EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

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MESTRADO EM TECNOLOGIAS LABORATORIAIS EM CIÊNCIAS FORENSES

ASSESSMENT OF THE VIABILITY OF USING SEMIQUANT PSA QUANTIFICATION WITH SERATEC® SERAQUANT™ AS A CONFIRMATORY TEST FOR TRACES OF SEMEN IN A FORENSIC GENETICS LABORATORY – IDEALIZATION, DEVELOPMENT AND VALIDATION OF A METHOD

Trabalho submetido por
Raquel Medeiros
para a obtenção do grau de Mestre em Tecnologias Laboratoriais em Ciências Forenses

novembro de 2024

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Trabalho orientado por
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novembro de 2024

To Hop,
Thank you,
for the last six years.
Without you, I wouldn't
have made it here.
I'll always love you.

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Declaração de Honra

IMP-EM-EI-12

Declaro, por minha honra, que o presente trabalho académico é original e foi elaborado por mim própria, Raquel Alexandra Nunes Colaço Medeiros, não se tendo recorrido a quaisquer outras fontes, para além das indicadas, usadas, adotadas literalmente ou adaptadas a partir dos seus originais (em fontes impressas, não impressas ou na internet) e encontram-se adequadas, identificadas e citadas, com observância das convenções do trabalho académico em vigor.

Mais declaro que esta Tese, Assessment of the viability of using Semiquant PSA Quantification with SERATEC® SeraQuant™ as a confirmatory test for traces of semen in a Forensic Genetics Laboratory – Idealization, Development and Validation of a Method, não foi apresentada, para efeitos de avaliação, a qualquer outra entidade ou instituição, para além da(s) diretamente envolvida(s) na sua elaboração, e que os conteúdos das versões impressa e eletrónica são inteiramente coincidentes.

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Data: 22/11/2024

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Eu Raquel Alexandra Nunes Colaço Medeiros, referente a Tese, Assessment of the viability of using Semiquant PSA Quantification with SERATEC® SeraQuant™ as a confirmatory test for traces of semen in a Forensic Genetics Laboratory – Idealization, Development and Validation of a Method, declaro que não possuo conflitos de interesse de ordem pessoal, comercial, académica, política ou financeira.

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Resumo

Os crimes sexuais representam uma grave violação dos direitos humanos e têm um impacto profundo nas vítimas e na sociedade. Eles correspondem a 10% dos crimes investigados pela Polícia Judiciária (PJ). Conforme especificado no Código Penal português (CP), os crimes de natureza sexual são distinguidos em dois grupos: crimes contra a liberdade sexual (artigos 163.º a 170.º) e crimes contra a autodeterminação sexual (artigos 171.º a 176.º-C). Ao realizar um exame forense de um crime sexual, o vestígio biológico mais importante a se procurar é o sémen. Este fluído é essencial nestes tipos de crimes, pois fornece uma prova incontestável de que um ato sexual foi cometido. Por esta razão, o Setor de Biologia (BBG) do Laboratório de Polícia Científica da Polícia Judiciária (LPC-PJ) tem investido em novos métodos e equipamentos para facilitar a identificação de sémen pelos peritos forenses.

Neste trabalho, foi desenvolvido um método utilizando o instrumento SERATEC® SeraQuant Reader para os testes semi-quantitativos do Antígeno Prostático Específico (PSA). Para avaliar se este método poderia-se tornar um teste confirmatório, amostras de casos reais foram analisadas na fase de observação do fluxo de trabalho do BBG e os resultados obtidos nessa fase foram registados. Quarenta e oito amostras foram adquiridas de 25 exames de crimes sexuais e uma série de estudos de concordância foram realizados entre os resultados dos testes preliminares, o teste confirmatório e os perfis obtidos na análise de ácido desoxirribonucleico (ADN), com o objetivo de identificar possíveis relações. Além disso, o instrumento foi validado internamente realizando-se testes de sensibilidade, especificidade, reprodutibilidade e repetibilidade.

Para as 48 amostras dos casos reais, os estudos de concordância demonstraram que, entre os valores de quantificação do PSA e os perfis da fração masculina (FM), cerca de 89% das amostras com alta concentração de PSA (acima de 4,1 ng/mL) estavam em concordância com o perfil de ADN obtido na FM (masculino ou mistura). Por outro lado, quase 87% das amostras que foram negativas para sémen apresentaram resultados positivos para PSA. Os resultados obtidos nos testes de sensibilidade, especificidade, reprodutibilidade e repetibilidade permitiram validar internamente o SeraQuant Reader.

Palavras-Chave: Sémen, Antígeno Prostático Específico, validação interna, crimes sexuais e SERATEC®.

Abstract

Sexual crimes represent a serious violation of human rights and have a profound impact on the victims and in society, they represent 10% of the crimes investigated by the *Polícia Judiciária* (PJ). As specified in the Portuguese *Código Penal* (CP) crimes of sexual nature are distinguished in two groups: crimes against sexual freedom (article 163.º to 170.º) and crimes against sexual self-determination (article 171.º to 176.º-C).

When performing a forensic exam of a sexual crime, the most important biological trace to look for is semen. This fluid is essential in these types of crimes, because it gives undeniable proof that a sexual act was committed. Therefore, the Biology Sector (BBG) of the *Laboratório de Polícia Científica da Polícia Judiciária* (LPC-PJ) has been investing new methods and equipment is to ease the identification of semen for the forensic experts.

In this work, a method using the SERATEC® SeraQuant Reader instrument was developed for the Prostate-specific Antigen (PSA) Semiquant tests. In order to evaluate if this method could become a confirmatory test, real casework samples were analysed in the observation stage of the BBG workflow and the results obtained in this stage were recorded. Forty-eight samples were acquired from 25 exams of sexual crimes and a series of concordance studies were made between the preliminary tests results, the confirmatory test and the profiles obtained in the deoxyribonucleic acid (DNA) analysis with the aim of identifying possible relations. Furthermore, the instrument was internally validated by performing sensitivity, specificity, reproducibility and repeatability tests.

For the 48 real casework samples, the concordance studies demonstrated that between the PSA quantification values and the Male fraction (MF) profiles, nearly 89% of the samples with high PSA concentration (above 4,1 ng/mL) were in concordance with the male or mixed DNA profile that was obtained in the MF on the other hand, almost 87% of the samples that were negative for semen had positive results for PSA. The results obtained for sensitivity, specificity, reproducibility and repeatability tests allowed to internally validate the SeraQuant Reader.

Keywords: Semen, Prostate-specific Antigen, internal validation, sexual crimes and SERATEC®.

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List of Abbreviations

AICEF - Academia Iberoamericana de Criminalística y Estudios Forenses

AP - Acid phosphatase

AP test - Acid phosphatase test

APAV - Associação Portuguesa de Apoio à Vítima

AS - Assay Setup

BBG – Biology Sector

BBG-LPC - Biology Sector of the Laboratório da Polícia Científica

bps – Base pairs

CE - Capillary Electrophoresis

CF- Cellular Fraction

CP - Código Penal

CPP - Código de 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

INMLCF - Instituto Nacional de Medicina Legal e Ciências Forenses

ISFG - International Society for Forensic Genetics

LPC - Laboratório da Polícia Científica

LT-DNA - low-template-DNA

MAC - Maximum Allele Count

MF - Male Fraction

PCR - Polymerase Chain Reaction

PJ - Polícia Judiciária

PK - Proteinase K

PSA - Prostate-Specific Antigen

PSA test - Prostate-Specific Antigen test

QA - Quality assurance

QC - Quality control

qPCR - Quantitative Polymerase Chain Reaction

RFLP - Restriction Fragment Length Polymorphism

RFUs - Relative fluorescent units

SDS - Sodium Dodecyl Sulfate

SNP - single nucleotide polymorphism

SP – Sample Preparation

SPE - Solid-phase extraction

STRs - Short Tandem Repeats

VNTRs - Variable Number Tandem Repeats

I. Introduction

1. Forensic Genetics

Forensic Genetics has been defined by Jobling and Gill in 2004 as “the application of genetics for the resolution of legal cases” [1].

1.1. Brief History of Forensic Genetics

Forensic Genetics is a field within the Forensic Science that is in constant development and evolution. It began in 1900 when Karl Landsteiner discovered the ABO human blood groups which based on four blood groups (A, B, AB and O). This discovery could help in identifying the blood type of individuals involved in criminal cases as well as help solving paternity tests. The ABO system started then being used in forensic laboratories, but since it needs a high amount of biological material and the fact that the proteins present in this material, start to degrade when in contact with the environment, it has limited discriminatory efficacy. [2–4]

It wasn’t until 1985 when Professor Alec Jeffreys described in a Nature article the term “Deoxyribonucleic acid (DNA) Fingerprinting” that Forensic DNA analysis was introduced. Professor Jeffreys realized that the DNA had hypervariable sequences containing genetic information (polymorphisms that allow for human identification), known as Minisatellites or Variable Number Tandem Repeats (VNTRs) (less than 100 base pairs - bp and greater than ten bp), and that the number of repeats varies amongst individuals. Therefore, he developed a technique that determines the size of VNTRs using the Restriction Fragment Length Polymorphism (RFLP) thus creating a distinct genetic profile for each individual. This technique was first applied in an immigration case in March 1985. Two years later, in 1987, DNA Fingerprinting was successfully used for the first time in a criminal investigation of a double rape and homicide in the United Kingdom. [1,5–8]

Nowadays, the genetic polymorphisms more commonly used in Forensic Genetics are the Microsatellites or Short Tandem Repeats (STRs) since they are non-coding DNA sequences that have between two to six bp. This makes them the markers of choice for a DNA analysis since they have a smaller size of the repeated sequence. The analysis of

autosomal STR is highly discriminant and sensitive, because the number of repeated sequences varies between individuals, which is caused by random mutations. However, sometimes STRs don't determine complete DNA profiles or there is a lack of results from the samples analysis. This is due to DNA degradation, for as it begins to take place, this polymorphism becomes progressively more fragmented, and the production of profiles becomes more difficult. [9,10]

1.2. Science and Law

Edmond Locard, a french criminologist, proposed in 1910 the 'Locard is Exchange Principle' which states that "every contact leaves a trace". This principle established the grounds for forensic science as we know it. [2,11,12]

