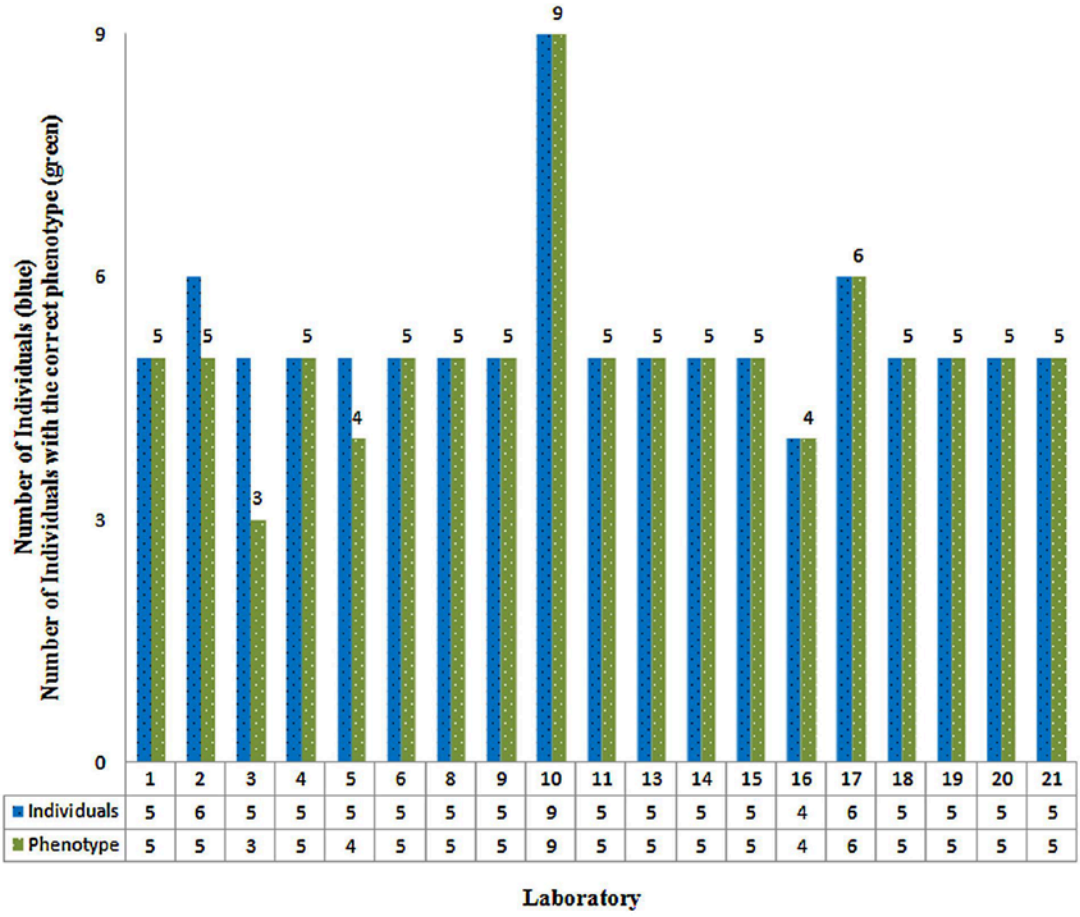


Fig. 6. Accuracy of the IrisPlex-based eye colour phenotype prediction of the samples selected by all 19 laboratories participating in Task 3 (Lab #7 did not participate in this task). Lab #12 though performed the task, did not include images and hence was not considered here. The correct eye colour phenotype was assessed by the host laboratory from inspection of the eye images provided, and compared with the eye colour phenotype reported by the participants based on IrisPlex analysis. Blue indicates the number of individuals that were genotyped and green indicates the number of individuals for which the correct phenotype was reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

blue and/or brown eyes is comparatively higher than intermediate in the population, which could explain the rarity of volunteers with intermediate eye colour phenotype used in Task 3.

The overall performances of the participating laboratories in all 3 tasks, is shown in Supplementary Table 4.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.04.006.

