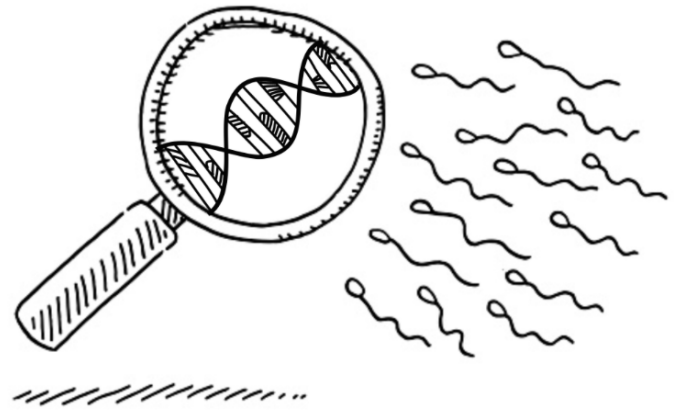
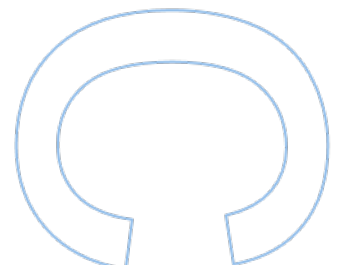
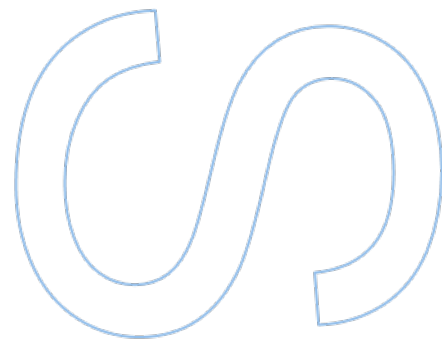
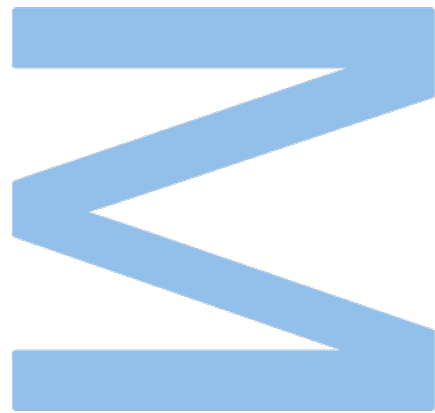




Implementation of a preliminary test for the detection of semen traces



Ana Rita Martins Santos

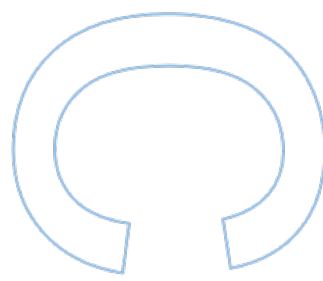
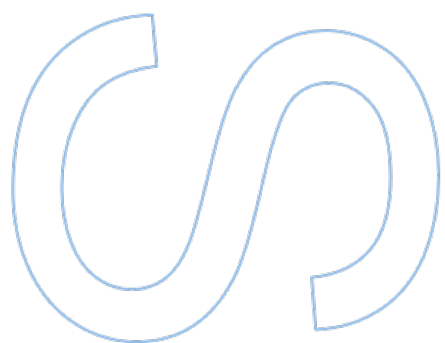
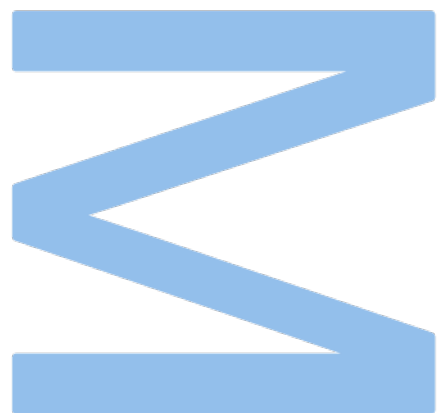
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2022/2023

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Acknowledgements

I would like to express my gratitude to all individuals and institutions that contributed to the successful completion of this study. Their support and valuable insights have been instrumental in the completion of another cycle of studies.

Firstly, I would like to acknowledge the invaluable support of my family. I am grateful for their unconditional love, and encouragement and for inspiring me to pursue my aspirations. Without their unwavering backing, this accomplishment would not have been possible.

To Aunt Ana for her support in legal matters.

To my co-supervisor, Iva Gomes, for accepting me as her master's student and reviewing this work.

To Faculdade de Ciências da Universidade do Porto and the respective city for their warm welcome.

To my classmates and now friends, Inês and Alejandra, thank you for making this journey in Invicta so much more beautiful.

To the Polícia Judiciária and the LPC for becoming home over the last year.

A special thanks to Sandra Costa, the boss of BBG, for entrusting me with this project and for the informative "5 minutos à Benfica" meetings.

To Paulo Miguel, my supervisor, thank you for your guidance throughout my project, and for all the mantras given to me, especially the one that goes "If you don't have a dog, you hunt with a cat."

The BBG team welcomed me and provided an opportunity to learn about the area that brings me joy, resulting in a memorable experience. A special thanks to Marco and Carolina for collaborating on my study.

To Ana Ramalhete, my PJ mummy, for all the support and dedication in this joint adventure and all the affection, but also all the "ralhetes".

To my colleagues "Patinhas" Ana and Bia, I am grateful for the memories we have shared both in and out of room 1.043. The internship year would not have been as enjoyable without your company.

To AXO Science, particularly Florian Tharin, for supporting my research and affording me the chance to present at their STK Sperm Tracker Workshop conference held in Cergy.

To Clínica IERA Lisboa Quirón Salud, I extend my gratitude, particularly to Mafalda Rato, Catarina Mello, and Marta Ramos for their contribution in providing and preparing the samples for my work.

I am thankful to my friends for their invaluable emotional support during my master's degree program, particularly during the thesis writing process.

And finally, I would like to express my appreciation to my Best Friend Mariana for her unwavering support during this process and for her assistance.

Resumo

Os crimes de natureza sexual consistem em todos os atos sexuais, consumados ou tentados, realizados sem o consentimento ou contra a vontade de uma pessoa. Este tipo de crimes tem vindo a aumentar ao longo dos anos e, consequentemente, as perícias técnico-científicas do sector BBG do LPC-PJ também têm vindo a progredir. Nos casos de crimes sexuais, o sémen é o vestígio biológico mais comum nas cenas de crime. A identificação deste fluido corporal é crucial, uma vez que a sua deteção constitui uma prova extremamente valiosa para comprovar a ocorrência de atividade sexual.

De forma a melhorar as metodologias na etapa de observação, especificamente na deteção de vestígios de sémen, este estudo teve como objetivo avaliar a eficácia das metodologias STK[®] Lab e STK[®] Spray, em superfícies porosas, de forma a determinar a sua potencial implementação e uma possível substituição do teste AP. Adicionalmente, o estudo teve como objetivo validar a eficácia da metodologia STK[®] Spray em superfícies não porosas, validar o método de microscopia ótica, coloração CT, e avaliar a possibilidade de substituir o método de microscopia ótica frequentemente utilizado no laboratório, coloração HE.

Para determinar o melhor *workflow* para os casos que envolvem este tipo de vestígios, foram efetuados dois testes. Um teste, onde foram utilizadas 66 amostras reais, adquiridas de casos de crimes sexuais. Estas amostras foram submetidas ao *workflow* utilizado pelo laboratório para este tipo de casos e utilizou-se adicionalmente o STK[®] Lab e STK[®] Spray e o teste de confirmação de sémen, coloração CT. Paralelamente, foi realizado um teste comparativo com amostras simuladas para aferir a eficácia das metodologias, com o objetivo de estudar diferentes características. Assim, foi subdividido em vários testes, tais como: Sensibilidade; Especificidade; Repetibilidade e Reprodutibilidade; Durabilidade; Verificação da qualidade do perfil de DNA; Teste STK[®] Spray e Teste de Coloração de Lâminas.

Os resultados indicam que as metodologias STK[®] Lab e STK[®] Spray são mais sensíveis, específicas, fáceis de utilizar e não tóxicas em comparação com o teste AP. Além disso, o STK[®] Lab é a abordagem mais adequada para superfícies porosas, enquanto o STK[®] Spray é mais adequado para superfícies não porosas. Este produto tem o potencial de melhorar o fluxo de trabalho da análise de vestígios de sémen, facilitando um trabalho mais rápido e eficiente no domínio dos crimes sexuais no sector BBG do LPC-PJ.

Palavras-chave: Crimes sexuais, sémen, testes preliminares, teste AP, STK[®] Lab, STK[®] Spray

Abstract

Crimes of a sexual nature consist of all sexual acts, consummated or attempted, carried out without a person's consent or against their will. These types of offences have been increasing over the years, and consequently, technical-scientific expertise in the BBG sector of the LPC-PJ in these cases has also progressed. In sexual offence criminal cases, semen is the most common biological trace at crime scenes. The identification of this body fluid is crucial, as detecting it provides extremely valuable evidence to prove the occurrence of sexual activity.

In order to improve methodologies at the observation stage, specifically in the detection of traces of semen, this study aimed to assess the efficacy of the STK[®] Lab and STK[®] Spray methodologies, on porous surfaces. The goal was to determine their potential implementation and potential replacement of the AP test. Additionally, the study aimed to validate the effectiveness of the STK[®] Spray methodology on non-porous surfaces, validate the optical microscopy method, CT staining, and evaluate the possibility of replacing the optical microscopy method frequently used in the laboratory, HE staining.

To determine the best workflow for cases involving this type of trace, two tests were conducted. In the first test, a total of sixty-six samples were used, acquired from cases involving sexual offences. These samples were submitted to the workflow used by the laboratory for this type of case and additionally used the STK[®] Lab and STK[®] Spray and the semen confirmation test, CT. At the same time, a comparative test was carried out with simulated samples to ascertain the methodologies' efficacy, with the aim of studying different characteristics. It was thus subdivided into various tests, such as: Sensitivity; Specificity; Repeatability and Reproducibility; Durability; Verification of the quality of the DNA profile; STK[®] Spray Test and Slide Staining Test.

The results indicate that the STK[®] Lab and STK[®] Spray methodologies are more sensitive, specific, user-friendly and non-toxic compared to the AP test. Additionally, STK[®] Lab appears to be the preferred approach for porous surfaces, while STK[®] Spray is more suitable for non-porous surfaces. This product has the potential to enhance the semen trace analysis workflow, facilitating faster and more efficient work within the domain of sexual crimes in the BBG sector of the LPC-PJ.

Keywords: Sexual crimes, semen, preliminary tests, AP test, STK[®] Lab, STK[®] Spray

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List of Abbreviations

ALS – Alternative Light Source

AP – Acid Phosphatase

AP test – Acid Phosphatase test

APAV – Associação Portuguesa de Apoio à Vítima

AS – Assay Setup

BBG – Biology sector

bps – Base pairs

CE – Capillary Electrophoresis

CF – Cellular fraction

cm – Centimetres

CPP – Código Processo Penal

CT – Christmas Tree

C_T – Cycle Threshold

DNA – Deoxyribonucleic acid

dNTPs – Deoxyribonucleotide triphosphates

DTT – Dithiothreitol

ENFSI – European Network of Forensic Science Institutes

EPG – Electropherogram

HE – Hematin/Eosin

IERA – Instituto Extremeño de Reproducción Asistida Lisboa Quirón Salud

INMLCF – Instituto Nacional de Medicina Legal e Ciências Forenses

IPC – Internal PCR Control

ISFG – International Society for Forensic Genetics

LPC – Laboratório de Polícia Científica

MF – Male fraction

mg – Miligram

ml – Millilitres

NC – Negative Control

nm – Nanometres

PC – Positive Control

PCR – Polymerase Chain Reaction

PJ – Polícia Judiciária

PK – Proteinase K

PSA – Prostatic Specific Antigen

PSA test – Prostatic Specific Antigen test

qPCR – Quantitative PCR

RFUS – Relative Fluorescence Units

rpm – Rotations per minute

SNPs – Single Nucleotide Polymorphisms

SP – Sample Preparation

SPE – Solid Phase Extraction

STK[®] – STK[®] Sperm Tracker

STK[®] Lab – STK[®] Sperm Tracker Lab

STK[®] Spray – STK[®] Sperm Tracker Spray

STRs – Short Tandem Repeats

UV – Ultraviolet

VNTR – Variable Number Tandem Repeats

µl – Microlitres

°C – Degree Celsius

Chapter I – Forensic Genetics - History and the Portuguese Case

1. Forensic Genetics

The history of Forensic Genetics began in 1900, when the Austrian scientist Karl Landsteiner discovered that human beings could be divided into four different blood groups based on their blood type: A, B, AB and O. Landsteiner discovered the ABO system of blood groups, which unfortunately has limited discriminatory efficacy [1].

In the 1980s, Dr Alec Jeffreys of the University of Leicester in the UK made a breakthrough discovery about the true nature of Deoxyribonucleic Acid (DNA). Contrary to previous understanding, DNA is a highly complex molecule consisting of two chains connected by chemical bonds, containing genetic information necessary for the evolution and functioning of organisms. During an experiment with samples from members of his technician's family, it was noted that there were both similarities and differences in their DNAs. Upon discovering that DNA sequences varied in number and order for each individual, Jeffery's developed a technique that utilises specific DNA sequence variations to create a distinct genetic profile for each individual. The DNA Fingerprinting technique led to the emergence of a new field in Forensic Science. Its primary objective is to compare profiles obtained from samples recovered at crime scenes with reference samples of individuals who may have been involved in the case. This aids in making a match or exclusion. The implementation of this technique has introduced novel methodologies for obtaining evidence, which assist criminal investigation [2,3].

The first forensic case where DNA Fingerprinting was instrumental in solving occurred in 1987 when two adolescent females were raped and murdered in distinct incidents, one in 1983 and the other in 1986, in neighbouring English towns. The resemblances between the two crime scenes convinced the authorities that the same individual was responsible despite the lapse of three years. Presently in police custody, an individual named Richard Buckland reportedly confessed to committing the second crime. Using Jeffreys' method, an analysis was conducted on the suspect's reference sample and compared with the samples obtained from the crime scenes. The analysis concluded that they were from the same person but not compatible with the suspect, thus disproving him as the killer they were searching for. To identify the true suspect, a comprehensive search of the area was undertaken, and all adult males were requested to provide a reference sample for testing. By using this method, a profile could be obtained and then cross-referenced against the profile derived from the semen samples.

Over five thousand samples were scrutinized, yet none aligned with the killer's profile, hence why both the authorities and the public became disenchanted with this pioneering method of individual identification. Six months into the investigation, a woman reported overhearing a conversation about giving a reference sample on behalf of Colin Pitchfork, a former colleague. Upon matching the DNA profile found at the crime scenes, Colin was detained and ultimately confessed to both crimes [2,4].

Around 1987, companies and medical, legal, and judicial institutions embarked on improving and applying the DNA Fingerprinting technique. The Lister Institute in Leicester, where Jeffery's worked, was the only institution practising it at the time. The 1990s saw an increase in its usage, and the technique has been constantly optimised to the present day. DNA evidence has become a fundamental tool since it provides highly specific and unique evidence for each individual (except for monozygotic twins). The use of this technique can be applied to various types of scenarios, including paternity tests, cadaver identification, etc. This allows for the identification of potential culprits and the provision of evidence in support of the court's decision [3].

2. Portuguese legislation on DNA and forensic ethics

According to article 1.º of the *Código de Processo Penal (CPP)* in the Portuguese legal system, a crime is defined as "the set of assumptions which the application of a criminal penalty or security measure to the perpetrator depends on." [5,6].

To solve crimes, the criminal investigation and the court require evidence that proves or disproves the "existence or non-existence of the crime" and relates to the suspect so that they can be punished or absolved [7,8]. Expert evidence, as stated in article 151.º of the CPP, is considered a recognised method of proof in an investigation that which requires technical, scientific or artistic knowledge [9]. This expertise can only be conducted in appropriate official establishments, such as the *Laboratório de Polícia Científica (LPC)*, which is autonomous and independent at a technical and scientific level, as specified in article 16.º of Law n.º 42/2009 of 12th February, since it has the competence to "research, collect, process, record traces and carry out expertise in the various fields of forensic science" [10,11].

Law n.º 90/2017, article 2.º, defines the reference sample as "the sample used for comparison" and the problem sample as "the sample, under investigation, whose identification is intended to be established". The sample refers to "any biological trace of human origin intended for DNA analysis, obtained directly from a person or collected from a corpse, part of a corpse, animal, thing or place where it is collected for

identification purposes". These samples are used to obtain DNA profiles, which are defined as "the result of analysing the sample using a DNA marker obtained according to scientifically validated and internationally recommended techniques" [12].

DNA databases are indispensable and useful tools for criminal investigations nowadays. They allow cooperation between countries to exchange data with the aim of deterring crime, which has led several countries to update their legislation on the subject. Nonetheless, legislative approaches vary among nations [13].

With the aim of civil and criminal identification, a DNA database was created in Portugal and approved in 2008. Initially regulated by Law n.º 5/2008 of 12th February, it has already suffered two amendments to date, with the latest being Law n.º 90/2017, which is currently in use [14].

This database consists of DNA profiles, which are obtained by the LPC or the *Instituto Nacional de Medicina Legal e Ciências Forenses (INMLCF)* [15]. The insertion and removal of profiles is the responsibility of the INMLCF, as mentioned in article 16.º of Law n.º 90/2017, and is carried out according to guidelines designed for the proper functioning of the database [16,17]. For the insertion of DNA profiles into the database, there are markers that, according to Order n.º 161/2018 of 6th July, are mandatory, which are the autosomal Short Tandem Repeats (STRs): vWA, THO1, D21S11, FGA, D8S1179, D3S1358, D18S51 and the gender determining gene, the amelogenin [18]. Law n.º 90/2017 also applies to the processing of samples are processed: collection, preservation, analysis and profiling, as well as the method to be used when comparing profiles [19].

A survey carried out in Portugal regarding the voluntary provision of DNA samples and their inclusion in the National Database concluded that while this practice would have social benefits in that it would add value to the fight against crime, but the lack of legislation on data protection is concerning [20].

Chapter II – Biology Sector - sample processing in the laboratory

The Biology Sector (BBG) is one of the sectors that make up the Biotoxicology area of the LPC, which belongs to the Central Units of Specialised Technical-Scientific Support of the *Polícia Judiciária (PJ)*, as can be seen in Figure 1. This sector is responsible for forensic activities involving biological material, alongside the crime scene sector, but also for the implementation of new work methodologies [21].

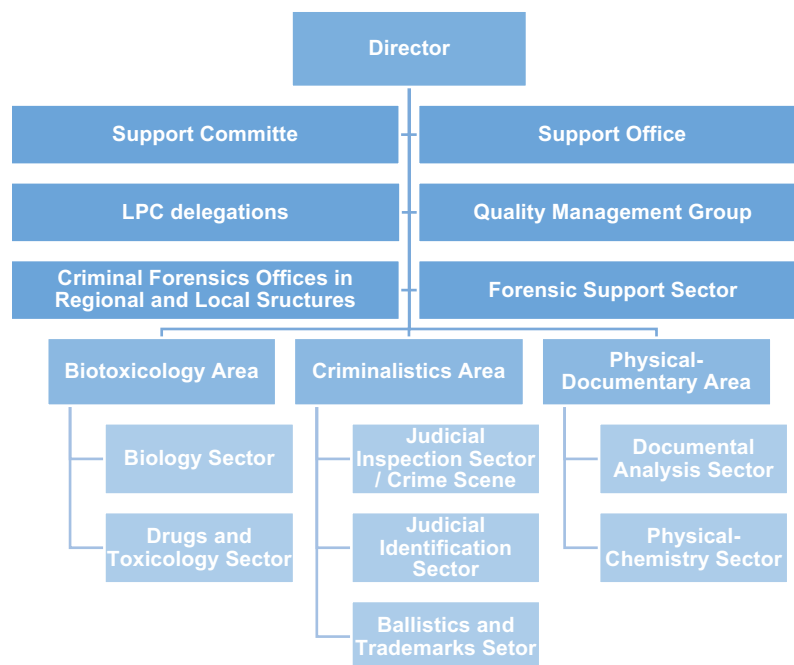


Figure 1 - Organizational chart of the LPC - PJ. [Adapted from: Instrução Permanente de Serviço 2/2016].

Belonging to forensic groups such as the ENFSI (European Network of Forensic Science Institutes) [22] and the ISFG (International Society for Forensic Genetics) [23], forensic experts attend regular training sessions, meetings, and proficiency tests so that their individual work, as a sector, and collective work, as a laboratory, are evaluated against predetermined standards. This sector is therefore prepared to respond to various types of queries, such as: the identification of biological traces; determination of genetic profiles from reference and problem samples using autosomal and sex markers (Y chromosome); comparative analysis of DNA profiles; use of statistical calculations to obtain more reliable results; paternity tests in the criminal justice field and participation

in the contribution of DNA profiles to the database as stipulated in Law n.º 5/2008 of 12th February [21]. In this way, it is possible to resolve the requests made by criminal investigations through the practice of this expert activity, biology and criminal genetics, where the most important ingredient is DNA.

1. DNA structure

DNA is a molecule that contains all the genetic information for the functioning and reproduction of cells in a living organism. This double-stranded, helical-shaped molecule, a model first described by Watson and Crick in 1953, is located in the nucleus of cells [24]. DNA is made up of a set of nucleotides, each composed of one of the four nitrogenous bases, purines (adenine and guanine) or pyrimidines (cytosine and thymine), a five-carbon sugar (deoxyribose) and a phosphate group (Figure 2). The two chains are held together through complementary pairing by hydrogen bridges, two, of which are established between adenine and thymine, and three between guanine and cytosine [25].