The application of science to the legal system has the purpose to assist the court and the criminal investigation by providing expertise in legal disputes (criminal or civil) so that they can be resolved. The forensic analysis starts with a legal question, asked by the court or by the criminal investigators, that will determine the way the forensic expert must take in the analysis of the physical evidence in hand. The answer (when there is one) will contribute with information in determining what may have happened, where it happened, when and who was involved, but not in determining why it happened. [13–15]

1.2.1. Portuguese Legislation

In the Portuguese legal system, crime is defined in the article 1.º of the *Código de Processo Penal (CPP)* as "the set of assumptions which the application of a criminal penalty or security measure to the perpetrator depends on.". [16,17]

As said in the previous point, science is now used in court and criminal investigations to help resolve legal disputes, as expert evidence. This evidence, as foreseen in the CPP article 151.º, is required when the perception or appreciation of the facts involves technical, scientific or artistic knowledge. Expert evidence can only be performed by experts in appropriate official establishments such as, *Laboratório da Polícia Científica* (LPC) – Article 41.º of Decree-law n.º 137/2019 of September 13th and *Instituto Nacional de Medicina Legal e Ciências Forenses* (INMLCF) – Chapter I *'Natureza e atribuições'* of Decree-law n.º 96/2001 of March 26th . [18–21]

Decree-law n.º 137/2019 of September 13th defines the LPC as a central unit of specialized technical-scientific support to criminal investigation. It acts in all national territory with technical and scientific autonomy, in accordance with the law, and it is responsible for "carrying out expertise in the various fields of forensic science, namely audio and sound, ballistics, biology, criminalistics, documents and currency, drugs and toxicology, handwriting, criminalistic imaging, information technology and telecommunications, physics, lofoscopy, marks and tools and chemistry". [20]

1.2.1.1. Portuguese DNA Legislation

DNA databases are important for civil and criminal identification, as they allow the comparison between profiles that were obtained on a case and profiles that were previously included in the database, which leads to the resolution of current investigations. With this in head, Law n.º 5/2008 was approved on February 12th 2008 in Portugal for the creation of a national DNA database. Since then, it has suffered two amendments, being the latest Law n.º 90/2017 of August 22nd. This law states the principles and conditions of the database function and use. [22,23]

In article 16.º, of Law n.º 90/2017, INML is defined as "the entity responsible for the DNA profile database and the operations applicable to it" meaning that the DNA profiles obtained by LPC and INMLCF have to be inserted and removed by the latest. According to Ordinance n.º 161/2018 of July 6th, eight specific genetic markers are required for a DNA profile to be considered valid. These markers are: vWA, THO1, D21S11, FGA, D8S1179, D3S1358, D18S51 and Amelogenin. [23,24]

The genetic profiles obtained by DNA analysis come from reference and problem samples, which are defined by Law n.º 90/2017, article 2.º, as "«Reference Sample» the sample used for comparison" and "«Problem Sample» the sample, under investigation, whose identification is intended to be established". [23]

2. Biology Sector – the workflow of a Forensic Genetics Laboratory

In the LPC there are three main areas: Biotoxicology, which includes the Biology Sector (BBG) and Drugs and Toxicology sector; Physical-Documentary, which encompasses the Documental, Currency and Handwriting Analysis and Physical

Chemistry sector; and Criminalistics, which brings together the Judicial Inspection (Crime Scene), Judicial Identification (Lofoscopy) and Ballistics and Trademarks sectors. [25]

The BBG is part of the European Network of Forensic Science Institutes (ENFSI), the International Society for Forensic Genetics (ISFG) and Academia Iberoamericana de Criminalística y Estudios Forenses (AICEF), which offers the forensic experts, workshops, training lessons and meetings for a constant growth in knowledge. It also provides random proficiency tests with the goal of evaluating the laboratory against their predetermined standards. Taking everything into account, it is the biology sector competence to search and identify biological traces, perform DNA analysis as well as comparative analysis of genetic profiles, paternity tests (in criminal investigations), contribute with DNA profiles to the national database, elaborate expert reports, attend court sessions when called upon and represent Portugal in ENFSI DNA Working Group. [26]

The workflow in a Forensic Genetics Laboratory must be divided in five main stages: Preservation, Observation, DNA analysis, Genetic Profile Interpretation and Expert Report (Figure 1).

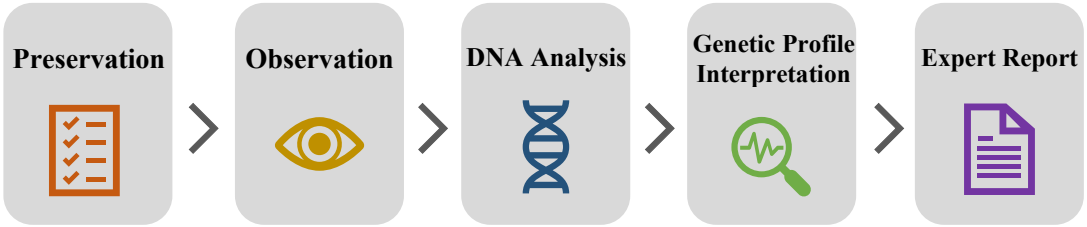


Figure 1 - Main stages of a Forensic Genetics Laboratory (Adapted from [30]).

2.1. Preservation

The first stage in the Biology sector of LPC (BBG-LPC) is Preservation where the evidence is delivered to the laboratory after it has been collected by the crime scene team or delivered to an inspector by the victim of a crime. This stage is fundamental for chain of custody verification, since it is where the expert confirms if all the evidence that was handed corresponds with the evidence listed in the court order of the requested exam. In this stage the reference samples and the problem samples are separated, seeing that they'll

be treated in different rooms (this subject will be addressed in the DNA Analysis Stage). [26]

The expert must verify if the evidence is properly identified and packaged, if this is not the case, the expert must carry out adequate conditioning. When packing DNA evidence, each item should be separate to an unused paper bag or breathable container. The use of plastic bags must be avoided as they create an environment susceptible to humidity, which will cause the degradation of DNA. The bag or container should be sealed, identified and stored in a dry and airy environment, until it is submitted for analysis. [27]

The type of crimes for which exams are requested at the LPC Biology sector is quite diverse but can mostly be grouped into homicides (which also includes attempts), rapes and sexual abuse, violent crimes (such as domestic violence or attacks on physical integrity), robberies/thefts and others. Within this last group, situations of abduction/kidnapping and paternity, for example.

2.2. Observation

As the name states, this stage consists of observing the criminal evidence already preserved and collect biological evidence for analysis. In the BBG a variety of biological traces are collected, including blood, semen, rooted hair, saliva, human fetus, faeces, amongst others.

For this stage there isn't a specific guideline to follow, since it depends on the experience, line of thought and the knowledge of the case, the expert responsible for the exam has. However, there are rules when it comes to the steps in the observation stage. To start with, the expert must carry out the observation with a personal protective equipment to avoid any type of contamination. Secondly, the expert must analyse one item at a time, a detailed description of the evidence is carried out and a photographic record with a forensic ruler, before carrying out any type of tests and collection, in order to guarantee the chain of custody. If there is any stain on the evidence, this also must be described since the condition and colour of it, may influence its analysis. [28,29]

The next step is to identify the type of biological evidence, for this the laboratory is equipped with forensic lights (for traces that are invisible to the naked eye), as well as preliminary and confirmatory tests for each type of biological trace. The BBG disposes

of two types of forensic lights the Crime-lite® ML2 (FOSTER & FREEMAN LIMITED, England) and their newest acquisition the t-ZOOM (Forenscope Scientific, Turkey. As for the preliminary tests the Kastle-Meyer is used for hematic traces, SERATEC® (which will be discussed with more detail in point 4 of present section) for human blood, menstrual blood, Prostate-Specific Antigen (PSA) for semen and saliva and the acid phosphatase (AP) test which is specific for semen. There is currently only one confirmatory test, the microscopic observation for human hair and spermatozoids research. [26,30]

The observation stage ends with the collection of samples (to labelled eppendorfs tubes) from the evidence originated from the crime scene, this step depends on the experience of the expert meaning that the collection of the sample must be a conscious decision regarding what to search and collect, where and how much trace to collect. This final step it is crucial to obtaining good results in the next stage. The collected samples must be stored in the cold until the DNA analysis.

In the end of the observation of an exam the expert must seal everything to maintain the chain of custody, as well as clean and disinfect all the material and workspace used. Once this is done the expert fills out a document (accessible to everyone in the sector) where it specifies the type of biological trace collected, this step is important for the DNA extraction process.

2.3. DNA Analysis

The DNA Analysis in the BBG-LPC is almost fully automated, this allows for the samples to be processed in batches with a fixed number of samples, through a defined internal procedure, reducing expert intervention and therefore reducing contamination. However, they must be able to know the theoretical foundations of each technique, understand automation processes and detect and resolve any technical problems. In this stage the problem samples go through four stages: extraction and purification, quantification, amplification and capillary electrophoresis. Reference samples are processed in a separate room (to avoid contamination with problem samples), in a flow with only three stages where extraction is immediately followed by amplification, since this samples have an “optimal” quantity of DNA, their quantification becomes redundant.

Semen samples are more complex since, before the extraction process, they require a preparation that separates fractions. [1,31,32]

Table 1 shows the commercial kits, equipment and software is the BBG-LPC utilizes for each of the DNA analysis steps.

Table 1 - Commercial Kits, equipment is and software used in each laboratory process necessary to determine DNA profiles.