4. Conclusions

Overall, the high level of consistency achieved throughout this collaborative effort in all 3 tasks illustrates the reliability of the IrisPlex assay in producing highly accurate 6-SNP genotypes and of the IrisPlex prediction model in producing accurate blue and brown eye colour phenotypes from IrisPlex genotypes. As shown here and previously [8], the IrisPlex assay provides reproducible results despite differing levels of experience of the laboratory personnel involved and differing DNA extraction and quantification methods used. The results obtained in this collaborative exercise demonstrate the robustness and reproducibility of DNA-based eye colour prediction when using the IrisPlex system in different forensic laboratories world-wide. As emphasised before [8–10], future focus shall be placed on improving DNA-based prediction of non-blue and non-brown eye colours, for which the IrisPlex system is less suitable than for blue and brown eye colour prediction from DNA.

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CHAPTER 8

Further Applications, Conclusions & Impact

Further Applications, Conclusions & Impact

8.1 Preface

The final chapter of this thesis discusses the rippling effect this research has had within the forensic science community, and the new directions for direct PCR research heading into the future. Given the number of variables a crime scene may produce, as far as the substrates and surfaces DNA may be found on, there are still many more applications of this technique to explore. *Section 8.2* describes these possibilities in depth, highlighting research already trialled and research currently underway. Specifically this section describes: the possibilities of using direct PCR with human teeth and bones, showcasing the preliminary results obtained during this candidature from human teeth samples; discussing the research projects that have been undertaken as a result of this candidature within the Flinders DNA Laboratory; and exploring the world wide use of this technique in other forensic areas such as explosive devices, and wildlife crime.

8.2 Further applications

As mentioned previously, there are many variables to explore using direct PCR, too many to research sufficiently in one candidature. Given the data from this thesis, it is obvious that certain substrates perform better than others due to physical properties and the presence of inhibitors. Keratin-based substrates such as human and animal hair, and human fingernails all consistently provided meaningful data. Keratin does not break down completely during direct PCR; hair and nail samples remain visibly intact after amplification, suggesting that known inhibitors such as melanin are not released into the PCR matrix. This is supported by the data as little to no inhibition is observed. Fibres however, are more difficult to navigate with this technique. There are endless blends and compositions of fibre types, synthetic or natural, as well as dyes. Fibre types and dyes can affect the release of the DNA from the fabric into the PCR matrix, as well as causing complete inhibition of amplification. Fibre variables are too high for direct PCR to produce consistent results when using samples such as clothing. The aim of using direct PCR is to maximise results by utilising all available DNA, and minimise costs by reducing the time spent processing samples for which there is little chance of generating a DNA profile. The data from this thesis can help determine what substrates are going to have beneficial outcomes, and what substrates are unsuitable for implementation.

8.2.1 Human teeth & bones

It is no secret that human bone and teeth are some of the most challenging substrates for DNA extraction and purification. The process is expensive, long and arduous, often requiring specific equipment or even specialised laboratories. The DNA from these samples is often highly degraded, commonly due to environmental exposure, with the composition of bone and teeth creating further challenges [1-4]. The samples must be decalcified in order to remove PCR inhibitors, and release the DNA from the internal matrices. There has been no shortage of research to simplify the process, to minimise the use of specialised and expensive equipment, minimise the use of toxic chemicals, reduce the risks of contamination, as well as maximising the quantity and quality of DNA obtained [5-14].

The successful direct amplification of these substrates would obviously drastically reduce time and costs involved, as well as reduce contamination opportunities, as there are fewer steps in which exogenous DNA could be unintentionally introduced. Similarly to all the substrates tested in this thesis, any DNA on the surface of a substrate would be released into the PCR matrix during the amplification process. Several initial direct PCR amplifications were conducted on human teeth samples during this candidature to assess if this was a viable technique for this substrate type.