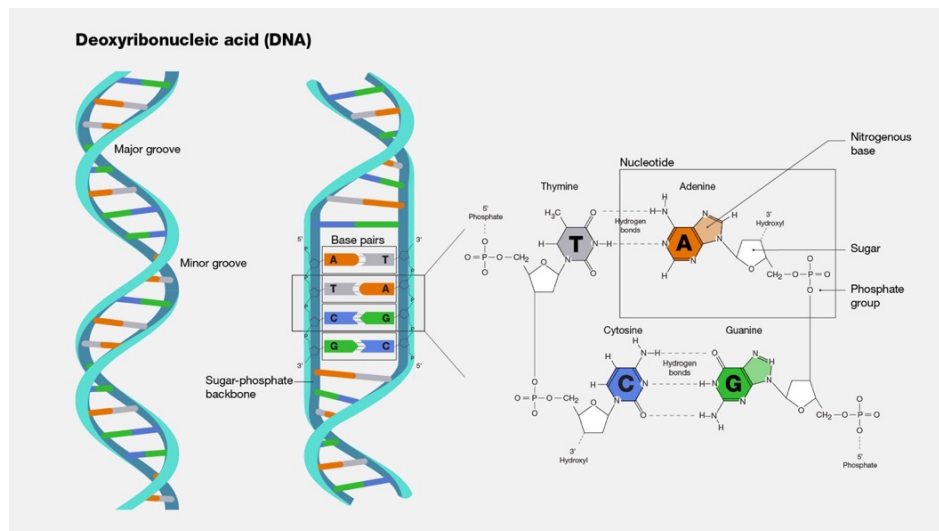


Figure 2 - Structure of the DNA molecule [Adapted from: www.genome.gov/genetics-glossary/Deoxyribonucleic-Acid].

This molecule is condensed and distributed over 23 pairs of hereditary chromosomes, 50 % of which come from the male parent and the other 50 % from the female parent. These sets of chromosomes are similar in both sexes and are found in 22 pairs of chromosomes, the autosomes, and the missing pair of chromosomes is the determinant of the individual's sex, the sex chromosomes (the X and Y chromosomes) [26]. All of the genetic information in the cell of a living being, which is about three billion

base pairs, is called the genome [27]. Biological diversity is explained by the variation in the order of the nitrogenous bases, which makes living beings different. In the *Homo sapiens* species, some regions are identical among all individuals and others differentiate them [26,28].

Of the human genome, the region that differs from individual to individual (polymorphic) is of real interest for forensic activity, and this part of the DNA, which does not code for protein synthesis, is located in the extragenic regions [24,29]. DNA is present in most of the cells that make up a human being's organism, it's unique to each person [29]. In this way, DNA has become an asset for forensic investigation in the field of biology, because we may leave our biological footprint behind or at a location. Therefore, it is possible to use this molecule as irrefutable evidence in the identification of those possibly involved, victim(s) and suspect(s), in a crime under investigation [30].

1.1. Types of genetic polymorphisms used in the forensic laboratory

In forensic practice, the sections of DNA that are studied are present in variable regions representing just 0.3 % of our total genetic code [26]. This small percentage allows differentiation between two individuals, except in the case of true twins, since in this portion, DNA analysis for each subject is variable when using recombinant markers. From generation to generation, DNA recombination occurs, resulting in random modifications to the genetic material inherited from each parent during meiosis [31]. This event occurs in the markers of the X chromosome and the autosomal chromosomes, leading to genetic diversity. This diversity makes it possible to determine the correlation between two samples, as knowledge of human DNA has allowed the development of markers from specific regions, which are analysed in the configuration of sequence and length polymorphisms [26,31].

The polymorphisms studied can be minisatellites or Variable Number Tandem Repeats (VNTR), Single Nucleotide Polymorphisms (SNPs) and microsatellites or STRs.

VNTRs were the first markers used in cases involving forensic genetics. These markers are length polymorphisms, ranging from six to one hundred base pairs (bps), which consist of repeated sequences in tandem form varying in different regions of the genome of a single individual and also between different individuals [32]. However, this type of polymorphism is no longer used due to the need of use of a large amount of DNA, and thus cannot be applied in cases of limited samples, with low amounts of DNA, or degraded samples [29].

Unlike the markers described above, SNPs are sequence polymorphisms. In this marker, the difference between the sequences of the samples analysed is only in the variation of a single nucleotide, resulting from a mutation during DNA replication. Since a high level of discriminatory power is essential, it is necessary to analyse a large number of these markers in order to use this type of polymorphism [29].

STRs, like VNTRs, are length polymorphisms. These are repeated in short tandem sequences ranging from one to six bps, with tetranucleotide repeats being the most commonly used in forensic applications. This polymorphism permits differentiation between individuals since the number of repeats is variable and is caused by random mutations [32]. STR polymorphisms have come to be used in the field of Forensic Genetics due to various characteristics, such as [33]:

- Found in large quantities in the human genome, particularly in the non-coding regions;
- Highly polymorphic markers;
- Possibility of amplification in a multiplex system, allowing several loci to be amplified simultaneously (in one single reaction);
- Possibility of automated analysis;
- Simple to interpret results;
- Possible use in cases of limited and degraded samples.

2. Workflow in a Forensic Biology Laboratory

The workflow in the BBG sector of the LPC–PJ is divided into three stages: Preservation, Observation and DNA Analysis. This workflow consists of processing DNA samples that need to pass through these three stages, which will be described below, to obtain results in the form of a genetic profile and consequently to be able to make comparisons between them.

2.1. Preservation

Preservation is the initial stage in the BBG sector, where the expert first comes into contact with the evidence after it has been collected by the crime scene team. At this stage, the expert is responsible for checking that all the samples included in the request for examination have been properly packed and identified. This stage also involves separating the reference samples from the problem samples. Problem samples

are samples with unknown origin and where the evidence is found that will be analysed [34]. Reference samples are samples that are taken from individuals that may be related to the case under investigation and used to carry out comparison studies to match or exclude suspects and victims from a determined crime scene [32]. Reference samples can also be obtained from direct family members in order to be used in investigative cases such as paternity tests, missing people, cadaver identification, etc. [35].

To properly pack biological evidence, the use of plasticised materials should be avoided, as DNA tends to degrade more quickly due to the creation of moisture [34]. Thus, packaging should be done in paper bags/envelopes to allow the traces to “breathe” [34]. They should be stored in dry places at an environmental temperature so that the DNA molecule can be preserved until it is submitted for analysis, thus keeping it stable and preventing its degradation [35,36]. When packed correctly, the envelopes must be sealed to avoid contamination and labelled to guarantee the so-called “chain of custody”. This consists of documenting the evidence’s complete journey to guarantee its authenticity [37].

2.2. Observation

The daily life of a forensic expert in the field of biology can be very challenging, coming across different types of crimes, from homicides to rapes and thefts, among others. Observation mirrors this reality, where reading a criminal case can determine what type of trace is to be researched and what methodology is to be used since we are faced with samples that we do not know the origin.

The first step in this stage is to carry out an unequivocal description of the evidence under analysis. This description must contain certain information, such as the name/number attributed to that evidence, and a photographic record before carrying out any type of testing and collection, among other information, in order to guarantee the chain of custody, mentioned in the previous section [36].

Biological traces that can be analysed in the laboratory include blood, semen, saliva, and rooted hair, among others [26]. Often these traces are “camouflaged” in the objects under observation, due to various aspects such as tone, porosity, and material, which makes them difficult to visualise and undetectable to the naked eye [38]. Checking the condition of the stain under examination is essential, as it can influence how it is tested and collected [36].

To eventually determine the type of biological fluid, there are various orientation and confirmation tests that can be carried out. Since some stains may not be noticeable

macroscopically, it is necessary to use an alternative light source, such as the Crime-lite® ML2 (FOSTER & FREEMAN LIMITED, England), which with a combination of wavelengths and filters, makes it possible to visualise some stains, proving to be an essential method in these cases [39]. In addition, preliminary tests are carried out, which serve only as a guide to determine the type of trace. For blood research, these are based on the enzyme-like activity of haemoglobin peroxidase [40], such as the Kastle Mayer test [41], and for semen research, the Acid Phosphatase test, and the chromatographic immunoassay SERATEC® PSA Semiquant (SERATEC®, Germany), which will be discussed in the next chapter. Confirmatory tests can also be carried out, which clearly indicate the type of trace, such as staining slides of semen samples to visualise the sperm cells using optical microscopy [42], performing Hematin/Eosin and Christmas Tree staining on the Panthera Cloud optical microscope (Motic, China), visualisation of hairs to identify the species of origin (human or animal) [43], and the chromatographic immunoassay for the specific detection of human blood using the SERATEC® HemDirect test (SERATEC®, Germany) [44].

To obtain a reportable genetic profile, it is necessary to collect the minimum amount possible that contains the maximum amount of DNA [36]. At this stage, the origin of the sample is known, but its quality and quantity are unknown.

2.3. DNA analysis

The DNA analysis stage in the BBG sector is subdivided into two stages: reference samples and problem samples. The workflow for reference samples is conducted over three steps: extraction, amplification and detection of the amplified DNA. Problem samples require prior preparation appropriate to each type of trace, and there is a specific protocol for samples containing hair or semen. Samples containing semen require a more complex preparation so that separation between fractions is possible [45]. The procedure for problem samples is conducted over four steps: extraction, quantification, amplification and detection of the amplified DNA [24].

2.3.1. Extraction

To obtain a genetic profile from a problem sample, the first thing to do is to purify the DNA. To do this, a DNA extraction is made, since in the biological evidence collected at a crime scene, other cellular components are always involved with the molecule of interest [26].

There are different extraction techniques: organic extraction, Chelex extraction and solid phase extraction (SPE) [46]. Organic extraction has been used for many years and is useful for extracting larger fragments. However, the organic reagent used to denature proteins, phenol-chloroform, proved to be toxic and the extraction process is complex and time-consuming [35,47]. Unlike the previously described extraction, Chelex extraction is a simple process that uses ion exchange resins, which act as a chelating group to create a stable medium by binding with metal ions [48]. In this method, PCR inhibitors are found in the extract, so there is a need to use another technique to minimise this problem. One of the most used extraction techniques in forensic laboratories today is SPE. This technique involves specifically binding the DNA molecule to a solid substrate, such as magnetic beads [49].

Since automated processing became a necessity due to the increase in the number of samples to be processed, extraction procedures began to be devised in the form of commercial kits that are currently used in forensic laboratories, such as the QIA Symphony DNA Investigator Kit (QIAGEN, Germany) allowing the consecutive extraction of up to 96 samples [50].

In the laboratory, sample processing begins with the collection being placed on the filter column that is inserted into the eppendorf. These samples are then placed in the QIAgility (QIAGEN, Germany) instrument, which distributes the reagents needed for each type of sample, as there is a specific preparation for each type of trace. This is followed by temperature agitation so that the cells are lysed and centrifugation so that all the contents are transferred from the filter column to the bottom of a tube. Then, in an automated process, the samples are transferred to the QIA Symphony equipment (QIAGEN, Germany) which contains specific compartments for all the reagents and samples subjected to purification, using magnetic beads in the Sample Preparation (SP) and the preparation of the samples for quantification and amplification in the Assay Setup (AS) [51]. The processing of the reference samples is similar to that of the abovementioned samples, except for the last step, the automated processing in the QIA Symphony equipment (QIAGEN, Germany), which is no longer carried out, since neither the purification and quantification steps are necessary because their origin is known [52].

2.3.1.1. Differential extraction

In the context of sexual crimes, it is necessary to carry out the method of differential extraction of semen samples in order to obtain profiles from epithelial cells, the cellular fraction (CF), and sperm cells, the male fraction (MF) [35]. The QIAcube equipment (QIAGEN, Germany) has facilitated the work of forensic experts by being able to carry out the entire differential separation process in an automated way [53].

Differential extraction is a method used by forensic laboratories in sexual assault cases to separate epithelial cells from sperm cells. This method begins by incubating the samples mixed with ATL Buffer mix (QIAGEN, Germany) with proteinase K (PK) in order to lyse the epithelial cells. It is then necessary to carry out a new, more violent lysis by adding Dithiothreitol (DTT) in the previously utilized mix, as the sperm cells are still not lysed. The addition of DTT to the mix is essential because it is capable of disintegrating the protein disulphide bridges that constitute the sperm cell membranes, since without it the sperm cells are impermeable to digestion [35,45].

In this way, it is possible to obtain two fractions that will generate two profiles, one obtained from epithelial cells and the other from sperm cells [35].

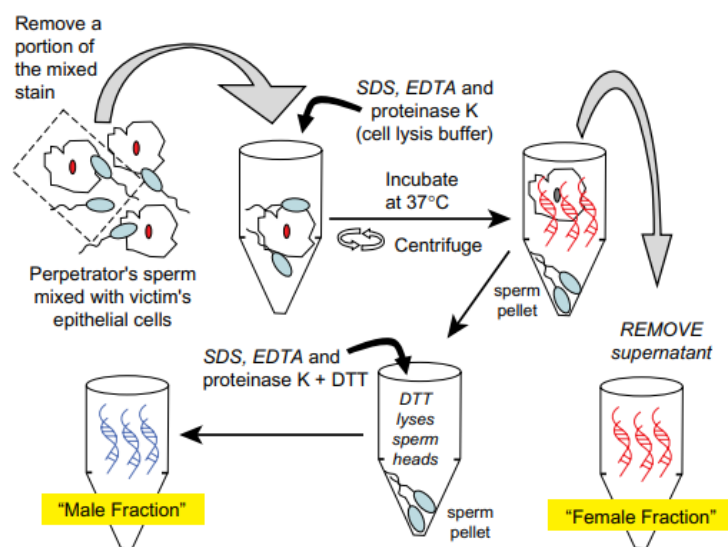


Figure 3 - Schematic illustration of differential extraction process used to separate male sperm cells from epithelial cells [34].

2.3.2. Quantification

Quantification is an indispensable stage in the processing of samples in the forensic area. This is especially important since this type of work is constantly presented with problem samples where the amount of DNA is unknown. In this way, an estimate is made of the amount of biological material in each sample analysed so that an appropriate amplification protocol can be carried out, avoiding any excess or absence of DNA [35].

The amount of DNA present in a sample makes it possible to determine the total product that can be amplified, using the minimum amount of DNA to obtain a complete profile. This tool is particularly useful when processing problem samples, where the amount of genetic material can vary and sometimes is limited, depending on the type of trace and its condition [54]. On the other hand, in reference samples, it is not common to carry out the quantification step, since this type of sample is theoretically of good quality and therefore of enough quantity [52].

Real-time PCR, also known as quantitative PCR (qPCR), is a technique that makes it possible to determine the relative quantity of samples based on a standard curve and also to indicate the degree of degradation. This technique is based on the use of a TaqMan probe with a reporter marker at one end and a quencher at the other end, which inhibits the fluorescence emission of the reporter marker when they are in proximity. There is complementarity between the probe and the sequence of the amplification product, pairing up during the PCR reaction. Then, during primer extension, the Taq polymerase enzyme cleaves the TaqMan probe leading to the separation of the reporter from the quencher, with the reporter marker emitting fluorescence, the intensity of the fluorescence produced being congruent with the amount of amplified DNA [35].

In this technique, as it is possible to monitor the production of amplified material in real-time, enables the determination of fluorescence intensity as a function of the number of cycles during the exponential phase, which is when the reaction components are in large quantities and the amplification products are being produced, and where the amplification is most consistent [55].

To determine the quantities, present in each sample, the value determined by the software of the number of cycles needed for the fluorescence to reach a specific level, known as the Cycle Threshold (C_T), is used. The C_T value and the initial amount of DNA present in a sample are disproportionate. The lower the C_T value, the bigger the initial amount of genetic material [35].

In the laboratory, this process begins in the AS module of the QIA Symphony equipment (QIAGEN, Germany) where an optical plate is prepared and placed in the QuantStudio equipment (Thermo Fisher Scientific, USA). In this process, the commercial

Quantifiler Trio kit (Thermo Fisher Scientific, USA) is used to amplify three specific markers: Large, Small and Y. By relating information acquired from the first two markers, Large and Small, it is possible to determine the level of degradation of the sample and the Y chromosome marker is useful in discriminating cases of results for where there are mixtures between male and female individuals [3,56]. The Internal PCR Control (IPC), which is also included in the abovementioned kit, is used to check for reaction inhibitors and to verify if the amplification reaction went as expected [56].

2.3.3. PCR amplification and Profiling

The advent of the Polymerase chain reaction (PCR) technique, described by Kary Mullis in the 1980s, enabled the amplification of samples containing small amounts of DNA and consequently obtained genetic profiles [35].

The PCR technique is based on the amplification of a specific fragment, resulting in a large number of copies of a particular DNA sequence [35]. In Forensic Genetics, it is an advantage, since the type of samples analysed are frequently limited samples with a low quantity of genetic material [35]. To perform this technique, two primers are used, forward and reverse, complementary to each end of the sequence of interest, deoxyribonucleotide triphosphates (dNTPs) which have proportionally the same number of nitrogenous bases, the thermostable DNA polymerase enzyme, Taq polymerase, and a buffer, magnesium ions, serves to keep the pH and the concentration of ions, for the ideal reaction [32].

The technique is processed is summarised in three stages: Denaturation, Annealing and Extension, which occurs in consecutive reaction and double the product of interest every cycle [32]. In the first stage, denaturation, the double strand of the DNA molecule is separated due to the high increase in temperature. This is followed by a decrease in temperature so that the primers can bind to the regions of interest; this stage is called annealing. Finally, the extension occurs, which consists of the polymerisation of a new DNA chain using the Taq polymerase enzyme.

In the laboratory, the amplification process begins with the acquisition the values obtained during the quantification stage, which are automatically calculated by the QuantStudio equipment (Thermo Fisher Scientific, USA). The data is conveyed between equipment's via file transfer informing the AS module of the QIA Symphony equipment (QIAGEN, Germany) where the amplification plate is prepared and how the samples are prepared. This process involves the use of commercial kits that have primers containing fluorophores useful in the fragment detection stage. The amplification process is also

automated through the use of a thermal cycler, such as the Veriti Thermal Cycler (Thermo Fisher, USA).

The amplified material of STRs are detected through length separation, in a process known as Capillary Electrophoresis (CE). Detection is achieved via fluorescence emissions [35]. The methodology is executed in an automated way on a 3500 Series Genetic Analyzer (Thermo Fisher Scientific, USA), which has capillaries where an electric current is formed due to the presence of the negative electrode, the cathode, and the positive electrode, the anode [35]. This electric current allows the DNA fragments to migrate from the negative to the positive pole, since DNA is a negatively charged molecule and opposite charges are attracted [35,57]. The migration of the fragments takes place in a dissipated process, due to the presence of a polymer in the capillaries that acts as a separation matrix and provides resistance for the fragments, causing the smaller ones to pass through first, as they move through the polymer pores faster than the larger fragments, and are therefore the first to be detected [57]. Detection of the fragments occurs when they pass through an optical detection window, where the fluorophores, which were added previously, are excited, emitting fluorescence by a projected laser [29]. This fluorescence is converted by software into electrical signals of relative fluorescence units (RFUs), which are proportional to the fluorescence intensity and therefore proportional to the number of fluorophores, which in turn is proportional to the number of amplified fragments [32,35]. To identify the size of each fragment, a size standard, which is made up of known fragments of different sizes (bps), is added to each sample, and the allelic ladder, which consists of an artificial mixture, made up of the most frequent fragments present in the human population, is placed separately but in the same assay [29,35].

2.3.4. Profile interpretation

The step of obtaining a genetic profile is completed by genotyping the samples through the use of a software program, GeneMapper ID-X 1.6 (Thermo Fisher Scientific, USA). This program has the capacity to assign each STR detected in the capillary electrophoresis and its corresponding allele [35]. The alleles are assigned by comparing the allelic ladder with the size of the standard size fragments of each sample [29]. The results are presented in an electropherogram (EPG) format, which includes all the alleles in the sample represented by peaks and organised by markers which, in turn, are arranged by fluorophores, as illustrated in Figure 4 [32].

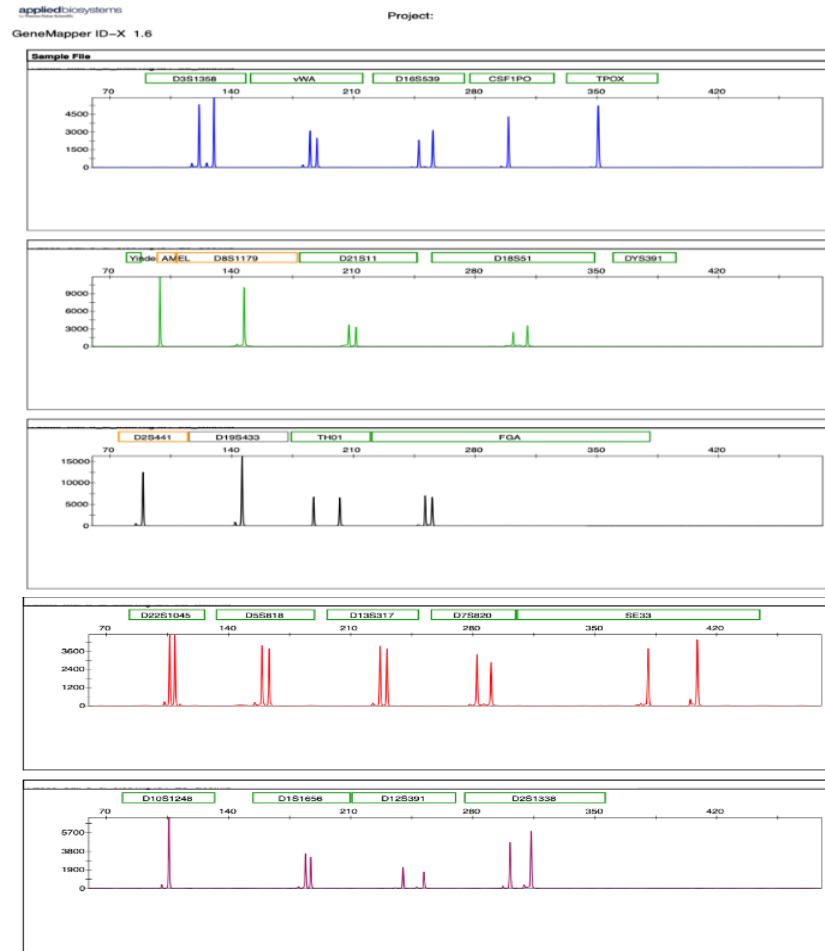


Figure 4 - Result of an EPG as an example of a single female DNA profile.