DNA Analysis Stage	Comercial Kits	Equipment	Software
Extraction	QIAsymphony DNA Investigator Kit	QIAgility QIAcube Connect QIASymphony SP	QIAGEN QIAgility Version 4.17.1 QIAsymphony Management Console
Quantification	Quantifiler® Trio DNA Quantification Kit	QIAsymphony AS QuantStudio™ 5 Real-Time PCR System for Human Identification	HID Real-Time PCR Analysis Software v1.3
Amplification	GlobalFiler™ PCR Amplification Kit GlobalFiler™ Express PCR Amplification Kit Yfiler™ Plus PCR Amplification Kit	QIAsymphony AS Veriti Thermal Cycler	QIAsymphony Management Console
Capillary Electrophoresis	N/A	QIAgility Applied Biosystem 3500xL Genetic Analyzer	3500 Series Data Collection Software v4.0.1

N/A – Not Applicable

2.3.1. Extraction

Before a genetic profile is obtained the DNA must be extracted and purified, since the evidence collected at the crime scene may contain other cellular components. [7]

There are different techniques for the extraction process, for example, organic extraction, Chelex extraction or solid-phase extraction (SPE). The first one is normally used for more challenging samples, it utilizes proteinase K (PK) for cell lysis with a detergent (sodium dodecyl sulfate – SDS) followed by phenol-chloroform to denature the proteins. Although this technique was used for years, it is not safe due to toxicity of phenol, and it is very complex and time-consuming which all led to its disuse. On the other hand, a simpler DNA extraction method is the Chelex technique which utilizes Chelex-100 chelating resin (Bio-Rad Laboratories, Hercules, CA) that binds with polyvalent metal ions, such as magnesium which acts as a catalyst in DNA degradation, its removal protects the DNA molecules. Nowadays, the most used extraction technique in forensic genetic laboratories is the SPE, since it decreases the risk of contamination and sample degradation. The SPE extraction method consists of binding a solid substrate to the DNA molecule. The solid phase most currently used is the magnetic beads, because they're faster and simpler when separating and isolating the DNA from the waste and debris. [31,33–37]

In the BBG-LPC, the extraction process starts with the preparation of the sample batch (which can go up to 90 samples) by the expert. The batch is then inserted in the QIAgility (QIAGEN, Germany), that distributes the reagents depending on the nature of the biological trace, seeing that the extraction process is specific to the type of trace. After this step, the samples are transferred to specific compartments within the QIAsymphony (QIAGEN, Germany), through an automated process. In this equipment there are two phases: the Sample Preparation (SP), where the DNA is purified using magnetic beads and the Assay Setup (AS), where there is a preparation of the samples for quantification and amplification. As referred previously, the process for the reference sample is similar, for the exception of the purification and quantification steps in the QIAsymphony, which are not performed. [26,38]

2.3.1.1. Differential Extraction

Differential Extraction is a method that was designed specifically for the separation of the epithelial cells – Cellular Fraction (CF) and semen – Male Fraction (MF), in a sexual assault exam sample, generating in the end two genetic profiles. For this the BBG-LPC is equipped is the QIAcube (QIAGEN, Germany), an automated equipment that performs the differential extraction of semen samples. [39,40]

Figure 2 illustrates this extraction method. It begins with the incubation of the samples with an extraction buffer, after which they undergo two lysis. The first lysis consists in the addition of PK to the incubation for the lysis of the epithelial cells, the mix it is then centrifuged and Dithiothreitol (DTT) is added to the pellet for the lysis of the sperm cells, since it lessens the disulfide bonds that make up the sperm nuclear membranes, which are resistant to digestion, thus releasing the sperm cell DNA. [40–42]

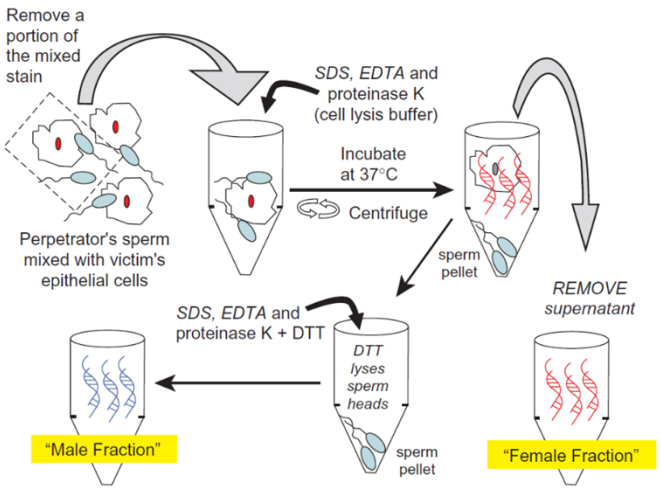


Figure 2 - Schematic illustration of the differential extraction method used to separate male spermatozoa from the epithelial cells (Adapted from [40]).

2.3.2. Quantification

This stage is essential to determine the amount of DNA that is present in the samples being processed, seeing that in the forensic field the amount of DNA in problem samples is always unknown. Quantification is also crucial for a obtaining a good genetic profile, because if there is too much or barely enough DNA in the sample, the profile will turn out difficult to interpret, since a narrow concentration (between 0,5 ng and 1 ng) works best with STRs during the Polymerase Chain Reaction (PCR). For this, samples may need to be diluted or concentrated, depending on the amount of DNA that was quantified, so that the input of the PCR can be adapted to the concentration of the samples to be amplified. [43,44]

For many years, different quantification methods have been used to determine the amount of DNA within a sample, hybridization by slot blot being one of those processes, but they required a significant amount of time, and the sensitivity and specificity were low. Nowadays, real-time PCR, also known as quantitative PCR (qPCR), is the most

commonly used method since it makes possible to determine the amount of DNA in each phase of the amplification in real-time, as well as it is degree of degradation. The qPCR method involves the monitoring of the amplification process through fluorescence techniques, where the amount of fluorescence emitted is directly correlated to the quantity of amplified DNA. The most common approach utilizes a target-specific probe – the TaqMan probe, which has two fluorescent dyes, a 5' fluorescent reporter dye and a 3' quencher dye that are specific to a sequence in the DNA and emit different wavelengths. There are four stages in the qPCR process: the baseline phase, the exponential phase, the linear phase and the plateau phase. It is in the exponential phase that the amplification occurs, and the quantity of DNA is measured through the calculation of the number of cycles that are required for the fluorescence to reach the threshold determined by the software – Cycle Threshold (C_T) (Figure 3). The initial amount of DNA present in the sample and the C_T value calculated by the software is irreversibly proportional, meaning that the higher the initial amount of DNA the lower the C_T value. [44–47]

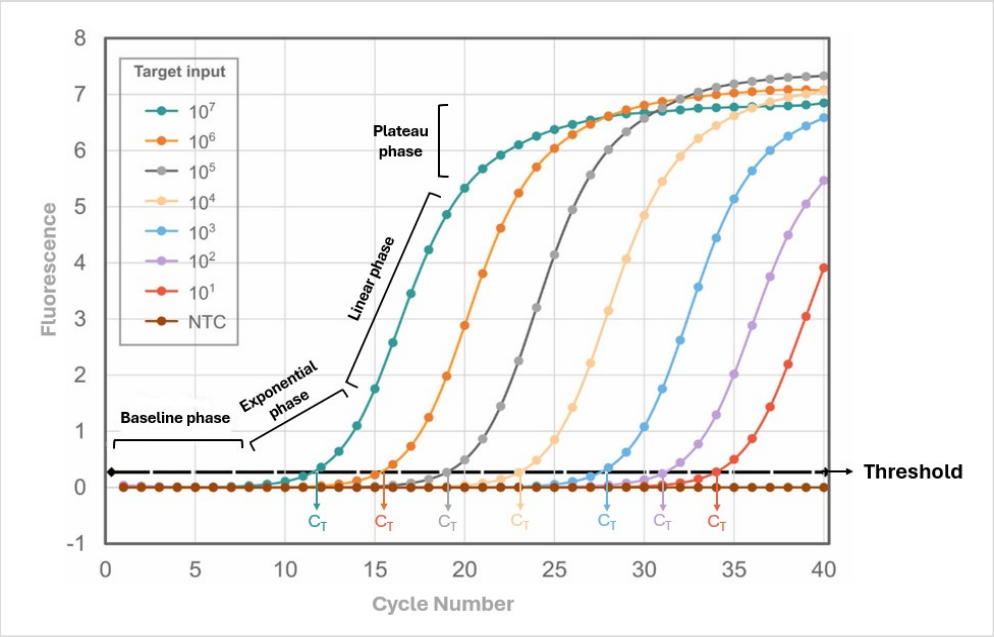


Figure 3 - Real-time PCR phases (Adapted from [45]).

In the biology sector of the LPC the quantification process starts in the QIAsymphony AS where the samples are prepared in an optical plate. After this first step, the plate is then placed in the QuantStudio™ (Thermo Fisher Scientific, USA) for the qPCR process. The experts in the BBG utilize the commercial Quantifiler® Trio DNA kit (Thermo Fisher Scientific, USA) to amplify three genetic markers: Large Autosomal, Small Autosomal

and the Y-chromosome, which allows them to determine the degradation level of the sample (by analysing the ratio of the large and small autosomal markers) and through the analysis of the Y-chromosome marker, the experts are able to determine the ratio of male and female in mixture samples. [48,49]

2.3.3. DNA Amplification

PCR was first invented in the 1985 by Kary Mullis, winning him The Nobel Prize in Chemistry in 1993. The PCR method allows for a small amount of a specific DNA fragment to be copied in large numbers in a short time frame. This discovery has been of extreme importance in the molecular biology field, especially in forensic genetics, where the quantities of DNA in the samples are very limited. [50]

Several years after the PCR discovery, the possibility of a Multiplex PCR was developed, it has been commonly used in forensic laboratories seeing that it amplifies multiple STR target sequences of the DNA simultaneously in the same reaction. For this technique two sets of primers are used, each pair (reverse primer and forward primer) has a fluorescent dye linked to one of its ends, it is also needed deoxyribonucleotide triphosphates (dNTPs), Taq polymerase since it is thermostable, a buffer and magnesium ions to allow an ideal reaction. This method has three stages: first occurs the denaturation of the DNA molecule, secondly the primers anneal to the DNA denatured strands and finally the elongation occurs, through the Taq polymerase where the synthetises of a new DNA strand happens (Figure 4). [47,51,52]

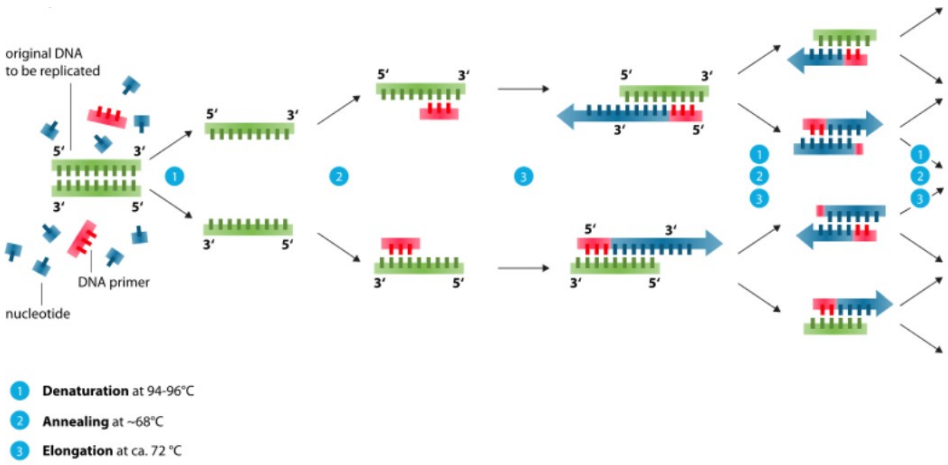


Figure 4 - Polymerase Chain reaction stages and cycles (Adapted from [51]).