Dr Laurence J Henbest of Perfect Smile in Adelaide, South Australia, donated human teeth samples, providing consent for the samples to be used for human identification for forensic science research purposes only. Samples were stored individually in Milton Antibacterial Solution: Hospital Grade (MSDS: 1 % NaOCl - bleach), and then rinsed in ethanol followed by a sterile water wash. Samples were left to dry before processing. The outer layer of the tooth was scraped and cleaned with a disposable scalpel to remove any residual bleach that may cause inhibition, followed by another sterile water wash. To fragment the tooth sample small enough to amplify directly, the tooth was wrapped in Kimwipes™ (Kimberley-Clark®) and then placed in a sterile ziplock bag. This package containing the tooth was then crushed in a clean vice. The crushed tooth was then emptied into a sterile plastic container.

The pulp of the tooth was removed and placed in a 1.5 mL centrifuge tube for extraction, to enable DNA comparison to any tooth fragments. The pulp was extracted using the QIAamp® DNA Micro Kit (Qiagen) following the manufacturer's protocol for tissue extraction. The sample was incubated overnight and eluted in a final volume of 50 µL. The DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies).

Direct PCR was conducted by placing powdered tooth sample into a 0.2 mL thin walled tube containing 10 µL of PCR master mix from the AmpFℓSTR® NGM™ kit (Life Technologies, Victoria, Australia) along with 5 µL of the primer mix and 1 µL (5 units) of AmpliTaq Gold® DNA polymerase (Figure 8.1). The addition of the AmpliTaq Gold® DNA polymerase is to increase the overall units of enzyme in the reaction to assist in overcoming inhibitors that may be present on the tooth. A further 9 µL of sterile H_2O were added to make the final volume 25 µL. The amplification was conducted in a GeneAmp® System 9600 thermal cycler (Life Technologies) using the manufacturer's recommended conditions. During PCR, 29 cycles was used for all reactions. The NGM™ kit amplifies 15 STR loci plus the amelogenin locus.

Capillary electrophoresis was performed on an ABI 3130*xl* Genetic Analyser (Life Technologies) using POP-4™ polymer (Applied Biosystems). An aliquot of 1 µL of the PCR sample was added to a solution of 0.5 µL of ABI GeneScan-600 LIZ® Size Standard and 9.5 µL of Hi-Di™ Formamide. Samples were then denatured at 95 °C for 3 min. Electrophoresis was conducted at 3 kV with a 10 s injection. The data were analysed using GeneMapper® v3.2. The detection threshold was set at 50 relative fluorescence units (RFU).

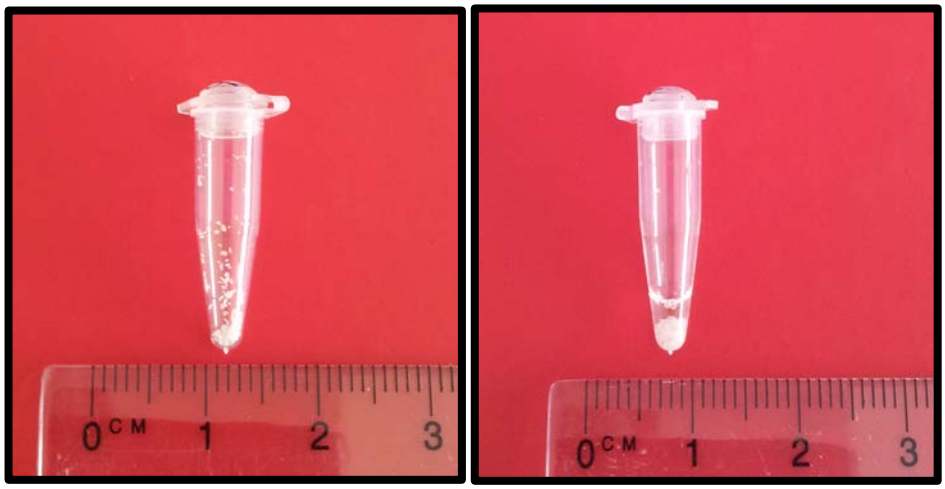

Figure 8.1 – Powdered human tooth sample in 0.2 mL thin walled tube. Left frame shows tooth powder prior to the addition of the PCR master mix, right frame shows the addition of 25 µL of NGM™ kit reagents after brief centrifugation.