The EPG is presented in the form of a graph, where the intensity of the fluorescence detected in each fragment, is represented by RFUs on the ordinate axis, which is related to the quantity of DNA. Meanwhile, the abscissa axis represents the fragment's detection time by the laser, which corresponds to its size [32]. The graphic arranges the fragments in ascending order of size, with the smallest fragments appearing in the markers on the left and the longest fragments on the right.

Before interpreting a profile, it is essential to check the quality control samples processed in the same assay, in order to verify their quality and whether they match to what is expected. Even if the samples have been correctly processed and the expected quality controls have been obtained, caution is warranted while interpreting the results of an EPG, since the presence of a peak may not necessarily correspond to an allele. These events may be artefacts, requiring expert manual review to identify and exclude them from the profile being analysed [58]. This step is performed for both problem and reference samples.

Chapter III – Forensic Expertise in Sexual Crimes

Crimes of a sexual nature encompass any sexual acts, whether consummated or attempted, performed without the consent or against the will of the victim. Such acts may manifest physical contact to verbal or interactions of a sexual nature. According to the Portuguese law, sexual acts with a child or young person up to the age of fourteen, even with their consent, are still considered a crime [59,60].

This kind of crime is independent of an individual's type, be it the victim or the aggressor, and affects people of every age, gender, religion, ethnicity, sexual orientation, social and familial background, and financial status. Invariably, its consequences are always haunting and long-lasting. The victim incurs numerous negative consequences from these crimes, impacting their physical, mental, emotional, and psychological wellbeing, as well as disrupting everyday and/or sexual behaviour [61,62].

According to the Portuguese Penal Code, crimes of a sexual nature are divided into two distinct categories: crimes against sexual freedom (Art.º 163.º to 170.º), which involves non-consensual sexual activities of any kind, and crimes against sexual self-determination (Art.º 171.º to 176.º-B), which includes non-consensual sexual crimes committed against minors [63,64].

A statistical study conducted by APAV (*Associação Portuguesa de Apoio à Vítima*) on sexual crimes between 2013 and 2018 revealed a rise in number of victims per year; 506 and 1167 victims were recorded, respectively, representing a percentage increase of 130%. Females accounted for a higher percentage of victims (92%), while males made up a larger proportion of aggressors (94%) [65]. Comparing APAV's annual statistical reports for 2021 and 2022, there was a slight decrease of 4.2% in sexual crimes against children and young people, while an increase of approximately 34.7% was seen in cases against adults [66,67]. This information is confirmed by the annual Internal Security report for 2022, which is based on data obtained by the PJ, revealing that which reports that the majority of these crimes are child sexual abuse (39%), followed by rape (~20%) [68].

1. Biological Trace – Semen

Semen is a biological fluid ejaculated from the male reproductive organ after sexual stimulation. It is characterized by its alkaline pH, whitish and viscous appearance [69]. It is composed of seminal fluid, prostatic secretion and sperm cells which are mixed in the urethra during ejaculation [69,70]. The seminal fluid is produced in the seminal vesicles and functions to transport sperm from the testicles to the egg, allowing fertilization to occur [71]. Two markers commonly used in detecting semen for forensic purposes are acid phosphatase enzyme (AP) and prostate specific antigen (PSA). These markers are found in high concentrations in prostate secretion [57,72]. Sperm cells consist of three parts: the head, which contains the nucleus, acrosome, and centriole; the middle piece, where the mitochondria are located; and the flagellum or tail, which enables them to move, as can be seen in Figure 5 [57,70,73].

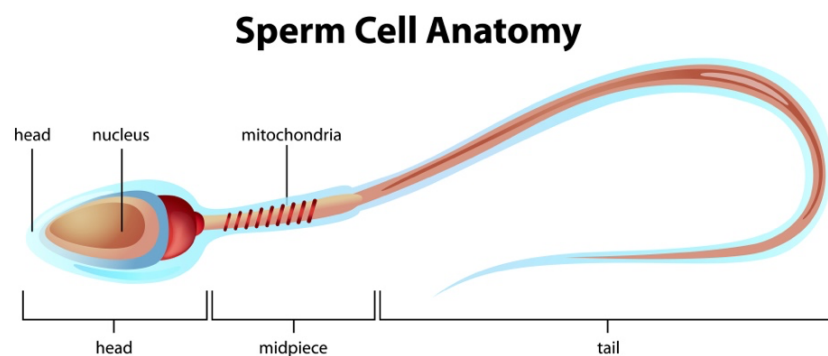


Figure 5 - Sperm Cell Structure [Adapted from: www.freepik.com/free-vector/sperm-cell-structure_2451326.htm].

Upon ejaculation, the released fluid typically ranges between 2 and 5 ml and contains an estimated 10 to 100 million sperm cells per ml in a healthy individual. However, individuals who have had a vasectomy (which consists of removing a fragment of the deferent canal) or who suffer from azoospermia (the complete absence of sperm cells in the semen) or oligospermia (the presence of sperm cells in the semen in very limited quantities) exhibit a different composition of semen [57,74–76].

2. Semen traces in sex crimes

In cases of sexual crimes, semen is the most frequently encountered biological trace found at the crime scene. Identifying this fluid is crucial in proving the occurrence of the sexual act. Its detection provides a valuable piece of evidence, which may be unique in some cases, and enables the establishment of a link between the story under investigation, the type of trace and the donor's DNA profile [70,77].

In criminal cases of this nature, it is important for there to be good communication between the criminal investigation and the assigned expert. Establishing a professional relationship should help the expert understand various facts of the story so that he can carry out an appropriate expert analysis. Before starting the exam, several aspects of the crime must be known, such as: whether it was a sexual assault or rape; how many people were involved and their gender; if males were involved, whether ejaculation occurred; what items are being analysed and who they belong to, and other aspects that might be of relevance [78]. Thus, with this complementary information, the expert will be more objective in his analysis and will be more certain of what he can find.

By following this approach, it is possible to direct the criminal investigation more accurately and help the court to be more assertive in its conclusions.

3. Approach to the methodologies used in the observation stage in the BBG sector of the PJ in the context of sexual crimes

As stated previously, the approach employed in the observation stage of each sex crime depends on various aspects of the story, but the methodology used is protocolized and must be applied in the correct way, starting with various preliminary tests and ending with tests of certainty.

3.1. Preliminary tests

In a forensic analysis of biological traces, preliminary tests must be carried out, which serve as an orientation on body fluids, before samples are taken for DNA extraction. These orientation tests serve as a screening method to identify the presence of specific fluids if the result is positive. They are characterized as sensitive, fast and simple to use. However, they are not specific and, therefore, are not considered conclusive as to the presence of a biological fluid [35,57,79].

3.1.1. Alternative light source – Crime-lite® ML2

Detecting biological traces is often impossible with the naked eye. The alternative light source (ALS), as depicted in Figure 6, is an auxiliary visualisation method that facilitates the detection of stains, acting as a preliminary test. It allows the expert to be guided on the type of stain and thus carry out the appropriate tests on the trace under analysis. The differentiation between types of traces is due to their fluorescence emission when exposed to a specific wavelength [57,80].

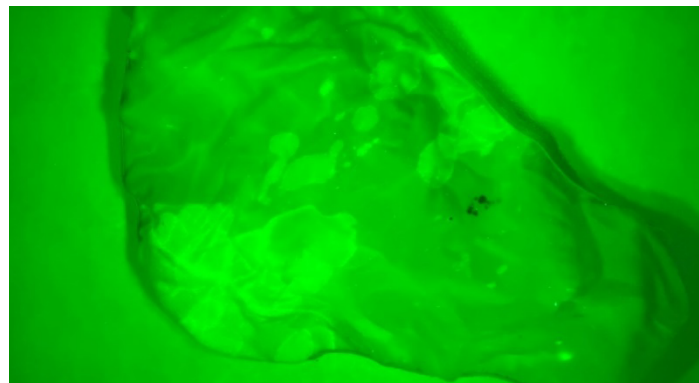


Figure 6 - Photograph taken during this study representing the visualisation of semen stains with Crime-lite® ML2.

An example of an ALS is the Crime-lite® ML2 (FOSTER & FREEMAN LIMITED, England). The manufacturer's recommendation for visualising biological fluids includes the wavelengths, as depicted in Figure 7: ultraviolet (UV) (350 - 380nm), violet (395 - 425nm), blue (420 - 470nm) and blue/green (445 - 510nm). The theoretical wavelength for detecting fluorescence from semen traces is between 450 and 495 nm. The sensitivity of this method is not high since fluorescence from other types of fluids may be emitted and the fluorescence intensity can be affected by the colour and material of the support on which the trace is deposited [57,81].

	W	UV	V	B	BG	G	O	R	IR
Blood	✓		✓	✓					✓
Body Fluids		✓	✓	✓	✓				
Bites & Bruising		✓	✓						
Bone & Tooth				✓	✓	✓			
Hair			✓	✓					

Figure 7 - Table used to determine the wavelength corresponding to the analysis needs. [64]

3.1.2. Acid Phosphatase Test

The AP enzyme is found in a significant quantity of semen and is released by the prostate into the seminal fluid [72]. This enzyme is utilised to detect semen traces since its presence is about 400 times more concentrated in seminal fluid when compared to other body fluids where it is also present, but in low amounts [35,79,82].

One of the most widely used preliminary tests to verify the presence of semen is the enzymatic method, the Acid Phosphatase test (AP test). This test is based on the reaction that the AP enzyme exerts on sodium α -naphthyl phosphate (as illustrated in Figure 8), causing the release of naphthol. When naphthol reacts with the Brentamine Fast Blue (Sigma-Aldrich, USA) solution, it results in a purple compound [35,70,82].

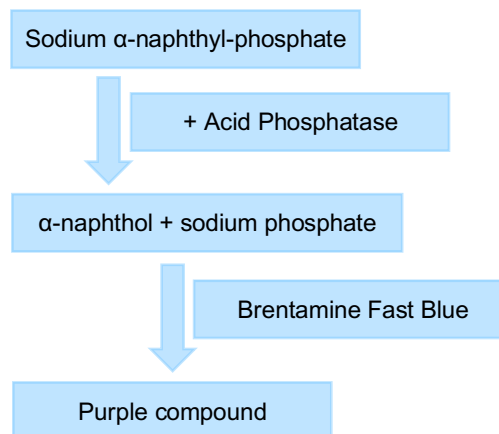


Figure 8 - Diagram of the AP test reaction.

Two types of results can be obtained in this test depending on the reaction time: if the reaction is rapid after placing the AP and produces a purple colour, the result is positive, as shown in Figure 9; and if the reaction does not show any colour change within 60 seconds, the result is negative. Despite its ease of use, this test has the disadvantages of being toxic, destructive of genetic material and having high rates of false positive results [70,79,82,83].



Figure 9 - Photograph taken during this study representing a positive AP test result.

3.1.3. SERATEC® PSA Semiquant

PSA is a glycoprotein synthesised in the prostate and secreted into the seminal fluid. Its main physiological function is to liquefy the seminal fluid to allow sperm cells to move [84,85].

This protein is found in high concentrations, 0.2 – 3.0 mg/ml, and is one of the most abundant in seminal fluid [86]. Although PSA can also be found in other body fluids, such as breast milk, sweat glands, vaginal secretions, among others, but in a very limited concentration [57,87].

One of the rapid immunochromatographic tests available for the semi-quantitative detection in the market is the SERATEC® PSA Semiquant (PSA test) (SERATEC®, Germany) [86]. This test based on the antibody's ability to bind to the PSA antigen and can have the following results (as illustrated in Figure 10): positive, if the antigen is detected, three lines will be seen: the sample result (right), the internal standard (centre) and the control (left); and negative, if only two lines are seen, the internal standard (centre) and the control (left) [84].



Figure 10 - Positive (left) and negative (right) SERATEC® PSA Semiquant test results (Adapted from: [70]).

Although this test is sensitive, it is not specific for detecting semen, as it may be present in other types of fluid. Nonetheless, the manufacturer recommends diluting the sample to reduce the likelihood of false positives [86]. One significant advantage of this method is the ability to detect seminal fluid in individuals who have undergone vasectomies or who are azoospermic, as the PSA test does not require the presence of sperm cells to detect it [84,85].

3.1.4. STK[®] – The innovative method

With the aim of optimizing working methodologies for forensic detection of semen traces in order to obtain more reliable results, the opportunity arose to implement the STK[®] Sperm Tracker (STK[®]) (AXO Science, France) method to replace the AP test.

The innovative STK[®] method is also a preliminary test designed to identify human semen stains based on the fundamental principle of AP detection, as in the AP test. The product was developed with the purpose of providing a simpler, faster, and more reliable method to detect semen stains [88,89].

In the laboratory, this product can be used in two ways: STK[®] Sperm Tracker Lab (STK[®] Lab) and STK[®] Sperm Tracker Spray (STK[®] Spray).

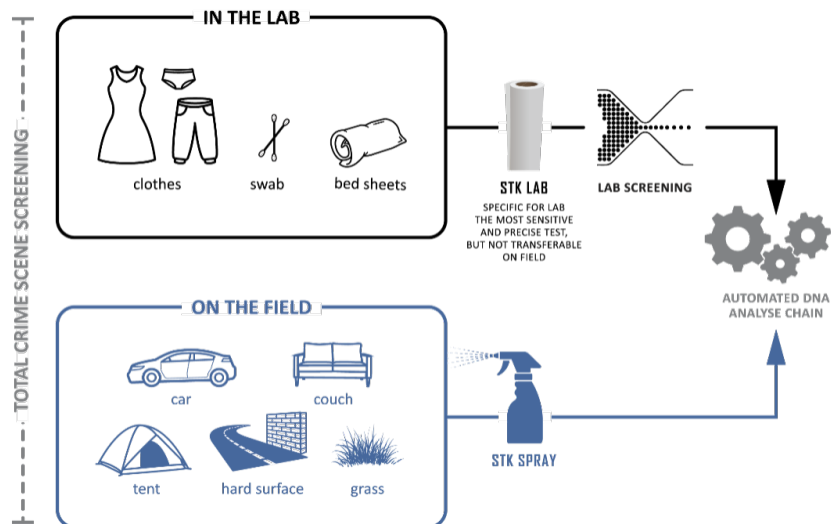


Figure 11 - Applications for the STK[®] Lab e STK[®] Spray methodologies [72].

STK[®] Lab is designed for laboratory applications and recommended for use on materials with porous surfaces, such as clothing and bed sheets. The methodology utilizes a paper impregnated with reagents, which react in the presence of semen when pressed against the suspect area using a homemade pressure table (Figure 12) [89,90].

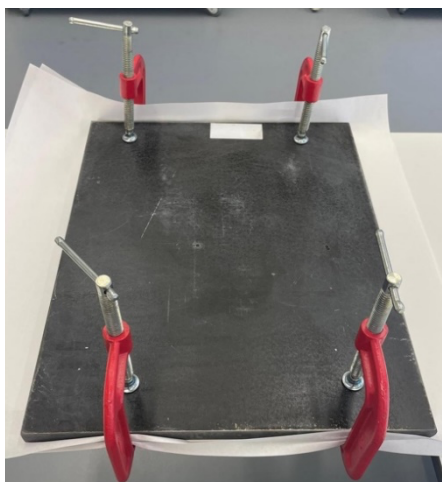


Figure 12 - Photograph taken during this study representing the homemade pressure table.

Although STK[®] Spray was designed for use at crime scenes, indoors and outdoors, and is recommended by the manufacturer for application to non-porous materials such as flooring, grass, vegetation leaves, preservatives, and others, but this type of item may appear for analysis in the laboratory. This method involves a mixture of soluble powder containing reagents in its composition, and distilled water. When sprayed on a suspected stain, it reacts with the presence of semen [89,91,92].

The results of these tests are visualised through the detection of fluorescence using an ALS, such as the VILBER VL-6.L (AXO Science, France), which is a 365 nm UV lamp. For better visibility, the analysis should be conducted in a dark place to intensify the signal, and the UV light should be positioned approximately 25 cm away. The results are considered positive when a bright, light blue luminescence is visualised and negative when no fluorescence is visualised or when it is homogeneous between the support and the suspicious stain [93,94].

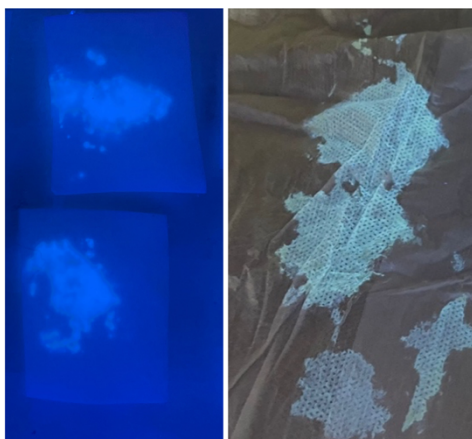


Figure 13 - Photograph taken during this study representing the positive results from the STK[®] Lab (left) and STK[®] Spray (right) methodologies.

This is an analysis methodology that has several advantages, such as [88,95]:

- Sensitivity, detecting diluted and old semen stains, which is an advantage in cold cases [96]. It is effective on different types of surfaces, once the protocol is appropriate.
- Specificity, detecting semen stains (only from humans) even when it is mixed with other fluids.
- Simplicity, since it is ready to use, and the protocol is easy to reproduce.
- Annual validity in the case of STK[®] Lab.
- It is non-toxic, does not contain carcinogenic reagents.
- It does not destroy the DNA, allowing for compatible with extraction techniques [97].
- It has a certificate, can be used within forensic laboratories as it has the appropriate grade.

3.2. Certainty tests – Staining

In sexual crime cases involving male individuals, it is important to verify the presence of sperm cells, which is a crucial factor in confirming the presence of semen. The certainty test is carried out to verify their existence using staining methods which, in the case of semen analysis, are based on viewing samples under an optical microscope after they have been stained to identify and prove the presence of sperm cells. This is a confirmatory test, as the possibility of identifying just one whole sperm cell is enough to confirm the presence of semen [42,70].

As mentioned previously, the sperm cell consists of a head, midpiece and flagellum/tail and after ejaculation, these cells tend to start degrading due to adverse conditions, starting with the loss of the tails being the most vulnerable part. Since this work is based on the routine context of a forensic laboratory, where traces are exposed to a various uncontrolled condition, the microscopic visualisation of sperm cells can be difficult. In most cases, only the heads and other cells such as epithelial cells can be seen [70].

This confirmation test has two possible results: positive if heads or a whole sperm are visualised, and negative if none of the above elements are visualised. Training on standard slides is crucial to analyse the sample in order to accurately distinguish the heads of sperm cells from other types of cells such as epithelial cells, since their nucleus is also stained and can, therefore, result in a false positive. This method proves unfavourable in situations where the sample is derived from individuals with health

issues, such as azoospermia and oligospermia, or who have had a vasectomy or have not ejaculated or used condoms, as there are few or no sperm cells present for visualisation [70].

3.2.1. Hematin/Eosin staining

Hematin/Eosin staining (HE staining) is a method commonly used to stain sperm cells [70]. This technique uses hematin and eosin as its principal dyes. Hematin is a basic dye which attracts acidic substances (basophilic), such as the cell nucleus. This dye has a purplish colour and is responsible for staining the sperm cells nuclei by binding to the chromatin. After staining with hematin, ammonia is added to the sample. The purpose of this chemical compound is to make the medium basic so that cellular components such as proteins present in the cytoplasm become positively charged and, with the addition of eosin, stained. Eosin is an acidic dye with a pink colour that attracts basic cellular components (acidophilic). [98]



Figure 14 - Photograph taken during this study representing the visualisation of sperm cells using the HE staining under an optical microscope at 40X resolution.

3.2.2. Christmas Tree staining

Christmas Tree staining (CT staining) is a method used to identify sperm cells under a microscope. This staining method comprises two reagents: Nuclear Fast Red and Picro-Indigo-Carmine, which are coloured red and green respectively. The Nuclear Fast Red reagent is responsible for staining the head of sperm cell red and the acrosomal cap pink. The solution composed of picric acid and indigo carmine that results in the Picro-indigo-carmine reagent gives the tail and midpiece of the sperm a green and blue tint, respectively, as shown in Figure 15 [57,70,99].

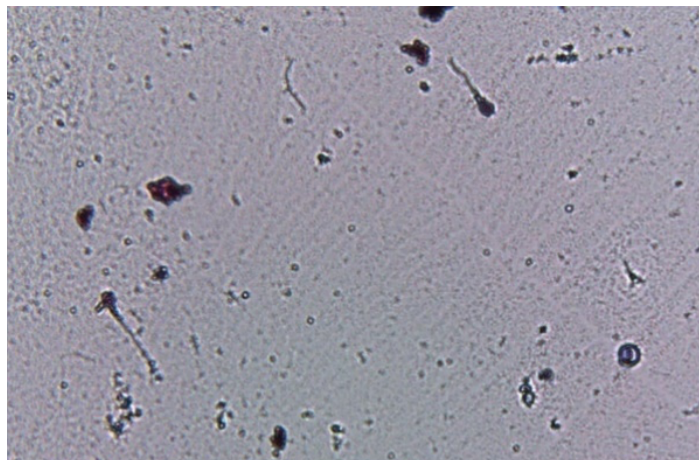


Figure 15 - Photograph taken during this study representing the visualisation of sperm cells using the CT staining under an optical microscope at 40X resolution.

Aims

In order to improve the efficacy of trace detection in evidence found at crime scenes, in particular in sexual assaults, it is of high importance to test new methodologies and be updated regarding the most recent developments as well as new technologies.