In the BBG-LPC, the DNA amplification starts with the preparation of the amplification optical plate in the QIASymphony AS based on the values provided from the quantification step, which are automatically calculated by the QuantStudio. The plate is then inserted in the Veriti Thermal Cycler (Thermo Fisher, USA) for the PCR. [26]

Afterwards, and before the genotyping, the amplified STRs must be separated by length and the STR markers detected, which combined will establish the STR profile. Currently, Capillary Electrophoresis (CE) is the chosen method by the laboratory for this process, the detection of the markers is presently fluorescent based. The BBG-LPC is equipped with a 3500 Series Genetic Analyzer (Thermo Fisher Scientific, USA), making this step fully automated. This equipment has a capillary tube that is filled with a polymer solution which assists in the separation of the DNA fragments by length through an electric voltage, that is applied through the capillary, allowing its migration. A laser-induced fluorescence light is located nearly at the end of the tube detecting when a DNA molecule passes by, due to the fluorochromes bound to it. Correlating the time span since the sample was injected until the laser detects it at the end of the capillary, the equipment is capable of knowing the fragment size. For the CE to identify alleles from different loci overlapping in size ranges and distinguish them, the fluorescent dye linked to the primers used in the amplification is excited by the laser light and emits fluorescence in different regions. In order to recognise the size of each fragment, the 3500 Series Data Collection Software has an internal size standard made by known DNA fragments labelled with different dyes. The information generated in the software is saved and it will be used to obtain the STR profile. [28,53–56]

*

As an alternative to the extraction, quantification and amplification processes described above, the BBG-LPC is equipped with two RapidHIT™ ID System (ThermoFisher Scientific, USA) which allow for a quick DNA analysis. These equipments make it possible to obtain a genetic profile in just under two hours, being essential in cases where a quick response is needed. However, despite being advantageous due to its speed and the little human intervention it requires, only traces in considerable quantities can be processed by this method, as it is necessary to guarantee that, in the

absence of results, a new collection can be made, and the sample can continue through the conventional extraction process.

2.4. STR Genotyping and Genetic Profile Interpretation

The data obtained in the CE are analysed in the GeneMapper™ ID-X software, which is capable of determining the alleles size and assign the corresponding alleles to each STRs detected. In this software a control is also carried out regarding contamination, both between samples from the same batch, as well as possible contamination in the collection of the biological traces, at the crime scene and in processing the samples in the laboratory.

All of the detected alleles are then arranged by marker and fluorophores. The final result is presented in an electropherogram (EPG) format, where each peak corresponds to an allele (Figure 5). These peaks are traced by the correlation of the fluorescent intensity detected, which is measured in relative fluorescent units (RFUs), with the detection time of the fragments on the CE (the fragment size). Afterwards, the experts must perform a revision on the EPG seeing that there may be peaks that need to be identified and edited by hand. [53,55,57]

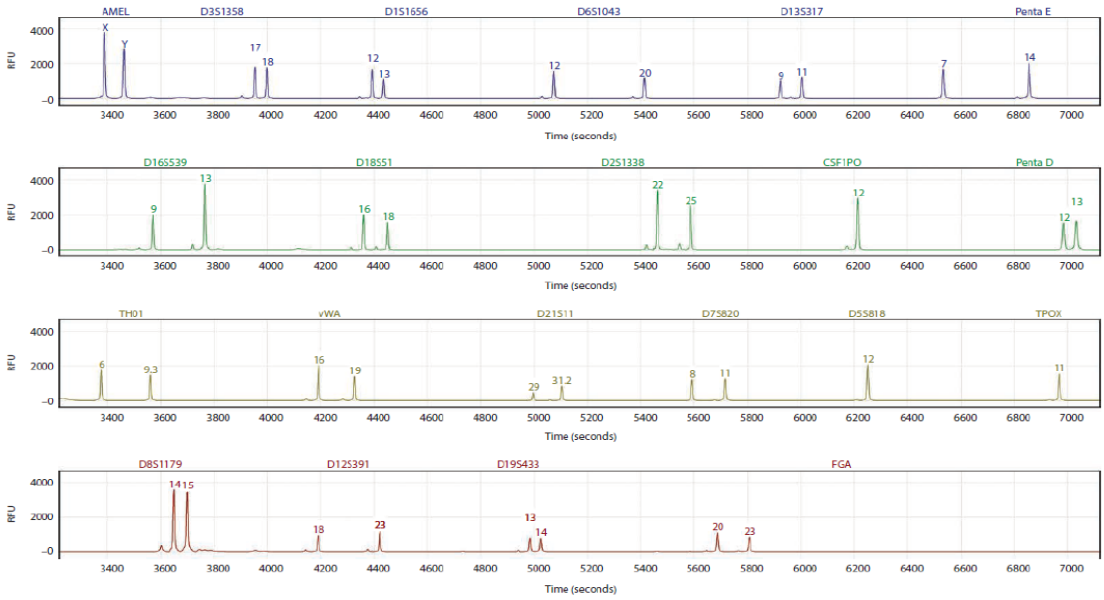


Figure 5 - Electropherogram demonstrating a genetic profile with 20 STRs (Adapted from [43]).

The final genetic profile is subsequently interpreted by the experts, two statistical softwares are provided in case the expert needs assistance in this task, the STRmix™ (which is used for complex samples) and the *Familias 3*.

There are four types of profile results: Single Female Profile, Single Male Profile, Mixed Profile and Inconclusive Profile. The single profiles can be identified by two alleles per locus and by one pair of sex chromosomes. On the other end, when a profile is inconclusive no result was obtained, meaning that the sample may be insufficient or of low quality. A mixture is the most common profile result in forensic genetics, and it is detected on an EPG by the presence of more than two alleles per locus. However, a careful examination of the extra alleles ought to be carried out as to identify if they do belong to another individual or if they are artifacts or stochastic effects. After the detection of a mixture, it is important to determine the number of contributors that are present in it (this is always an estimated parameter), they can either be equal (1:1) or unequal (major and minor contributor). For this the experts focus on the locus that has the highest number of alleles to determine the minimum number of contributors – Maximum Allele Count (MAC), as well as in the analysis of the relative heights of the alleles. [58–60]

In the end, a comparison of the profile obtained from the traces collected in the observation stage and the profile from the reference sample - the victim or the suspect (when there is one) – is carried out by the expert responsible for the exam and there is two possible conclusions: the individual cannot be excluded, or the individual is excluded.

2.4.1. Strategies for complex samples

It is common for forensic genetic samples to have a limited quantity (low-template DNA) or degraded DNA and sometimes it can result in poor genetic profiles, the biology sector has defined some strategies for these two cases.

Nowadays, with low-template-DNA (LT-DNA), the BBG has commercial kits that are able to reamplify the LT-DNA by increasing the number of cycles in the PCR to 34, resulting in an improvement of the sensitivity. On the other hand, with degraded DNA samples the developed strategy involves the use of mini-STRs or SNP, seeing that due to their small size, they are less susceptible to fragmentation and thus can provide a complete genetic profile. [52,61,62]

It is worth to note that in samples collected in a crime context there will be samples which contain biological material from more than one individual. For complex mixed profiles the biology sector is equipped with the STRmix™ specifically designed for this kind of genetic profiles. The DNA commission of ISFG provides a recommended guideline for the interpretation of mixtures. [63,64]

3. Forensic Analysis in Sexual Crimes

Sexual crimes represent a serious violation of human rights and have a profound impact on the victims and in society. These crimes refer to a category of offenses involving acts of a sexual nature, attempted or consummated, that are committed without the victim is consent. Crimes like these can go from physical contact to interactions, such as verbal, of a sexual nature and it affects every kind of person leaving them with long-lasting emotional scars, as well as physical and psychological traumas on both the victims, friends and family. [65]

As specified in the Portuguese *Código Penal* (CP) crimes of sexual nature are distinguished in two groups: crimes against sexual freedom (article 163.º to 170.º) which penalize any type of sexual activity committed without the victims consent regardless of the age, and crimes against sexual self-determination (article 171.º to 176.º-C) that penalize any type of sexual activity with minors and whose existence is directly linked to the need to protect the free development of the child or young person is personality in the sexual domain. [66,67]

In the last five years, the *Associação Portuguesa de Apoio à Vítima* (APAV) has registered 6776 sexual crimes against children and young people. In 2023 the number of crimes of sexual nature increased in more than 30% comparing to the previous year, 1760 sexual crimes against minors and 459 sexual abuses against adults were reported, giving a total of 2219 victims of crimes of sexual nature (in these numbers sexual coercion and harassment complaints are not included). The sexual crimes represent 10% of the crimes investigated by the *Polícia Judiciária* (PJ), of which 65% to 70% of these cases concern sexual abuse of children and young people and around 51% of these are committed within the family. [68–70]

3.1. Biological Traces in Sexual Crime

When doing a forensic exam of a sexual crime, the most important biological trace to look for is semen. This fluid is essential in these types of crimes, because it gives undeniable proof that a sexual act was committed. It is equally important to know the story behind the crime, to know what to look for in the evidence that was submitted for exam. For this, the assigned expert and the criminal inspector of the case should establish a good line of communication to help with the facts of the story, so that the expert knows what to look for, for example, if it was a rape or sexual assault, how many people were involved in the crime, if it was oral, anal or vaginal and whether there was ejaculation or not. By knowing these details, the expert will be able to conduct a more efficient exam on the evidence and collect the right biological traces from the materials.

