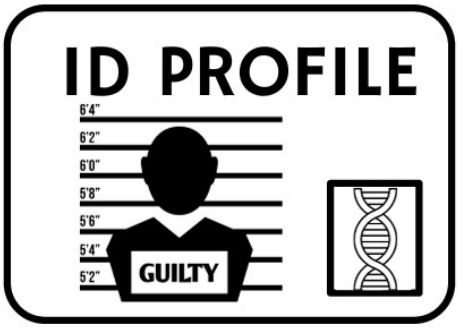

Development and validation of a protocol to analyse in STAR Q Punch reference samples collected with a swab

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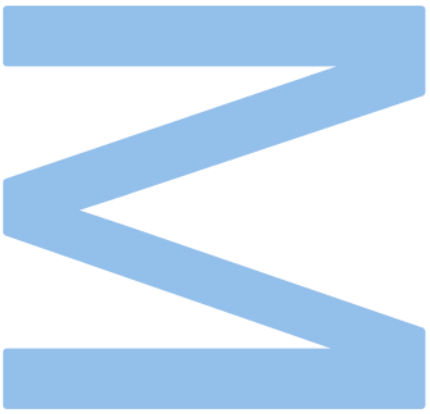

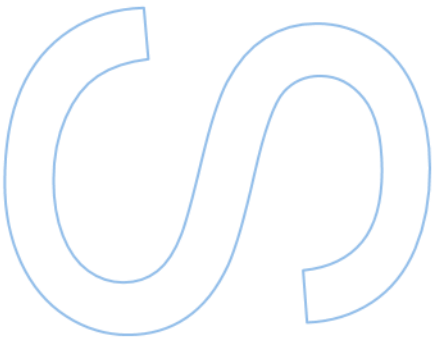

Dedicated to my grandfather,

Vitorino Fonseca

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Resumo

Numa investigação criminal, a comparação de vestígios recolhidos numa cena de crime com as amostras referência é parte fundamental do processo. Estas correspondem por norma a amostras de células bucais recolhidas através de zaragatoas e permitem que seja estabelecida a ligação de um suspeito a um crime.

Neste momento e a nível nacional, são utilizados dois tipos de zaragatoas: maioritariamente as de aspeto serrilhado, com material absorvente, [OmniSwab (Whatman, GE HealthCare Technologies Inc, Reino Unido)] e as de algodão. Após a sua chegada ao laboratório, estas são cortadas e processadas com o auxílio do equipamento QiAgility (QIAGEN, Alemanha) que pipeta o reagente SwabSolution (Promega Corporation, EUA), permitindo que o ADN seja eluído e a sua posterior análise seja feita.

O laboratório adquiriu um equipamento STAR Q Punch AS (QIAGEN, Alemanha), que permite o processamento das amostras referência de uma forma mais automatizada e a preparação da placa PCR para a sua amplificação. No entanto, o equipamento apenas processa amostras em cartões FTA que podem ser obtidas a partir da sua recolha com o EasiCollect (GE HealthCare Technologies Inc, Reino Unido) em que a amostra fica num cartão FTA. Assim sendo, é necessário implementar este novo método de recolha pelo país para se poder começar a utilizar este equipamento devido a todas as vantagens que este oferece.

Com a implementação do novo método, haverá um período de tempo em que irão chegar ao laboratório os 3 tipos de recolha, porém, apenas os cartões podem ser utilizados no STAR Q Punch AS. Uma vez que não se pretende manter duas linhas de trabalho para o mesmo tipo de amostra e para evitar o *backlog* das amostras referência, o objetivo deste trabalho é que seja implementada a passagem das amostras em zaragatoas de algodão e de aspeto serrilhado para novos cartões FTA.

Neste estudo, foram efetuadas recolhas de amostras de saliva a um total de 12 voluntários com os 2 diferentes suportes disponíveis. Após a sua secagem, foram testados 2 tampões diferentes e água [SwabSolution kit (Promega Corporation, EUA), TE (Illinois Tool Works, EUA) e água esterilizada desionizada (diH_2O)] para voltar a humedecer as zaragatoas de algodão e serrilhadas e, esfregando num cartão FTA novo,

passar a amostra para este. Foram ainda recolhidas amostras coletadas com o EasiCollect que serviram como cartões controlo para a análise posterior.

Na amplificação, utilizaram-se dois kits: o Investigator 24plex GO! (QIAGEN, Germany) e o GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, EUA) em que os parâmetros variáveis foram: o volume da *master mix* utilizada (volume recomendado pelo fabricante e metade desse volume) e o número de ciclos da reação de PCR.

A combinação que apresentou melhor resultados com o kit da QIAGEN, o Investigator 24plex GO!, foi o volume total de reagentes com 26 ciclos para a reação de PCR. Foi possível obter perfis reportáveis com os três tampões testados, sendo que as amostras mais consistentes foram as que provieram das zaragatoas serrilhadas.

Relativamente ao kit da Applied Biosystems, o GlobalFiler Express, os resultados foram inconclusivos, uma vez que não se conseguiu obter perfis reportáveis de modo consistente nas corridas (de eletroforese capilar) realizadas.

Palavras-chave: [Genética Forense, ADN, amostras referência, PCR]

Abstract

In the realm of criminal investigations, the comparative analysis of traces gathered from a crime scene against reference samples stands as a pivotal component of the investigative process. These reference samples predominantly comprise buccal cell samples obtained through the utilization of swabs, thereby enabling the establishment of a connection between a suspect and the commission of a crime.

Currently, at the national level, two distinct types of swabs find prevalent use in this endeavour: the majority are OmniSwab (Whatman, GE HealthCare Technologies Inc, United Kingdom) – brush type with absorbent material –, and the rest cotton swabs. Upon their arrival at the forensic laboratory, these swabs are cut and processed through the QiAgility (QIAGEN, Germany). This specialized instrumentation meticulously pipettes the SwabSolution reagent (Promega Corporation, USA), thereby facilitating the elution of DNA from the swabs. Subsequently, this eluted genetic material is subjected to analytical scrutiny.

The laboratory has acquired a STAR Q Punch AS (QIAGEN, Germany), that allows the automated processing of reference samples and the preparation of PCR plates for subsequent amplification. This equipment exclusively processes samples contained on FTA cards, which can be collected with the EasiCollect device (GE HealthCare Technologies Inc, UK). With this method, the sample remains securely affixed to an FTA card. Consequently, in order to fully harness the myriad advantages presented by this advanced equipment, it becomes imperative to institute the adoption of this new collection methodology nationwide.

The introduction of the new methodology entails a transitional phase during which all three types of sample collection will be received at the laboratory. However, only the cards can be effectively processed through the STAR Q Punch AS equipment. In the interest of avoiding two parallel workflows for identical sample types and preventing the reference samples backlog, the primary objective of this study is to facilitate the transfer of samples originally collected on cotton swabs and OmniSwabs onto new FTA cards.

In the context of this study, samples were collected from 12 consenting individuals, utilizing the two distinct available support mediums. Subsequently, following the drying phase, an investigation was conducted to evaluate the efficacy of two different buffers and water, namely the SwabSolution kit (Promega Corporation, USA), TE solution (Illinois Tool Works, USA), and sterile deionized water (diH_2O), for the purpose of

rehydrating both cotton swabs and OmniSwabs. This rehydration process was undertaken to facilitate the transfer of the collected samples onto new FTA cards through gentle rubbing. Samples acquired via the EasiCollect device were also employed as control cards, serving as a reference for subsequent analytical assessments in this study.

Two distinct amplification kits were employed: the Investigator 24plex GO! kit (QIAGEN, Germany) and the GlobalFiler Express kit (Applied Biosystems, ThermoFisher Scientific, USA). The experimental variables under consideration encompassed the volume of the master mix used, which included both the manufacturer-recommended volume and half of that volume, as well as the number of cycles employed in the PCR reaction.

In the case of the QIAGEN kit, the most promising outcome was achieved when utilizing the full volume of reagents in conjunction with 26 cycles for the PCR reaction. This particular combination yielded consistently reportable profiles. Notably, these favorable results were obtained across all three tested buffers, with the most reliable samples originating from the OmniSwabs.

Conversely, in the context of the Applied Biosystems kit, the outcomes proved inconclusive. Regrettably, the study did not yield consistently reportable profiles in the capillary electrophoresis runs conducted with this particular kit.

Keywords: [Forensic Genetics, DNA, reference samples, PCR]

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List of Abbreviations

ALS	ALTERNATE LIGHT SOURCES
AT	ANALYTICAL THRESHOLD
CE	CAPILLARY ELECTROPHORESIS
CODIS	COMBINED DNA INDEX SYSTEM
CT	CYCLE THRESHOLD
DNA	DEOXYRIBONUCLEIC ACID
DTT	DITHIOTHREITOL
dNTPS	DEOXYNUCLEOTIDE TRIPHOSPHATES
ESS	EUROPEAN STANDARD SET
EPG	ELECTROPHEROGRAM
HGP	HUMAN GENOME PROJECT
IPC	INTERNAL PCR CONTROL
KM	KASTLE-MEYER
LPC	SCIENTIFIC POLICE LABORATORY
NIJ	NATIONAL INSTITUTE OF JUSTICE
NTC	NO-TEMPLATE CONTROL
PCR	POLIMERASE CHAIN REACTION
PSA	PROSTATE-SPECIFIC ANTIGEN
PK	PROTEINASE K
PTC	POSITIVE TEMPLATE CONTROL
RFLP	RESTRICTION FRAGMENT LENGTH POLYMORPHISM
RFUs	RELATIVE FLUORESCENT UNITS
SDS	SODIUM DODECYL SULFATE
SNPs	SINGLE NUCLEOTIDE POLYMORPHISMS
SPA	SEMINAL ACID PHOSPHATASE
SPE	SOLID-PHASE EXTRACTION
STR	SHORT TANDEM REPEATS
VNTRs	VARIABLE NUMBER TANDEM REPEATS

1. Introduction

1.1. History of Forensic Genetics

Forensic Genetics is defined as “the application of genetics to human and non-human material (in the sense of a science with the purpose of studying inherited characteristics for the analysis of inter- and intra-specific variations in populations) for the resolution of legal conflicts” by the *Forensic Science International: Genetics journal* [1]. Its main purpose is to link a suspect to a crime, resorting to the employment of scientific techniques for evidential analysis. The main tool is the use of deoxyribonucleic acid (DNA) from human biological traces such as those obtained from crime scenes, victims, suspects, and convicted offenders. Saliva, blood, semen, and hair are the most common types of samples due to their effectiveness in creating a DNA profile.

In 1900, Karl Landsteiner classified human blood into four different categories (A, B, AB, and O) describing the ABO blood system group [3]. This became the first genetic tool used in forensics with the purpose of establishing paternity. Additionally, the inclusion of alternative blood group markers and soluble blood serum protein markers allowed for the exclusion of individuals whose profiles did not correspond. Nevertheless, these techniques had significant limitations and were not very informative [2-4].

In 1985, Alec Jeffreys discovered that particular regions of DNA consisted of short tandem-repetitive sequences – minisatellites [5] – and that its number of copies differed between individuals. Based on this finding, he developed a technique, later called Restriction Fragment Length Polymorphism (RFLP), which allowed him to create human identity tests. He performed DNA digestion using a restriction enzyme, separated the fragments using agarose electrophoresis, transferred them to a nitrocellulose membrane, and conducted hybridization with complementary probes. The length polymorphism observed in repetitive DNA regions from different sources allowed for specific individual identification. He became the first geneticist to describe “DNA fingerprinting”, currently known as DNA typing [6]. This method was first applied in an immigration case in the same year and two years later in a criminal case [7, 8].

Techniques employing RFLP analysis encountered certain drawbacks concerning DNA quality and quantity, as well as challenges in reliably comparing genetic profiles, which lead to a gradual replacement by methods based on the Polymerase Chain Reaction (PCR) [9]. Since then, DNA-based applications in forensic science have come a long way and evolved into new and improved technologies.

Nowadays, the method of choice for forensic DNA profiling are multiplex systems based on Short Tandem Repeats (STRs). STR typing conveys the analysis of a panel of multi-allelic STR markers that are shorter than the original minisatellites and therefore easier to amplify [10]. Commercial kits available include the STR markers requested by the criminal databases: the European Standard Set (ESS) of loci [11] and the US Combined DNA Index System (CODIS) [12] increasing the discrimination power of this DNA typing technique.

1.2. DNA Structure and Organization

DNA was described in 1953 by Watson and Crick as a double-stranded molecule [13]. It is located in the nucleus and mitochondria of cells in eukaryotes holding the coding data for cell replication and protein synthesis as well as the information required for future generations to inherit genetic traits.

This molecule is formed by nucleotide units comprising a nucleobase, a deoxyribose sugar, and a triphosphate group. The last two form the backbone structure of DNA and the nucleobase conveys the variation in each nucleotide unit, resulting in the four bases: adenine (A), thymine (T), cytosine (C), and guanine (G). These pair up with their complementary base – adenine and thymine are connected by two hydrogen bonds and cytosine and guanine are linked by three [14]. This makes the two DNA strands to be in an ‘anti-parallel’ helical shape, which means one of the strands is oriented 5’ to 3’ and the other one in the opposite direction [15]. The diversity among humans is attributed to the presence of nearly three billion base pairs that give rise to countless possibilities of nucleotide sequences.