Therefore, this study aimed to:

- 1) Evaluate the effectiveness of the STK[®] Sperm Tracker Lab (STK[®] Lab) and STK[®] Sperm Tracker Spray (STK[®] Spray) (AXO Science, France) methods when applied to textiles;
- 2) Comparisons were made with the Acid Phosphatase test method with the potential to replace it and establish a new working methodology to improve workflow in the detection of semen traces in criminal investigations related to sexual offences;
- 3) To validate the use of the STK[®] Sperm Tracker Spray (STK[®] Spray) (AXO Science, France) on non-porous surfaces, to validate the optical microscopy method, Christmas Tree staining, and to assess the potential to replace the commonly used laboratory method, Hematin/Eosin staining.

Methods

To implement a new working methodology for the detection of semen traces, it was necessary to carry out various types of tests on the STK[®] Lab and STK[®] Spray methodologies, and the AP test, commonly used in the laboratory, to compare and find out which is the best workflow to apply in cases involving this type of trace.

The validation of a product requires the use of standard samples handled in controlled environments to avoid contamination, to test, in this case, semen traces in various conditions. These types of samples are not in the same conditions as samples from real cases and there is a difficulty in reproducing these environments, due to the complexity of various related factors. Therefore, since these are the ones that are processed in this professional context, it is essential to test real samples.

In order to be able to draw more concrete conclusions, it was used both types of samples, real and simulates, combining the best that each could offer real working conditions and tests variability, respectively.

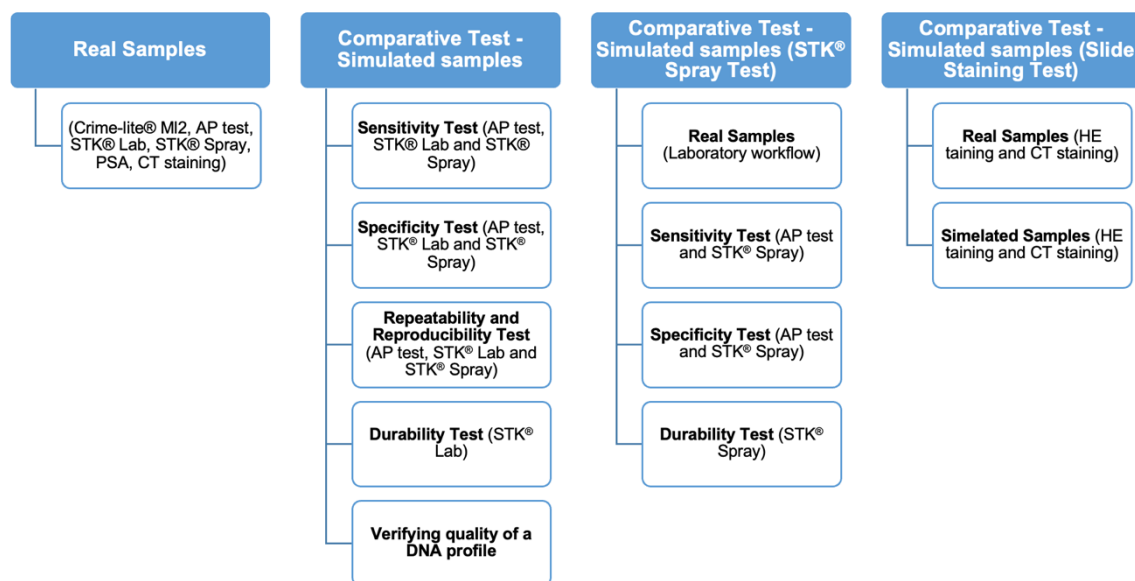


Figure 16 - Schematic illustration of the methodology used in the study.

1. Real Samples

Sixty-six samples of various types of textile materials, such as elastane, polyester and mainly cotton, were used, especially from clothing and household textiles. These samples were obtained from real cases of sexual assault which, according to the expert's request, required the detection of traces of semen.

With the aim of testing new preliminary methods for the detection of traces of semen, in particular STK[®] Lab and STK[®] Spray, and to compare them with the current laboratory test, the AP test, these samples were subjected to the workflow used by the laboratory for this type of case. In addition, the STK[®] Lab and STK[®] Spray methodologies were carried out and a semen confirmation test, the CT staining, was included in the performance.

The protocol used to search for possible traces of semen begins with the search for suggestive stains with the aid of an ALS, in this case, the Crime-lite[®] ML2 (FOSTER & FREEMAN LIMITED, England) with the combination of "Blue light" (420 - 470 nm) and the 550 nm filter, specific for the visualisation of biological fluids. Upon locating the stains, they were marked with a pen so that, without using the search light, they could be used to carry out the necessary tests. Once the entire trace had been visualised, the preliminary tests began.

First, the AP test was performed. A piece of filter paper was moistened with distilled water, placed over the suspicious stain and tapped with the hand in the shape of a fist to transfer the sample to the paper. In cases where there was a significant amount of trace, a small piece of fabric was cut off, placed on a piece of filter paper already moistened with distilled water and compressed with the fingers. The AP solution was spread over the filter paper and after one minute the result was seen.

Next, the STK[®] Lab method was performed (refer to Figure 17). Pieces of STK[®] Lab paper were cut to the size of the suspicious stains, moistened, and pressed with a homemade pressure table for five minutes. After the elapsed time, the STK[®] Lab paper was removed and visualised the results using the ALS (365 nm), VILBER VL-6.L (AXO Science, France).

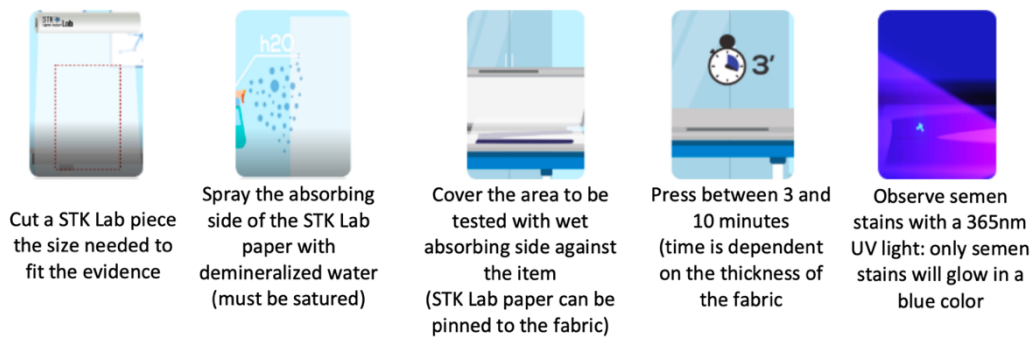


Figure 17 - Protocol summary of STK® Lab method procedure [92].

In the third preliminary test, the STK® Spray method (refer to Figure 18), the reagent was prepared by combining the powdered reagent with 100 ml of distilled water in a sprayer. The suspect stain was sprayed and the results were visualised using the ALS (365 nm), VILBER VL-6.L (AXO Science, France).

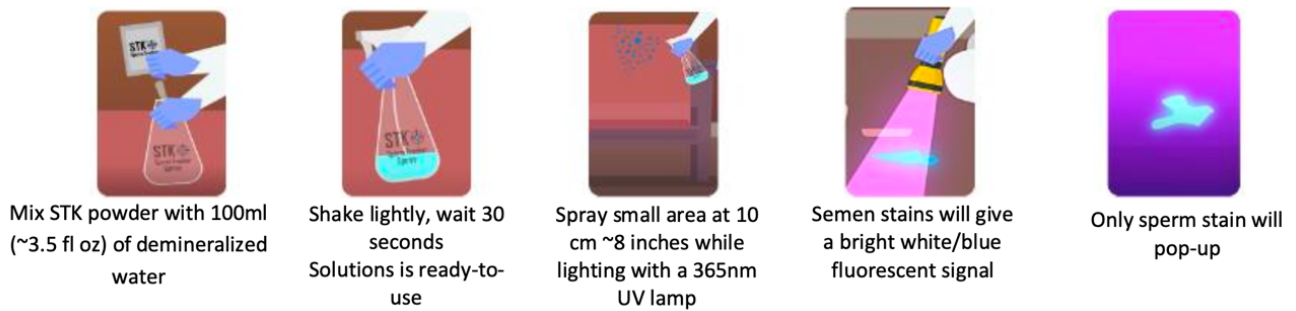


Figure 18 - Protocol Summary of STK® Spray method procedure [90].

To carry out the last preliminary test, the PSA test (Figure 19), a piece was cut from the area where the suspicious stain was located, placed in an eppendorf tube and added to the extraction buffer. After waiting ten minutes, four drops were pipetted into the rapid test and the result was displayed.

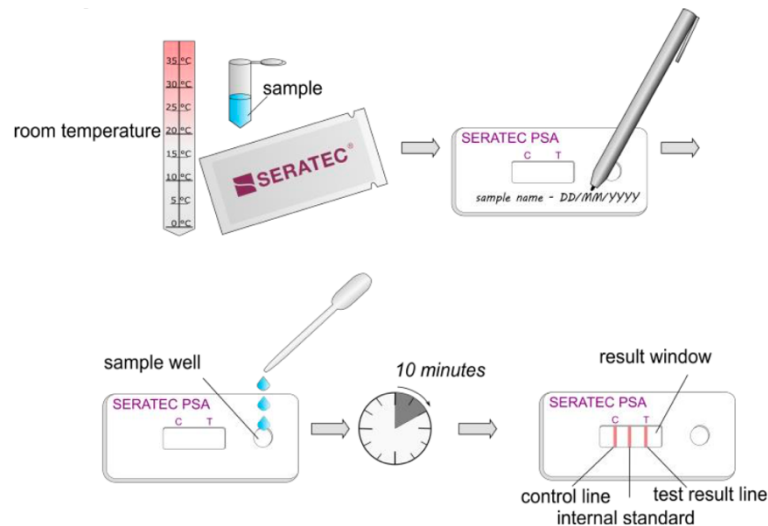


Figure 19 - PSA test procedure [70].

If the results of the preliminary tests were negative, a piece of the suggestive stain was cut out and sent to the biological trace extraction workflow.

If the results were positive, a piece of the tested stain was cut out and sent to the semen sample extraction workflow.

What was left in the PSA test eppendorf tube was saved for use in preparing the CT staining slide.

The CT staining method began by removing the tissue sample from the eppendorf tube using tweezers, squeezing out all of the liquid it contained. The samples were then centrifuged at 1500 rpm for 5 minutes to obtain a pellet and the excess supernatant was carefully removed. 20 µl of the pellet sample was pipetted onto a slide and fixed using a flame. The sample was covered with Fast red dye and the slide was placed in a damp chamber, improvised with a petri dish and kitchen paper, prepared in advance, and left to act for 15 minutes. Following this, the sample was washed with distilled water and then the Picro-indigo-carmin dye was pipetted on to cover the entire sample for 15 to 30 seconds. Finally, it was washed with 90% ethanol, previously diluted in the laboratory, left to dry and viewed under a microscope using 40x and 60x objectives.

2. Comparative Test – Simulated samples

To carry out a comparative test to determine the product's efficacy, semen and seminal fluid samples were needed. These samples came from males, whose identity and genetic history are anonymous, and were acquired through a collaboration protocol between the BBG sector and the *IERA (Instituto Extremeño de Reproducción Asistida) Lisboa Quirón Salud* fertilization clinic. The samples used were pure and diluted according to the needs of each test. This meant that the samples had to be handled carefully to avoid contamination, as this could interfere with the results obtained.

The comparative test aimed to study different characteristics. It was therefore subdivided into various tests, such as sensitivity, specificity, repeatability and reproducibility, durability and verifying the quality of a DNA profile; each test has a different study objective.

Despite the different samples and ways of preparing each test, the methods for detecting traces of semen were the same, except for the following tests: Durability Test; Verifying the quality of a DNA profile; STK[®] Spray Test; and Slide Staining Test. The AP test was started by moistening a piece of filter paper with distilled water, placing it on top of the support and tapping it in the shape of a fist to transfer the sample to the paper. To better visualise the results, the areas previously made on each support were marked out on the paper. The AP solution was spread over the entire filter paper and after a minute the results were displayed. Following this, the STK[®] Lab method was carried out: small pieces of STK[®] Lab paper were cut to the size of the fractions outlined on each support, moistened with distilled water, the pieces were taped together and the supports were placed on a homemade pressure table for five minutes. After the time had elapsed, each support was removed and the results were visualised using the ALS (365 nm), VILBER VL-6.L (AXO Science, France). Finally, the STK[®] Spray method was used: the reagent was prepared by adding the powdered reagent to 100 ml of distilled water in a sprayer. The support was sprayed and the results were visualised using the ALS, VILBER VL-6.L (AXO Science, France).

2.1. Sensitivity Test

The sensitivity test was conducted in two phases. The first phase aimed to verify the effectiveness of the product in different type of concentrations on different types of textiles and to see which of the three preliminary tests had the best results on each type of material and concentrations, and the second phase, aimed to see if the STK[®] Lab and STK[®] Spray also had the sensitivity to detect samples from azoospermic individuals.

2.1.1. Different textiles

To carry out this test, different types of textiles were used as a support: a green cotton fabric, pink polyester, dark green elastane, black lace, brown wool, blue denim, white cotton, pink satin and beige linen, obtained from old clothes in the house or bought in stores. To test the sensitivity of the three methodologies, namely AP test, STK[®] Lab and STK[®] Spray, to the different states of semen on different textiles, three pieces of each fabric were used to test each methodology, which were subdivided into ten areas, each for the deposition of the different semen samples, in pure and diluted state (1/10, 1/100 and 1/1000), and negative control (distilled water), in duplicates, as can be seen in Figure 20.

After marking the ten fractions on each tissue, 50 µl of distilled water (NC), pure semen (PS), semen diluted at 1/10 (D 1/10), semen diluted at 1/100 (D 1/100) and semen diluted at 1/1000 (D 1/1000) were deposited, in the order described. The samples were then left to dry and tested the following day.

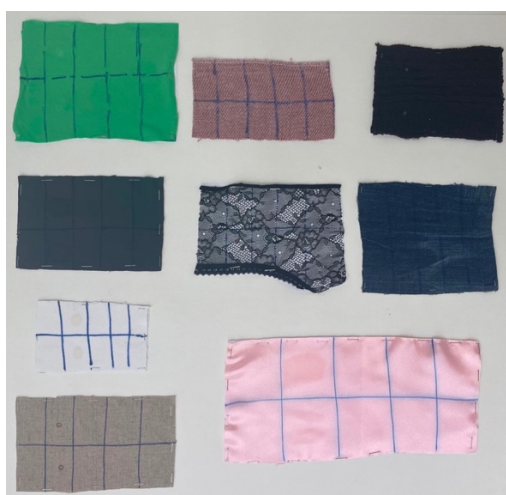


Figure 20 - Fabric samples used as support in the Sensitivity Test carried out in textile diversification testing.

2.1.2. Azoospermic samples

To check that the product could also detect seminal fluid traces, samples from azoospermic individuals were requested from the clinic. For this test, a green cotton fabric was used as a support, obtained from old clothes in the house. To test the sensitivity of the three methodologies to different concentrations of seminal fluid, three fabric supports were used to test the AP test, STK[®] Lab and STK[®] Spray. These were subdivided into ten areas, each for the deposition of the different samples, semen in pure and diluted state (1/10, 1/100 and 1/1000), and negative control (distilled water), in duplicates, as shown in Figure 21.

After marking the ten fractions on each support, 50 µl of distilled water (NC), pure semen (PS), diluted semen at 1/10 (D 1/10), diluted semen at 1/100 (D 1/100) and diluted semen at 1/1000 (D 1/1000) were deposited, in the order described. The samples were then left to air dry and tested the following day.

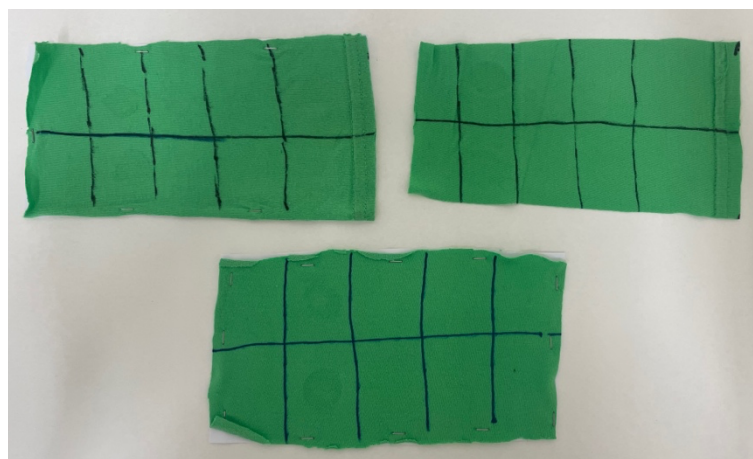


Figure 21 - Tissue samples used as support in the Sensitivity Test carried out with samples from azoospermic individuals.

2.2. Specificity Test

The purpose of the specificity test was to find out whether the STK[®] Lab and STK[®] Spray also react to other fluids, such as blood, saliva, urine, faeces and vaginal fluid, and to check the product's ability to detect seminal fluid when it is mixed with other fluids.

To carry out this test, cotton fabric was used as a support, obtained from old clothes in the house. To test the specificity of the three methodologies on the different types of fluids, three pieces of fabric were used to test the three methodologies, AP test,

STK[®] Lab and STK[®] Spray, which were subdivided into ten areas. Each area is for the deposition of the different semen samples, in a pure state, a sample of the fluid under test (blood, saliva, urine, faeces and vaginal fluid), a mixture between the two fluids in proportions of 1: 1 and 1:10, and negative control (distilled water), in duplicates, as shown in Figure 22.

After demarcating the ten fractions in each tissue, 50 µl of distilled water (NC), pure semen (PS), a sample of the fluid under test (TS), a 1:1 mixture (M 1:1) and a 1:10 mixture (M 1:10) were deposited, in the order described. The samples were then left to air dry and the tests were carried out the on following day.

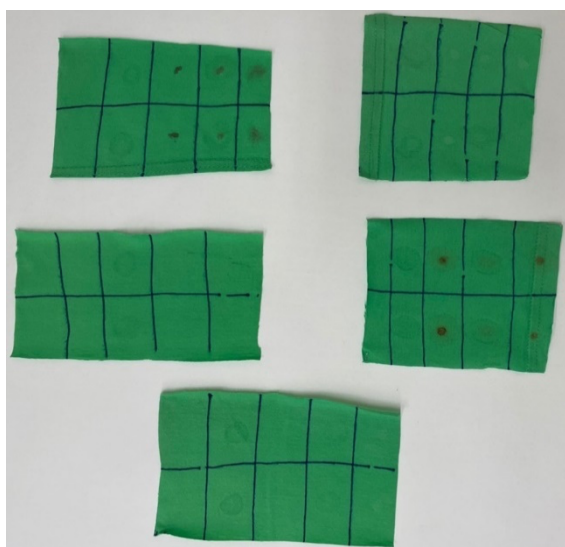


Figure 22 - Tissue samples used as support in the Specificity Test.

2.3. Repeatability and Reproducibility Test

The purpose of the repeatability and reproducibility test was to check whether the same methodology carried out by different people at different times, but with the samples subjected to the same conditions, had an impact on the results.

It was, therefore, necessary to choose five people from the laboratory to carry out the same test. These participants included: a trainee with no experience and knowledge of the product, a trainee with no experience and no knowledge of the product's methodology, a forensic expert with little experience and no knowledge of the product's methodology, an expert with experience and no knowledge of the product and an expert with experience and knowledge of the methodology.

The tests were carried out one day, one week and one month after the samples were placed on the supports. The support used for all the tests was a green cotton fabric,

obtained from old clothes in the house. To evaluate the repeatability and reproducibility, three pieces of fabric were used for each experimental day. Each piece was used in one of the methodologies, STK[®] Spray, STK[®] Lab and AP test, being subdivided into ten fractions and the different samples deposited in each. After demarcating the ten fractions on each support, 50 µl of distilled water (NC), pure semen (PS), diluted semen at 1/10 (D 1/10), diluted semen at 1/100 (D 1/100) and diluted semen at 1/1000 (D 1/1000) were deposited, in the order described, as shown in Figure 23. The samples were then left to air dry and tested the following day.

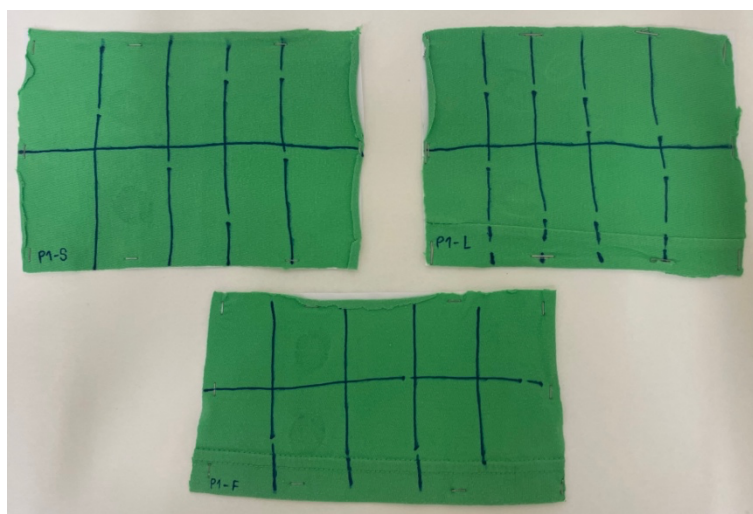


Figure 23 - Tissue samples used as support in the Repeatability and Reproducibility Test.

2.4. Durability Test

The aim of this test was to evaluate the durability of the STK[®] Lab product after opening it. The product was analysed one day, one week and one month after opening the package. It was necessary to prepare the STK[®] Lab paper and cotton support correctly for pipetting the semen samples. Small pieces of STK[®] Lab paper were cut to the size of the fractions outlined on each support. The pieces were moistened and secured with adhesive tape. Next, the supports were positioned on a homemade pressure table for a duration of five minutes. After the time had elapsed, each support was removed and the results were visualised using the ALS, VILBER VL-6.L (AXO Science, France). Photographs were captured to verify the efficiency of the product, and a comparison was made between the fluorescence obtained to see if it persisted or faded over time.