3.1.1. Semen

Semen, also known as sperm, is a viscous whitish liquid that is made up of different secretions, produced in the different structures of the male genital tract, which mix in the urethra at the time of ejaculation, giving it an alkaline pH. The three main secretions are the sperm cells (spermatozoa) produced in the testicles, the prostatic secretion produced in the prostate and the seminal fluid produced in the seminal vesicles, which is the most important secretion for the forensic biology field, because it contains the two markers which are used to detect semen in forensic exams, the PSA and the AP. Spermatozoa have two main parts, that are functionally and morphologically distinct: the head, which contains the genetic information in the nucleus, the acrosome and the centriole; and the tail, which facilitates their motion. The last one is divided in four parts: the connecting piece (neck), the mid piece (where the mitochondria are based), the principal piece (which has an outer dense fibre surrounded by a fibrous sheath) and the end piece. A schematic illustration of a sperm cell structure is shown in Figure 6. [49,71–74]

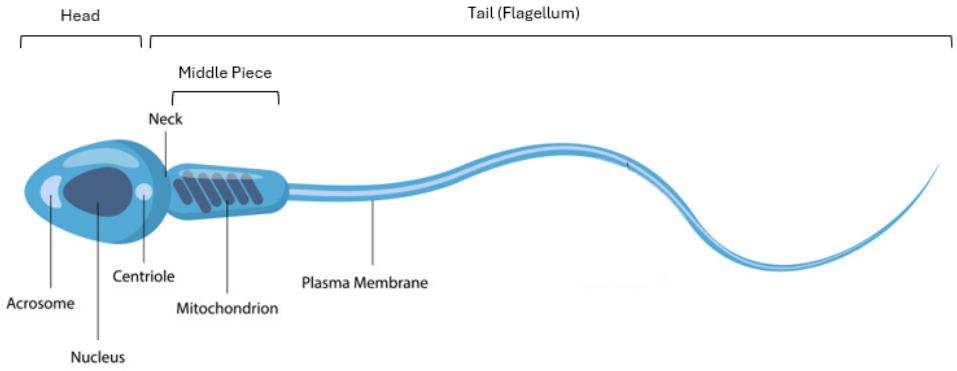


Figure 6 – Sperm cell structure (Adapted from [74]).

It is important to know that the constitution of the semen, upon ejaculation, varies from man to man, due to their habits and health. A normal healthy individual, typically releases from 2 to 5mL of semen, where approximately 90% consists of fluid from the prostate the seminal vesicles and only a small fraction is composed by the spermatozoa, which can be from 200 to 300 million per ejaculation. On the other hand, there are three cases that should be considered in the forensic field, such as individuals that suffer from oligospermia (limited quantities of spermatozoa in the semen) or from azoospermia (absence of spermatozoa in the semen) and individual that have had a vasectomy. [75–78]

3.2. The BBG sector methodologies used for sexual crimes in the observation stage

As stated in 2.2. the laboratory is equipped with forensic lights, preliminary and confirmatory tests for each type of biological trace. Although in the case of sexual crimes exams the approach used by the experts depends on the mainly on the story, the methods used are standardized and must follow the correct order, starting with the preliminary tests and ending with confirmatory tests.

3.2.1. Preliminary tests for semen

Preliminary or presumptive tests are characterized as initial evaluations to identify the biological traces nature and to guide the expert on the next decisions. These tests are fast and simple to apply, but they are not considered conclusive regarding the presence of biological fluids.

For semen, the BBG has three preliminary tests: the forensic light, the Acid Phosphatase Test (AP test) and the SERATEC® PSA Semiquant immunochromatographic test (SERATEC®, Germany).

3.2.1.1. Forensic Light Source – t-ZOOM

Forensic lights sources have been used for identification of biological traces, naked to the eye, for years since it is a simple non-destructive method. This is possible due to certain characteristics of the biological fluids such as fluorescent effect (semen, urine and saliva) and light absorption (blood). [79,80]

Nowadays, the BBG-LPC uses as a forensic light source the t-ZOOM, which is the latest multispectral portable camera. It provides a combination of wavelengths (from 330nm to 1100nm), has an automatic real-time image and video recording focus, RAW capture and advanced ring-lights filter system fully automated where the expert selects the type of trace that is searching for, and the device will adjust the ring-light and filter wheel settings, automatically. [81]

3.2.1.2. Acid Phosphatase Test

AP enzyme is found in high concentrations in the seminal fluid when compared with other body fluids. For this reason, its identification provides a quick way to detect a possible semen stain. [82,83]

The application of the AP test is one of the most common preliminary tests for semen detection. This specific test is based on a chromatic reaction between the AP enzyme and sodium α-naphthyl phosphate releasing naphthol which in turn reacts with the Brentamine Fast Blue (Sigma-Aldrich, USA) solution. The AP test requires pressing a damped filter paper onto the suspected stain on the item being analysed as to transfer a portion of any seminal fluid. The paper is then tested with the phosphatase reagent and there could be two types of results: there is a reaction and the paper changes to a dark purple meaning the result is positive or if the paper does not change colour within 60 seconds, the result is negative. Although it is an easy, quick and low-cost method, it does detect other substances, causing it to have high rates of false positive reactions. [84–86]

3.2.1.3. SERATEC® PSA Semiquant

PSA is a glycoprotein generated by the prostate gland (by both normal and malignant cells), which has the function of liquefying semen to allow the spermatozoa to move. It is the main protein found in the seminal fluid, with a concentration between 0,5 and 3,0 mg/mL in a healthy individual. PSA can be found in other body fluids such as vaginal secretions, sweat, urine, blood, faeces and etc. but the concentrations are very low when compared to semen. [87–91]

The most common method for PSA detection in forensic laboratories is an immunochromatographic test - SERATEC® PSA Semiquant – for a rapid semi-quantitative determination. It is based on the antigen (analyte) being capture in the test membrane by an immobilized PSA antibody. The SERATEC® PSA Semiquant has an internal standard line with a PSA concentration of 4ng/mL. There are three possible results (illustrated in Figure 7): negative (only the control – C and the internal standard appear), positive (the C, internal standard and test result line – T appear) and inconclusive (the C and/or internal standard line do not appear). [89,92,93]

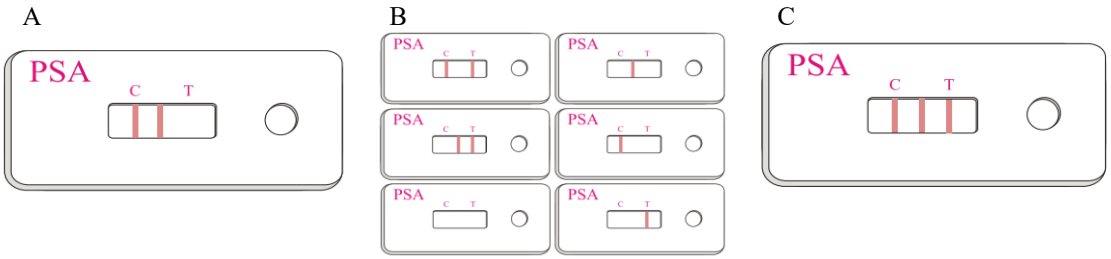


Figure 7 - Three possible results in the SERATEC® PSA Semiquant - A: negative result; B: inconclusive result; C: positive result (Adapted from [89]).

This test has the advantage of identifying PSA in cases where no spermatozoa can be found (for example azoospermic or vasectomized men). Nonetheless it is worth to note that this test it is not specific for semen, but for PSA protein. [89]

3.2.2. Confirmatory test for semen

Confirmatory or certainty tests are the tests required to confirm or rule out the nature of a biological trace. These types of tests are more sensitive, specific and they take longer to obtain results.

The BBG sector has currently only one confirmatory test for semen: the observation of spermatozoa through staining.

3.2.2.1. Microscopy observation – Christmas Tree Staining

In sexual crimes concerning men as the perpetrator, the observation of spermatozoa under the microscope, is irrefutable evidence that a crime of sexual nature has happened. For this the samples are stained with the Christmas Tree staining (CT staining) method, which involves two reagents: the Picro-Indigo-Carmine (green coloured) and the Nuclear Fast Red (red coloured). The first reagent stains the tail of the spermatozoa in green and the midpiece in blue, the Nuclear Fast Red on the other hand, will give the acrosomal a cap pink colour and the head a red colour. [73,94,95]

Although it is a great confirmatory test, it is not a simple one as there are many factors that make the spermatozoa observation harder. For example, as any biological trace, the spermatozoa after ejaculation will start to degrade principally the tails, since they are the most vulnerable parts to unfavourable conditions. It is also important to note that the evidence that comes in the laboratory arrives in conditions that are impossible to control, meaning that upon the collection of the sample for staining, there will be other components such as epithelial cells or fibres that will make the spermatozoa visualization more complicated. This confirmatory test is unfavourable in cases where the sample comes from individuals with health problems, such as oligospermia and azoospermia, or who have undergone a vasectomy, have not ejaculated or used condom in the crime, as there will be few or no spermatozoa to view. [73,96,97]

The CT staining method has two possible results: positive, if the expert is able to identify at least on head of spermatozoa or one full sperm cell, or negative if no element of a spermatozoa is observed.

4. SERATEC®

SERATEC® Gesellschaft für Biotechnologie mbH (SERATEC® GmbH) is a company located in Germany that specializes in both qualitative and quantitative rapid testing in areas like Point-of-care testing (healthcare) and Forensic Biology. They are directly

involved in the product development and testing, and they are all manufactured in accordance with the quality standards of ISO 9001. [98,99]

In the Forensic Biology area SERATEC® GmbH offers a range of products for body fluids detection that are suitable for both crime scene and laboratory. Some of these products are:

- Amylase Paper - mapping tool for the preliminary detection of saliva; [100]
- Amylase test for laboratory and crime scene - confirms the presence of the human saliva by detecting alpha-amylase; [101]
- HemDirect for laboratory and crime scene - hemoglobin test for a rapid identification of human blood; [102]
- PMB Test: Menstrual Blood Test - combines the detection of human hemoglobin and D-dime for a rapid identification of menstrual blood; [103]
- PSA Semiquant for laboratory and crime scene.