In humans, nuclear DNA consists of 23 pairs of chromosomes (22 autosomal matched pairs and 1 sex-determining pair), each chromosome having been inherited from each individual’s parent. Notwithstanding, owing to the interchanging of genetic material between chromosomes during meiotic crossing-over, they do not contain identical genetic information. The genome corresponds to the entire DNA in a cell. The Human Genome Project (HGP) focused on studying the human genome, having sequenced 99% of euchromatic DNA [16]. The researchers involved also realized that the number of protein-coding genes was lower than expected [17]. While the entire human genome is vast, it is interesting to point out that more than 98% of it consists of nonprotein coding DNA [18]. Although these regions were once considered to be "junk DNA" with no functional purpose, scientific research has shown that noncoding DNA can include

regulatory elements, such as promoters and enhancers, which control the activity of genes. While the targeted regions do not allow for phenotypic inference, i.e., information about the individual's appearance, it is important to note that the designation of these regions was primarily driven by the presence of appropriate DNA sections, with ethical concerns also being taken into account [19, 20].

1.3. Types of genetic polymorphisms

Only approximately 0.3% of our DNA is variable, and nonetheless, it allows for two individuals to be different and unique, with the exception of identical twins. The two main variability sources are genetic recombination and mutations. Genetic recombination, also called genetic reshuffling, occurs during meiosis and leads to the production of an offspring with a set of genetic information that differs from those found in either parent. Mutations comprise any change in the DNA nucleotide sequence and these usually lead to the generation of new alleles [21].

Having several types of variability is valuable for forensic matters such as kinship and human identification, allowing to determine whether there is a match between two samples or not. Tandem repeats are repeated DNA sequences. These repeats can be categorized based on the length of their repeating unit. Specifically, microsatellites or short tandem repeats have repeating units that are typically less than 10 base pairs in length, although some studies consider it can be as short as 2-6 base pairs. On the other hand, minisatellites or Variable Number Tandem Repeats (VNTRs) have repeating units ranging from 10 to 100 base pairs. Lastly, satellite DNA consists of tandem repeats with repeating units longer than 100 base pairs. [22].

1.3.1. VNTRS

Variable Number Tandem Repeats, or minisatellites, are length polymorphisms and were the first to be used in DNA profiling and in forensic casework [23]. These are usually located in subtelomeric regions of chromosomes with core repeat sequences ranging from 6bp to 100bp, making some alleles achieving a length as long as 30kb [2, 24]. New alleles can arise due to recombination events in the germline as a consequence of interallelic conversion and intra-allelic rearrangements. Occasionally, they can appear derived from the crossover pathway. In somatic cells, new allele generation is less

frequent than in the germline and can originate from intra-allelic duplications and deletions [24, 25].

Nonetheless, the use of minisatellites was limited by the high quantity of DNA required for a successful analysis and by the difficulty in the results interpretation. They have now been replaced by STRs.

1.3.2. STRs

Short Tandem Repeats, or microsatellites, are also length polymorphisms but smaller than VNTRs. They have a core sequence repeat with less than 10bp, being tetranucleotide repeats (4bp), the most common in forensic genetics. The number of repetitions of the core sequence repeat allows us to distinguish between two individuals: STR alleles can have between 50bp and 500bp [26].

Microsatellites showcased to be more sensitive and advantageous, satisfying many requirements, thereby becoming the forensic marker of choice: (a) they are dispersed in the genome, present both in the 22 somatic chromosomes and in the XY sex-determining chromosomes and, particularly, in non-coding regions [27]; (b) allow high throughput via multiplex (amplification of various loci in the same reaction) and consequently are more discriminatory [28, 29]; (c) high mutation rate of about 10^{-3} and 10^{-4}; (d) high intrapopulational diversity due to the numerous allelic possibilities; (e) low interpopulational diversity which allows for similar allelic frequencies; (f) simple interpretation of the profiles obtained due to the discrete alleles; (g) unlike VNTRs, STRs analysis is suitable for degraded and limited amount of DNA [30].

The increasing use of commercial STR commercial kits capable of multiplex amplification came to improve the DNA typing process and helped establish consistency and reproducibility across laboratories [31, 32].

1.3.3. SNPs

Single Nucleotide Polymorphisms (SNPs) are single base variation sequences between individuals which arise from mutations occurring from the DNA replication stage of the meiosis. They are extremely abundant in the human genome, wherein the less common allele has a frequency of 1% or greater [33, 34], and therefore it is theoretically possible to type hundreds of them and increase its discrimination power. PCR products of SNPs

can have less than 100bp, enabling higher success rates with degraded DNA samples whereas STR amplicons are usually between 300 and 400bp [35].

The majority of the SNPs are bi-allelic, and consequently only three different genotypes are possible. Therefore, these types of markers are far less informative than STRs which means it is necessary to use many more SNPs to withstand the same discrimination power of the STRs [36]. However, SNPs have found applicability in different areas of forensic science such as kinship analysis [37, 38], geographic ancestry and phenotypic traits prediction [39], due to the lower mutation rate of 10^{-8} but also due to other characteristics (as mentioned above) such as amplification of small size multiplexes which are very useful for degraded samples.

1.3.4. Indels

The second most common polymorphism are indels, characterized by the insertion or deletion of DNA segments of one or more nucleotides [40]. Most of indels are bi-allelic, though it is also possible to find multiallelic. STRs have been considered one of these since its variation is based on the insertion or deletion of tandem repeats [41], despite this is not a common classification of STRs.

Although the mutation rate of indels is lower than STRs and therefore less polymorphic, these have also been implemented in genetic populational studies before [42]. The small indels are also easily genotyped and allow for its analysis in short amplicons, becoming advantageous when dealing with degraded DNA in forensics [43]. This led to the need for its processing to be equally streamlined and automated [44].

The most frequent variation observed between alleles involves small nucleotide differences; nevertheless, bi-allelic indels, which are length polymorphisms, can occasionally occur due to the inversion of a retroposon like Alu.

1.4. Processing forensic samples at the Portuguese Scientific Police Laboratory (LPC)

The following scheme (Figure 1) is a general representation of the routine workflow of the Portuguese Scientific Police Laboratory (LPC). The major processes here represented are further described in detail in the sections below.

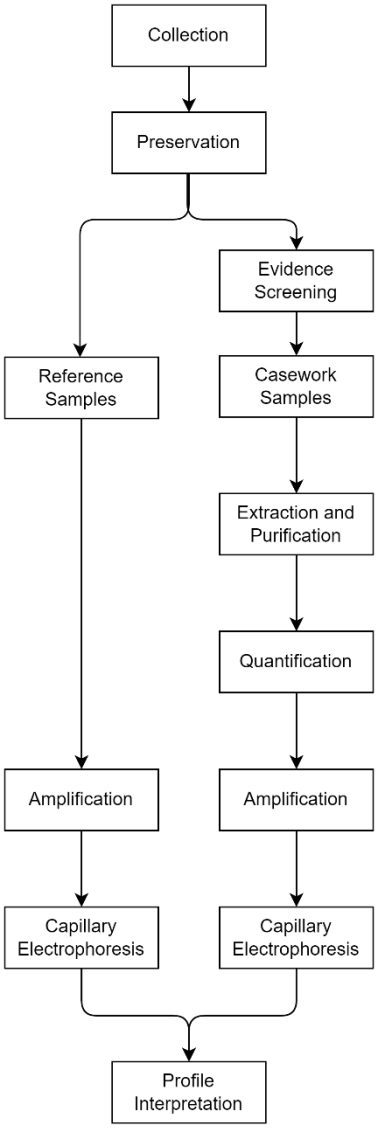

Figure 1 - Illustrative scheme of the biology sector of the Portuguese Scientific Police Laboratory (LPC) workflow.

1.4.1. Collection

It is possible to recover DNA from almost every crime scene due to its presence in every nucleated cell and therefore in every biological material. A diversity of evidence can be collected such as cigarette butts, clothing, shoes, weapons, gloves, etc. The biological materials usually analysed in forensic laboratories are blood, semen, rooted hair and epithelial cells. Reference samples from the victims and suspects should also be collected when possible.

With the PCR discovery in 1985 by Kary Mullis [45], it is possible to obtain a genetic profile from small DNA quantities due to the sensitive and multiplexing capabilities of the amplification process hence the ability to analyse a wide variety of samples in short time.

It is imperative that all materials retrieved from a crime scene are meticulously collected with a specific degree of precision, aimed at minimizing the inclusion of redundant evidence during their transport to the laboratory, preserved, stored and transported for posterior analysis. The collection and handling of the material should be carried out with extreme cautiousness to prevent any kind of contamination. A chain of custody is established in order to produce genetic profiles with significance in court [2, 6].

1.4.2. Preservation

When evidences enter the biology sector of the LPC the first step is to preserve it. The expert confirms whether all the material mentioned in the official letter has arrived and is properly stored or not, and classifies it according to the type of evidence and the laboratory's norms. Reference samples are then separated from the rest of the material since analyses are performed differently.

1.4.3. Evidence Screening

All of the material to be processed is cautiously screened by specialists in properly equipped laboratories, in which they will collect casework samples taking into account the type of crime and the requests present in the official letter.

Biological stains are not always visible to the naked eye and can raise some doubts. Presumptive tests are performed as a preliminary evaluation prior to sending swabs or cuttings of the material for forward analysis to attest for the presence of biological fluids.

The primary issue associated with these screening tests pertains to the damage caused to the sample. Therefore, it is of utmost importance to examine the small amount of biological evidence using methods that do not destroy it [46].

Alternate light sources (ALS) are a helpful method especially when trying to locate biological stains in bigger pieces, such as blankets, and when the stains are invisible. They detect these through the emission of light in different wavelengths in which the diverse fluids fluoresce [47].

Presumptive tests are ubiquitous, with widespread usage encompassing the following examples: Kastle-Meyer (KM) which relies on the peroxidase-like activity of haemoglobin for blood identification [48]; seminal acid phosphatase (SPA) test which detects the presence of the enzyme by assessing its catalytic capability in hydrolysing organic phosphate compounds; prostate-specific antigen (PSA, also known as p30) test for seminal fluid detection as well [49-52]. With the use of presumptive tests, specialists can effectively streamline and gather suitable casework samples for subsequent DNA analysis.

The only confirmatory test available for detection of semen uses microscopic identification of sperm cells. Two staining methods are suitable for application: the Christmas tree staining procedure which stains the heads red and the tails green; and hematoxylin and eosin which stains purple and pink, respectively.

1.4.4. Reference Samples

To conduct comparative DNA analysis between crime scene samples and potential suspects or victims, it is necessary for the police personnel to collect reference samples from the individuals involved. These samples can either be blood or buccal cells. However, according to Portuguese legislation, sample collection “must be done with a non-invasive method, where the dignity and the individual’s moral and physical integrity is respected” [53], which consequently leads to the preference of buccal cells due to its ease of collection and ability to mitigate health risks. Blood samples can alternatively be collected when justified.

Various types of tools are available for saliva sample collection such as buccal swabs [54], which are the most common and, more recently, Whatman FTA cards, have also become common for this type of body fluid collection. Both procedures require swabbing the inner surfaces of both cheeks to gather buccal cells. However, the treated paper

method entails an additional step of transferring the biological sample from the swab to the designated area on the FTA card. These also need to be left drying before handling. Very rarely, cytobrushes or mouthwash [55] can also be regarded as reference samples, though these are likely to be quite harsh and ineffective, particularly among certain populations like children [56].

FTA is a type of paper (Whatman, GE HealthCare Technologies Inc, United Kingdom) made from cellulose that has been infused with a unique chemical formula. This formula effectively protects DNA from degradation caused by nucleases, oxidation, UV damage, and the growth of bacteria or fungi [57]. As a result, DNA integrity remains preserved even when stored at room temperature on FTA paper, as long as it is kept dry. This allows for longer preservation compared to regular buccal swabs. Some studies have demonstrated that years later, samples remain feasible to analyse with an equivalent level of success [58, 59].

1.4.5. DNA extraction

DNA extraction represents the first step of the STR profiling process. Most of the times, samples collected from crime scenes are of unpredicted nature depicting a challenge for the process. The main goals are to maximize the yield of DNA obtained from this type of samples and to extract sufficient, pure DNA for subsequent analysis.

This process consists in DNA isolation from inside the cells by lysing it – causing the membrane to burst, protein denaturation, and consequent DNA liberation from the denatured proteins and other cell components.

Throughout the years, a variety of extraction techniques have been performed in forensic laboratories such as, for example, organic, Chelex and solid-phase extraction-based procedures.

Phenol-chloroform-based extraction, also known as organic extraction, relies on phenol-chloroform, which is an organic solution capable of denaturing proteins. Since it is toxic and very time-consuming, this method fell into common disuse [60].

Chelex 100 is a chelating resin which is added as a suspension in the form of beads to a sample and boiled allowing the disruption of cell membranes and proteins [61]. It became really popular in forensic science communities because it is fast, low-cost, simple, and, in contrast to the method previously described, does not use organic solvents and diminishes the cross-transfer of samples and contaminations [62].

However, this procedure does not include a purifying step which can result in the presence of PCR inhibitors and possible DNA degradation for long-term storage.

Even though FTA cards were developed with the purpose of collecting and archiving samples as mentioned before, they have been included in the extraction methods. In contact with the treated paper, the cell lysis and the DNA binds to the paper. This method is typically used with blood samples, but over time it is also becoming really useful to store reference samples due to its ability to preserve DNA for a long period of time. Previously, there were some concerns about the dry punched papers static electricity, however, owing to new technologies, such as the STAR Q Punch AS [63] equipment (QIAGEN, Germany) employed in this study, it is possible to overcome this issue. Another advantage of this method is the possibility to proceed direct amplification without a pre-treatment step owing to the characteristics of the FTA paper [64].

Solid-phase extraction (SPE) involves selective binding of the DNA molecules to a solid support matrix which can be silica-based matrices, magnetic beads, amongst others. The choice of the matrix is based on factors such as DNA yield and compatibility with downstream applications. This technique allows the separation between nucleic acids and other cellular components and impurities that can be PCR inhibitory. The chemistry of the DNA extraction kits is also important for the DNA quality and quantity. Solid-phase extraction gained a lot of attention and popularity amongst the forensic science society due to its automation and is the current method utilized in LPC for casework samples. In a laboratory workflow, every sample should be processed to the highest possible standard, especially when working with samples recovered from crime scenes since they have, most of the time, very little template DNA. Automated systems bring a lot of advantages such as the analysis of a large number of samples, a hands-free operation minimizing operator errors, low throughput, reduce cross-contaminations and enhance standardization and reproducibility [65, 66].