It should be noted that this test is not feasible within real parameters, since the product being used are product samples provided by the company for the study of this work.

2.5. Verifying the quality of a DNA profile

To verify the quality of a DNA profile after using STK[®] Spray, it was necessary to extract the samples to make sure that the reagents that make up the product did not affect the DNA. The assessment used fifteen samples: five samples from the sensitivity testing on different textiles, five samples from the sensitivity test on azoospermic samples and five samples from real cases, with and without spray, were used. A piece of each sample was taken, and the processing workflow of the semen samples was carried out for later comparison.

Initially, the samples were subjected to differential extraction in the QIAcube equipment (QIAGEN, Germany) to separate the CF from the MF. To accomplish this, 270 µl of ATL Buffer and 30 µl of PK enzyme were pipetted into each Eppendorf tube and the samples were placed in a heater shaker, at 900 rpm and 56°C for 15 minutes to lyse the epithelial cells. Then, they were placed in the centrifuge at 12.000 rpm for 2 minutes so that the liquid descended from the basket into the tube. After this, the equipment started an automated program in which it was consecutively centrifuged and washed four times to remove the supernatant, CF, into a new eppendorf, leaving the pellet, MF, so that they could be analysed separately. Finally, the equipment placed the mix, made up of G2 buffer, PK and DTT, in the male fraction tubes so that when they were placed in the heater shaker at 900 rpm and 70°C for 10 minutes, for lyse the sperm cells.

After the differential extraction procedure, the samples were placed in the QIA Symphony equipment (QIAGEN, Germany) for purification in SP and preparation of the samples for quantification and amplification in AS. To quantify the samples, the optical plate was placed in the QuantStudio equipment (Thermo Fisher Scientific, USA) and then an automatic file was transferred to the QIA Symphony AS equipment (QIAGEN, Germany) with the information on the quantities of each sample. This allow the equipment to prepare them for amplification and check whether there was a need to make dilutions if they contained an excess of DNA. The samples were amplified in the Veriti Thermal Cycler (Thermo Fisher Scientific, USA) and prepared for capillary electrophoresis with the GlobalFiler[™] kit (AB, Thermo Fisher Scientific, USA) in the QIAgility (QIAGEN, Germany). The Veriti Thermal Cycler (Thermo Fisher Scientific,

USA) was used again to denature the DNA chains and the plate was placed in the 3500 Series Genetic Analyzer (Thermo Fisher Scientific, USA) for capillary electrophoresis. Finally, when the run was complete, GeneMapper ID-X 1.6 software (Thermo Fisher Scientific, USA) was used to clean the samples so that could be determined and interpreted.

2.6. STK[®] Spray Test

This test was carried out with the aim of validating the use of the STK[®] Spray (AXO Science, France) methodology, on non-porous surfaces. As a complement to the study carried out, these tests were adapted to the interests of the laboratory.

The STK[®] Spray test also used real samples from sexual cases. Throughout the tests, it was possible to study six samples made up of non-porous materials, such as plastic, diaper components and, above all, latex. To achieve the objective of this complementary test, a procedure like the real samples from the main test was carried out, except that the STK[®] Lab product was not tested.

Despite the different samples and preparation methods for each test, the methods for detecting traces of semen were the same, except for the Durability Test. The AP test was started by moistening small pieces of filter paper with distilled water and rubbing them on the areas where the samples under study were located. The AP solution was spread over each piece of filter paper and after a minute the results were displayed. Following this, the STK[®] Spray method was used: the reagent was prepared by adding the powdered reagent to 100 ml of distilled water in a sprayer. The support was sprayed and the results were visualised using the ALS, VILBER VL-6.L (AXO Science, France).

2.6.1. Sensitivity Test

To carry out this test, different types of non-porous materials were used as supports, with the aim of testing the sensitivity of the two products on different types of materials with different states of semen concentration. The supports used were rubber, plastic, latex, cement, metal, vegetation, and components of a diaper, which were obtained from household or the laboratory. To test the sensitivity of the two methodologies, STK[®] Spray and the AP test, in the different types of semen states and on the different types of materials, two pieces of each support were used. Each support was subdivided into eight areas, each for the deposition of the different semen samples,

in pure and diluted state (1/10 and 1/20), and negative control (distilled water), in duplicates, as shown in Figure 24.

After marking the eight fractions on each support, 50 µl of distilled water (NC), pure semen (PS), diluted semen at 1/10 (D 1/10) and diluted semen at 1/20 (D 1/20) were deposited in the order described. The samples were then left to dry and tested the following day.

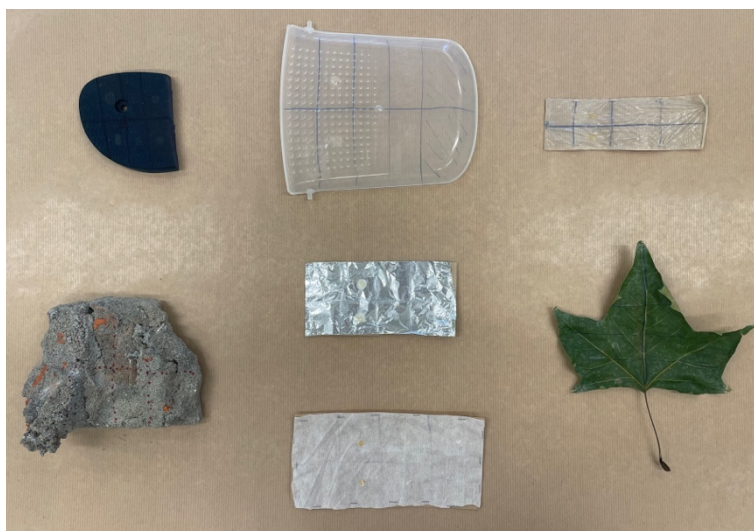


Figure 24 - Samples of non-porous materials used as support in the Sensitivity Test of STK® Spray Test.

2.6.2. Specificity Test

The purpose of the specificity test was to find out whether it also reacts to other fluids, such as blood, saliva, and urine, and to check the product's ability to detect seminal fluid when it is mixed with the aforementioned fluids.

Non-porous materials were used to carry out this test, specifically rubber and latex as a support, which were obtained from existing materials at home or at the laboratory. To test the specificity of the two methodologies STK® Spray and the AP test in the different types of fluids, two pieces of material were used. Each support was subdivided into ten areas, each for the deposition of the different samples, semen in a pure state, a sample of the fluid under test (blood, saliva, urine), a mixture between the two fluids in proportions of 1:1 and 1:10, and negative control (distilled water), in duplicates, as shown in Figure 25.

After marking out the ten fractions on each support, 50 µl of distilled water (NC), pure semen (PS), a sample of the fluid under test (TS), a 1:1 mixture (M 1:1) and a 1:10

mixture (M 1:10) were deposited, in the order described. The samples were then left to dry and the tests were carried out the following day.

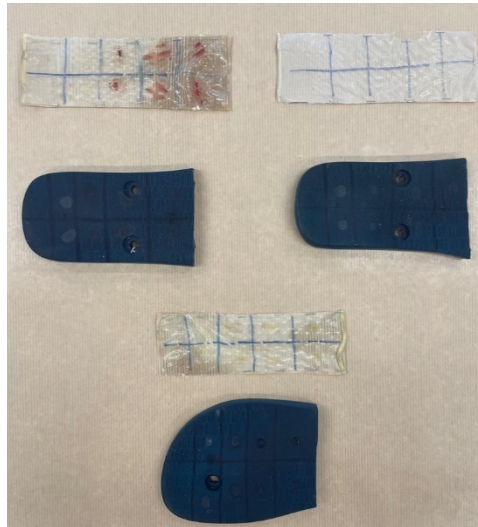


Figure 25 - Samples of non-porous materials used to perform the Specificity Test of STK® Spray Test.

2.6.3. Durability Test

The aim of this test was to verify the durability of the STK® Spray product after its creation. To carry out this test, the reagent was prepared by combining the powdered reagent with 100 ml of distilled water in a sprayer, and positive controls, as illustrated in Figure 26. For a period of fifteen days, positive control was sprayed daily to check the effectiveness of the product and to compare the fluorescence obtained and see if it remained or faded over time.

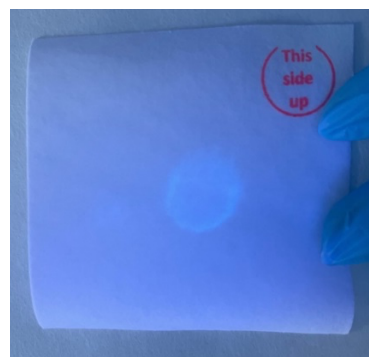


Figure 26 - Photograph taken during this study representing a positive control.

2.7. Slide Staining Test

This test was carried out with the aim of validating the certainty test, CT staining, by staining sperm cells on a slide for microscope visualising. As a complement to the study carried out, this test was adapted to the interests of the laboratory.

Fourteen different tissue supports were used to carry out this test. Seven as a negative control (NC), containing no semen traces, and seven as a positive control (PC), containing semen samples. Real samples from sexual cases were also used in this test. Throughout the tests, it was possible to carry out both stains, for comparison, on fifteen samples of the traces being analysed. The supports were cut into similar pieces to conduct a comparison test that could be carried out with the stain already used in the laboratory, HE staining. The slides were identified with a description of the sample number and the respective stain.

The first staining test, HE staining, was carried out by pipetting a drop of hydrochloric acid onto the piece of tissue to undo, using tweezers and scissors to help. It was then fixed to the flame using a lamp. Hematin solution was pipetted onto the sample and waited ten minutes. The sample was washed with distilled water and a few drops of diluted ammonia were pipetted on and immediately washed again with distilled water. Finally, the sample was covered with eosin solution, left to act for one minute and washed with distilled water. When dry, it was viewed under a microscope with 40x and 60x objectives.

The CT staining method began by removing the tissue sample from the PSA test eppendorf using tweezers, squeezing out all the liquid it contained. Afterwards, it was centrifuged at 1500 rpm for 5 minutes to obtain a pellet and the excess supernatant was carefully removed. A volume of 20 µl of the sample was pipetted onto a slide and fixed in the flame. The sample was covered with Fast red dye and the slide was placed in a damp chamber, improvised with a petri dish and kitchen paper, prepared in advance, and left to act for 15 minutes. The sample was washed with distilled water and then the Picro-indico-carmin dye was pipetted on to cover the entire sample for 15 to 30 seconds. Finally, it was washed with 90% ethanol, previously diluted in the laboratory, allowed to dry and viewed under a microscope with 40x and 60x objectives.

Results and discussion

1. Real Samples

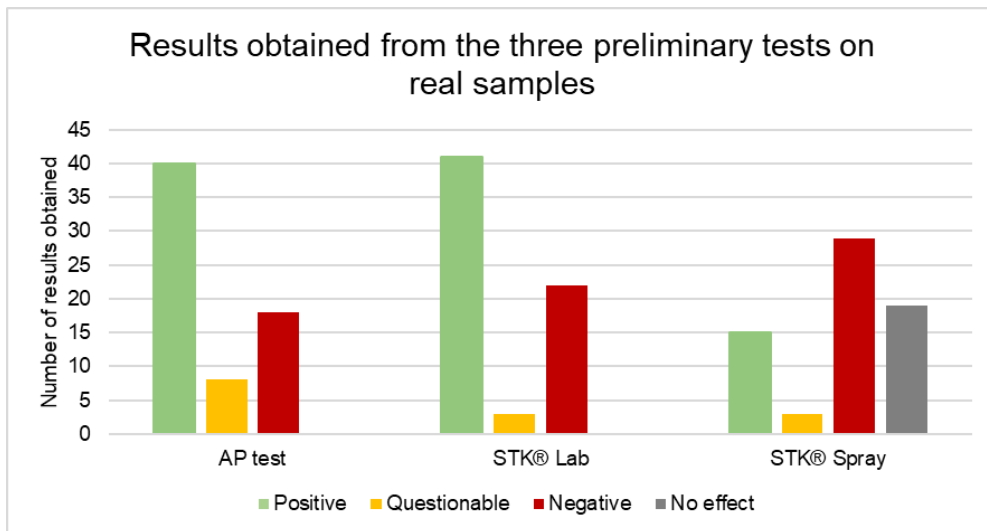
Sixty-six real samples were analysed, twenty-six of these came from home textiles (~40%) (bed linens; bath sheets; etc.), twenty-three from underwear (~35%) (briefs; boxers; bikinis; etc.), ten from clothing (~15.15%) (t-shirts; pants; etc.), five from hygiene products (~7.50%) (pads and diapers) and two from other types of trace (~3%) (for example, swabs). These samples were located on different types of textiles such as cotton (~75.80%), synthetic (~7.60%), diapers material (~7.60%), polyester (~4.55%), as well as microfiber, denim, and lace (~4.55%). The fabrics analysed had different shades, namely white (~27.30%), blue (~15.15%), black (~12.12%), and beige (~10.60%), among others, as detailed in the table in Attachment 1.

The results obtained were different and a colour was assigned to each type of result as following:

- 1) The green colour corresponds to positive results, which are those where there was a positive reaction, the appearance of a purple hue in the case of the AP test, and the detection of strong fluorescence in the case of the STK[®] Lab and STK[®] Spray.
- 2) The yellow colour corresponds to questionable results, which are those that incited doubt, a weaker fluorescence in the case of the STK[®] Lab and STK[®] Spray and a softer tone in the AP test.
- 3) The red colour corresponds to negative results, which are those where there was no reaction of any kind.
- 4) The grey colour corresponds to the results that were not carried out, referred to as "no effect". The last results mentioned were not obtained by: nineteen, because the STK[®] Spray methodology was not carried out due to the limitation of biological material for analysis and thus, in order not to jeopardize the expertise, it was decided to carry out the remaining tests; three, due to the framework of the case, the amount of sample for analysis and the results obtained in the remaining preliminary tests, the expert chose not to carry out this test; and seventeen and eleven, in the CT staining and the MF profile, respectively, since the preliminary results were all negative and the analysis of semen samples was not pursued.

1.1. Results obtained from the three preliminary tests on real samples

Graphic 1 shows the results of the real samples from the three preliminary tests, namely the AP test, STK® Lab and STK® Spray.



Graphic 1 - Total of the positive, questionable, negative, and no-effect results obtained from carrying out the preliminary tests under study, AP test, STK® Lab and STK® Spray, on real samples.

Out of the sixty-six samples analysed, forty tested positive in the AP test, forty-one in the STK® Lab method and fifteen in the STK® Spray method, corresponding to 60.61%, 62.12% and 22.73% respectively. There were eight questionable results in the AP test and three in each of the STK® Lab and STK® Spray methodologies. These figures represent 12.12% and 4.55% respectively. About negative results, eighteen were obtained in the AP test (27.27%), twenty-two in the STK® Lab method (33.33%), and twenty-nine in the STK® Spray method (43.94%).

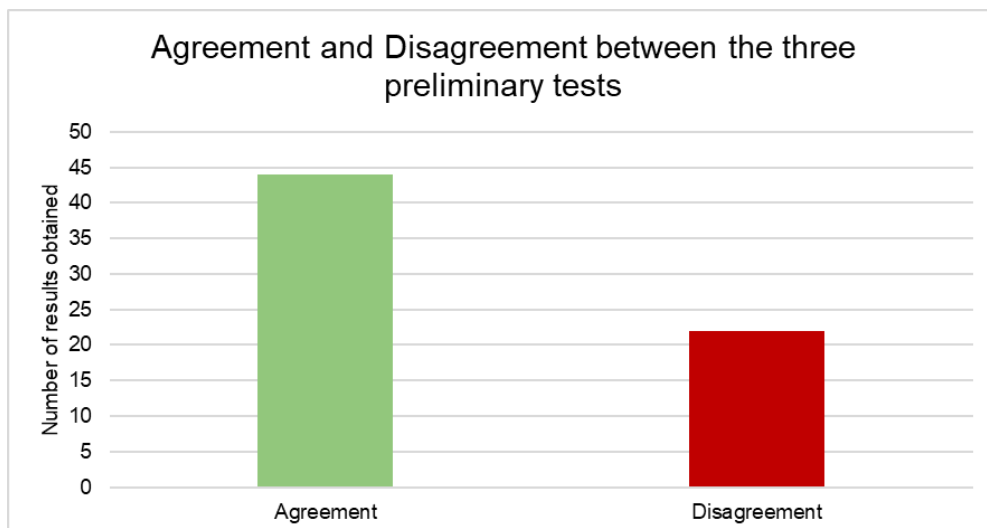
Only in the STK® Spray method had no effect results, nineteen representing to 28.79%.

Comparing the percentages obtained only from the screened results of the sixty-six samples, STK® Lab method has a higher percentage of positive results in comparison to the other two tests. About questionable results, the STK® Spray method has the same percentage, and the AP test has a higher percentage. As for the negative results, STK® Spray was the method with the highest percentage compared to the other two tests.

The following comparisons of the results of the real samples were made using agreements and disagreements. "Agreements" indicate consistency between the results and "disagreements" indicate inconsistencies in the results, which can be with just one difference or more. In these comparisons, there are also "no effect comparisons", which are comparisons that were not carried out due to the non-performance of the preliminary tests (AP test, STK[®] Lab, STK[®] Spray and PSA test), CT staining and differential extraction to obtain the MF profile, which are due to the reasons described above.

1.2. Agreement and Disagreement between the three preliminary tests

Graphic 2 shows the number of agreements and disagreements obtained when comparing the results of the real samples between the three preliminary tests, the AP test, STK[®] Lab and STK[®] Spray.



Graphic 2 - Total of the results of the agreements and disagreements obtained between the three preliminary tests in the analysis of sixty-six real samples.

Of the sixty-six samples analysed, forty-four agreements were obtained between the results of the three preliminary tests under study, of which two had questionable results, twenty-four had positive results and eighteen had negative results. Of these forty-four agreements, sixteen comparisons were made only between the AP test and the STK[®] Lab, since the STK[®] Spray was not carried out as the samples being analysed were limited. In the remaining twenty-two samples, disagreements were obtained. Of these disagreements, only three occurred between the AP test and the STK[®] Lab.

The disagreements obtained were different in all twenty-three samples:

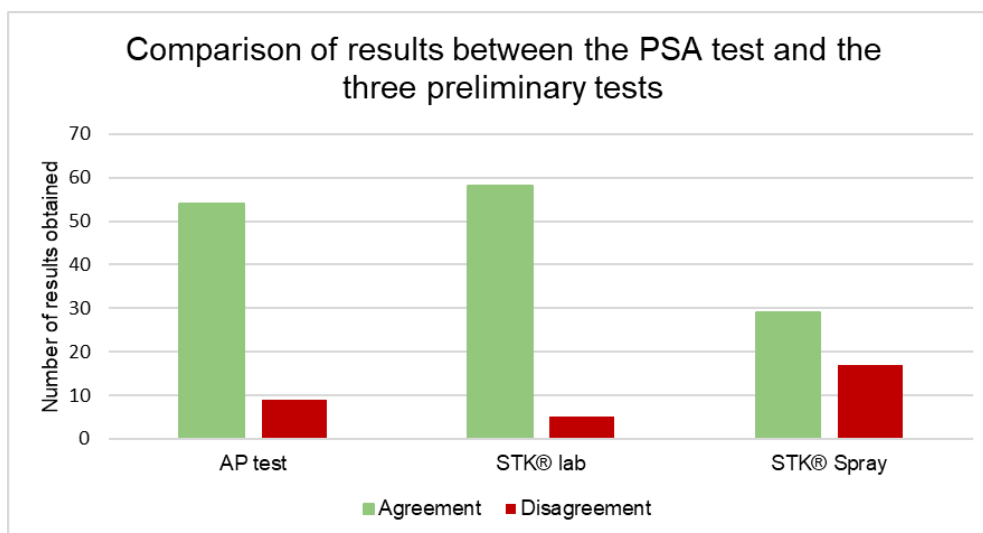
- Fourteen samples, in which STK[®] Spray result was negative and the results of AP test and STK[®] Lab were positive. The disagreement obtained in these samples can be explained by the fact that most of them come from white fabrics and when the fabric absorbs the product's liquid, it emits a homogeneous fluorescence throughout the fabric, making it impossible to differentiate between the suspicious stain and the rest.
- Four samples, in which the AP test result was questionable, and the remaining results were negative. This can be explained by the fact that this test can react to other types of fluid.
- Two samples in which the AP test result was questionable and the STK[®] results were positive. This could be due to the technician not applying the test correctly, not transferring enough samples to the filter paper and obtaining a questionable result.
- One sample in which the AP and STK[®] Lab test results were positive and the STK[®] Spray result was questionable. This disagreement can be explained by the possible absorption of the product by the tissue, resulting in homogeneous fluorescence, with no discernible differentiation between the suspicious stain and the rest.
- One sample in which the AP test result was positive and the STK[®] Lab result was questionable. This disagreement can be explained by the operator's incorrect execution of the STK[®] Lab technique.

1.3. Comparison of results between the other tests used frequently in sexual crime cases and the three preliminary tests

Next, to verify the results of the real samples, a comparison was made between the results obtained from each orientation tests under study and the results obtained from the other frequent tests used in sexual crime cases.

1.3.1. Comparison of results between the PSA test and the three preliminary tests

The first comparison was made between the PSA test and the three preliminary tests under study, the AP test, STK® Lab and STK® Spray, where graphic 3 shows the total number of agreements and disagreements obtained from the comparison.



Graphic 3 - Total results of the agreements and disagreements obtained from the comparison between the three preliminary tests under study and the PSA test.

This comparison could only be carried out on sixty-three samples with the AP test and STK® Lab and forty-six with the STK® Spray test. Three comparisons had no effect due to the PSA test not being carried out and twenty comparisons had no effect due to the PSA test and STK® Spray not being carried out, respectively.

From the comparison between the results obtained from the frequently used test, the AP test, and the PSA test, there were fifty-four agreements and nine disagreements. These figures equate to a percentage of 85.71% and 14.29%, respectively, in the total of sixty-three samples compared.