Tooth samples were trialled from 13 individuals, targeting different areas of the tooth (such as the root dentine, and the crown enamel) with varying success. The results indicated that tooth itself is not a PCR inhibitor, as DNA profiles could be obtained when directly amplified. Full profiles could be obtained from tooth powder (Figure 8.2), that matched the corresponding tooths pulp DNA profile. Eight samples returned DNA data ranging from just a few alleles to full profiles. Five of these samples returned profiles of five complete loci or more.

Most tooth samples showed signs of over amplification; with high RFU values and the occasional split peaks. Clearly there is opportunity to optimise the methods, and determine the best section of tooth for direct amplification. Due to limitations in time and equipment, tooth as a substrate for direct amplification was not researched further in this candidature. Gaining STR information from forensic samples provides the highest discrimination power in terms of DNA analysis. Teeth and bone samples are often analysed for mitochondrial DNA or SNP information due to degradation factors that make STR information difficult to obtain from these substrates. STR information was successfully generated from teeth samples in this preliminary work when amplified directly. With new forensic SNPs being researched and implemented, teeth and bone samples would make an ideal substrate for direct amplification using SNPs as they amplify smaller fragments than STRs. Further investigation into human teeth and human bone samples using STR and SNP analysis would undoubtedly be a significant research project on its own.