Currently, QIAsymphony SP/AS (QIAGEN, Gemany) [67] is implemented for casework samples analysis at the LPC. This requires a manual pre-treatment in which samples are incubated with a lysis buffer that contains a detergent (usually sodium dodecyl sulfate - SDS) and with proteinase K (PK). Moreover, this will destabilize the membrane and cause its disruption, releasing the DNA [2].

As for reference samples, the SwabSolution kit (Promega Corporation, USA) [68] is used for rapid processing of swabs. Due to the samples' characteristics, a step for extraction and purification is not necessary, therefore samples are directly amplified.

Differential extraction is mainly used in sexual assault cases in which female and male mixtures are recurrent. It was first described by Peter Gill and his colleagues in 1985 [69], aiming to separate epithelial cells (female fraction) and sperm cells (male fraction). Firstly, samples are incubated with SDS and PK only leading to the epithelial cell's lysis. Subsequently, SDS, PK and dithiothreitol (DTT) are added and the spermatozoa nucleus breaks-open, releasing the DNA. Epithelial cells are possible to lysate in mild conditions. In contrast, the spermatozoa nucleus is capped by the acrosome which has a large number of disulphide bonds, protecting it. With the addition of DTT, a reducing agent, it becomes possible to break down these bonds.

1.4.6. Quantification

The next step for STR analysis workstream is the quantification of the casework DNA samples. Reference samples are considered optimal samples, which means they come from a well-known source and usually collected in a controlled environment, therefore they do not need to be submitted to this step.

The purpose of DNA quantification is to determine the amount of DNA present in the extracts in order to include the appropriate amount in the PCR reaction aiming to obtain a good quality electropherogram (EPG). Insufficient DNA tends to result in allele loss due to stochastic effects and EPGs with STR imbalance. Too much DNA leads to overblown EPG making its interpretation very difficult, challenging and time consuming. Quantification results will allow the normalization of DNA concentration by dilution or concentration of the extract.

Methods for quantification have also been evolving. Initially, they were not species-selective, meaning every template DNA present (human and non-human) would be quantified, had low sensitivity and specificity and were more laborious.

The current method most employed is real time PCR, also called quantitative PCR, and was first described during the 1990s [70]. It is possible to monitor the amplicons production process in real time, using fluorescence techniques. The emission of fluorescence is proportional to the DNA amount amplified and its signal will generate an amplification curve with different phases (Figure 2): (a) baseline - an initial stage and no product has been formed, so nothing is measured; (b) exponential phase - a lot of the reaction reagents are present and amplicons are being produced, doubling at every cycle; (c) linear phase - the reaction slows down due to the scarce of reagents; (d)

plateau phase - the end of the reaction. The number of cycles required to reach a particular threshold is called cycle threshold (CT) value (Figure 2). The increase of PCR products is related to the initial amount of DNA present in the samples. The less cycles necessary for it to emit fluorescence, the more DNA is present. The final curves are subsequently compared with standard curves.

Besides quantifying, this process also gives information about the quantity of the 3 genomic targets: small autosomal, large autosomal and Y chromosomal portions. The ratio between small and large autosomal indicates the degradation level of the samples. If the proportions are similar and the index close to one, it is a high-quality sample since large autosomal portions would be difficult to remain intact if the sample is degraded. Lastly, it helps to evaluate male and female mixtures by comparing the Y-chromosomal with the autosomal portions.

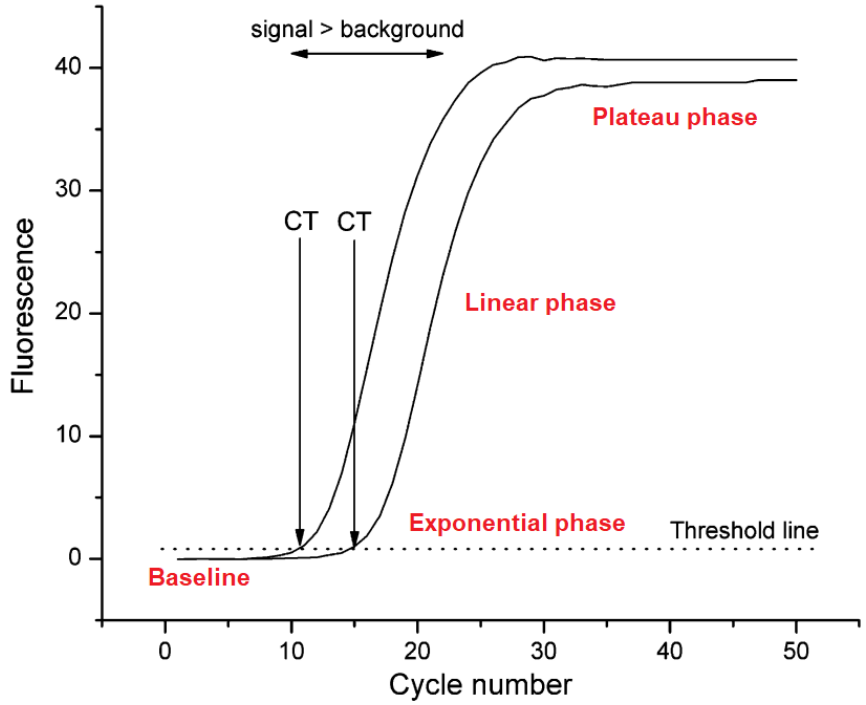

Figure 2 – Real-time PCR phases. (Adapted from M. Kubista et al. [71]). CT= Cycle Threshold.

Commercial kits, like Quantifiler Trio (Thermofisher Scientific, USA), include two controls: 1) the no-template control (NTC) which is a negative control containing the same reagents but without the DNA, and so it is possible to detect primer-dimers and contaminations; 2) The internal PCR control (IPC) that has synthetic template DNA which tests for the presence of inhibitors.

1.4.7. Amplification

The discovery of specific restriction endonucleases paved the way for the isolation of DNA sequences [71]. As mentioned, in 1985, PCR discovery, revolutionized the genetic field by enabling the multiplication of a specific DNA sequence [45]. This process is accomplished through repetitive cycles of DNA denaturation, primer annealing and polymerase extension. It is based on the cell cycle, particularly, on the duplication of the genetic material.

The analysis of casework samples is frequently constrained by limitations in both their quality and quantity; nevertheless, the advent of PCR has empowered their examination, facilitating the analysis and interpretation of such samples despite these challenges. Differing from RFLP methods, PCR is not very limited by the quality of the DNA, and it is rather fast and sensitive.

Every PCR reaction contains (a) template DNA which is copied; (b) DNA polymerase, an enzyme that extends the DNA strand which is thermostable, so it does not fall apart during the denaturation temperature process. The most commonly used is Taq polymerase that is isolated from thermophilic bacteria; (c) primers which are short oligonucleotides designed to match sequences flanking the target region; (d) deoxynucleotide triphosphates (dNTPS) that correspond to the building blocks of DNA molecules with the four nitrogenous bases (adenine, thymine, cytosine and guanine) [72].

Different temperatures rule the different phases of the PCR (Figure 3): (a) the PCR starts with a high-temperature step called DNA denaturation in which the double stranded DNA template separates; (b) the temperature decreases to enable primer annealing and the primers bind to their complementary sites on the denatured DNA strands; (c) an optimal temperature is reached for the DNA polymerase to extend the primers by adding complementary dNTPs and synthesizing new DNA strands. These temperature variations are reached by using a thermal cycler which controls the timing and temperature for each step. A typical PCR reaction consists of 20-40 cycles, doubling the amount of DNA in each cycle.

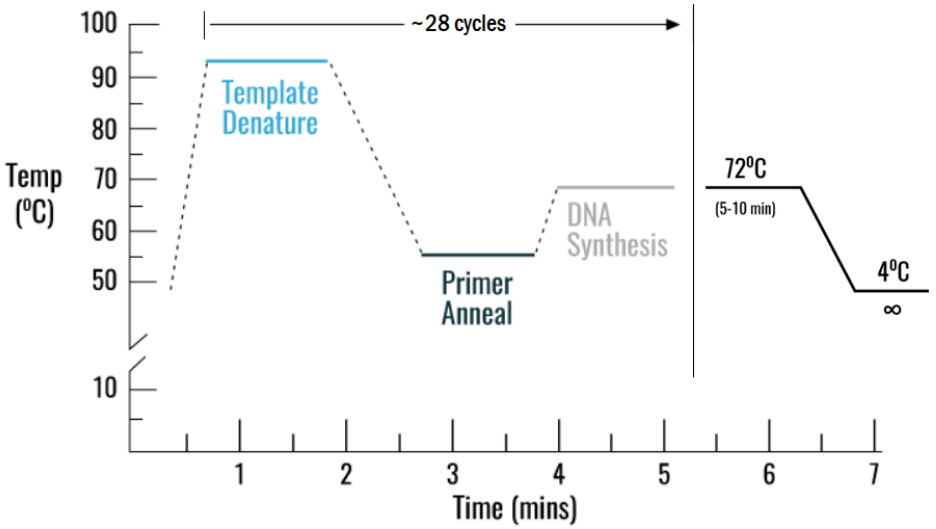

Figure 3 – Polymerase chain reaction profile (Adapted from https://www.bosterbio.com/protocol-and-troubleshooting/pcr-protocol).

PCR multiplexing was developed in order to amplify multiple target sequences simultaneously in a single reaction. In forensic analysis, a set of primers specific to multiple STR loci are included and labelled with distinct fluorescent dyes to simplify the detection and distinction of the amplicons during subsequent analysis [73].

Nowadays, STR multiplex commercial kits [74-76] provide pre-formulated reagents and optimized protocols simplifying PCR workflows and increasing the efficiency and accuracy of DNA amplification. Moreover, these kits often incorporate specialized features, such as master mixes, and quality control measures, further enhancing the reliability and performance of PCR reactions [77]. It is important that pre- and post-PCR procedures are executed in different locations.

1.4.8. Separation and detection

After amplification, it is necessary to separate and detect the twenty or more DNA fragments which will constitute the STR profiles.

Formerly, separation was carried out in polyacrylamide gels and detected with silver-staining. However, nowadays, capillary electrophoresis (CE) replaced gel-based electrophoresis because it is less laborious, without the need to pour gels and load the DNA, offers greater automation and, consequently, an increased number of samples can be analysed, only requiring a small fraction of the sample which can be retested if needed.

PCR products undergo separation based on their size and dye colour through the process of electrophoresis, followed by laser-induced fluorescence detection.

Sample preparation for CE implies diluting a small portion of the PCR product into either water or deionized formamide to denature it and obtain the single-stranded DNA. To ensure complete denaturation, a common practice involves subjecting the sample to rapid heating at 95ºC, followed by immediate cooling.

This type of electrophoresis employs a technique known as electrokinetic injection to introduce charged molecules from the sample into the capillary - a thin glass tube filled with a polymer solution. In the case of DNA, which carries a negative charge, a voltage is applied to facilitate its molecules migration into the capillary. It is possible to detect DNA fragments overlapping in size through a laser light placed at the end of the capillary since they were previously labelled with different dyes. Smaller fragments migrate more quickly, so there is a direct correlation between size and the time taken from sample injection to detection.

In a CE system such as the 3500xL Genetic Analyzer (Applied Biosystems, Thermofisher Scientific, USA) [78] employed at the LPC, standard markers like internal standard and allelic ladder are mandatory.

1.4.9. STR genotyping

Some software programs are available for analysis of the raw data output from the CE. The GeneMapper software (Applied Biosystems, Thermofisher Scientific, USA) is commonly used to process data from the 3500xL Genetic Analyzer. The internal size standard comprising DNA fragments of known size and labelled with a distinct dye is typically co-electrophoresed with each sample to ensure accurate sizing. The resulting data are then analysed using the software that automatically determines the sizes of the STR alleles by comparison with a standard curve generated from the size standard. STR genotyping involves comparing the allele sizes within each sample to the sizes of alleles present in an allelic ladder, which spans all the common alleles of each locus sequenced until now [79]. The STR markers detected are assigned to each respective allele that consists in the number of repeats [80].

The final result is an EPG with every allele detected in a peak form and organized by marker size and by dye colour forming a STR profile; a combination of every loci genotypes. In CE, the data point of an EPG corresponds to the detected signal intensity

at specific time points or migration distances along the capillary. These data points are typically obtained as the separated molecules pass through a detection window of the capillary. Once again, smaller sized fragments are detected first and therefore have smaller data points. The peaks height is positively correlated to the DNA quantity present in the samples and is measured in relative fluorescent units (RFUs) which indicates fluorescence intensity.

1.4.10. Profile Interpretation

Not every peak in an EPG corresponds to an allele. Sometimes these appear as artifacts such as pull-ups, background noise or PCR by-products [81]. Experts must analyse STR profiles carefully by watching out for wrongly assigned alleles and edit them if necessary. To avoid uncertainty and validate the results, these are usually assessed by two analysts separately.

Each laboratory should define limits of detection - an analytical threshold (AT) - in order to differentiate background and analytical fluorescence [82]. However, these must be picked with caution because a high AT can lead to allele loss and false negatives, and a very low one can show too much background noise and artifacts. Another major issue associated with this are the homozygous loci that are wrongly detected when it is deemed that an allele loss (drop-out) happened. Hence, some laboratories have implemented a second threshold named interpretation or stochastic threshold [83]. Whenever a single peak is present and its height is above the threshold, the data is considered reliable and free from stochastic effects and so a homozygous peak.

Due to the challenges associated with STR profiling interpretation, proper training of the analysts is crucial for its accurate interpretation and assessment.