Fifty-eight agreements were obtained between the STK® Lab and the PSA test, giving an equivalence of 92.06%, and five disagreements, equivalent to 7.94%, out of a total of sixty-three samples compared.

The last comparison was between the results obtained from the samples tested with the STK® Spray product and the PSA test. Twenty-nine results were in agreement, equivalent to 63.04%, and seventeen disagreements, corresponding to 25.76%, out of forty-six samples compared.

Thus, comparing the percentages obtained from the results of the three preliminary tests under study, the method with the highest percentage of agreements and consequently the lowest percentage of disagreements with the results obtained from the PSA test was STK[®] Lab.

The disagreements obtained during these three comparisons were different, and were classified by type of result, as presented in Table 1.

Table 1 - Type and number of occurrences of disagreements between the three preliminary tests under study and the PSA test.

Types of disagreements	Results		Number of Occurrences		
	Preliminary tests under study	PSA test	AP test	STK [®] Lab	STK [®] Spray
1			5	2	2
2			3	1	1
3			1	1	0
4			0	1	14

The first type of disagreement, in which the results of the preliminary tests under study were questionable and the PSA test was negative, can be explained by the fact that the preliminary tests under study may have been reacting to another type of fluid.

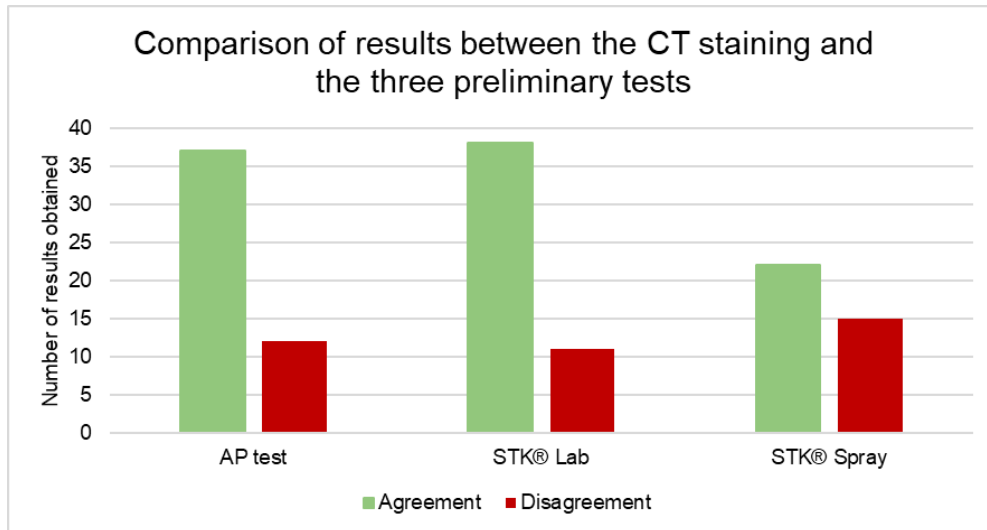
The second type of disagreement, in which the results of the preliminary tests under study were questionable and the PSA test was positive, can be explained by the possible incorrect application of the tests by the technician.

The third type of disagreement, in which the results of the preliminary tests under study were positive and the PSA test was negative, can be explained by the fact that the results of the preliminary tests are false positives, since they can react to other types of fluids, and this is confirmed by the negative result of the PSA test, which is more specific.

The fourth type of disagreement, in which the results of the preliminary tests under study were negative and the PSA test was positive, can be explained, in the case of STK[®] Lab, by the incorrect application of the test by the technician, with samples not being transferred to the STK[®] Lab paper and a negative result being obtained. In the case of the STK[®] Spray, it could be because these samples came from white or light-coloured fabrics. As a result, when the fabric absorbed the liquid from the product, it emitted a homogeneous fluorescence throughout the fabric, making it impossible to differentiate between the suspicious stain and the rest.

1.3.2. Comparison of results between the CT staining and the three preliminary tests

The second comparison was between the CT staining and the three preliminary tests under study, the AP test, STK[®] Lab and STK[®] Spray, where graphic 4 shows the total number of agreements and disagreements obtained from the comparison.



Graphic 4 - Total of the results of the agreements and disagreements obtained from the comparison between the three preliminary tests under study and the CT staining.

This comparison could only be carried out on forty-nine samples with the AP and STK[®] Lab tests and thirty-seven with the STK[®] Spray test, with seventeen comparisons having no effect due to the CT staining not being performed and twenty-nine comparisons having no effect due to the CT staining and STK[®] Spray tests not being carried out, respectively.

The comparison between the results obtained from the commonly used laboratory test, the AP test, and the CT staining showed that there were thirty-seven agreements, equivalent to 75.51%, and twelve disagreements, corresponding to 24.49% of the forty-nine samples compared.

Thirty-eight agreements and eleven disagreements were obtained from the results obtained when comparing the STK[®] Lab method and the CT staining, corresponding to 77.55% and 22.45%, respectively, of the forty-nine samples compared.

In the comparison between the results obtained from the STK[®] Spray method and the CT staining, twenty-two agreements were obtained, equivalent to 59.46%, and fifteen disagreements, representing 40.54%, of the thirty-seven samples compared.

Thus, comparing the percentages obtained from the results of the three preliminary tests under study shows that the method with the highest percentage of agreement and consequently the lowest percentage of disagreement with the results obtained from the CT staining was STK[®] Lab.

The disagreements obtained during these three comparisons were different, and were classified by type of result, as can be seen in Table 2.

Table 2 - Type and number of occurrences of disagreements between the three preliminary tests under study and the CT staining.

Types of disagreements	Results		Number of Occurrences		
	Preliminary tests under study	CT test	AP test	STK [®] Lab	STK [®] Spray
1			4	2	2
2			1	1	1
3			7	8	2
4			0	0	10

The first type of disagreement, in which the results of the preliminary tests under study were questionable and the CT staining was negative, can be explained by the fact that the preliminary tests under study may have been reacting to another type of fluid.

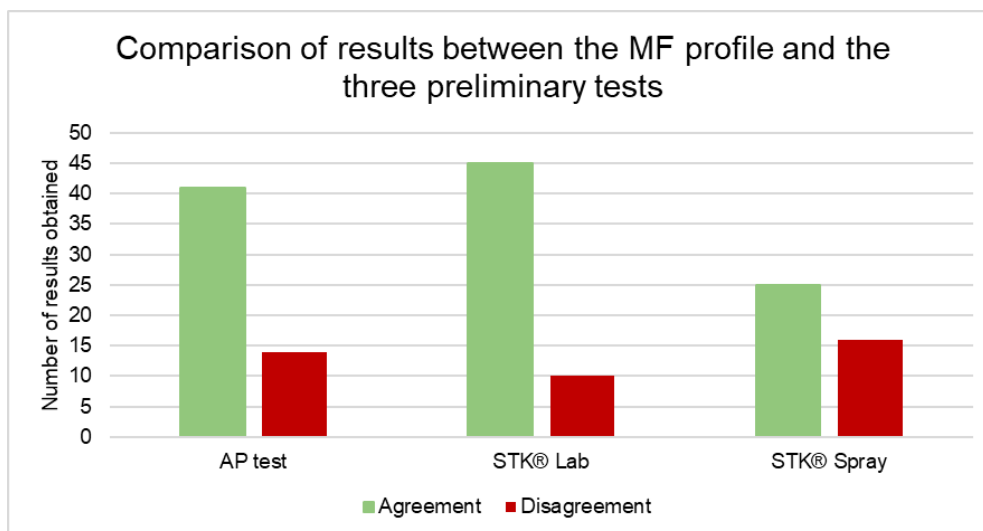
The second type of disagreement, in which the results of the preliminary tests under study were questionable and the CT staining was positive, can be explained by the possible incorrect application of the tests by the technician.

The third type of disagreement, in which the results of the preliminary tests under study were positive and the CT staining was negative, can be explained by the fact that the results of the preliminary tests are false positives, since they can react to other types of fluids or be samples from azoospermic, oligospermic or vasectomized individuals.

The fourth type of mismatch, in which the results of the preliminary tests under study were negative and the CT staining was positive, can be explained by the fact that these samples come from white or light-coloured fabrics and so, when the fabric absorbs the liquid from the STK[®] Spray product, it emits a homogeneous fluorescence throughout the fabric, making it impossible to differentiate between the suspicious stain and the rest.

1.3.3. Comparison of results between the MF profile and the three preliminary tests

The third comparison was between the results obtained from the MF profiles and the results obtained from the three preliminary tests under study, the AP test, STK® Lab and STK® Spray, where graphic 5 shows the total number of agreements and disagreements obtained from the comparison.



Graphic 5 - Total of the results of the agreements and disagreements obtained from the comparison between the three preliminary tests under study and the MF profile.

This comparison could only be carried out on fifty-five samples with the AP test and STK® Lab and forty-one with the STK® Spray, with eleven comparisons having no effect due to the MF profiles not being carried out and twenty-five comparisons having no effect due to the MF and STK® Spray profiles not being carried out, respectively.

The comparison between the results obtained from the MF profiles and the AP test produced forty-one agreements, equivalent to 74.55%, and fourteen disagreements, corresponding to 25.45% of the fifty-five samples compared.

The comparison between the results obtained from the STK® Lab methodology and the profiles obtained from MF resulted in forty-five agreements and ten disagreements. These figures represent 81.82% and 18.18% respectively of the total of fifty-five samples compared.

Finally, in the comparison between the results obtained from the STK® Spray methodology and the MF profiles, twenty-five agreements were obtained, equivalent to 60.98%, and sixteen disagreements representing 39.02% of the forty-one samples compared.

Thus, comparing the percentages obtained from the results of the three preliminary tests under study, the method with the highest percentage of agreements

and consequently the lowest percentage of disagreements with the results obtained from the MF profiles was STK[®] Lab.

The disagreements obtained during these three comparisons were different, and were classified by type of result, as can be seen in Table 3.

Table 3 - Type and number of occurrences of disagreements between the three preliminary tests under study and the MF profile.

Types of disagreements	Results		Number of Occurrences		
	Preliminary tests under study	FM	AP test	STK [®] Lab	STK [®] Spray
1			6	3	2
2			1	0	1
3			7	7	2
4			0	0	11

The first type of disagreement, in which the results of the preliminary tests under study were questionable and the MF profile was negative, can be explained by the fact that the preliminary tests under study may have been reacting to another type of fluid.

The second type of disagreement, in which the results of the preliminary tests under study were questionable and the MF profile was positive, can be justified by the possible incorrect application of the tests by the technician.

The third type of disagreement, in which the results of the preliminary tests under study were positive and the MF profile was negative, can be explained by the fact that the results of the preliminary tests are false positives, since they may react to other types of fluids or be samples from azoospermic, oligospermic or vasectomized individuals.

The fourth type of mismatch, in which the results of the preliminary tests under study were negative and the MF profile was positive, can be explained by the fact that these samples come from white or light-coloured fabrics and so when the fabric absorbs the liquid from the STK[®] Spray product, it emits a homogeneous fluorescence throughout the fabric, so that it is not possible to differentiate between the suspicious stain and the rest.

1.4. Comparison of all the results obtained from the real irregular samples

A comparison between all the results obtained in the preliminary tests (AP test, STK[®] Lab, STK[®] Spray and PSA test), the CT staining and the MF profile revealed some irregular results that should be highlighted, as shown in Table 4.

Table 4 - Comparison of all the results obtained from five real irregular samples.

Sample	AP test	STK [®] Lab	STK [®] Spray	PSA test	CT test	FM
1						
20						
23						
30						
31						

In samples 1 and 20, the STK[®] products is more sensitive than the AP test, since in sample 1 the remaining results are positive and in sample 20 the remaining results of the preliminary tests are positive and the CT staining and the MF profile are negative, possibly originating from an azoospermic, oligospermic or vasectomized individual.

In sample 23, only the AP test and the STK[®] Lab have positive results; these could be false positives, as they are reacting to another type of fluid.

In sample 30, the PSA test is a false positive result, since all the other results, except the AP test, are negative and therefore confirm the questionable result obtained in the AP test.

Sample 31 showed positive results for the AP test, STK[®] Spray and the CT staining. The questionable result obtained in the STK[®] Lab may be due to a possible incorrect application of the test by the technician, as not enough sample was transferred to the STK[®] Lab paper. On the other hand, the negative result in the MF profile could be due to the incorrect execution or visualisation of the sperm staining, or the fact that the sample came from an oligospermic individual, so there were few sperm and consequently, it was not possible to obtain a good MF profile.

2. Comparative Test – Simulated Samples

The results obtained were different and a colour was assigned to each type of result. The green colour corresponds to positive results, which are those that showed a positive reaction, the appearance of a purple hue in the case of the AP test, and the detection of strong fluorescence in the case of the STK[®] Lab and STK[®] Spray. Yellow corresponds to questionable results, which are those that incited doubt, a weaker fluorescence in the case of the STK[®] Lab and STK[®] Spray and a softer tone in the AP test. The red colour corresponds to negative results, which are those where there was no reaction of any kind. These types of results can be seen in the tests: Sensitivity on different textiles; Sensitivity on Azoospermic Samples; Specificity; Repeatability and Reproducibility; Spray: Real Samples, Sensitivity and Specificity and Slide Staining. Also in these tests, except the STK[®] Spray Test: Real Samples, two types of comparisons were made: a first comparison in which the test with the best success rate in each of the tissues, fluids or testing days was verified by counting them (except the Sensitivity Test on Azoospermic Samples and the Slide Staining Test); and a second comparison in which the success rate of each test was averaged and compared. To do this, a correspondence of values was made: one value for expected results, half a value for questionable results and zero for unexpected results and multiplied by one hundred to be presented as a percentage.

2.1. Sensitivity Test on different textiles

Attachment 2 shows the results obtained from the sensitivity test carried out on different textiles using the AP test.

On the green cotton fabric, positive results were obtained on samples PS (A and B) and D 1/10 (A). However, sample D 1/10 (B) had a questionable result, since a colour change was visible, but to pink. The NC (A and B), samples D 1/100 (A and B) and D 1/1000 (A and B) had no colour change.

In denim, positive results were obtained in the PS samples (A and B) and a sample of D 1/10 (A). Negative results were obtained for the remaining samples.

For polyester, positive results were obtained in samples PS (A and B) and D 1/10 (B). Sample D 1/10 (A), D 1/100 (A and B) and D 1/1000 (A and B) had questionable results, which meant that only the NCs did not change colour.

For lace, white cotton, elastane and satin, positive results were obtained for samples PS (A and B) and D 1/10 (A and B). The remaining samples showed no colour changes, so they obtained negative results.

In the wool fabric, positive reactions were detected in samples PS (A and B), D 1/10 (A and B), D 1/100 (A and B) and D 1/1000 (A). The D 1/1000 (B) sample showed a questionable pink colour.

On the linen support, positive results were only obtained on the PS samples (A and B).

Attachment 3 contains the results obtained from the sensitivity test carried out on different textiles using the STK[®] Lab methodology.

In the green cotton fabric, fluorescence was observed in the PS samples (A and B) and D 1/10 sample (B). Questionable fluorescence was seen in sample D 1/10 (A) and NC (A and B), and no fluorescence was obtained in samples D 1/100 (A and B) and D 1/1000 (A and B).

In denim, fluorescence was only detected in the semen samples. In sample D 1/10 (A) there was questionable fluorescence and in the remaining samples, there was no fluorescence.

In polyester, fluorescence was observed in samples PS (A and B), D 1/10 (A and B) and sample D 1/100 (B). Questionable fluorescence was detected in sample D 1/100 (A) and no fluorescence was seen in the remaining samples, NC (A and B) and D 1/1000 (A and B).

In the lace, fluorescence was detected in the PS (A and B), D 1/10 (A and B) and D 1/100 (A). No fluorescence was seen in the NC (A and B), D 1/100 (B) and D 1/1000 (A and B) samples.

In the fabric composed of woollen fabric, fluorescence was obtained in samples PS (A and B), D 1/10 (A and B), D 1/100 (A and B) and D 1/1000 (A). No fluorescence of any kind was observed in the NC (A and B) and D 1/1000 (B) samples.

On the white cotton support, fluorescence was only obtained in the PS samples (A and B). Questionable fluorescence was seen in samples D 1/10 (A and B) and no fluorescence was seen in the remaining samples, NC (A and B), samples D 1/100 (A and B) and D 1/1000 (A and B).

Elastane, satin and linen obtained the same results, while fluorescence was obtained in PS (A and B) and D 1/10 (A and B) samples. No fluorescence was obtained in NC (A and B), D 1/100 (A and B) and D 1/1000 (A and B).

Attachment 4 contains the results obtained from the sensitivity test carried out on different textiles using the STK[®] Spray method.

The same results were obtained on green cotton and polyester fabrics. Samples PS (A and B), D 1/10 (A and B) and D 1/100 (A and B) showed fluorescence. The NC (A and B) and D 1/1000 (A and B) samples showed no fluorescence.

In denim, questionable fluorescence was obtained in the PS samples (A and B) and D 1/10 sample (A). No fluorescence was observed in the remaining samples, NC and in samples D 1/10 (B), D 1/100 (A and B) and D 1/1000 (A and B).

On the lace, white cotton and elastane fabric supports, no fluorescence was seen in all the test samples.

On the wool support, fluorescence was seen in all the PS and diluted samples. No fluorescence of any kind was observed in the NCs.

Satin and linen obtained the same results, with fluorescence in the PS (A and B) and D 1/10 (A and B) samples. No fluorescence was obtained in the remaining samples.

2.1.1. Comparison of preliminary tests results

Table 5 shows all the results of the three preliminary tests for detecting traces of semen in the sensitivity test, described above.

Table 5 - Comparison of the results of the Sensitivity Test on different textiles using the three methods.

Sensitivity Test	Green Cotton			Denim			Polyester			Lace			Wool			White Cotton			Elastane			Satin			Linen		
	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray
NC (A)																											
NC (B)																											
PS (A)																											
PS (B)																											
D 1/10 (A)																											
D 1/10 (B)																											
D 1/100 (A)																											
D 1/100 (B)																											
D 1/1000 (A)																											
D 1/1000 (B)																											
Success rate (%)	55	55	80	50	45	35	75	75	80	60	70	10	95	90	100	60	50	10	60	60	10	60	60	60	40	60	60

The first comparison shows that on green cotton, polyester and wool, the best methodology is STK[®] Spray. For denim and white cotton, it's the AP test. For lace, it's STK[®] Lab, for elastane it's the AP test and STK[®] Lab, for linen it's both STK[®] product methods and for satin all three tests obtained the same percentage. Thus, the STK[®] Spray method is the best solution as it has the highest success rates in five of the nine fabrics analysed.

To carry out the second comparison, the average percentages for each test were calculated, giving an average of 61.67% for the AP test, 62.78% for STK[®] Lab and 49.40% for STK[®] Spray. This shows that the STK[®] Lab method has the highest average percentage.

2.2. Sensitivity Test on azoospermic samples

Table 6 shows the results obtained from the sensitivity test on samples from azoospermic individuals.

Table 6 - Comparison of the results of the Sensitivity Test on azoospermic samples using the three methods.

Sensitivity Test	AP test	STK [®] Lab	STK [®] Spray
NC (A)			
NC (B)			
PS (A)			
PS (B)			
D 1/10 (A)			
D 1/10 (B)			
D 1/100 (A)			
D 1/100 (B)			
D 1/1000 (A)			
D 1/1000 (B)			
Success rate (%)	60	80	65

In the AP test, positive results were obtained in the PS (A and B) and D1/10 (A and B) samples. Negative results were obtained for NC (A and B), D 1/100 (A and B) and D1/1000 (A and B).

When testing the STK[®] Lab methodology, fluorescence was obtained in the PS (A and B) and D 1/10 (A and B) samples. Questionable fluorescence was detected in the D 1/100 (A and B) and D 1/1000 (A and B) samples, and there was no fluorescence only in the NC (A and B).

In the STK[®] Spray methodology, fluorescence was found in samples PS (A and B) and D 1/10 (A and B). Sample D 1/100 (A) showed suggestive fluorescence and samples D 1/100 (B), D 1/1000 (A and B), and NC (A and B) showed no fluorescence.

Comparing the results of this test through the percentages of success rates, with an average of 60% in the AP test, 80% in the STK[®] Lab and 65% in the STK[®] Spray, the STK[®] Lab method has the highest average percentage.

2.3. Specificity Test

Attachment 5 shows the results obtained by performing the AP test to detect semen in the specificity test. Diverse results were obtained for the different types of biological traces studied.

In blood, positive results were obtained in the PS samples (A and B) and the M 1:1 samples (A and B). No colour changes were observed in the NC (A and B) and TS (A and B) samples and questionable results were obtained in the M 1:10 (A and B) samples.

In saliva, positive results were obtained in the PS samples (A and B), in a TS sample (A), the M 1:1 (A and B) and M 1:10 (A) samples. Negative results were obtained in NC (A and B), TS (B) and M 1:10 (B).

In urine, positive results were found in the PS (A and B), M 1:1 (A and B) and M 1:10 (B) samples. In the M 1:10 (B) sample, a questionable pink result was obtained and, in the NC, (A and B) and TS (A and B) samples, negative results were obtained.

In vaginal fluid, positive results were obtained in the PS samples (A and B), the M 1:1 (A and B) and M 1:10 (A and B) samples. Negative results were obtained in NC (A and B) and TS (A and B).

In the faeces, positive results were obtained in the PS samples (A and B) and the M 1:1 samples (A and B) and in the remaining samples no change in tone was detected.

Attachment 6 shows the results obtained by using the STK[®] Lab method to detect semen in the specificity test.

The same type of results was obtained in blood, vaginal fluid and faeces. Fluorescence was detected in the PS samples (A and B), the M 1:1 (A and B) and M 1:10 (A and B) samples. No fluorescence of any kind was found in the NC (A and B) and TS (A and B).