The BBG-LPC uses the last four SERATEC® rapid tests (or immunoassay cards) on a daily basis.

4.1. SERATEC® SeraQuant Reader

In the last few years SERATEC® GmbH has been developing an instrument – The SeraQuant Reader – for a computer-based quantification of PSA, Hemoglobin and Amylase concentration by reading the coloured band of the respective immunochromatographic rapid test and develop an automatic result document (see Appendix I for a result document example). [104]

The SeraQuant Reader instrument is a cube-shaped device, on its left front side the is a round turn on/off button and on the right side a tray for de SERATEC® immunoassay cards (Figure 8). The interior of the instrument consists of two different units: an optical unit and a computational unit. The optical unit is a sealed and dust-proof compartment with light absorbing inner coating and it is equipped with two grounded cameras and LED elements for the scanning of the card surface (membrane) through spectrophotometry as well as the barcode that contains batch data of a SERATEC® card, for example the batch expiration date and the batch number. The computational unit is equipped with USB, DVI and LAN ports for the connection to input/output devices as well as the local network,

whereas the SeraQuant Software uses a multi-step approach based on sophisticated statistical methods for an accurate and objective outcome of the protein quantitation. [104,105]

Figure 8 - SERATEC® SeraQuant instrument.

The usage of this instrument in forensic laboratories has various advantages such as, a sophisticated algorithm that can distinguish accurately between positive results at very low concentrations and negative results, a measurement protocol which contains all relevant data including raw images and calibration details ensuring the complete traceability of results and calibration procedures, and it is possible for each laboratory to measure calibration curves and set their own standards of calibration and measurement quality. [104]

5. Validation in forensic laboratories

Implementing new instruments, methods, procedures, kits or software in forensic laboratories, with the aim of obtaining scientific evidence that could lead to the acquittal or conviction of a suspect, ought to be evaluated and validated thoroughly. [106]

It is important to discuss quality assurance (QA) and quality control (QC), in order to maintain a good laboratory practice and obtain precise results. QA involves the systematic or planned actions taken preceding data collection, which are necessary to ensure that a product or service meets certain quality standards. On the other hand, QC procedures take

place after the data collection, and generally refer to the daily activities and operational techniques that are used to achieve the quality requirements. [106,107]

The validation of instruments and methods used in the forensic area is a crucial procedure that guarantees their reliability, that is the results accuracy. This process involves systematic analysis of methods and instruments to ensure that they produce consistent and reproducible results, meaning that in each time the method or instrument is used, the results obtained should by similar or the same. Validation encompasses several steps, such as performance assessment, sensitivity and specificity tests, and checking the robustness of the instruments under different conditions, in order for the results to be reliable. In addition, validation contributes to the continuous development of forensic practices, promoting the implementation of new technologies and methods that can improve criminal investigation, this can either be done by an external organization (development validation) or by someone in the laboratory (internal validation). [108,109]

The BBG-LPC follows ENFSI Guidelines which state, for Instrumental-based methods validation, that the performance parameters related to method validation are, in fact, evaluated during the method development. After this initial phase is completed, the laboratory must document the measurement procedure in detail. Before the validation begins, a thorough internal verification of this procedure is required. [110]

Aims

This work was carried out in the context of semen research in sexual crimes in the BBG-LPC, as a way to ease the experts with the current semen confirmatory test, assist them in their forensic reports' conclusions and increase the laboratory efficiency.

Hence, the aims of the present study were:

1. Development of a method in forensic genetics using SERATEC® PSA Semiquant quantification of real casework sexual crimes with the SeraQuant Reader.
2. The internal validation, in BBG-LPC laboratory, of the developed method according to ENFSI Guidelines for Instrumental-based methods.
3. Evaluation of the results and the possibility of turning the PSA Semiquant rapid test into a confirmatory test, using the SeraQuant Reader equipment for quantification.

II. Material and Methods

The results for this study were collected in the observation stage of the BBG-LPC workflow (with the exception of the genetic profiles). The three preliminary tests performed were the observation with the t-Zoom, the AP test and the PSA test, as for the confirmatory test it was the microscopy observation with CT Staining. All the tests (preliminary and confirmatory) were done by experts in forensic biology.

Since the main interest in this work is the study of semen traces, when working with the SeraQuant Reader, only the PSA program was used.

1. Calibration Testing

PSA standard was provided by SERATEC® GmbH, for this study, and kept at -4ºC. The standard consists in a 100 µL solution with a PSA concentration of 500 ng/mL.

Dilutions of the PSA standard were prepared using the SERATEC® PSA buffer (containing the following ingredients (in 1L distilled H_2O): 8,0 g NaCl; 0,2 g KCl; 1,44 g $Na_2HPO_4 \bullet 2H_2O$; 0,24 g KH_2PO_4; 0,1 ml 10 wt% NaN_3; pH 7,4) according to the protocol for Calibration Curve #4 provided by the company, since it gives a more robust and accurate results. For this calibration protocol 38 PSA cards (from the same batch) are needed, and seven dilutions were performed, starting with a concentration of 10 ng/mL down to a PSA concentration of 0,00 ng/mL. Since SERATEC® PSA standard has an initial concentration of 500 ng/mL an initial 1:12,5 dilution was prepared in order to obtain a concentration of 10 ng/mL from which a serial dilution was carried out, meaning that only 40 µL of the PSA standard is required (these dilutions were also used in the validation protocol). It is important to note that these dilutions can be kept at low temperatures for up to three months.

A protocol for the BBG was designed with the dilutions, and it can be seen in Appendix 2. Table 2 shows the final volume and number of repetitions for each concentration.

Table 2 – Calibration Curve repetitions and their respective Prostate-specific antigen (PSA) concentrations and volumes.

PSA Concentration (ng/mL)	10	4	2	1	0,5	0,25	0,00
Final Volume (mL)	2	1	1	1	1	1	1
Number of repetitions	3	5	5	5	5	5	10

Eighty µL (approx. three drops with a small Pasteur pipette) from each dilution were added to the membrane of the PSA Semiquant and the cards were read in the SeraQuant Reader after 10-12 minutes, as stated in the user is manual. The results were recorded with the calibration software, incorporated in the SeraQuant Reader, were the values read correspond to a relative intensity. [104]

2. Real Casework Samples

The samples used in this study were all from real criminal investigations, where the forensic exam was performed in the BBG-LPC, given that these present a multifactorial complexity that is difficult to reproduce. All samples were taken in the observation stage.

Following the protocol for observation of sexual crimes exams (preliminary tests first), the search for semen traces started with a naked eye examination of the items for suggestive stains assisted with the t-ZOOM in the body fluids program (specific filter and light). It is normal to see various types of stains with the forensic light, that is why the experts experience is important to distinguish semen stains (they emit more light) from other types of body fluids. Suggestive semen stains (as seen in Figure 9) located with the t-ZOOM are circled with a pen, since they are invisible to naked eye.

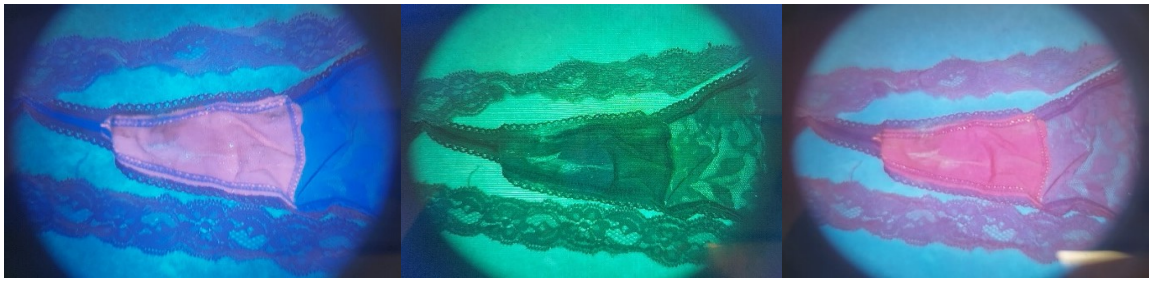


Figure 9 – Photograph taken during this study representative of suggestive semen stains visualized with t-ZOOM in the "body fluids" program in blue, green and white light.

Succeeding the forensic light, the AP test is performed. As explained in 3.2.1.2. this test requires a damp filter paper which is then pressed on the circled stains in order to transfer possible traces onto it. In items were there is a sizable amount of trace, the experts end up cutting a small piece of it and place it directly onto the filter paper instead of pressing it, since it will give more accurate results. The paper is then tested with the phosphatase reagent, a positive result for the AP test is represented in Figure 10.

Figure 10 - Photograph taken during this study representative of a positive Acid Phosphatase test.

The PSA Semiquant test is carried out, as the last preliminary test (Figure 11). A piece of the stain is cut (the size of it depends on the amount of trace) and placed in an eppendorf tube with 300 µL of the PSA buffer. After ten minutes three to four drops from the solution with the sample are added, with a small Pasteur pipette, to the card membrane.

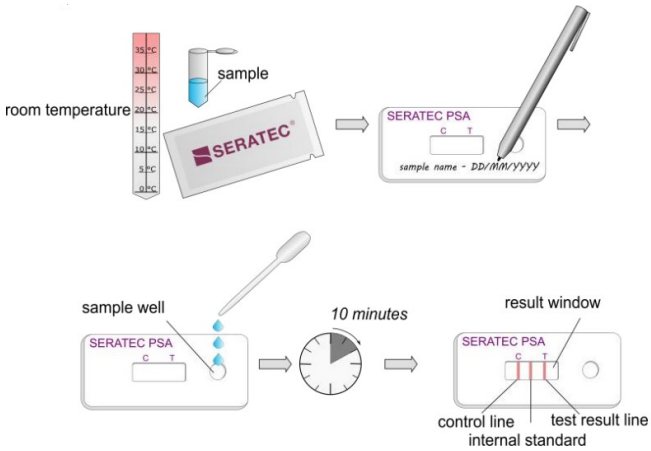


Figure 11 – SERATEC® Prostate-specific antigen (PSA) Semiquant test procedure

After 10-12 minutes, the card is placed inside the SeraQuant Reader instrument, for quantification (Figure 12), where an internal threshold of 0,5 ng/mL was established, for the time being, based on the experts naked eye results of this test. The values registered were all recorded in automatic result documents.