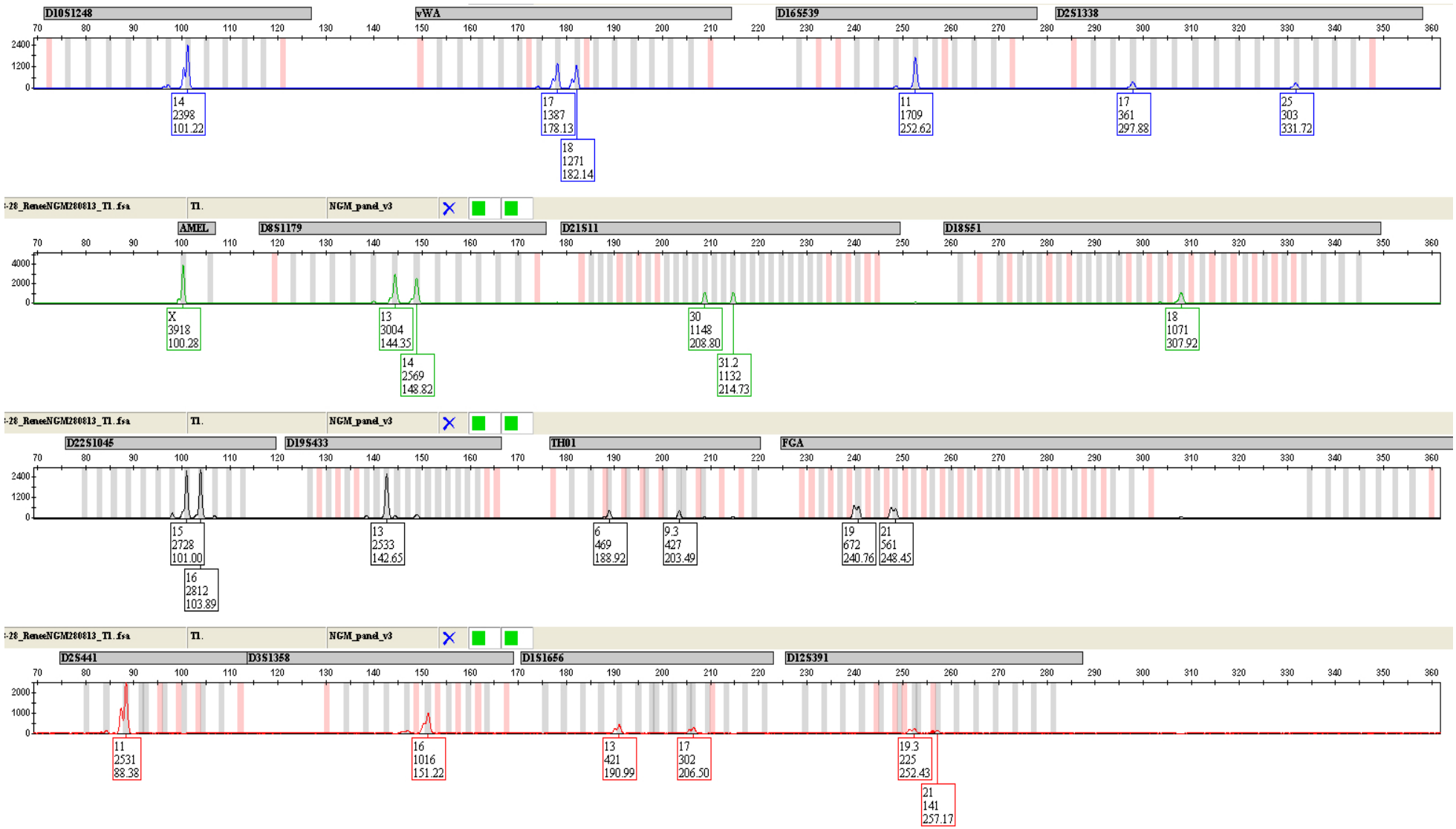

Figure 8.2 – Chromatogram of nuclear DNA from tooth powder amplified directly using AmpFℓSTR® NGM™ kit at 29 cycles on a GeneAmp® System 9600 thermal cycler. Sample was injected on an Applied Biosystems 3130*xl* Genetic Analyser at 3 kV for 10 s.

8.2.2 Flinders DNA Laboratory projects

Early on into this candidature, it was clear that the spectrum of substrates to research using direct PCR was too large to properly investigate in one research project. The second direct PCR candidature, undertaken by Jennifer Templeton, focuses on the use of swabs with touch DNA. Swab fibres are amplified directly after swabbing an area of interest. Unlike fibres from clothing and textiles, variables can be kept constant when using forensic swabs. Preliminary data showed nylon FLOQswabs™ to give the best DNA profile results when compared to various other swab types, such as cotton and foam. Using control DNA deposited onto brass, plastic and glass, swabbing with FLOQswabs™ and amplifying the swab fibres directly was shown to improve DNA recovery when compared to standard extraction processes [15]. Following the results of this experiment, a mock case study was developed to determine if DNA from fingermarks could be obtained from various substrates using direct PCR [16]. The method summary was published, reporting the generation of interpretable profiles from 71 % of 170 fingermarks [17]. The method is rapid, allowing for a reduction in associated costs, and eliminates the need to increase PCR cycle number. The technique also provides a new tool to obtain DNA from fingermarks, where previously fingermarks may have only been submitted for fingerprint analysis.

Fingermarks or touch DNA present at crime scenes can be challenging for collection as their location is mostly assumed, meaning DNA is not always present at the areas targeted for swabbing. The flow on effect is that many touch DNA samples submitted for analysis contain little or no DNA. The candidature undertaken by Alicia Haines investigates the *in situ* detection of latent DNA using DNA-binding dyes [18]. The fluorescence of the dyes where DNA is present can be observed easily using a Polilight®, allowing for a more targeted approach in the collection of touch DNA. With the right concentration of dye, ridge detail within a fingermark can also be observed allowing for fingermark comparisons to be made [19]. The dyes investigated have no significant effect on DNA extraction, amplification (direct or standard), and STR typing [20, 21]. Applying this technique to single hairs allows for rapid screening to determine if sufficient DNA is present for successful profiling [22, 23]. Combining this technique with direct PCR has further improved the DNA typing success rate of this difficult sample type [21].

The combination of techniques being developed in these three candidatures is creating powerful new tools for the rapid detection and targeting of trace DNA from difficult substrates with high DNA typing success rates. These techniques all adhere to current manufacturer protocol recommendations, without the increase of amplification cycle number, providing results fast and at a lower cost.