1.4.10.1. Artifacts

Stutters are the most common PCR by-products. Due to a slip in the extension of a new strand by the DNA polymerase where this detaches from the DNA, a loop is formed either on the primer or on the template strand. When it is formed in the extending strand, the new fragment will be longer by one repeat [84]. These are also called forward stutters, and do not appear very often. However, back stutters, which result from a loop in the template strand, and translate to smaller fragments, are very common [85]. They appear later in the amplification process and tend to be less than 15% of the main peak. Although

they are easy to detect, stutters can become troublesome in mixed samples when a minor contributor is present. Notwithstanding, they can also be advantageous when identifying other artifacts because only true alleles can have a stutter peak [86].

Split peaks result as a natural occurrence from the addition of an adenine to the 3'-end of PCR products by the polymerase. However, when a large amount of DNA is present, the result is incomplete adenylation and some molecules from the same allele will be shorter than others [87].

Other artifacts are usually pull-ups, dye blobs and spikes and are related to the technology of separation and detection of the STRs [6]. Pull-ups come from increased DNA amounts, where the range of detection in CE is exceeded due to spectral overlap [81]. The inability of the instrument to resolve the dye colours leads to a bleeding of these to another channel. Some artifacts are residual-dye molecules [88]. They appear in EPGs due to incomplete attachment of the dye colours whilst primer extension of the new strand leading to its release. The dyes end up migrating through the capillary and the resulting peak is fairly broad and generally easy to distinguish. It is possible to remove them through a filtration column. Spikes are sharp peaks transcending all colour panels as a result from air bubbles in the capillary, crystal formation in the polymer or voltage discharges [89]. The problem is effortlessly solved by reinjection of the sample.

1.4.10.2. Degraded DNA samples

Due to the immense environmental variables to which samples may be subjected, it is quite ordinary to find some with degraded DNA. The extension of its decay and genotyping quality is dependent on the environmental conditions, such as humidity, pH, temperature, soil chemistry; and the time exposure to these circumstances [90]. Degraded samples can pose an enormous challenge to the identification of markers available for forensic analyses [91].

Extreme conditions are followed by chemical reactions that cause unsteadiness in the DNA molecule leading to loss of bases, their modification and single/double strand breakage. In these cases, smaller amplicons are preferably amplified, since larger markers are more susceptible to fragmentation due to their bigger size as degradation occurs [92, 93]. This pattern of behaviour results in a very typical profile of a degraded DNA sample, as revealed in figure 4.

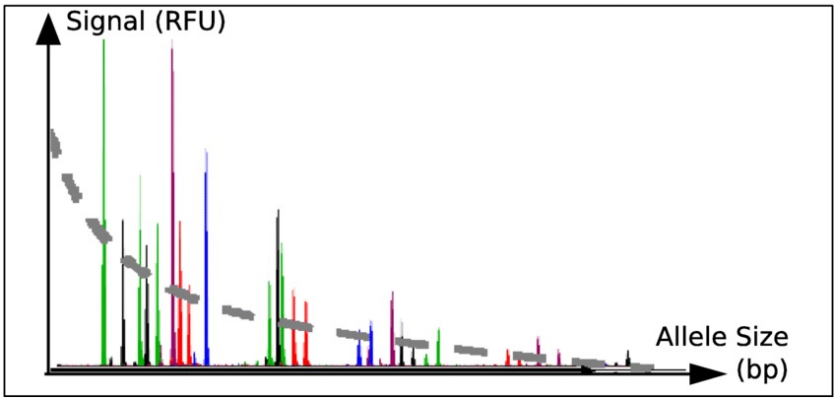

Figure 4 – Representation of the effect of DNA degradation on STR profiles with progressive decreasing in the peak height of the PCR products as the size of the amplicon increases (From Karkar, S., Alfonse, L.E., Grgicak, C.M. et al. [94]).

To enhance the analysis of severely degraded DNA, researchers have developed alternatives like mini-STRs, SNPs and INDELs [35, 95, 96]. These methods involve smaller amplicons, which are less prone to fragmentation and enable more complete DNA profiles.

1.5. Sample Backlog

To date, DNA sample backlogs are a significant issue in many forensic laboratories. The definition of backlog was only standardized in 2011 when the National Institute of Justice (NIJ), characterized a forensic biology/DNA backlog as a case that remains unfinished beyond a period of 30 days from the time it is received at the laboratory [97].

Backlogs can result in delays in criminal investigations, prolonged court proceedings, and potential injustice for victims and the accused. The backlog can also hinder the identification of suspects, slowing down the resolution of cases. Some strategies implemented by many forensic laboratories to overcome this problem were streamlining laboratory processes, hiring additional staff, and investing in updated technology and equipment. Some even collaborated with law enforcement agencies or implemented outsourcing measures to help mitigate the backlog [98].

A study made in 2006, estimated a backlog of over five hundred thousand unsolved cases through a nationally representative sample of local enforcement agencies [99]. Although DNA sample backlogs are not currently a pressing issue in Portugal due to the country's criminal reality, it is still important to explore potential solutions and conduct

research to ensure that investment in new equipment can be implemented gradually and proactively to avoid significant delays in case processing.

2. Aims

The STAR Q Punch AS instrument (QIAGEN, Germany) along with the buccal sample collection device EasiCollect (GE HealthCare Technologies Inc, United Kingdom) [100] for reference samples, offers many advantages over the previous collection methods such as the possibility of easiness for tracing of the samples, increased stability in DNA storage, greater level of automation and, consequently, less room for human errors and cross-contamination.

Prior to a full-scale implementation of the EasiCollect device (GE HealthCare Technologies Inc, United Kingdom), all sorts of tools in addition to the latter one [for example, the OmniSwab (Whatman, GE HealthCare Technologies Inc, United Kingdom) – brush type with absorbent material – and cotton swabs] are expected to be received at the Portuguese Scientific Police Laboratory, given the nationwide stock (still) available.

In order to avoid having two workflows operating at once, specific aims were established in this work:

- To determine whether it is feasible to transfer forensic samples obtained using conventional techniques (buccal swabs) to FTA cards with the joint use of STAR Q Punch AS automated sample punching and STR assay setup instrument by analysing STR profiling results;

- To test several solutions [TE buffer (Illinois Tool Works, USA), Ultrapure water and SwabSolution kit (Promega Corporation, USA)] for humidification of dried swabs for sample transfer;
- To amplify the autosomal STR kits Investigator 24plex GO! (QIAGEN, Germany) and GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA), commonly used for human identification in routine casework and infer on the completeness of profiles obtained through the different transfer procedures;

- To optimize the best workflow (best STR profiling results) and suggest an implementation protocol for an easiness and automated workflow of reference samples.

3. Materials and Methods

3.1. Sample Collection and Preparation

Three collection methods (cotton swabs, OmniSwabs and FTA cards) were used in order to evaluate the feasibility of transferring the sample from the current collection methods (cotton swabs and OmniSwabs) to an FTA card. Initially, buccal cells were collected from twelve different, unrelated volunteer individuals, under informed consent of the content of the research experiment (Table 1), one with a sterile OmniSwab (Whatman, GE HealthCare Technologies Inc, United Kingdom) and the other with regular cotton swab. These types of swabs make up the majority of those received in the laboratory on a daily basis.

Four card controls were also included by collection with the EasiCollect device (GE HealthCare Technologies Inc, United Kingdom). The buffers applied for wetting the swabs were the SwabSolution (Promega Corporation, USA), which is the buffer currently implemented for reference samples analysis; and TE buffer (Illinois Tool Works, USA), since it is a universal buffer. All samples were dried at room temperature for at least 30 minutes before handling. Table 1 illustrates the samples included and the buffers applied to each one.

Table 1 – Table listing every sample used for initial analysis, its method of collection and the buffer applied to each one. Control FTA cards were obtained through collection with the EasiCollect device (GE HealthCare Technologies Inc, United Kingdom).

Sample Name	**SwabSolution**		**TE**		**Control FTA Card**
	Cotton Swab	OmniSwab	Cotton Swab	OmniSwab	
S1	X	X			
S2	X	X			
S3	X	X			
S4	X	X			
S5	X	X			
S6			X	X	
S7			X	X	X
S8			X	X	
S9			X	X	
S10			X	X	X
S11					X
S12					X

Once the results of the first samples had been analysed and in order to optimise the number of cycles of the PCR reaction, the experimental design was revised due to the inherent costs and time constraints. Hence, the number of individuals was reduced to three volunteers (Table 2). Compared to previous analyses, this method led to more consistent and comparable results amongst the varying buffers employed.

Just as in the first set of samples, buccal cells were collected with sterile OmniSwabs (Whatman, GE HealthCare Technologies Inc, United Kingdom), regular cotton swabs, and with the EasiCollect device (GE HealthCare Technologies Inc, United Kingdom). Each swab collection was repeated three times to assess which buffer would yield the best results. The buffers implemented were SwabSolution (Promega Corporation, USA), TE (Illinois Tool Works, USA) and sterilised deionized water (diH_2O) was added because it is of general use in every laboratory.

These samples dried at room temperature for at least 30 minutes before handling.

In both the first set of samples and the second, the methodology adopted was identical. The process involved wetting the swabs with approximately 100µL of each buffer: (a) SwabSolution (Promega Corporation, USA); (b) TE buffer (Illinois Tool Works, USA); (c)

sterilised deionized water – diH_2O; and rubbing it onto the card carefully since the card could rupture extremely easily when too much strength is applied.

Control cards were also included and left to dry for 30 minutes at least as well as the other samples already transferred to an FTA card (Table 2).

Target and control samples were treated separately to avoid contamination.

Table 2 – Table listing every sample used for the latest analysis, its method of collection and the buffer applied to each one. Control FTA cards were obtained through collection with the EasiCollect device (GE HealthCare Technologies Inc, United Kingdom).

Sample Name	**SwabSolution**		**TE**		**diH_2O**		**Control FTA Card**
	Cotton Swab	OmniSwab	Cotton Swab	OmniSwab	Cotton Swab	OmniSwab	
S12	X	X	X	X	X	X	X
S6	X	X	X	X	X	X	X
S11	X	X	X	X	X	X	X

3.2. Sample Analysis

3.2.1. Direct Amplification

The assay setup was processed through the STAR Q Punch AS instrument [102], (QIAGEN, Germany). EasiCollect's (GE HealthCare Technologies Inc, United Kingdom) cards enable direct amplification and therefore no extraction step is needed which leads to the preparation of a PCR reaction plate with the punches from the sample cards.

PCR master mixes were prepared manually previously according to each kit's manufacturer's instructions [103, 104] and loaded onto the equipment along with control DNA, also provided by the commercial kits. In addition, the magazines with the FTA cards collected are loaded as well as blank card magazines for cleaning punches, the tips for the automated pipette system and a reaction plate, in this case the PCR microplate (Axygen Scientific, USA) was used. These master mixes can either be prepared for a full-volume or a half-volume reaction which is predefined by the user before starting the run. An amplification kit is typically manufactured with a specified capacity for a particular number of samples. When the kit is utilized at its recommended

full volume, it is capable of analysing the designated quantity of samples. Conversely, if only half of the recommended volume is employed, it is suggested that the same kit may theoretically amplify twice the number of samples. It is important to note, however, that achieving consistently accurate results under such conditions may vary, necessitating preliminary testing to ensure reliability.

Investigator 24plex GO! (QIAGEN, Germany) and GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) were the two PCR amplification kits employed for the assay setup. The components of each one are mentioned in table 3.

Table 3 – Table summarizing the components of each STR amplification kit used in this study. Also mentioned in parenthesis is the respective volume, in µL, of said component, per full-volume and half-volume reaction.

	PCR kit name *Company*			
Kit components *(volume per reaction in µL)*	**Investigator 24plex GO!** *QIAGEN*		**GlobalFiler Express** *Applied Biosystems*	
	Full-volume	Half-volume	Full-volume	Half-volume
Master Mix	Fast Reaction Mix 2.0 *(7.5)*	Fast Reaction Mix 2.0 *(3.75)*	GlobalFiler Express Master Mix *(6.0)*	GlobalFiler Express Master Mix *(3.0)*
Primer Mix	Primer Mix 24plex GO! *(12.5)*	Primer Mix 24plex GO! *(6.25)*	GlobalFiler Express Primer Set *(6.0)*	GlobalFiler Express Primer Set *(3.0)*
Buffer	Investigator STR GO! Punch Buffer *(2.0)*	Investigator STR GO! Punch Buffer *(1.0)*	Low-TE Buffer *(3.0)*	Low-TE Buffer *(1.5)*
Control DNA	Control DNA 9948 5ng/µL *(1.0)*		Control DNA 007 2ng/µL *(1.0)*	

After inserting all components, the instrument proceeds to transfer the defined volumes of the master mix onto the plate's wells, and the control DNA. A negative control is also added composed only by the master mix as well as the positive control consisting of 1µL of the DNA control and master mix. Neither of these contain any card punches.

After the liquid handling step, the instrument transports the plate to the punching area. The punch diameter is set to 1.2mm for the STAR Q Punch AS (QIAGEN, Germany). Before each sample, a cleaning punch is performed to avoid possible cross-contaminations. It is the user who defines whether to use separate blank cards for the cleaning punches or the sample cards themselves, usually in an area outside of the black circle and determined by the software camera as being free of sample. In this study, a separate blank card was used for cleaning punches.

The instrument has a camera system that illuminates the card and captures an image, allowing the analysis of the sample area. The presence of a pink pigment on these specific cards, which changes colour to white upon contact with saliva fluids, allows the imaging software to identify the region within the sample area (represented by a black circle on the card) with the highest likelihood of containing DNA. Once detected, the software proceeds to puncture the card into the well, and aspirates any surplus particles that may be present. Whenever the software cannot fetch any colour changing, the punching strategy is configured as "centre of sample". This imaging feature simultaneously scans a barcode while capturing the image of the sample area, allowing easy sample tracking.