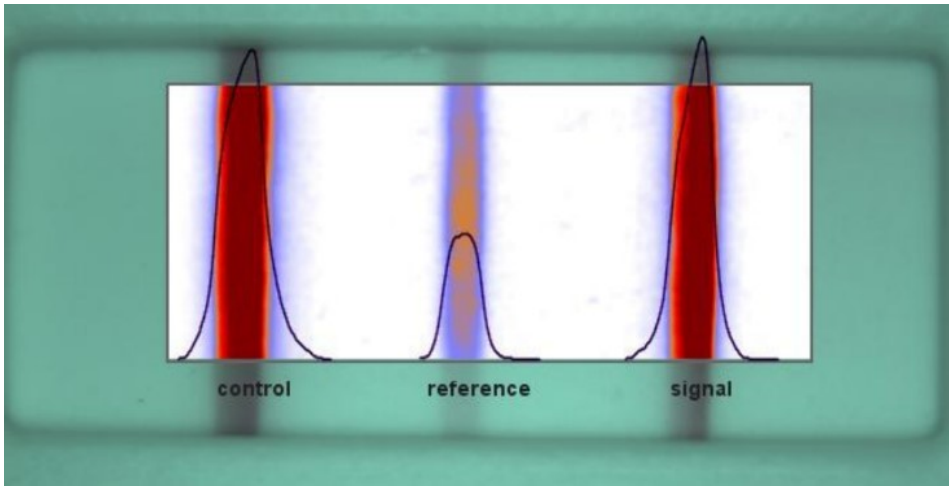


Figure 12 - Photograph taken during this study representative of the Prostate-specific antigen (PSA) card being quantified inside the SeraQuant Reader.

In the case that preliminary tests results are negative a piece of the trace stain is sent for normal DNA analysis workflow, on the other hand if the results from the tests are positive, a piece of the trace stain is sent to differential extraction and the PSA buffer eppendorf tube is stored at low temperatures for posterior use in the CT staining (confirmatory test).

The CT staining method starts by removing the item sample from the eppendorf tube using sterilized tweezers. The tube is then centrifuged at 1500 rpm for five minutes, the is supernatant discarded and 20 µL of the pellet are deposited on a glass slide and fixed with flame. Once is fixed, fast red dye is added to the sample until its fully covered and the glass slide is placed in a damp chamber (which consists of a petri dish with a wet kitchen paper) for 15 minutes. Afterwards the glass slide in washed with distilled water, the picro-indigo-carmine dye is added until the sample is fully covered and after 15 to 30 seconds its washed with 90% ethanol and left to dry. As soon as the sample is dry it is observed under the optical microscope, Figure 13 represents spermatozoa visualization with CT Staining. [49,95]

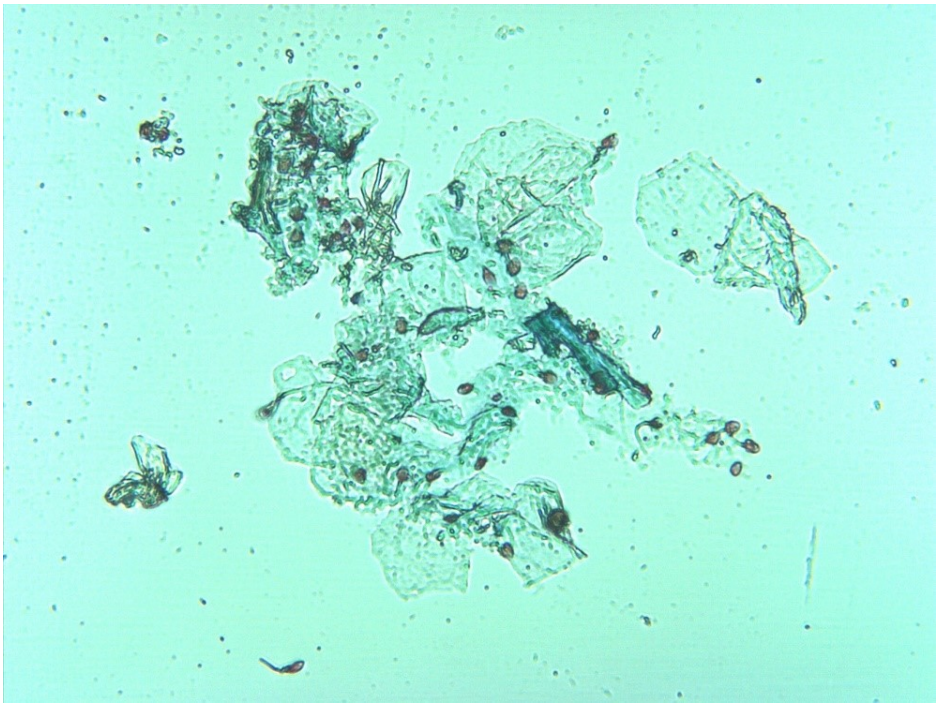


Figure 13 – Photograph taken during this study representative of a full spermatozoa and spermatozoa heads with CT staining under an optical microscope at 40X resolution.

After the DNA analysis semen workflow, the genetic profiles obtained are interpreted and classified as male, female, mixed or inconclusive. This step was accomplished with the help of the forensic experts.

3. Internal validation

The validation procedure, for the SeraQuant Reader, followed ENFSI Guidelines for Instrument-based methods. The parameters analysed were the minimum required by ENFSI, encompassing sensitivity, specificity, reproducibility and repeatability. [110]

As referred in point 1, the dilutions used for this protocol were the same as the calibration curve but were made anew since the internal validation occurred five months after the calibration curve was performed. The internal validation was all performed in the same day.

Furthermore, for each validation parameter 80 µL (approx. three drops with a small Pasteur pipette) from each dilution were added to the membrane of the PSA Semiquant and the cards were read after 10-12 minutes, as stated in the user is manual. The results were recorded with the reading software, incorporated in the SeraQuant Reader.

3.1. Sensitivity

As the word suggests, sensitivity tests are performed to assess how "sensitive" a method can be, in order to evaluate its robustness which reflects the ability of the method to remain unchanged in the face of small intentional variations. [111]

The sensitivity test was performed through a series of six dilutions of PSA standard (from 10 ng/mL to 0,25 ng/mL), tested in triplicate.

3.2. Specificity

Specificity is the ability of a method to exclusively measure the element of interest, without being affected by other sample components, such as degradants, impurities or matrix substances, that may be present. [112]

In order to study this parameter, a dilution with a 2 ng/mL concentration of PSA was analysed as well as, PSA Semiquant cards with SERATEC® PSA buffer. This was done in triplicate.

3.3. Reproducibility and Repeatability

Reproducibility tests are carried out to assess the variation in the average results obtained by different operators, with the same measuring instrument and measuring the same sample multiple times. Repeatability tests aim to evaluate the variation in measurements made by a single operator, with the same method and instrument, when measuring the same sample multiple times. [113,114]

For the reproducibility test, four replicates of a 0,5 and 2 ng/mL PSA concentration were made by two different operators.

The repeatability test was done through a series of dilutions by a single operator in different times of the day (morning and afternoon) separated by five hours. In between the readings the dilutions were kept at low temperatures.

III. Results

1. Calibration Testing

Only one calibration curve is required by the manufacturer for each PSA Semiquant batch. During this study, only one batch was used, therefore only one calibration curve was designed. Figure 14 shows the values obtained from the quantification of the seven PSA concentration dilutions, by using 38 PSA Semiquant cards.

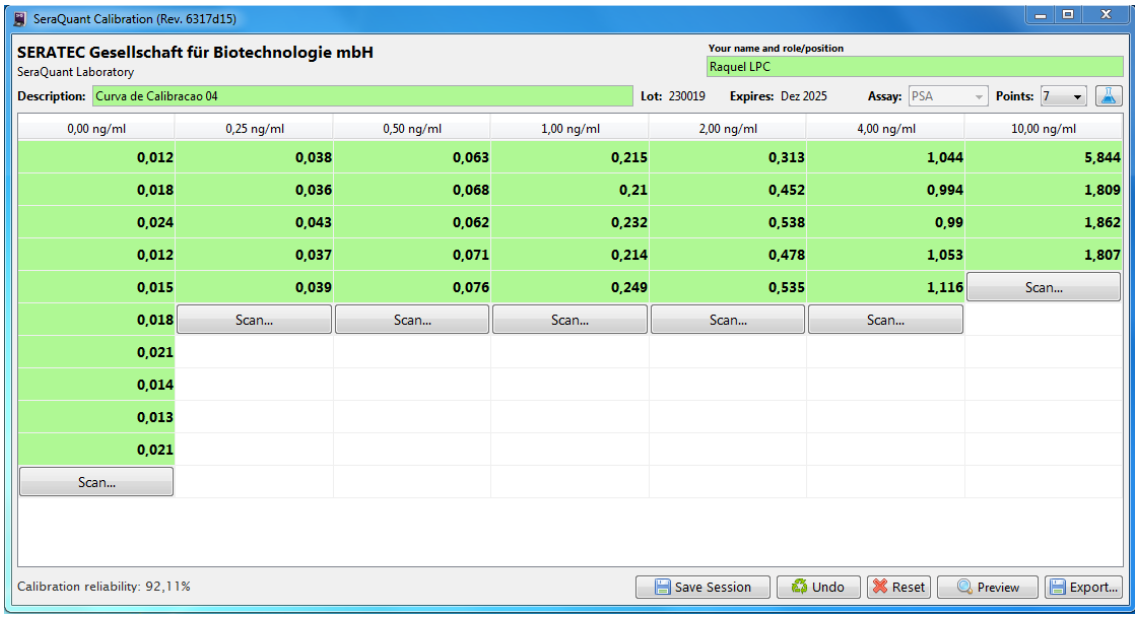


Figure 14 – Calibration curve values obtained per Prostate-specific antigen (PSA) concentration, using 38 PSA Semiquant cards.