8.2.3 Explosive devices

Explosive devices used in terrorist attacks and bombings present a great challenge in investigations to identify the perpetrator, as the strong blasts often create incredibly small fragments of evidence [24]. Touch DNA is likely to be present on the components of explosive devices, transferred during assembly. The Omagh car bombing of 1998 (*Sean Hoey v. R*) was one of the first cases where LCN was implemented, generating a DNA profile from the wires associated with the bomb. Explosive devices are often made from household items, utilising electrical tapes and wires, batteries, and mobile phones, with containment in items such as PVC pipes, cookware, and backpacks [25-27]. The success rates for DNA STR typing of explosive device components are low [25, 27, 28], with the explosion itself likely to cause DNA degradation [25, 26]. Direct PCR has shown it can successfully amplify DNA from small fragments of fibres, keratin (hair and nails), and plastic (tape lifting), improving success rates in all cases. A recent study implemented direct PCR on simulated explosive device components, electrical tape and copper wire, as part of a larger study to determine the most efficient collection methods with explosive devices [29]. The sample size is low with only ten electrical tape samples, and ten copper wire samples amplified directly, yielding DNA recovery percentages of 20 – 35 %. The success rates are likely to increase if direct swabbing techniques [15], or a Triton™ X buffer soak (amplified directly) [16], are also implemented.

8.2.4 Wildlife crime

The illegal wildlife trade has a devastating and cascading effect on many animals, plants and their natural habitats. The UN estimates that 500 million shipping containers travel the world each year, and that less than 2 % are physically inspected [30]. Much of these containers are originating from under-developed countries and arriving at under-monitored ports, making it extremely difficult to determine the scale of illegal wildlife trade [31]; however, the wildlife forensic community estimates the trade at $20 billion per year [32, 33]. The discipline is greatly underfunded and is not prioritised in most forensic laboratories [33, 34].

Direct PCR is now becoming a popular tool to help combat illegal wildlife trade, as it is fast, cost effective, only requires a minute sample size, and is effective with trace DNA samples. Direct PCR of keratin substrates has been greatly successful throughout this candidature. Keratin substrates are also common in the animal kingdom, making up a large portion of illegal wildlife trade. Alpha keratins can be found in hair (wool), horns, nails and claws of mammals. Harder beta keratins are found in: the nails, scales and claws of reptiles; the shells of turtles and tortoises; the feathers, beaks and claws of birds; and the quills of porcupines, to name a few.

Mammalian samples of bone, ivory, horn, feces, urine, dried skin, 30-year old hair, muscle tissue, and antler have all successfully been amplified using direct PCR [35-37]. Direct PCR can also aid with species identification by enabling DNA to be obtained from the smallest of museum samples, samples that typically need to remain intact [38]. Traditional extraction methods would require too large of a sample in most cases, causing damage to the original item. Since the beginning of this candidature, other science disciplines have also begun to utilise direct PCR. DNA has been successfully amplified directly from leaf and stem tissues from fibre crops [39, 40], woody plants [41], and insect skins [42]. These substrates are all relevant to wildlife crime analysis.

Furthermore, animal SNP markers are becoming an increasing focus in the wildlife forensic field to help assist in species identification and the amplification of degraded DNA samples [34]. As previously shown, using direct SNP methods are successful on difficult keratin samples and would be another means of fast and cost effective identification within this field.

8.3 Final statement

The research contained within this thesis has undoubtedly highlighted the huge advantage direct PCR techniques has had within the forensic science community and beyond. The published data has facilitated new projects, not only within our own laboratory, but nationally and worldwide [15, 21, 34-45]. The stepping-stones for multidisciplinary collaboration have been paved, with the combination of techniques providing new and powerful methods for data collection and analysis. The ultimate goal of any forensic research is to improve methods so as to gain more valuable information during investigations. Not only has direct PCR enabled better DNA recovery for difficult substrates, the process is also faster and cheaper than current standard methodologies. As the technique does not require validated protocols to be adjusted, it can be introduced with ease, with many laboratories now implementing and researching direct PCR techniques for casework purposes [46-54].

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