In saliva, fluorescence was obtained in the PS (A and B) and M 1:1 (A and B) samples. Questionable fluorescence was obtained in the M 1:10 samples (A and B) and no fluorescence was obtained in the NC (A and B) and TS samples.

Urine showed similar results to the three fluids described, except that no fluorescence was detected in the M 1:10 (B) sample.

Attachment 7 shows the results obtained by using the STK[®] Spray method to detect semen in the specificity test.

In blood, fluorescence was obtained in the PS samples (A and B) and the M 1:1 samples (A and B). Questionable fluorescence was detected in the M 1:10 sample (A and B) and no fluorescence was observed in the NC and TS samples (A and B).

In saliva, fluorescence was detected in the PS samples (A and B), in the TS samples (A and B), the M 1:1 (A and B) and M 1:10 (A and B) samples, meaning that only in the NCs was no fluorescence observed.

In the urine, fluorescence was observed in the PS samples (A and B), the M 1:1 (A and B) and M 1:10 (A and B) samples. No fluorescence was obtained in the NC (A and B) and TS (A and B) samples.

In the vaginal fluid, fluorescence was obtained in the PS samples (A and B), the M 1:1 (A and B) and M 1:10 (A and B) samples. Questionable fluorescence was obtained in the TS (A and B) and no fluorescence was detected in the NC (A and B).

Finally, in the faeces, fluorescence was obtained in the PS samples (A and B), M 1:1 (A and B) and M 1:10 (A) samples. No fluorescence was detected in the NC (A and B), TS (A and B) and M 1:10 (B) samples.

2.3.1. Comparison of preliminary tests results

Table 7 shows all the results described above from the three preliminary tests for detecting traces of semen in the specificity test.

Table 7 - Comparison of the results of the Specificity Test with the execution of the three methods.

Specificity Test	Blood			Saliva			Urine			Vaginal fluid			Faeces		
	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray
NC (A)															
NC (B)															
PS (A)															
PS (B)															
TS (A)															
TS (B)															
M 1:1 (A)															
M 1:1 (B)															
M 1:10 (A)															
M 1:10 (B)															
Success rate (%)	90	100	90	80	90	80	95	90	100	100	100	90	80	100	90

To highlight some of the results obtained: in the saliva test samples in the AP test and the STK[®] Spray, there were positive results for the AP test in one sample and fluorescence detection in the STK[®] Spray in two samples. However, it is not possible to verify with existing laboratory techniques whether the mixing results are positive/questionable due to the presence of seminal fluid or saliva. The same is true of vaginal fluid: the two test samples, in the STK[®] Spray methodology, result in the detection of questionable fluorescence.

The first comparison shows that in blood, saliva and faeces the best methodology is STK[®] Lab. In urine, it's STK[®] Spray and in vaginal fluid, it's the AP test and STK[®] Lab. In this way, the STK[®] Lab method is the best solution, since it has a higher number of success rates in four of the five biological fluids studied.

To carry out the second comparison, the average percentages for each test were calculated, giving an average of 89% for the AP test, 96% for STK[®] Lab and 90% for STK[®] Spray. This also shows that the STK[®] Lab method has the highest average percentage.

2.4. Repeatability and Reproducibility Test

2.4.1. Test 1

Table 8 shows the results obtained from the test carried out by Person 1 - No experience but knowledgeable about the product.

Table 8 - Comparison of the results of the first test of the Repeatability and Reproducibility Test with the execution of the three methods.

Repeatability and Reproducibility Test	1 Day			1 Week			1 Month		
	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									
Success rate (%)	60	65	100	60	40	60	60	60	70

On the first day of testing, positive results were obtained in the AP test on samples PS (A and B) and D 1/10 (A and B), and negative results were obtained on the remaining samples. In the STK[®] product Lab methodology, fluorescence was observed in samples PS (A and B) and D 1/10 (A and B). Questionable fluorescence was obtained in sample D 1/100 (A) and no fluorescence was detected in samples D 1/100 (B), D 1/1000 (A and B) and NC (A and B). In the STK[®] Spray methodology, fluorescence was detected in all the samples except the NCs, where there was no fluorescence.

In the test carried out one week after the samples were deposited, the AP test only produced positive results for the PS samples (A and B) and the D 1/10 samples (A and B). No colour changes were observed in the remaining samples, leading to negative results. In the STK[®] Lab methodology, fluorescence was only detected in the PS samples

(A and B) and in the STK[®] Spray methodology, fluorescence was observed in the PS samples (A and B) and the D 1/10 samples (A and B).

When the three methodologies were tested after one month, it was found that the AP test and the STK[®] Lab methodology yielded the same type of results. In the AP test, positive results were obtained in the PS (A and B) and D 1/10 (A and B) samples. Negative results were obtained in the NC (A and B), D 1/100 (A and B) and D 1/1000 (A and B) samples. In the STK[®] Lab methodology, fluorescence was obtained in samples PS (A and B) and D 1/10 (A and B). No fluorescence was observed in the NC (A and B), D 1/100 (A and B) and D 1/1000 (A and B) samples. In the STK[®] Spray methodology, fluorescence was observed in samples PS (A and B) and D 1/10 (A and B). Questionable fluorescence was detected in the D 1/100 (A and B) samples and no fluorescence was observed in the NC and D 1/1000 (A and B) samples.

To highlight some of the results obtained, the samples tested after one week, D 1/10 (A and B) in the STK[®] Lab test, and D 1/100 (A and B) in the STK[®] Spray test are considered stochastic, as they go from negative to positive and from negative to questionable, respectively. This event may have been caused by the technician's incorrect execution of the method.

The first comparison showed that on the first day and after a month, the best method is STK[®] Spray. In the test carried out after one week, the AP test matches the results of STK[®] Spray. Thus, the STK[®] Spray method is the best solution as it has a higher number of success rates on three of the three days tested.

To perform the second comparison, the average percentages for each test were calculated, giving an average of 60% for the AP test, 55% for STK[®] Lab and 76.67% for STK[®] Spray. This also shows that the STK[®] Spray method has the highest average percentage.

2.4.2. Test 2

Table 9 shows the results obtained from the test carried out by Person 2 - No experience and no knowledge of the product.

Table 9 - Comparison of the results of the second test of the Repeatability and Reproducibility Test with the execution of the three methods.

Repeatability and Reproducibility Test	1 Day			1 Week			1 Month		
	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									
Success rate (%)	60	65	80	60	50	60	50	50	60

On the first day of testing, the AP test showed colour changes in the PS samples (A and B) and the D 1/10 samples (A and B). Negative results were obtained for the remaining samples. In the STK[®] Lab methodology, fluorescence was observed in the PS samples (A and B) and the D1/10 samples (A and B). Questionable fluorescence was obtained in sample D 1/100 (A) and no fluorescence was detected in samples D 1/100 (B), D 1/1000 (A and B) and NC (A and B). In the STK[®] Spray methodology, fluorescence was detected in samples PS (A and B), D 1/10 (A and B) and D 1/100 (A and B). No fluorescence was observed in the NC (A and B) and D 1/1000 (A and B) samples.

In the test carried out a week after the samples were deposited, the AP test showed the same results as the STK[®] Spray methodology. In the AP test, positive results were obtained in the PS (A and B) and D 1/10 (A and B) samples. The remaining samples were negative. In the STK[®] Spray methodology, fluorescence was detected in samples PS (A and B) and D 1/10 (A and B). No fluorescence was detected in the remaining samples. In the STK[®] Lab methodology, fluorescence was only detected in samples PS

(A and B) and D 1/10 (A). No fluorescence was detected in the NC (A and B) and the D 1/10 (B), D 1/100 (A and B) and D 1/1000 (A and B) samples.

When the three methodologies were tested after one month, it was found that the AP test and the STK[®] Lab methodology yielded the same type of results. In the AP test, positive results were obtained in the PS samples (A and B) and a questionable result in the D 1/10 samples (A and B). Negative results were obtained in the NC (A and B), D 1/100 (A and B) and D 1/1000 (A and B). In the STK[®] Lab test, fluorescence was obtained in the PS samples (A and B) and questionable fluorescence in the D 1/10 samples (A and B). No fluorescence was observed in the NC (A and B), D 1/100 (A and B) and D 1/1000 (A and B). In the STK[®] Spray methodology, fluorescence was found in samples PS (A and B) and D 1/10 (A and B). No fluorescence was seen in the D 1/100 (A and B), D 1/1000 (A and B) and NC samples.

To highlight one of the results obtained, the sample tested after one week, D 1/10 (B) in the STK[®] Lab test, is considered stochastic, as it changes from negative to questionable after one month. This event can be explained by the technician's incorrect execution of the method.

The first comparison shows that STK[®] Spray is the best method on the first day and after one month. In the test carried out after a week, the AP test matches the results of the STK[®] Spray. Therefore, the STK[®] Spray method is the best solution because it has a higher success rate on three of the three days tested.

To perform the second comparison, the average percentages for each test were calculated, giving an average of 56.67% for the AP test, 55% for STK[®] Lab and 66.67% for STK[®] Spray. This also shows that the STK[®] Spray method has the highest average percentage.

2.4.3. Test 3

Table 10 shows the results obtained from the test carried out by Person 3 - With experience and no knowledge of the product.

Table 10 - Comparison of the results of the third test of the Repeatability and Reproducibility Test with the execution of the three methods.

Repeatability and Reproducibility Test	1 Day			1 Week			1 Month		
	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray	AP test	STK® Lab	STK® Spray
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									
Success rate (%)	55	70	70	60	60	60	60	65	75

On the first day of testing, the AP test, positive results were obtained in samples PS (A and B) and D 1/10 (A). A questionable result was obtained in sample D 1/10 (B) and negative results were obtained in the remaining samples. The STK® Lab and STK® Spray methodologies obtained the same results. No fluorescence was found in the NC (A and B) and D 1/1000 (A and B) samples and questionable fluorescence was detected in the D 1/100 (A and B) samples. In the PS (A and B) and D 1/10 (A and B) samples, fluorescence was obtained for both methodologies of the STK® product and the presence of a purple hue in the AP test.

In the results of the test carried out a week after the preparation of the study test, the three preliminary tests had the same type of results. Positive results were obtained for the PS samples (A and B) and the D 1/10 samples (A and B). Negative results were obtained for the remaining samples.

When the three methodologies were tested after a month, the opposite of the previous test occurred and the results were different. In the AP test, they remained the

same as the results of the test carried out after one week, with a purple hue observed in the PS (A and B) and D 1/10 (A and B) samples. In the STK[®] Lab methodology, fluorescence was obtained in samples PS (A and B) and D 1/10 (A and B). Questionable fluorescence was obtained in sample D 1/100 (A) and no fluorescence was obtained in the remaining samples. Finally, in the STK[®] Spray methodology, fluorescence was observed in samples PS (A and B), D 1/10 (A and B) and D 1/100 (B). Questionable fluorescence was obtained in sample D 1/100 (A) and no fluorescence was observed in samples D1/1000 (A and B) and NC (A and B).

To highlight some of the results obtained, the samples tested after one week, D 1/100 (A) in the STK[®] Lab test, and D 1/100 (A and B) in the STK[®] Spray test are considered stochastic because, in the test after one month, samples D 1/100 (A) from the STK[®] Lab and D 1/100 (A) from the STK[®] Spray go from negative to questionable, and sample D 1/100 (B) changes from negative to positive. This can be explained by the fact that the technician incorrectly performed the methods.

The first comparison shows that on the first day, both STK[®] methodologies yielded the same results. In the test carried out after one week, the three orientation tests had equal results and after one month the methodology with the best success rate was STK[®] Spray. Thus, the STK[®] Spray method is the best solution since it had higher number of success rates on three of the three days tested.

For the second comparison, the average percentages for each test were calculated, giving an average of 58.30% for the AP test, 65% for STK[®] Lab and 68.33% for STK[®] Spray. This also shows that the STK[®] Spray method has the highest average percentage success rate.

2.4.4. Test 4

Table 11 shows the results obtained by Person 4 - an experienced forensic expert who was not familiar with the product's methodology.

Table 11 - Comparison of the results of the fourth test of the Repeatability and Reproducibility Test with the execution of the three methods.

Repeatability and Reproducibility Test	1 Day			1 Week			1 Month		
	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									
Success rate (%)	70	60	80	60	60	80	40	40	70

In this test after one day, the results of the AP test were positive in samples PS (A and B) and D 1/10 (A and B). In the D1/100 (A and B) samples, a pinkish-orange colour was obtained, which makes to these results being classified as questionable. In the NC (A and B) and D 1/1000 (A and B) samples were negative results for the test performed. In the STK[®] Lab and STK[®] Spray methodologies, similar results to the AP test were obtained, with the difference that in the STK[®] Lab methodology no fluorescence was detected in the D 1/100 samples (A and B), giving negative results, and in the STK[®] Spray methodology the same samples showed positive fluorescence.

After a week, the results obtained from the AP test were similar to those of the one-day test, with the difference that the D 1/100 samples (A and B) went from questionable to negative. The results of the STK[®] Lab and STK[®] Spray methodologies remained the same as those of the one-day test.

In the one-month test, there was only one change in the AP test compared to the results of the one-week test, where samples D 1/10 (A and B) changed from positive to

negative. In the STK[®] Lab methodology, the only change was also in the D 1/10 samples (A and B), where no fluorescence of any kind was detected. The result went from positive to negative compared to the samples tested in the one-week test. Finally, in the STK[®] Spray methodology, there was only one change, but in D 1/100 (A and B) samples, where questionable fluorescence was observed.

The first comparison shows that the STK[®] Spray method has the highest success rate on all three test days. Thus, the STK[®] Spray method is the best method since it has a higher number of success rates on three of the three days tested.

For the second comparison, the average percentages for each test were calculated, giving an average of 56.67% for the AP test, 53.33% for STK[®] Lab and 76.67% for STK[®] Spray. This also shows that the STK[®] Spray method has the highest average percentage success rate.

2.4.5. Test 5

In the Repeatability and Reproducibility Test, carried out by Person 5 - an experienced expert who knows the STK[®] product methodology - the results shown in Table 12 were obtained.

Table 12 - Comparison of the results of the fifth test of the Repeatability and Reproducibility Test with the execution of the three methods.

Repeatability and Reproducibility Test	1 Day			1 Week			1 Month		
	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray	AP test	STK [®] Lab	STK [®] Spray
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									
Success rate (%)	70	70	75	55	60	80	60	60	75

In the test carried out one day after the samples were deposited, in the AP test, positive results were obtained for the presence of semen in PS (A and B) and D 1/10 (A and B). Questionable results were obtained for the D 1/100 samples (A and B) since the shade reproduced from the reaction of the reagent when it comes into contact with the acid phosphatase enzyme did not correspond to the colour obtained. The NCs (A and B) and the D 1/1000 samples (A and B) obtained negative results. In the STK[®] Lab methodology, the same results were obtained as in the AP test, except that fluorescence analysis is used in this method. The results obtained using the STK[®] Spray method were similar to those of the previously described tests. However, a positive fluorescence was observed in D1/100 (B) sample.

In the tests carried out after one week, there were changes across all methodologies. In the AP test, compared to the one-day test, there was a change in sample D 1/10 (B), which went from a positive result to a questionable result, and samples D 1/100 (A and B) went from a questionable result to a negative result. In the STK[®] Lab methodology samples D (A and B) obtained a negative result and in the STK[®] Spray methodology sample D 1/100 (A) emitted fluorescence, obtaining a positive result.

Finally, in the test carried out one month later, there was a change in the AP test in sample D 1/10 (B), which became positive. In the STK[®] Lab methodology, the results remained the same as in the one-week test. In the STK[®] Spray methodology, there was only one change in sample D 1/100 (A), equalling the results obtained of the one-day test.

The first comparison shows that on all three testing days, the STK[®] Spray method has the highest success rate. In this way, the STK[®] Spray method is the best methodology since it has a higher number of success rates on three of the three days tested.

To carry out the second comparison, the average percentages for each test were calculated, giving an average of 61.67% for the AP test, 63.33% for STK[®] Lab and 76.67% for STK[®] Spray. This also shows that the STK[®] Spray method has the highest average percentage success rate.

2.4.6. Comparison between tests

By conducting a final comparison between the five tests during the Repeatability and Reproducibility Test, it is possible to verify, by separating the results for each preliminary test and for each day tested, that the results are generally similar, except for the stochastic results mentioned in each test carried out.

2.5. Durability Test

To test the durability of the STK[®] Lab paper, three tests were carried out at different intervals: one day after opening the envelope containing the STK[®] Lab paper, the leftmost stain; one week after, the central stain; one month after, the rightmost stain, as shown in Figure 27. The fluorescence obtained were consistent across three tests. Hence, It can concluded that the STK[®] Lab paper can produce good results one month after opening the packaging.



Figure 27 - STK[®] Lab test results after one day, one week and one month.

2.6. Verifying the quality of a DNA profile

A total of thirty male fraction profiles were analysed from five sprayed and five unsprayed samples from each of the tests: Real samples, Sensitivity test on different textiles and Sensitivity test of azoospermic samples. To verify the quality of a DNA profile after using STK[®] Spray. Then, comparisons were made between the samples with and without spray from the same tests.

In the real samples, DNA profiles were obtained in all samples with and without spray. It was found that the profiles obtained were the same, with the same number of homozygotes/heterozygotes in the same markers and consequently the same number of alleles.

In the Sensitivity Test samples, a negative result was obtained in the negative control samples, with and without spray, as expected. In the pure semen samples and with dilutions of 1/10 and 1/100 with and without spray, complete profiles were obtained. In the 1/1000 diluted semen sample without spray, a complete profile was obtained and in the one with spray, an incomplete profile was obtained. A comparison of the profiles showed that the negative control samples, pure semen and semen diluted 1/10 and

1/100 were the same, with the same number of homozygotes/heterozygotes for the same markers and consequently the same number of alleles. Only in the comparison between the 1/1000 diluted semen samples with and without spray was there a difference between the profiles obtained, with the absence of four alleles in the sample with spray, out of a profile with thirty-nine alleles in total.

In the Sensitivity Test of azoospermic samples, no DNA profiles were obtained in all the samples with and without the spray, as was expected, since they belong to individuals with azoospermia, who do not have spermatozoa and so there is no male genetic material to extract to obtain a genetic profile.

2.7. STK[®] Spray Test

2.7.1. Real Samples

Six real samples of non-porous materials were analysed, four of which came from sexual products (condoms), one from hygiene products (diapers) and one from decoration item (e.g., vases). These samples were situated on different types of surfaces such as latex, diaper material and plastic. The analysed surfaces varied in shade, such as transparent, white and brown, as can be seen in the table in Attachment 8.

Comparing all the results obtained from the real samples of non-porous materials in the preliminary tests (AP test, STK[®] Spray and PSA test), the CT staining and the MF profile, the following results are shown in Table 13.

Table 13 - Comparison of all the results obtained from the six samples analysed.

Sample	AP test	STK [®] Spray	PSA test	CT test	FM
1					
2					
3					
4					
5					
6					

The following comparison of the results of the real samples was conducted using agreements and disagreements. “Agreements” indicate consistency between the results and “disagreements” indicate inconsistency in the results, which can be with just one difference or more.

In the six samples of non-porous materials obtained from real cases, there was disagreement in only one sample and agreement in the remaining five. In sample 3, where there was disagreement, the STK® Spray method is more specific than the AP test since, while all the results are negative, including the STK® Spray result, only the AP test result is questionable, and there may have been a reaction with another type of fluid.

2.7.2. Sensitivity Test

Table 14 shows the results of the sensitivity test carried out to detect traces of semen on non-porous materials.

Table 14 - Comparison of the results of the Sensitivity Test on different non-porous material supports using the two orientation methods, AP Test and STK® Spray.

Sensitivity Test	Rubber		Plastic		Latex		Cement		Aluminium		Vegetation		Components of a diaper	
	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray
NC (A)														
NC (B)														
PS (A)														
PS (B)														
D 1/10 (A)														
D 1/10 (B)														
D 1/20 (A)														
D 1/20 (B)														
Success rate (%)	100	100	87,5	100	100	100	81,25	100	100	100	100	100	100	100

For the first material used, rubber, both tests obtained the same results. In the AP test, positive results were obtained in the PS (A and B), D 1/10 (A and B) and D 1/20 (A and B) samples and negative results only in the NC (A and B). In the STK® Spray methodology, fluorescence was present in all the PS (A and B), D 1/10 (A and B) and D 1/20 (A and B) samples and absent only in the NC (A and B). As with this material, the same results were obtained on latex, aluminium, vegetation and the components of a diaper.

In the AP test carried out on plastic, positive results were obtained on samples PS (A and B) and D 1/10 (A and B). A questionable result was obtained on samples D 1/20 (A and B), which had a pink colour, and negative results were obtained on NC (A and B).

In cement, the AP tests obtained were positive for PS (A and B) and sample D 1/10 (A). Questionable results were obtained for samples D 1/10 (B) and D 1/20 (A and B), since there is a reaction, but the colour obtained is pink.

The same results were obtained with the STK[®] Spray method on the plastic and wall materials: positive on all the semen samples, pure and diluted, and negative on the NCs (A and B).

In the first comparison, on rubber, latex, aluminium, vegetation and diaper components both tests have the same results. However, on plastic and cement, the methodology that stood out with the best success rate was STK[®] Spray. The STK[®] Spray method is the best solution as it has the highest success rates on seven of the seven substrates analysed.

To carry out the second comparison, the average percentages for each test were calculated, giving an average of 95.54% for the AP test and 100% for STK[®] Spray. This shows that the STK[®] Spray method has the highest average percentage.

2.7.3. Specificity Test

Table 15 shows the results of the specificity test carried out on the non-porous latex material.

Table 15 - Comparison of the results of the Spray Specificity Test with the execution of the two methods on a Latex surface.

Specificity Test	Blood		Saliva		Urine	
	AP test	STK® Spray	AP test	STK® Spray	AP test	STK® Spray
NC (A)						
NC (B)						
PS (A)						
PS (B)						
TS (A)						
TS (B)						
M 1:1 (A)						
M 1:1 (B)						
M 1:10 (A)						
M 1:10 (B)						
Success rate (%)	100	100	90	90	80	80

In the blood test, the results obtained were as expected in both preliminary tests. In the AP test, positive results were only obtained in samples containing semen and in the STK® Spray methodology, fluorescence was also found only in samples containing semen.