PCR reactions were performed through a Veriti 96-well Thermal cycler (Applied Biosystems, Thermofisher Scientific, USA), following the kit manufacturer's protocols for the PCR cycles temperature, described in table 4. The number of cycles had to be adapted to the study with several attempts.

Table 4 – Polymerase Chain Reaction (PCR) protocols for each amplification kit used in this study.

	Polymerase Chain Reaction Steps			
PCR kit name *Company*	**Initiation**	**Denaturation, Annealing and Elongation**	**Final Elongation**	**Hold**
Investigator 24plex GO! *QIAGEN*	98.0ºC for 30'' 64.0ºC for 40'' 72.0ºC for 5'' X3 times	96.0ºC for 10'' 61.0ºC for 40'' 72.0ºC for 5'' X24 times*	68.0ºC for 2' 60.0ºC for 2'	10.0ºC For ∞
GlobalFiler Express *Applied Biosystems*	95.0ºC for 60''	94.0ºC for 3'' 60.0ºC for 30'' X23 times*	60.0ºC for 8'	4.0ºC For ∞

* This cycle number corresponds to the recommended by the manufacturer and used in the current method. It was the number with which we started the study, but had to be increased further on.

3.2.2. Capillary Electrophoresis and Data Analysis

Thereafter, with the QIAgility (QIAGEN, Germany), 1µL of each sample was pipetted into a new plate (Axygen Scientific, USA), together with the size standard of the respective kit and diluted with Hi-Di Formamide (Applied Biosystems, ThermoFisher Scientific, USA). Table 5 describes the amounts and components used.

Once the final plate was ready, a septa designed for 96-well plates was utilized to cover the wells. Subsequently, the samples underwent denaturation also in the Veriti 96-well Thermal cycler (Applied Biosystems, ThermoFisher Scientific, USA) before proceeding with capillary electrophoresis. This step was crucial to mitigate mobility complications arising from base mismatches. The samples were heated to 95ºC for three minutes and cooled at 4º C for another three minutes.

Finally, the plate was analysed through the 3500xL Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific, USA). This is a 24-capillary sequencing instrument and an allelic ladder was added at every injection.

Table 5 – Table outlining the specific components of different amplification kits required for each well of the microplate used in capillary electrophoresis. It was mandatory for every well to include formamide as a diluent and a size standard. For all the kits, the Hi-Fi Formamide (Applied Biosystems, ThermoFisher Scientific, USA), was utilized.

	PCR kit name *Company*	
Well Components for Capillary Electrophoresis	**Investigator 24plex GO!** *QIAGEN*	**GlobalFiler Express** *Applied Biosystems™*
Size Standard *Volume*	DNA Size Standard 24plex (BTO) *0.5µL*	GeneScan™ 600 LIZ™ Size Standard v2.0 *0.5µL*
Formamide *Volume*	*12.0µL*	*9.5µL*
Sample or Allelic Ladder *Volume*	Sample or Allelic Ladder 24plex *1.0µL*	Sample or GlobalFiler Express Allelic Ladder *1.0µL*

The raw data files were then analysed using the GeneMapper™ *ID-X* version 1.6 (Applied Biosystems, ThermoFisher Scientific, USA).

3.3. Previous Method

Reference samples are usually collected in duplicate with swabs. After arriving at the laboratory, they are processed and queued for analysis.

In this extraction method, about a third of the swabs is cut into an Eppendorf tube with a basket and stored in the freezer until the batch is complete for extraction. Afterwards, using the QIAgility (QIAGEN, Germany), 300µL of SwabSolution (Promega Corporation, USA) are pipetted into each basket and the tubes incubated in an Eppendorf Thermomixer for 30 minutes at 500 r.p.m. and 70º C and subsequently centrifuged at 15000rpm for 2 minutes. The basket is removed and the tubes are placed into a QIAgility (QIAGEN, Germany) liquid handler to prepare the PCR reaction plate with all the reagents from the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) and 1µL of the DNA extract from the sample following the manufacturer's recommendation.

Afterwards, the plate follows the same procedures as the STAR Q Punch AS one described above.

4. Results and Discussion

4.1. Sample Collection

As previously mentioned, reference saliva samples were collected from volunteer unrelated consenting individuals. After processing and transferring each sample, all FTA cards were left to dry at room temperature. These were then inserted into the STAR Q Punch AS instrument. An example of the images captured by the Hamilton imaging software before any card being punched is presented in figure 5.

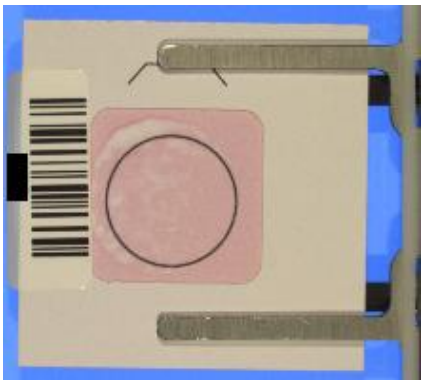

Figure 5 – Image captured by the Hamilton imaging software before the card being punched.

According to the manufacturer's guidelines, the areas where DNA is present are expected to change colour, which can be observed in the image above with a slightly lighter colouring. This is what allows the imaging software to recognize the area to be punched. Whenever the colour changing is not visible, the instrument is programmed to punch the card in its centre. Since we are working with DNA that is being transferred from swabs to a card, DNA losses are inevitable. This particular tendency of the instrument becomes a liability and a huge variable factor to this work due to the sensibility of the imaging software and because it is not possible for the user to manipulate the punching area.

4.2. Setting up a run

When starting a run in the STAR Q Punch AS, the user has to choose between a half-volume or a full-volume master mix. As mentioned previously, two PCR amplification kits were employed, the first one was the Investigator 24plex GO! (QIAGEN, Germany) in which the full-volume corresponds to 22µL of master mix per sample and the half-volume

to 11µL. In the second one, GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA), the full-volume is 15µL and half-volume is 7.5µL.

After the plate is prepared by the instrument with the master mix, the respective punches of the cards, the positive (PTC) and negative (NTC) controls, it undergoes the PCR, which has the cycles pre-defined as indicated by the manufacturer's instructions (Table 4).

Subsequently, in the QIAgility (QIAGEN, Germany), a mixture is prepared with Hi-Di formamide (Thermo Fisher Scientific, USA) and DNA size standard (BTO). The volumes of 12µL of formamide and 0.5µL of BTO are added per sample to a new plate. 1µL of sample or allelic ladder are aliquoted subsequently to the wells. One allelic ladder is added per injection, since the 3500xL Analyzer is a 24-capillary instrument. When the reaction plate is ready, it is inserted in the genetic analyser which will perform capillary electrophoresis.

The results obtained are then imported to the GeneMapper software to proceed STR genotyping.

4.3. DNA profiling results

Through this work, it was ultimately aimed to sought good results using half-volume master mix, as this would enable the kit to be optimised for double the number of samples. Half-volume and full-volume runs were tested alternatively to comprehend its effect on the results. Same applies to the increasing number of cycles.

The results are represented through tables grouped by different types of experiments and different shades of grey. The lighter grey corresponds to positive results, i.e., reportable profiles; the medium grey to profiles that do not meet the threshold and the darkest grey to negative results, in which there is no profile. The threshold previously defined for reference samples at the Scientific Police's laboratory is 300 RFUs, meaning only the peaks equal or above 300 RFUs are considered alleles by the software.

A reportable genetic profile must meet specific criteria for inclusion in an official report, which encompasses the following elements: having an adequate number of genetic markers, presenting clearly distinguishable peaks, and successfully passing rigorous quality control assessments (Figure 6). The category of "does not meet the threshold" (Figure 7) includes all the profiles in which was possible to see clear peaks under 300 RFUs by enlarging its axis in the GeneMapper software. Based on this observation, it

was then understood that the issue was the insufficient amount of DNA. When the DNA quantity was even more limited or non-existent, no peak was visible and therefore, no profile was present (Figure 8).

Figures 6, 7 and 8 are examples of STR profiles from the same individual defined according to the classification described before.

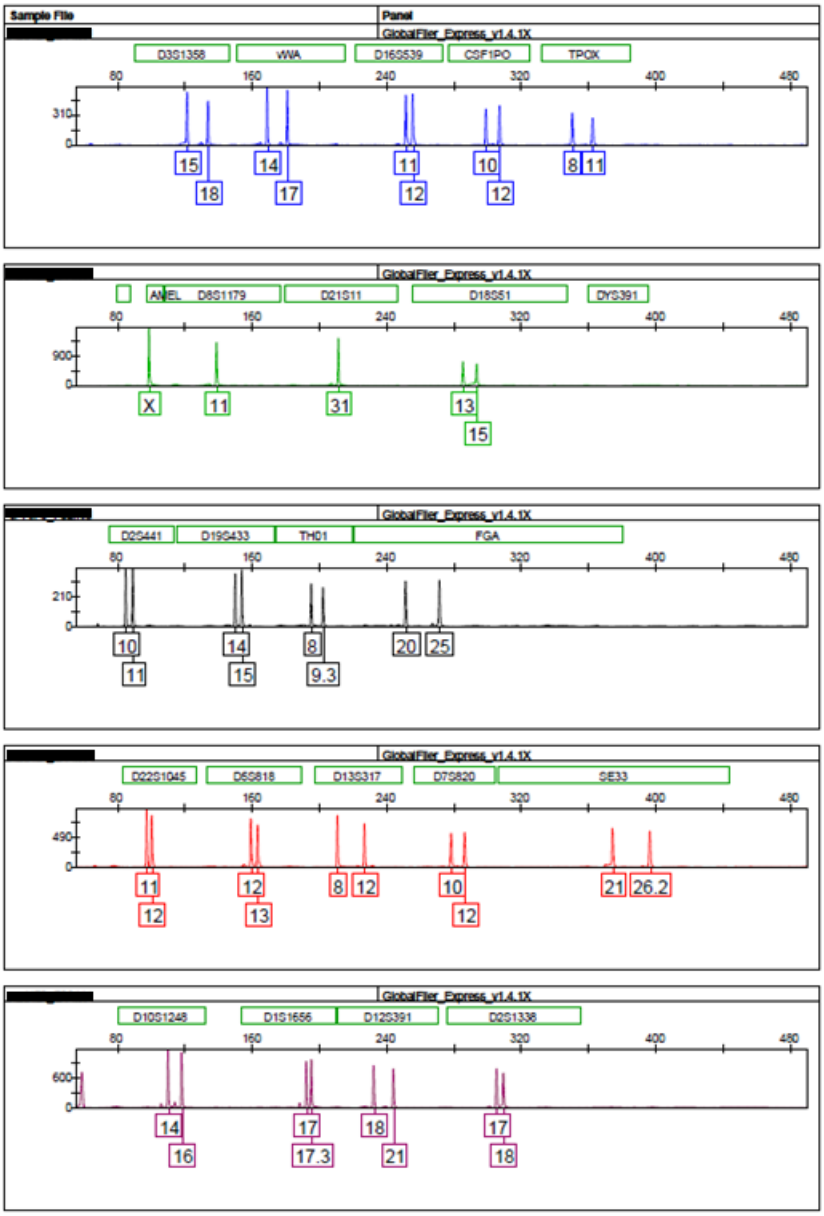

Figure 6 – STR profile from sample S12 classified as “reportable profile”. The kit implemented was the Investigator 24plex GO!.

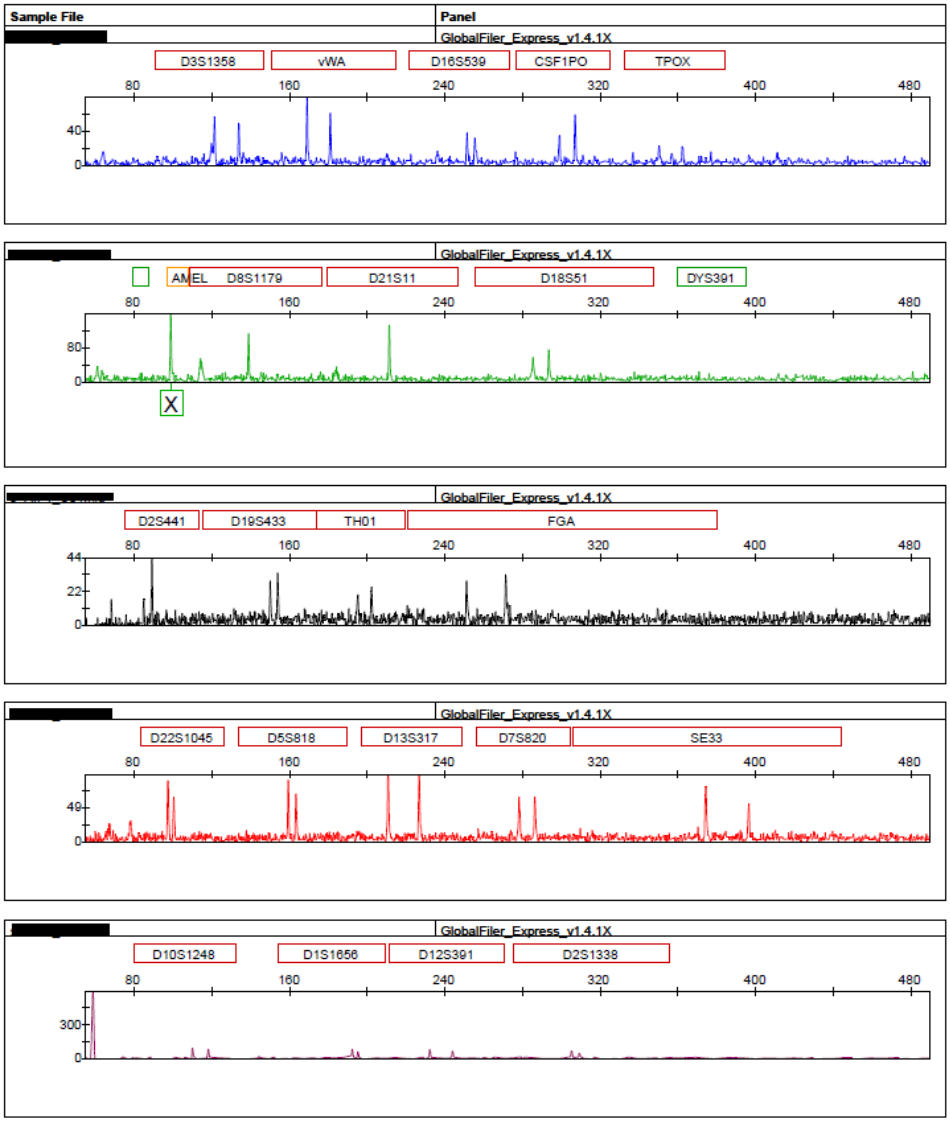

Figure 7 – STR profile from sample S12 classified as “does not meet the threshold”. The kit implemented was the Investigator 24plex GO!.