In the saliva test, the results were also identical in both preliminary tests. In the AP test, only the samples containing semen were positive. The TS samples (A and B), in this case, saliva, showed a change when the reagent came into contact with the swab, but this is not the colour corresponding to a positive result. In the STK® Spray methodology, only positive fluorescence was detected in the samples containing semen. Questionable fluorescence was detected in the TS samples (A and B).

Finally, in the test carried out with the urine test sample, the results obtained in both preliminary tests were similar. In the AP test, positive results were obtained for the PS (A and B) and M 1:1 (A and B) samples. In the TS (A and B) and M 1:10 (A and B), questionable results were obtained because the colour obtained did not correspond to the colour of a positive result. In the STK® Spray methodology, fluorescence was

detected in the samples containing semen, except the M 1:10 samples (A and B), where no fluorescence was observed.

Looking at the results obtained from the TS (A and B) saliva and TS (A and B) urine samples, the AP test shows questionable results. In the case of the STK[®] Spray, only the TS (A and B) saliva samples showed this type of result. This raises the question of whether the positive/questionable results obtained in the samples with mixtures might not be influenced by some reaction with the other fluid.

Making the first comparison, in the three biological fluids under study, both orientation tests, the AP test and STK[®] Spray have the same results. Both preliminary tests are therefore suitable for this type of testing.

To carry out the second comparison, the average percentages for each test were calculated, giving an average of 90% for the AP test and also 90% for the STK[®] Spray. This also shows that both tests are suitable. However, the STK[®] Spray does not react to urine, so it can be considered the most suitable test for testing fluid mixture stains on non-porous surfaces.

Table 16 shows the results obtained in the specificity test carried out on the non-porous support, rubber, using two preliminary tests, the AP test and STK[®] Spray method.

Table 16 - Comparison of the results of the Spray Specificity Test with the execution of the two methods on a Rubber surface.

Specificity Test	Blood		Saliva		Urine	
	AP test	STK [®] Spray	AP test	STK [®] Spray	AP test	STK [®] Spray
NC (A)						
NC (B)						
PS (A)						
PS (B)						
TS (A)						
TS (B)						
M 1:1 (A)						
M 1:1 (B)						
M 1:10 (A)						
M 1:10 (B)						
Success rate (%)	100	100	90	85	80	85

In the blood test, the same results were obtained in both tests. In the samples where semen is present, the result was positive.

In the test carried out on saliva, in the AP test, the PS (A and B), M 1:1 (A and B) and M 1:10 (A and B) samples obtained positive results, as in the TS (A) sample. In the STK[®] Spray methodology, fluorescence was detected in the PS (A and B), M 1:1 (A and B) and M 1:10 (B) samples. Fluorescence was obtained in the TS samples (A and B) and the M 1:10 sample (A), but it did not correspond to positive fluorescence.

Finally, in the urine test, positive results were obtained for the AP test in the PS (A and B) and M 1:1 (A and B) samples. Questionable results were obtained for the TS (A and B) and M 1:10 (A and B) samples. In STK[®] Spray, fluorescence was detected in the PS samples (A and B) and the M 1:1 samples (A and B). Questionable fluorescence was obtained in the M 1:10 sample (A) and the TS sample (A and B) no fluorescence was obtained.

Looking at the results obtained from the TS (A) saliva and TS (A and B) urine samples, the AP test shows a positive and questionable result, respectively. In the case of the STK[®] Spray, the TS (A and B) saliva samples showed questionable results. This raises the question of whether the results obtained in the mixed samples might be influenced by some reaction with the other fluid.

The first comparison showed that in blood, both the AP test and the STK[®] Spray have the same results. In saliva, the AP test has a higher success rate and in urine, the methodology that stood out was STK[®] Spray. In this way, both preliminary tests are suitable for this type of testing since they both had higher success rates in two of the three biological fluids analysed on the rubber support.

To carry out the second comparison, the average percentages for each test were calculated and an average of 90% was obtained for the AP test and also 90% for the STK[®] Spray. This also shows that both tests are adequate. However, the STK[®] Spray does not react to urine and compared to the AP test, it does not have positive results for the biological fluid, saliva, so this can be considered the most suitable test for testing stains from fluid mixtures on non-porous surfaces.

2.7.4. Durability Test

To test the durability of the sprayable solution of the STK[®] Spray product, fifteen tests were carried out at different intervals. Daily tests were performed on a positive control over a fifteen days period. Figure 28 shows the results obtained: at the end of one day of product preparation, the leftmost stain; after seven days, the central stain and on the last day of testing, fifteen days later, the rightmost stain. The fluorescence measured in the first two tests shown in this figure, displayed no notable difference from the first to the seventh day. Whereas, the fifteenth day image demonstrated a greater drag, as if the sample were diluting. These results suggest that the STK[®] Spray test's sprayable solution can reproduce reliable results for up to a seven day after preparation. However, this use is not recommended by the manufacturer.

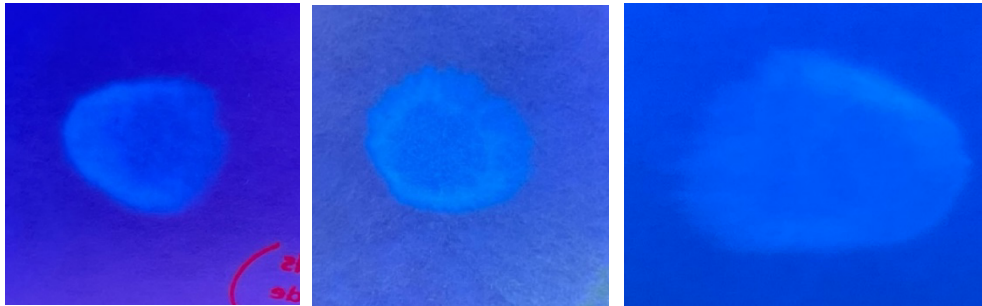


Figure 28 - Test results with STK[®] Spray after one, seven and fifteen days.

2.8. Slide Staining Test

Table 17 shows the results obtained from the HE and CT staining certainty tests. In slides A to G of both tests, derived from negative control samples, no whole spermatozoa or only their heads were visualised. However, slides prepared from standard semen samples, from H to N, showed whole sperm cell or their heads.

Table 17 - Results of the slide staining certainty tests carried out with HE and CT on simulated samples.

Slide Staining Test	HE	CT
NC (A)		
NC (B)		
NC (C)		
NC (D)		
NC (E)		
NC (F)		
NC (G)		
PC (H)		
PC (I)		
PC (J)		
PC (K)		
PC (L)		
PC (M)		
PC (N)		
Success rate (%)	100	100

Table 18 shows the results obtained from the HE and CT staining certainty tests on samples from real cases. On slides 1-6, 8-12 and 14 of both tests, whole sperm cell or their heads were visualised. On slides 7, 13 and 15, either no whole spermatozoa or only their heads were visualised. These results can be confirmed by comparing them to the results obtained from the differential extraction MF profile, resulting in complete agreement between the tests.

Table 18 - Results of the slide staining certainty tests carried out with HE and CT on samples from real cases.

Slide staining Test samples	HE	CT	FM
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

The comparison revealed that identical results were obtained in both staining tests on simulated samples and real samples.

Although the same type of results was obtained on the fourteen simulated sample slides and the fifteen real sample slides, it should be noted that the slides stained with the HE staining process consumed more time to view compared to the slides stained with the CH stain. This is due to various factors, such as: dye's shade, and therefore their contrast, and the way the slide is prepared.

Each staining method employs different dyes with varying shades to visualise the sperm cells and there is greater contrast in CT staining. During HE staining the medium and the spermatozoa are stained with very similar shades, making it difficult to differentiate between other cells and the spermatozoa. However, in CT staining the medium is not affected by the dyes, only the spermatozoa, acquiring a red hue in the nucleus, which makes it easier to see.

The preparation of the slides with the HE staining also affects their observation, since when it is necessary to "undo" the tissue where the suggestive stain is located, the sample under analysis is spread over the slide when the tissue fluid is released, covering a larger area. Afterwards, it will have to be fully visualised and the release of fibres means

that they are easily burnt when dried by the lamp, thus making the slide dirtier. In CT staining, using the rest of the sample from the PSA test and pipetting the sample prevents these two problems mentioned in HE staining. Once the tissue has been soaked in the PSA test buffer, it simply needs to be removed, leaving no fibres behind, and by pipetting only the required amount of pellet, the sample is concentrated.

Conclusion

The observation stage is crucial in criminal expertise, thus necessitating the optimisation of biological trace detection methods.

This study sought to compare a variety of methodologies in detecting semen traces. The results revealing varying results across different parameters and analysed showed that:

- Real Samples

The most effective methodology was the STK[®] Lab as it produced the most pragmatic results, which were less open to questionable results than those produced by the AP test (AP test = 12.12%; STK[®] Lab = 4.55%; STK[®] Spray = 4.55%) and no no-effect results, as opposed to STK[®] Spray (AP test = 0%; STK[®] Lab = 0%; STK[®] Spray = 28.79%). Among the tests compared, this methodology exhibited the highest degree of agreement in its results (PSA test: AP test = 85.71%; STK[®] Lab = 92.06%; STK[®] Spray = 63.04%), (CT staining: AP test = 75.51%; STK[®] Lab = 77.55%; STK[®] Spray = 59.46%) and (FM: AP test = 74.55%; STK[®] Lab = 81.82%; STK[®] Spray = 60.98%).

- Comparative Test – Simulated Samples

In the Sensitivity Test conducted on different textiles, it was found that the STK[®] Spray method demonstrated a superior success rate on a larger selection of fabrics when compared to the other two methodologies. However, the STK[®] Lab method displayed a higher overall success percentage (AP test = 61.67%; STK[®] Lab = 62.78%; STK[®] Spray = 49.40%). Although the STK[®] Spray performed better than the other methods in some tissue samples, it proved ineffective in others. As a result, the STK[®] Lab is deemed more sensitive and secure as its outcomes have greater consistency across all tissues. The most successful methodology for azoospermic samples was found to be the STK[®] Lab (AP test = 60%; STK[®] Lab = 80%; STK[®] Spray = 65%). This demonstrates the effectiveness of the methodology for individuals who are azoospermic, vasectomised, or oligospermic, supporting that the presence of sperm is not necessary factor for detection.

In the Specificity Test, the methodology that acquired the most excellent outcomes was the STK[®] Lab (AP test = 89%; STK[®] Lab = 96%; STK[®] Spray = 90%). These results show that this methodology is more specific, detecting semen even when mixed with other biological fluids.

In the Repeatability and Reproducibility Test, the STK® Spray emerged as the methodology with the superior results in both comparisons across all five tests. The findings suggest that this user-friendly product does not require knowledge of its methodology or professional experience, while still yielding similar results.

During the STK® Lab Durability Test, it was determined that the product remained efficient in producing results for a minimum of fifteen days following its initial use.

During the assessment of DNA profile quality, the anticipated outcomes were achieved, and all the profiles obtained are reportable, demonstrating compatibility with the current laboratory extraction techniques.

Following the manufacturer's guidelines, it was discovered that the STK® Spray is unsuitable for porous materials as a higher proportion of results proved negative. Nevertheless, it demonstrated a favourable success rate on certain supports tested. Further investigation into different fabrics is required to ascertain conclusive findings.

A comparison of the methodologies for porous materials, the AP test and the STK® Lab, showed that STK® Lab generated superior results. The STK® Lab approach had better outcomes in the Sensitivity Test on different textiles, outperforming the AP test, and in the Real Samples test, as well as in the Sensitivity Test on azoospermic samples, and in the Specificity Test. It is therefore possible to conclude that the STK® Lab approach is more sensitive and more specific than the AP test approach.

- STK® Spray Test

In the Real Samples, Sensitivity Test and Specificity Test, the STK® Spray yielded superior results as compared to the AP test. Furthermore, the requirement to rub the filter paper on the stain and then bring it in contact with the AP solution jeopardizes the quantity of the sample, which can be crucial when the samples are limited.

In the STK® Spray Durability Test, the findings suggest that the product remains effective for obtaining results for up to seven days after its creation. It is worth noting that the manufacturer does not recommend using it beyond the intended timeframe.

This test reveals that the STK® Spray method is better suited for non-porous surfaces. Nevertheless, unlike samples collected from porous surfaces, this samples type is not routinely dispatched to the BBG sector.

According to the results above, the STK® product methodologies demonstrate increased sensitivity and specificity, simplicity of use, and non-toxicity for both the operator and the sample. The STK® Lab is suitable for use on porous surfaces, while

the STK® Spray is more appropriate for non-porous surfaces. However, the product is more expensive and necessitates the use of an ALS to visualise the results. Additionally, false positive results have decreased, but have not been resolved yet.

- Slide Staining Test

During the trial, CT staining demonstrated superior results to HE staining. This finding is attributed to the stark contrast, allowing for better visibility of the spermatozoa, hence reducing the time spent examining them. Additionally, using the same collection from the PSA test for CT staining eliminates further consumption of samples, particularly significant in limited samples contexts.

In Forensic Genetics investigation, it is vital to conduct various tests, including preliminary and confirmatory ones, and obtain DNA profiles, to enable a comprehensive analysis of the findings. This approach leads to a more conclusive and objective outcome.

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Attachments

Attachment 1 – Real Samples.

Sample	Surface	Type of material	Colour	AP test	STK® Lab	STK® Spray	PSA test	CT staining	FM
1	Underwear	Cotton	White	Questionable	Positive	Positive	Positive	Positive	Positive
2	Underwear	Cotton	White	Positive	Positive	Positive	Positive	Positive	Positive
3	Home textiles	Cotton	White	Positive	Positive	Positive	Positive	Positive	Positive
4	Underwear	Synthetic	Pink	Positive	Positive	Positive	Positive	Positive	Positive
5	Underwear	Polyester	Black	Positive	Positive	Positive	Positive	Positive	Positive
6	Underwear	Cotton	Beige	Positive	Positive	Positive	Positive	Positive	Positive
7	Underwear	Cotton	White	Questionable	Questionable	Questionable	Negative	Negative	Negative
8	Underwear	Cotton	Red	Positive	Positive	Questionable	Positive	Positive	Positive
9	Underwear	Synthetic	Blue	Positive	Positive	Positive	Positive	Positive	Positive
10	Underwear	Synthetic	Black	Positive	Positive	Positive	Positive	Positive	Positive
11	Home textiles	Cotton	White	Positive	Positive	Positive	Positive	Positive	Positive
12	Underwear	Cotton	Beige	Questionable	Questionable	Questionable	Negative	Negative	Negative
13	Hygiene products	Diaper Material	White	Positive	Positive	Positive	Positive	Positive	Positive
14	Underwear	Cotton	White	Negative	Negative	Negative	Negative	Negative	
15	Underwear	Cotton	White	Negative	Negative	Negative	Negative	Negative	Negative
16	Clothing	Polyester	Blue	Negative	Negative	Negative	Negative		
17	Home textiles	Microfiber	Purple	Negative	Negative	Negative	Negative		Negative
18	Clothing	Synthetic	Multicoloured	Positive	Positive		Positive	Positive	Positive
19	Clothing	Denim	Blue	Negative	Negative	Negative			
20	Underwear	Cotton	Blue	Questionable	Positive	Positive	Positive	Negative	Negative
21	Clothing	Cotton	Black	Negative	Negative				
22	Underwear	Synthetic	Black	Negative	Negative				
23	Home textiles	Cotton	White	Positive	Positive	Negative	Negative	Negative	Negative
24	Home textiles	Cotton	Blue	Positive	Positive		Positive	Positive	Positive
25	Home textiles	Cotton	White	Negative	Negative	Negative	Negative		Negative
26	Home textiles	Cotton	Beige	Positive	Positive	Negative	Positive	Positive	Positive
27	Underwear	Cotton	Pink	Questionable	Negative		Negative		Negative
28	Hygiene products	Diaper Material	White	Positive	Positive	Positive	Positive	Negative	Negative
29	Home textiles	Cotton	White	Negative	Negative	Negative	Negative	Negative	Negative
30	Hygiene products	Diaper Material	White	Questionable	Negative	Negative	Positive	Negative	Negative
31	Underwear	Cotton	Black	Positive	Questionable		Positive	Positive	Negative
32	Home textiles	Cotton	Green	Negative	Negative		Negative		
33	Underwear	Cotton	Red	Negative	Negative	Negative	Negative		Negative

34	Others (Feces)	Cotton	White	Questionable	Negative		Negative		
35	Others (Swab)	Cotton	Yellow	Negative	Negative	Negative	Negative		Negative
36	Home textiles	Cotton	Blue	Positive	Positive		Positive	Negative	Negative
37	Home textiles	Cotton	Green	Positive	Positive	Negative	Positive	Positive	Positive
38	Home textiles	Cotton	Grey	Positive	Positive		Positive	Positive	Positive
39	Home textiles	Cotton	Pink	Positive	Positive	Negative	Positive	Positive	Positive
40	Hygiene products	Diaper Material	White	Negative	Negative	Negative	Negative	Negative	Negative
41	Home textiles	Cotton	Pink	Positive	Positive		Positive	Positive	Positive
42	Hygiene products	Diaper Material	White	Positive	Positive	Negative	Positive	Negative	Positive
43	Underwear	Lace	Blue	Positive	Positive		Positive	Positive	Positive
44	Home textiles	Cotton	White	Positive	Positive	Negative	Positive	Positive	Positive
45	Underwear	Cotton	Blue	Positive	Positive		Positive	Positive	Positive
46	Clothing	Cotton	White	Positive	Positive	Negative	Positive	Positive	Positive
47	Home textiles	Polyester	Grey	Positive	Positive	Negative	Positive	Positive	Positive
48	Home textiles	Cotton	Multicolored	Negative	Negative		Negative		
49	Clothing	Cotton	Blue	Positive	Positive		Positive	Negative	Negative
50	Clothing	Cotton	Blue	Positive	Positive		Positive	Positive	Positive
51	Underwear	Cotton	Black	Positive	Positive		Positive	Positive	Positive
52	Clothing	Cotton	Red	Negative	Negative		Negative		
53	Home textiles	Cotton	Multicolored	Negative	Negative	Negative	Negative		
54	Home textiles	Cotton	Yellow	Positive	Positive	Negative	Positive	Negative	Negative
55	Home textiles	Cotton	Yellow	Positive	Positive	Negative	Positive	Positive	Positive
56	Home textiles	Cotton	Yellow	Positive	Positive	Negative	Positive	Positive	Positive
57	Home textiles	Cotton	Yellow	Positive	Positive	Negative	Positive	Negative	Negative
58	Home textiles	Cotton	Yellow	Questionable	Negative	Negative	Negative		Negative
59	Clothing	Cotton	Pink	Positive	Positive	Positive	Positive	Positive	Positive
60	Underwear	Cotton	Black	Negative	Negative	Negative	Negative		
61	Clothing	Cotton	Beige	Negative	Negative	Negative	Negative		
62	Underwear	Cotton	Grey	Positive	Positive	Positive	Positive	Positive	Positive
63	Underwear	Cotton	Black	Positive	Positive	Positive	Positive	Positive	Positive
64	Home textiles	Cotton	Beige	Positive	Positive	Negative	Positive	Positive	Positive
65	Home textiles	Cotton	Beige	Positive	Positive	Negative	Positive	Positive	Positive
66	Home textiles	Cotton	Beige	Positive	Positive		Positive	Positive	Positive

Attachment 2 – Sensitivity Test results performed on different textiles using the AP test.

[illegible]

Attachment 3 – Sensitivity Test results performed on different textiles using the STK® Lab method.

[illegible]

Attachment 4 – Sensitivity Test results performed on different textiles using the STK®
Spray method.

Sensitivity Test	Green Cotton	Denim	Polyester	Lace	Wool	White Cotton	Elastane	Satin	Linen
NC (A)									
NC (B)									
PS (A)									
PS (B)									
D 1/10 (A)									
D 1/10 (B)									
D 1/100 (A)									
D 1/100 (B)									
D 1/1000 (A)									
D 1/1000 (B)									

Attachment 5 – Specificity Test results performed with the AP test.

Specificity Test	Blood	Saliva	Urine	Vaginal fluid	Faeces
NC (A)					
NC (B)					
PS (A)					
PS (B)					
TS (A)					
TS (B)					
M 1:1 (A)					
M 1:1 (B)					
M 1:10 (A)					
M 1:10 (B)					

Attachment 6 – Sensitivity Test results performed with the STK[®] Lab method.

Specificity Test	Blood	Saliva	Urine	Vaginal fluid	Faeces
NC (A)					
NC (B)					
PS (A)					
PS (B)					
TS (A)					
TS (B)					
M 1:1 (A)					
M 1:1 (B)					
M 1:10 (A)					
M 1:10 (B)					

Attachment 7 – Sensitivity Test results performed with the STK[®] Spray method.

Specificity Test	Blood	Saliva	Urine	Vaginal fluid	Faeces
NC (A)					
NC (B)					
PS (A)					
PS (B)					
TS (A)					
TS (B)					
M 1:1 (A)					
M 1:1 (B)					
M 1:10 (A)					
M 1:10 (B)					

Attachment 8 – Real Samples of STK® Spray Test.

Sample	Surface	Type of material	Colour	AP test	STK® Spray	PSA test	CT staining	FM
1	Sexual products	Latex	Transparent	Positive	Positive	Positive	Positive	Positive
2	Hygiene products	Diaper Material	White	Positive	Positive	Positive	Positive	Positive
3	Sexual products	Latex	Transparent	Questionable	Negative	Negative	Negative	Negative
4	Sexual products	Latex	Transparent	Negative	Negative	Negative		Negative
5	Sexual products	Latex	Transparent	Negative	Negative	Negative		
6	Decoration	Plastic	Brown	Negative	Negative	Negative	Negative	Negative