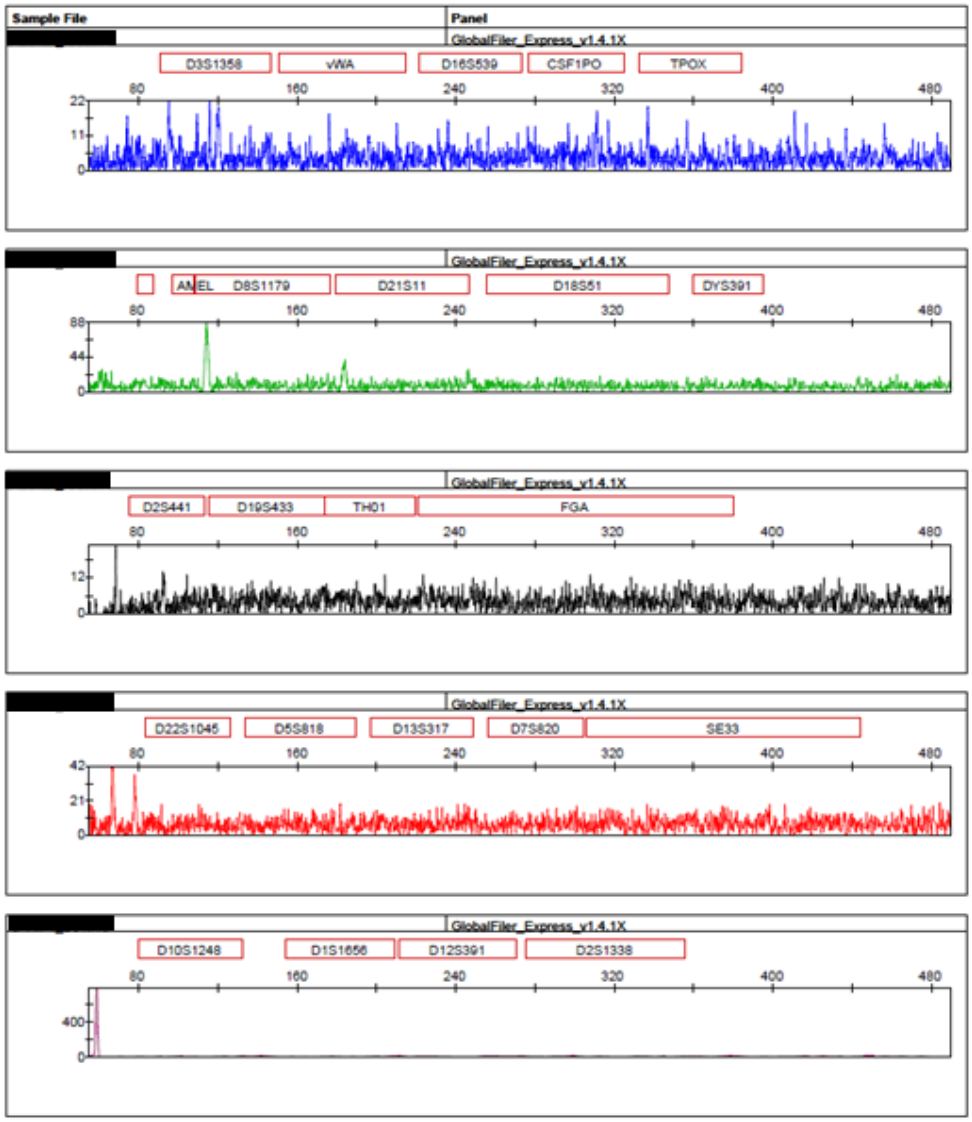

Figure 8 – STR profile from sample S12 classified as “no profile”. The kit implemented was the Investigator 24plex GO!.

4.3.1. Investigator 24plex GO!

This work relied heavily on trial and error, in which the variable parameters were: 1) the volume of master mix employed and 2) the number of cycles of the PCR reaction. As such, the results obtained for the Investigator 24plex GO! (QIAGEN, Germany) kit are shown below in the various tables.

For run number 1, a reaction was prepared to half-volume and the number of cycles of the PCR reaction was set accordingly to the manufacturer’s protocol, which stated 24 cycles. The results are shown below in table 6.

Table 6 – Illustrative table of the results obtained in run number 1. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a half-volume master mix and a PCR reaction with 24 cycles.

Sample Name	SwabSolution		TE		Control FTA Card
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					

reportable profiles profiles that do not meet the threshold no profile

A total of 4 samples did not have results in which 2 of them were transferred with SwabSolution and the other 2 with TE. Likewise, 4 samples had reportable profiles and 4 samples did not meet the threshold, in both buffers. Although it was not possible to conclude which of the buffers performed better, this first run demonstrated that this method of transferring buccal cells from swabs to FTA cards was viable since a total of 8 samples presented reported profiles and 8 of them had EPGs with peaks bellow the threshold.

Another run was performed, with the same individuals and type of samples, but the master mix was prepared for full-volume, to test for the possibility of having better results (Table 7).

Table 7 – Illustrative table of the results obtained in run number 2. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a full-volume master mix and a PCR reaction with 24 cycles.

Sample Name	SwabSolution		TE		Control FTA Card
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					

reportable profiles profiles that do not meet the threshold no profile

Overall, the results of this run improved with both buffers, with one more reportable profile and one more that did not meet the threshold. However, some samples had a downgrade in its result. Once again, a lot of variable aspects need to be taken into account such as the sensibility of the equipment mentioned earlier. Also, the fact that the original samples were collected by the own individuals, which can apply more or less pression with the swab, or spend more or less time with the collection itself, translating into possible different results each time.

Since many of the results still were profiles that did not meet the threshold, the number of cycles was slowly increased in the course of time. Table 8 represents the following run with one more cycle added, i.e., 25 cycles.

Table 8 – Illustrative table of the results obtained in run number 3. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a full-volume master mix and a PCR reaction with 25 cycles.

Sample Name	SwabSolution		TE		Control FTA Card
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S1					
S2					
S3					
S4					
S5					
S6					
S7					
S8					
S9					
S10					
S11					
S12					

reportable profiles profiles that do not meet the threshold no profile

The outcomes of this run (Table 8) do not show any significant disparities in comparison to the antecedent one. Nonetheless, it is discernible that there exists an additional profile deemed reportable, along with one less that fails to meet the established threshold criteria. Even though the control FTA card S12 had no profile, this might have been due to the sample harvest, handling or processing in this specific experiment, since it only happened in one of this kind of samples.

At this point, it was necessary to rearrange the experimental design due to the costs and time associated with processing the considerable number of samples. The number of individuals was reduced to 3 (S12, S6, S11) and for each of them, 7 samples were collected: 3 with a cotton swab, 3 with an OmniSwab and 1 with an FTA card, ensuring greater consistency when comparing the results. Sterilized deionized water was also included to the buffers employed.

After implementing this strategy, a run with the same characteristics as the previous one was performed for better understanding the outcome with different buffers. The results are displayed in table 9 below.

Table 9 – Illustrative table of the results obtained in run number 4. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a full-volume master mix and a PCR reaction with 25 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

Samples transferred with sterilized deionized water had better results overall, with 3 reportable profiles, one that did not meet the threshold and 2 from the same individual (S11) without results. However, the peaks heights are rather low as seen in figure 9.

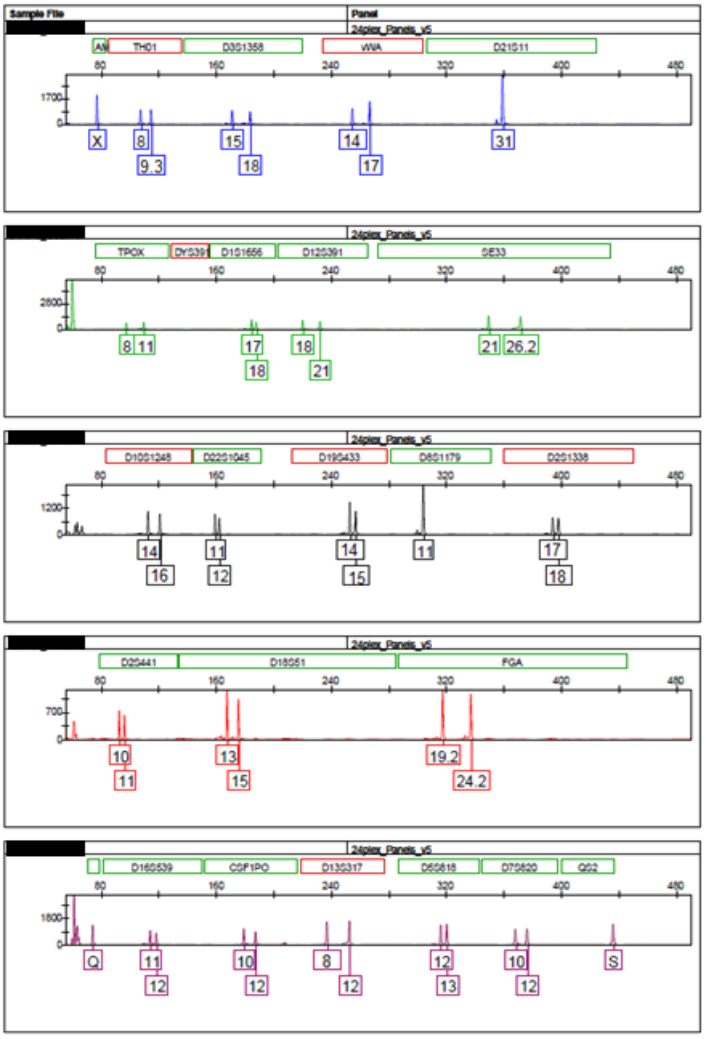

Figure 9 – Electropherogram of the sample transferred with diH$_2$O from an OminSwab from individual S11 on run number 4.

Samples from the individual S11 had consistently the worst results which perhaps can be explained for this particular case. The sample collection must be done at least 30 minutes after eating/drinking due to inhibitors that might be present in food and drinks, and even so, some inhibition can occur. Therefore, possibly the individual did not follow these rules or a “bad” swabbing was done not retrieving many buccal epithelial cells.

In the following run, the volume was changed to half (Table 10), since it was of interest to study the behaviour of the amount of DNA available under different volumes of master mix.

Table 10 – Illustrative table of the results obtained in run number 5. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a half-volume master mix and a PCR reaction with 25 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

In this transition, the improvement is noticeable with a decrease of the number of samples without a profile and the increase of the ones that do not meet the threshold. Under identical conditions of amplification cycles, it is noteworthy that superior outcomes were achieved when utilizing half the recommended volume (Table 10), which departs from conventional expectations. It is pertinent to reiterate that the recommended volume aligns with the guidelines provided by the suppliers of the amplification kits, and therefore, a reduction in volume would typically not be anticipated to yield equivalent or superior results compared to employing the full volume.

In this particular scenario, it is plausible that these outcomes could be attributed to stochastic effects or potential pipetting issues encountered during the reaction involving the full recommended volume.

Even though the results were getting better, these still were not reportable profiles, and therefore, they could not be used or considered a unique profile. This was an indication that the DNA amount present was not sufficient yet, hence one more cycle was added and the volume of the mix maintained (Table 11).

Table 11 – Illustrative table of the results obtained in run number 6. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a half-volume master mix and a PCR reaction with 26 cycles.

Sample Name	SwabSolution		TE		diH_2O		Control FTA Card
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12						*	
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

* The instrument did not punch the card corresponding to this sample. This kind of error would happen when a card was crooked, not found, when the barcode was not readable or due to some malfunction of the instrument.

After adding one more cycle, the results for the cotton swabs started to worsen (Table 11), but on the other hand the results for the OmniSwabs started to look more promising. After assessing the number of cotton swabs received in the laboratory in one week, these results were not as concerning as the number was rather low. Cotton swabs represent only about 20% of the reference samples received.

As a next attempt, the following run had a full-volume (Table 12), which overall allows the card punch to be better submersed in liquid in a plate, having a better performance.

Table 12 – Illustrative table of the results obtained in run number 7. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a full-volume master mix and a PCR reaction with 26 cycles.

Sample Name	SwabSolution		TE		diH_2O		Control FTA Card
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

Finally, with 26 cycles in the PCR reaction, and full-volume master mix (Table 12), it was possible to get a reportable profile for every OmniSwab transferred with the three buffers tested. As the amount of DNA increases with the number of cycles in the PCR reaction, there must be enough reagents, such as primers, for the reaction to proceed normally without running out of them. Therefore, the run is expectable to have better results when employing a full-volume, since it will have more of these products available.

OmniSwabs enable better oral sampling than regular cotton swabs due to its design of brush-like head and unique absorbent material. The OmniSwab has been optimized by the manufacturer for this type of sample collection and therefore it is not unexpected that the results tend to be superior when samples are collected with this type of swab.

To have confirmed the results obtained in run number 7, a new batch of samples was collected from 3 new individuals (S13, S14, S15). Exactly like the previous one, 7 samples were collected from each one: 3 with a cotton swab, 3 with an OmniSwab and 1 with an FTA card (Table 13).

Table 13 – Illustrative table of the results obtained in run number 8. The kit implemented was the Investigator 24plex GO! (QIAGEN, Germany) with a full-volume master mix and a PCR reaction with 26 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S13							*
S14							
S15				*			

reportable profiles profiles that do not meet the threshold no profile

* The instrument did not punch the card corresponding to this sample. This kind of error would happen when a card was crooked, not found, when the barcode was not readable or due to some malfunction of the instrument.

These were indeed the best results obtained with the methodology implemented, with all samples, cotton and OmniSwabs, with reportable profiles. Moreover, the electropherograms have good quality, with high RFUs.

4.3.2. GlobalFiler Express

The prevailing amplification kit employed for the analysis of reference samples at the LPC is the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA). Consequently, it was also important to assess the results of the method under study when utilizing this particular kit. As with the Investigator 24plex GO! (QIAGEN, Germany), the experimental procedure followed was the collection of 7 samples from each 3 individuals (S12, S6, S11), the re-moisten of the swabs with 3 different buffers [SwabSolution (Promega Corporation, USA), TE (Illinois Tool Works, USA) and sterilised deionized water (diH$_2$O)] and application into new FTA cards by scraping.

The recommended number of cycles for this kit is 23, so the first run was performed accordingly with full-volume (Table 14). All conditions applied to the previous method were maintained for this one.

Table 14 – Illustrative table of the results obtained in run number 9. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a full-volume master mix and a PCR reaction with 23 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

The findings (Table 14) unveiled identical concerns as those in the preceding analysis, with majority of samples revealing no profile or electropherograms with peaks bellow the threshold. Given that a similar observation had already been made using the alternative kit, it was decided to augment the number of cycles, starting with a half-volume master mix (Table 15).

Table 15 – Illustrative table of the results obtained in run number 10. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a half-volume master mix and a PCR reaction with 24 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

While there has not been a substantial enhancement, it is evident that certain profiles have exhibited improved performance (Table 15). Conversely, it should be noted that there have also been instances of worsening in the results obtained for some samples. Generally, there was a reduction of one sample without a discernible profile,

accompanied by an increase of one sample falling below the established threshold (<300 RFUs).

As a result, the samples underwent a subsequent analysis, during which the volumes of the master mix were adjusted to full-volume (Table 16).

Table 16 – Illustrative table of the results obtained in run number 11. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a full-volume master mix and a PCR reaction with 24 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

Once more, there is a lack of significant improvement (Table 16); instead, a decline of positive results is evident in the samples collected from the cotton swabs. This pattern is similarly observed in the runs conducted using the Investigator 24plex GO! (QIAGEN, Germany) kit, suggesting a potential commonality in the underlying cause, possibly related to disparities in the quality of the profiles obtained from both swab types.

The amplification cycle count was elevated with the expectation of achieving enhanced results, particularly in the case of profiles acquired through the use of OmniSwabs (Table 17).

Table 17 – Illustrative table of the results obtained in run number 12. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a full-volume master mix and a PCR reaction with 25 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

Under these specified conditions (Table 17), the outcomes are consistently paralleled with those acquired with the alternative kit. Regrettably, no reportable profiles were obtained in actuality, which remains an unfavourable outcome. Consequently, a subsequent analysis of the samples using half-volume was deemed impractical, as it was anticipated that the results would not substantially differ from the preceding iteration.

Subsequently, a fresh analysis was conducted, encompassing an additional cycle and the utilization of the full-volume, with the expectation that samples yielding electropherograms falling below the threshold might exhibit improved performance due to the augmented DNA quantity (Table 18).

Table 18 – Illustrative table of the results obtained in run number 13. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a full-volume master mix and a PCR reaction with 26 cycles.

Sample Name	**SwabSolution**		**TE**		**diH_2O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

The adjustment in the cycle count revealed that the SwabSolution and TE buffers possess a greater efficacy (Table 18) when compared to the use of diH_2O, for which no positive results were attained. Notably, only a single sample derived from the OmniSwabs yielded an unsatisfactory result. This outcome may be attributed to factors such as the sampling process itself, potential mishandling of the specimen, or the specific location from which the sample was extracted, possibly containing a diminished quantity of DNA.

Nevertheless, in light of the previously achieved positive outcomes with two of the employed buffers, the subsequent investigative phase would entail an exploration of the feasibility of employing half the volume of reagents while maintaining the same cycle count (Table 19).

Table 19 – Illustrative table of the results obtained in run number 14. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a half-volume master mix and a PCR reaction with 26 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

The findings reveal (Table 19) a discernible decline in performance (i.e., in obtainable profiles), wherein the majority of samples obtained from OmniSwabs exhibit electropherogram peaks falling below the established threshold, and one sample even manifests a negative outcome.

Additionally, it is noteworthy that the FTA control card for individual S11 exerted an influence on the resulting profile. These findings represent the outcome of augmenting the cycle count concurrently with a reduction in the volume of reagents. A comprehensive explanation of this phenomenon will be provided subsequent to the presentation of the results obtained from the forthcoming run, wherein an increased cycle count will be maintained alongside the current reagent volume (Table 20).

Table 20 – Illustrative table of the results obtained in run number 15. The kit implemented was the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) with a half-volume master mix and a PCR reaction with 27 cycles.

Sample Name	**SwabSolution**		**TE**		**diH$_2$O**		**Control FTA Card**
	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	Cotton Swab	Omni Swab	
S12							
S6							
S11							

reportable profiles profiles that do not meet the threshold no profile

The quality of the results (Table 20) exhibited a significant deterioration concurrent with an increase in the cycle count. Notably, the FTA control cards produced suboptimal outcomes, providing an indication of the challenges faced in achieving improved results. It is imperative to recognize that the augmentation of cycle count directly contributes to an exponential increase in the DNA quantity within the samples, doubling with each

cycle. Consequently, the concentration of the sample becomes excessive and this limitation resulted in the generation of incomplete DNA profiles and, in some instances, a complete absence of profiles and therefore cannot be applied.

5. Conclusion

The automation of diverse procedures within the confines of a Forensic Genetics laboratory constitutes a pivotal advancement in the field. This progressive automation not only facilitates the meticulous tracking of samples but also ensures a heightened level of stability in the storage of DNA. Moreover, it markedly enhances the overall efficiency and expeditiousness of procedures, resulting in a notable reduction in user errors and the potential for cross-contamination.

The primary objective underpinning the implementation of this novel instrument, STAR Q Punch AS (QIAGEN, Germany), resides in its capacity to supersede the existing method employed for processing reference samples within the Scientific Police Laboratory.

The core aspiration of this endeavour was to develop a methodological approach capable of seamlessly transferring reference samples, originally obtained via swabs, onto FTA cards and to allow future implementation into case work. In doing so, the laboratory seeks to streamline and reform its operational protocols, ultimately fortifying its efficacy and precision in forensic genetics analysis.

It is feasible to deduce that the primary goal of the study has been successfully attained. This achievement signifies the capability to effectively transfer swab samples onto FTA cards and subsequently process them utilizing the STAR Q Punch AS equipment (QIAGEN, Germany).

Utilizing the Investigator 24plex GO! (QIAGEN, Germany) kit, the acquisition of reportable profiles was achieved by adhering to the recommended volume of reagents stipulated by the suppliers for the master mix and configuring the PCR reaction to 26 cycles. It is imperative to underscore that these attributes can likewise be extended to the routine handling of samples received on FTA cards. This strategic approach enables the laboratory to streamline its workflow by consolidating reference sample processing exclusively on the STAR Q Punch AS (QIAGEN, Germany).

Substantial variations in the results obtained with the three distinct buffers are not discernible. Therefore, water presents itself as a favourable choice for this procedure, owing to its universal accessibility and cost-efficiency for the laboratory.

A notable observation emerged when an additional cycle was introduced between the fifth and sixth runs. It becomes evident that the results derived from the employment of

cotton swabs experience a significant deterioration. However, it is noteworthy that the final run utilizing this kit demonstrates that there remains the potential to obtain reportable profiles with these swabs.

In the context of the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) kit, the outcomes obtained were marked by their inconclusiveness. The methodology employed remained consistent throughout, yet the results manifested as unsatisfying, characterized by peaks falling below the established threshold. To address this discrepancy, systematic adjustments were made to both the number of amplification cycles and the volume of reagents employed.

As per the guidelines provided by the kit's manufacturers, treated cards, such as those employed in this study, do not require the incorporation of any supplementary buffer, in contrast to certain other kits. One potential approach to enhance the achieved results would be to explore the application of the protocol intended for untreated cards when working with these types of samples.

Significantly, the most favourable results were observed during the thirteenth run, characterized by the utilization of 26 amplification cycles in conjunction with the recommended reagent volume, as prescribed by the kit suppliers.

In stark contrast to the Investigator 24plex GO! (QIAGEN, Germany) kit, it is important to highlight that only samples collected using SwabSolution (Promega Corporation, USA) and TE solution (Illinois Tool Works, USA) buffers yielded reportable profiles when employing the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) kit. This divergence emphasises the distinct performance characteristics of these two forensic analysis kits, thereby accentuating the critical importance of selecting the appropriate kit for specific sample types and analytical objectives.

The present study was predicated upon a series of iterative trials, the overarching objective of which was to achieve reportable profiles from samples that were transitioned from swabs to FTA cards. As a logical progression, it is imperative that future endeavours entail the replication of this procedure with a more extensive sample cohort as this work represents preliminary data. An expanded investigation is essential to corroborate and validate the outcomes observed with the Investigator 24plex GO! (QIAGEN, Germany) kit, thus enhancing the robustness and generalizability of the findings. It will be essential to undertake further experimentation with the GlobalFiler Express (Applied Biosystems, ThermoFisher Scientific, USA) kit with the aim of comprehensively investigating the optimal conditions conducive to the replication of superior outcomes.

References

1. *Forensic Science International: Genetics*. Available from: https://www.fsigenetics.com/.

2. Goodwin, W., A. Linacre, and S. Hadi, *An introduction to forensic genetics*. 2010: John Wiley & Sons.

3. Landsteiner, K. and P. Levine, *On individual differences in human blood.* The Journal of experimental medicine, 1928. **47**(5): p. 757.

4. *Karl Landsteiner – Biographical. NobelPrize.org.*; Available from: https://www.nobelprize.org/prizes/medicine/1930/landsteiner/biographical/.

5. Jeffreys, A.J., V. Wilson, and S.L. Thein, *Individual-specific 'fingerprints' of human DNA.* Nature, 1985. **316**(6023): p. 76-79.

6. Butler, J.M., *Fundamentals of forensic DNA typing*. 2009, Academic press. p. 2-3.

7. Jeffreys, A.J., J.F. Brookfield, and R. Semeonoff, *Positive identification of an immigration test-case using human DNA fingerprints.* Nature, 1985. **317**(6040): p. 818-9.

8. Aronson, J.D., *DNA fingerprinting on trial: the dramatic early history of a new forensic technique.* Endeavour, 2005. **29**(3): p. 126-31.

9. Roewer, L., *DNA fingerprinting in forensics: past, present, future.* Investig Genet, 2013. **4**(1): p. 22.

10. Edwards, A., et al., *DNA typing and genetic mapping with trimeric and tetrameric tandem repeats.* Am J Hum Genet, 1991. **49**(4): p. 746-56.

11. Gill, P., et al., *The evolution of DNA databases--recommendations for new European STR loci.* Forensic Sci Int, 2006. **156**(2-3): p. 242-4.

12. Budowle, B., et al. *CODIS and PCR-based short tandem repeat loci: law enforcement tools*. in *Second European symposium on human identification*. 1998. Promega Corporation, Madison, Wisconsin.

13. Watson, J.D. and F.H. Crick, *Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid.* Nature, 1953. **171**(4356): p. 737-738.

14. Watson, J.D. and F.H. Crick. *The structure of DNA*. in *Cold Spring Harbor symposia on quantitative biology*. 1953. Cold Spring Harbor Laboratory Press.

15. Travers, A. and G. Muskhelishvili, *DNA structure and function.* The FEBS journal, 2015. **282**(12): p. 2279-2295.

16. Consortium, I.H.G.S., *Finishing the euchromatic sequence of the human genome.* Nature, 2004. **431**(7011): p. 931-945.

17. Pennisi, E., *The Human Genome.* Science, 2001. **291**(5507): p. 1177-1180.

18. Asthana, S., et al., *Widely distributed noncoding purifying selection in the human genome.* Proc Natl Acad Sci U S A, 2007. **104**(30): p. 12410-5.

19. Benecke, M., *Coding or non-coding, that is the question: having solved the last technical hurdles to extract DNA information from virtually any biological material, forensic biologists now have to ponder the ethical and social questions of using information from exonic DNA.* EMBO Rep, 2002. **3**(6): p. 498-502.

20. Schneider, P.M., *Basic issues in forensic DNA typing.* Forensic Science International, 1997. **88**(1): p. 17-22.

21. Amorim, A. and B. Budowle, *Handbook of forensic genetics: biodiversity and heredity in civil and criminal investigation*. Vol. 2. 2016: World Scientific.

22. Lim, K.G., et al., *Review of tandem repeat search tools: a systematic approach to evaluating algorithmic performance.* Briefings in bioinformatics, 2013. **14**(1): p. 67-81.

23. Jeffreys, A.J., J.F. Brookfield, and R. Semeonoff, *Positive identification of an immigration test-case using human DNA fingerprints.* Nature, 1985. **317**(6040): p. 818-819.

24. Berg, I., et al., *Two modes of germline instability at human minisatellite MS1 (locus D1S7): complex rearrangements and paradoxical hyperdeletion.* The American Journal of Human Genetics, 2003. **72**(6): p. 1436-1447.

25. Jeffreys, A.J., V. Wilson, and S.L. Thein, *Hypervariable 'minisatellite' regions in human DNA.* Nature, 1985. **314**(6006): p. 67-73.

26. Ruitberg, C.M., D.J. Reeder, and J.M. Butler, *STRBase: a short tandem repeat DNA database for the human identity testing community.* Nucleic Acids Res, 2001. **29**(1): p. 320-2.

27. Ellegren, H., *Microsatellites: simple sequences with complex evolution.* Nature reviews genetics, 2004. **5**(6): p. 435-445.

28. Tamaki, K. and A.J. Jeffreys, *Human tandem repeat sequences in forensic DNA typing.* Legal Medicine, 2005. **7**(4): p. 244-250.

29. Lins, A.M., et al., *Multiplex sets for the amplification of polymorphic short tandem repeat loci--silver stain and fluorescence detection.* Biotechniques, 1996. **20**(5): p. 882-9.

30. Schneider, P.M., et al., *STR analysis of artificially degraded DNA-results of a collaborative European exercise.* Forensic Sci Int, 2004. **139**(2-3): p. 123-34.

31. Frank, W.E., et al., *Validation of the AmpFeSTR™ Profiler Plus PCR Amplification Kit for Use in Forensic Casework.* Journal of Forensic Sciences, 2001. **46**(3): p. 642-646.

32. Krenke, B.E., et al., *Validation of a 16-locus fluorescent multiplex system.* Journal of Forensic Sciences, 2002. **47**(4): p. 773-785.

33. Brookes, A.J., *The essence of SNPs.* Gene, 1999. **234**(2): p. 177-186.

34. McVean, G., C.C.A. Spencer, and R. Chaix, *Perspectives on human genetic variation from the HapMap Project.* PLoS Genetics, 2005. **1**(4): p. e54.

35. Børsting, C., H.S. Mogensen, and N. Morling, *Forensic genetic SNP typing of low-template DNA and highly degraded DNA from crime case samples.* Forensic Sci Int Genet, 2013. **7**(3): p. 345-52.

36. Gill, P., *An assessment of the utility of single nucleotide polymorphisms (SNPs) for forensic purposes.* Int J Legal Med, 2001. **114**(4-5): p. 204-10.

37. Amorim, A. and L. Pereira, *Pros and cons in the use of SNPs in forensic kinship investigation: a comparative analysis with STRs.* Forensic science international, 2005. **150**(1): p. 17-21.

38. Musgrave-Brown, E., et al., *Forensic validation of the SNPforID 52-plex assay.* Forensic Science International: Genetics, 2007. **1**(2): p. 186-190.

39. Xavier, C., et al., *Development and validation of the VISAGE AmpliSeq basic tool to predict appearance and ancestry from DNA.* Forensic Science International: Genetics, 2020. **48**: p. 102336.

40. Mullaney, J.M., et al., *Small insertions and deletions (INDELs) in human genomes.* Human molecular genetics, 2010. **19**(R2): p. R131-R136.

41. Weber, J.L., et al., *Human diallelic insertion/deletion polymorphisms.* Am J Hum Genet, 2002. **71**(4): p. 854-62.

42. Väli, Ü., et al., *Insertion-deletion polymorphisms (indels) as genetic markers in natural populations.* BMC genetics, 2008. **9**(1): p. 1-8.

43. Pereira, R. and L. Gusmão, *Capillary electrophoresis of 38 noncoding biallelic mini-Indels for degraded samples and as complementary tool in paternity testing.* DNA Electrophoresis Protocols for Forensic Genetics, 2012: p. 141-157.

44. Bhangale, T.R., M. Stephens, and D.A. Nickerson, *Automating resequencing-based detection of insertion-deletion polymorphisms.* Nat Genet, 2006. **38**(12): p. 1457-62.

45. Mullis, K., et al., *Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction.* Cold Spring Harb Symp Quant Biol, 1986. **51 Pt 1**: p. 263-73.

46. Virkler, K. and I.K. Lednev, *Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene.* Forensic science international, 2009. **188**(1-3): p. 1-17.

47. Sheppard, K., et al., *The adaptation of a 360° camera utilising an alternate light source (ALS) for the detection of biological fluids at crime scenes.* Sci Justice, 2017. **57**(4): p. 239-249.

48. Tobe, S.S., N. Watson, and N.N. Daéid, *Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA.* J Forensic Sci, 2007. **52**(1): p. 102-9.

49. Khaldi, N., et al., *Evaluation of three rapid detection methods for the forensic identification of seminal fluid in rape cases.* J Forensic Sci, 2004. **49**(4): p. 749-53.

50. Ballantyne, J., *SEROLOGY| Overview.* 2005.

51. Hochmeister, M.N., et al., *Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid.* J Forensic Sci, 1999. **44**(5): p. 1057-60.

52. Sakurada, K., K. Watanabe, and T. Akutsu, *Current methods for body fluid identification related to sexual crime: focusing on saliva, semen, and vaginal fluid.* Diagnostics, 2020. **10**(9): p. 693.

53. *Assembleia da República Portuguesa. Lei n.o 5/2008. 962–968 (Assembleia da República Portuguesa, 2008)*.

54. Walker, A.H., et al., *Collection of genomic DNA by buccal swabs for polymerase chain reaction-based biomarker assays.* Environ Health Perspect, 1999. **107**(7): p. 517-20.

55. García-Closas, M., et al., *Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(6): p. 687-96.

56. Milne, E., et al., *Buccal DNA collection: comparison of buccal swabs with FTA cards.* Cancer Epidemiol Biomarkers Prev, 2006. **15**(4): p. 816-9.

57. Smith, L.M. and L.A. Burgoyne, *Collecting, archiving and processing DNA from wildlife samples using FTA databasing paper.* BMC Ecol, 2004. **4**: p. 4.

58. Corradini, B., et al., *The importance of forensic storage support: DNA quality from 11-year-old saliva on FTA cards.* Int J Legal Med, 2019. **133**(6): p. 1743-1750.

59. Stangegaard, M., et al., *Repeated extraction of DNA from FTA cards.* Forensic Science International: Genetics Supplement Series, 2011. **3**(1): p. e345-e346.

60. Green, M.R. and J. Sambrook, *Isolation of High-Molecular-Weight DNA Using Organic Solvents.* Cold Spring Harb Protoc, 2017. **2017**(4): p. pdb.prot093450.

61. Walsh, P.S., D.A. Metzger, and R. Higuchi, *Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material.* Biotechniques, 1991. **10**(4): p. 506-13.

62. Burgos, G., et al., *Efficient preservation of DNA extracted from blood in FTA cards by Chelex method.* Forensic Science International: Genetics Supplement Series, 2019. **7**(1): p. 539-541.

63. QIAGEN, *STAR Q Punch AS User Manual*, in *Version 2*. 2022.

64. Hall, D.E. and R. Roy, *An evaluation of direct PCR amplification.* Croat Med J, 2014. **55**(6): p. 655-61.

65. Liu, J.Y., et al., *AutoMate Express™ forensic DNA extraction system for the extraction of genomic DNA from biological samples.* J Forensic Sci, 2012. **57**(4): p. 1022-30.

66. Barbosa, C., et al., *DNA extraction: finding the most suitable method*, in *Molecular microbial diagnostic methods*. 2016, Elsevier. p. 135-154.

67. QIAGEN. *QIAGEN Validation Report — Developmental validation of the forensic QIAsymphony workflow*. [cited 2023; Available from: https://www.qiagen.com/us/resources/resourcedetail?id=0042711e-316f-4a8b-b374-394f5d9e5934&lang=en.

68. Promega. *Promega Corporation Technical Manual #TMD037-SwabSolutionTM Kit*. [cited 2023; Available from: http://www.promega.com/resources/protocols/technical-manuals/101/swabsolution-kit-protocol.

69. Gill, P., A.J. Jeffreys, and D.J. Werrett, *Forensic application of DNA 'fingerprints'.* Nature, 1985. **318**(6046): p. 577-9.

70. Higuchi, R., et al., *Simultaneous amplification and detection of specific DNA sequences.* Biotechnology (N Y), 1992. **10**(4): p. 413-7.

71. Smith, H.O. and K.W. Wilcox, *A restriction enzyme from Hemophilus influenzae. I. Purification and general properties.* J Mol Biol, 1970. **51**(2): p. 379-91.

72. Canene-Adams, K., *General PCR.* Methods Enzymol, 2013. **529**: p. 291-8.

73. Chamberlain, J.S., et al., *Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification.* Nucleic Acids Res, 1988. **16**(23): p. 11141-56.

74. Cho, W.C., et al., *Validation and assessment of the Investigator 24plex QS kit for forensic casework application: Comparison with the PowerPlex fusion system and GlobalFiler™ PCR amplification kits.* Leg Med (Tokyo), 2021. **52**: p. 101902.

75. Ensenberger, M.G., et al., *Developmental validation of the PowerPlex() Fusion 6C System.* Forensic Sci Int Genet, 2016. **21**: p. 134-44.

76. Wang, D.Y., et al., *Developmental validation of the GlobalFiler() Express PCR Amplification Kit: A 6-dye multiplex assay for the direct amplification of reference samples.* Forensic Sci Int Genet, 2015. **19**: p. 148-155.

77. Edwards, M.C. and R.A. Gibbs, *Multiplex PCR: advantages, development, and applications.* PCR Methods Appl, 1994. **3**(4): p. S65-75.

78. Butts, E.L.R., et al., *NIST validation studies on the 3500 Genetic Analyzer.* Forensic Science International: Genetics Supplement Series, 2011. **3**(1): p. e184-e185.

79. Lazaruk, K., et al., *Genotyping of forensic short tandem repeat (STR) systems based on sizing precision in a capillary electrophoresis instrument.* Electrophoresis, 1998. **19**(1): p. 86-93.

80. Butler, J.M., et al., *Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis.* Electrophoresis, 2004. **25**(10-11): p. 1397-412.

81. Buckleton, J.S., J.-A. Bright, and D. Taylor, *Forensic DNA evidence interpretation*. 2018: CRC press.

82. Bregu, J., et al., *Analytical thresholds and sensitivity: establishing RFU thresholds for forensic DNA analysis.* J Forensic Sci, 2013. **58**(1): p. 120-9.

83. Gill, P., R. Puch-Solis, and J. Curran, *The low-template-DNA (stochastic) threshold—Its determination relative to risk analysis for national DNA databases.* Forensic Science International: Genetics, 2009. **3**(2): p. 104-111.

84. Gibb, A.J., et al., *Characterisation of forward stutter in the AmpFlSTR SGM Plus PCR.* Sci Justice, 2009. **49**(1): p. 24-31.

85. Walsh, P.S., N.J. Fildes, and R. Reynolds, *Sequence Analysis and Characterization of Stutter Products at the Tetranucleotide Repeat Locus VWA.* Nucleic Acids Research, 1996. **24**(14): p. 2807-2812.

86. Walsh, P.S., N.J. Fildes, and R. Reynolds, *Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA.* Nucleic Acids Res, 1996. **24**(14): p. 2807-12.

87. Magnuson, V.L., et al., *Substrate nucleotide-determined non-templated addition of adenine by Taq DNA polymerase: implications for PCR-based genotyping and cloning.* Biotechniques, 1996. **21**(4): p. 700-9.

88. Butler, J.M., Y. Shen, and B.R. McCord, *The development of reduced size STR amplicons as tools for analysis of degraded DNA.* J Forensic Sci, 2003. **48**(5): p. 1054-64.

89. Dash, H., P. Shrivastava, and S. Das, *Analysis of Capillary Electrophoresis Results by GeneMapper ID-X v 1.5 Software*. 2020. p. 213-237.

90. Burger, J., et al., *DNA preservation: a microsatellite-DNA study on ancient skeletal remains.* Electrophoresis, 1999. **20**(8): p. 1722-8.

91. Freire-Aradas, A., et al., *A new SNP assay for identification of highly degraded human DNA.* Forensic Sci Int Genet, 2012. **6**(3): p. 341-9.

92. Fondevila, M., et al., *Challenging DNA: Assessment of a range of genotyping approaches for highly degraded forensic samples.* Forensic Science International: Genetics Supplement Series, 2008. **1**(1): p. 26-28.

93. Dixon, L.A., et al., *Analysis of artificially degraded DNA using STRs and SNPs--results of a collaborative European (EDNAP) exercise.* Forensic Sci Int, 2006. **164**(1): p. 33-44.

94. Karkar, S., et al., *Statistical modeling of STR capillary electrophoresis signal.* BMC Bioinformatics, 2019. **20**(16): p. 584.

95. Hill, C.R., et al., *Characterization of 26 miniSTR loci for improved analysis of degraded DNA samples.* J Forensic Sci, 2008. **53**(1): p. 73-80.

96. Senge, T., et al., *STRs, mini STRs and SNPs--a comparative study for typing degraded DNA.* Leg Med (Tokyo), 2011. **13**(2): p. 68-74.

97. *Backlogs of Forensic DNA Evidence*. 2010 2023]; Available from: https://nij.ojp.gov/topics/articles/backlogs-forensic-dna-evidence.

98. Nelson, M.S., R. Chase, and L. DePalma, *Making sense of DNA backlogs, 2012: Myths vs. reality*. 2013: US Department of Justice, Office of Justice Programs, National Institute of Justice.

99. Pratt, T.C., et al., *This isn't CSI: Estimating the national backlog of forensic DNA cases and the barriers associated with case processing.* Criminal Justice Policy Review, 2006. **17**(1): p. 32-47.

100. Sciences, G.H.L., *Instructions for EasiCollect™.* 2013.