2. Real Casework Samples

Twenty-five sexual crimes exams were observed for this study and 48 samples were taken for different items, which are characterized in Table 3. From de exams observed, 48 SERATEC PSA Semiquant tests were quantified using the SeraQuant Reader. The result for the AP test and the PSA test, the confirmatory test done and the obtained profile, together with the total concentration values of both cellular and male fractions (including the Y chromosome) were recorded for future comparison (consult Appendix 3). From the 48 real casework samples, the AP test and the CT Staining weren't performed in two different samples each (due to the forensic expert is decision), meaning that the AP test was performed in 46 out of the 48 samples as well as CT Staining.

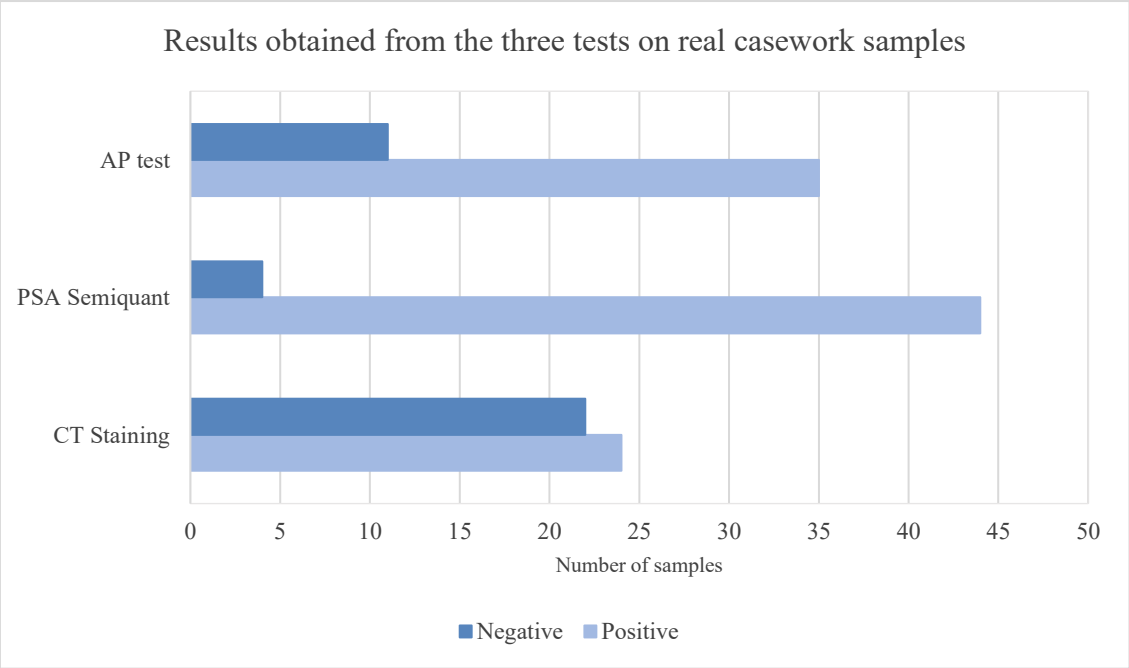
Out of the 48 values, only four were below the initial defined threshold (0,5 ng/mL) and only sample 29 (0,27 ng/mL) was positive for spermatozoa. Due to this the threshold defined in the beginning of this study was kept.

Table 3 – Characterization of the 48 samples with the respective item, from where they were collected.

Sample No.	Item	Sample No.	Item	Sample No.	Item	Sample No.	Item
1	Denim shorts	**13**	Paper tissue	**25**	Vaginal swab	**37**	Shorts
2	Trousers	**14**	Sweatshirt	**26**	Wipes	**38**	Underware
3	T-shirt	**15**	Sanitary pad	**27**	Underware	**39**	Sanitary pad
4	Underware	**16**	Underware	**28**	Vaginal swab	**40**	Vaginal swab
5	Underware	**17**	Tights	**29**	Vaginal swab	**41**	Boxers
6	Sheet	**18**	Underware	**30**	Sweater	**42**	Boxers
7	Sheet	**19**	Underware	**31**	Sweater	**43**	Duvet cover
8	Sheet	**20**	Blanket	**32**	Curtain	**44**	Duvet cover
9	Trousers	**21**	Underware	**33**	Curtain	**45**	Duvet cover
10	Trousers	**22**	Bra	**34**	Vaginal swab	**46**	Duvet cover
11	Underware	**23**	Underware	**35**	Sweater	**47**	Underware
12	Paper tissue	**24**	Vaginal swab	**36**	Underware	**48**	Hat

2.1. Results from the AP tests, PSA Semiquant and CT Staining on real casework samples

Graphic 1 shows the positive and negative results of the real casework samples from all three tests.

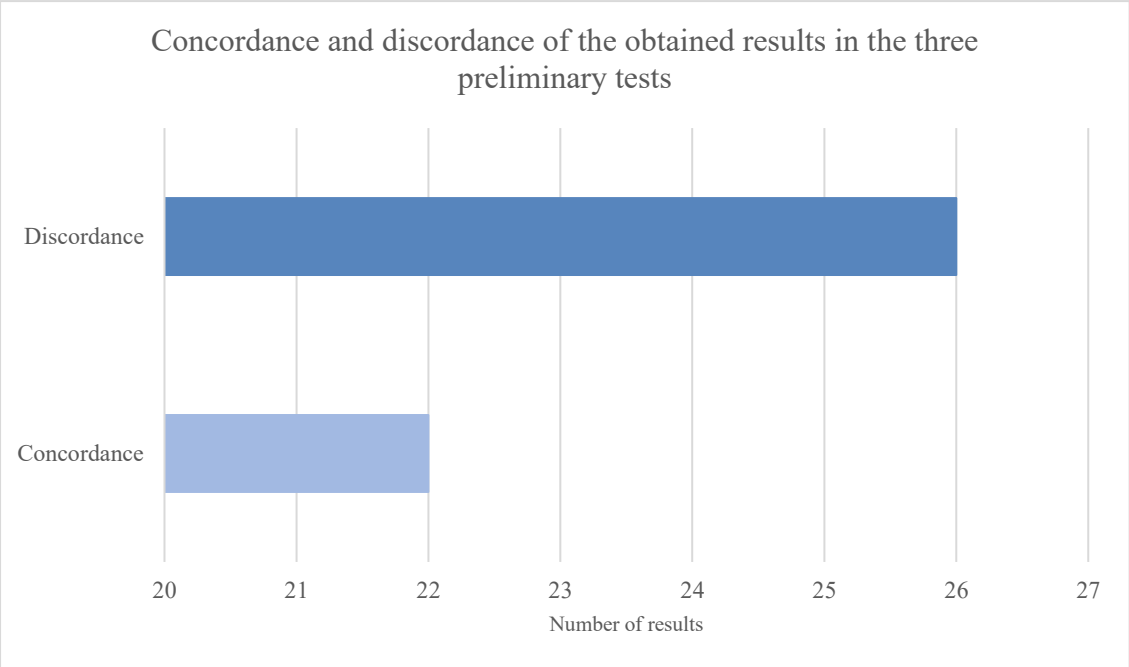


Graphic 1 – Positives and negatives total results, from the Acid-phosphatase test (AP test), the prostate-specific antigen test (PSA Semiquant) and the Christmas-tree staining (CT Staining) on real casework samples.

From the total 48 real casework samples 44 were positive for the PSA Semiquant (91,67%). Out of 46 samples, 35 were positive for the AP test (76,08%) and 24 for the CT Staining (52,17%). On the other hand, 11 results were negative for the AP test, four for the PSA Semiquant and 22 for the CT Staining, this makes 23,91%, 8,33% and 47,83% respectively.

2.2. Concordance and discordance between two preliminary tests and the confirmatory test

Graphic 2 represents the number of results that are in concordance and discordance between the three tests performed.

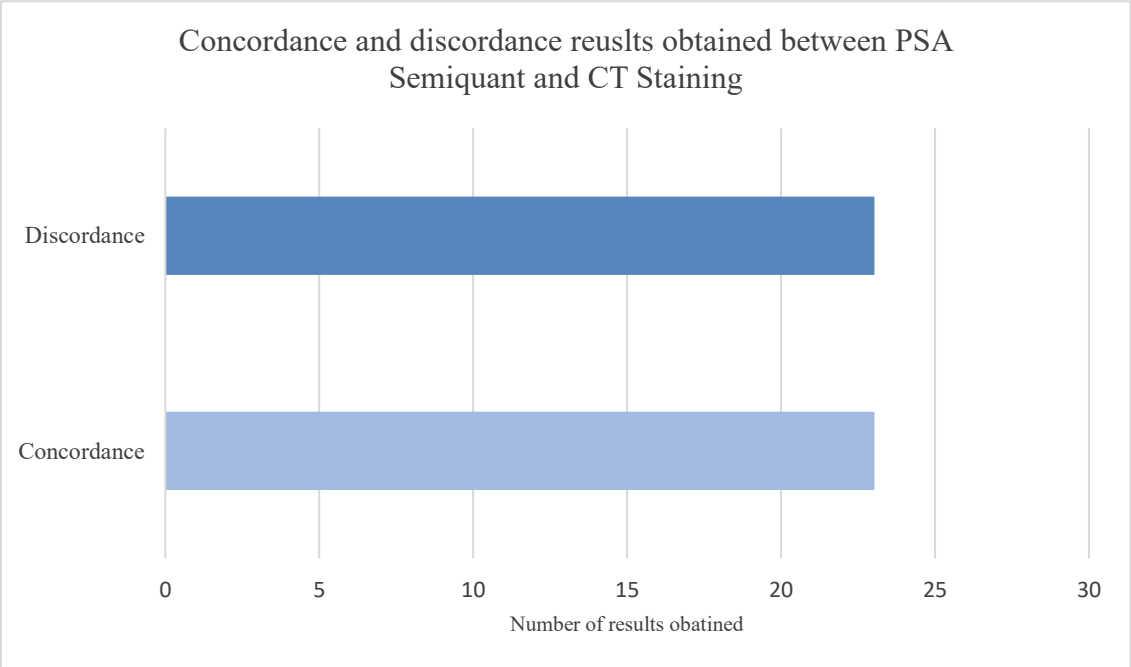


Graphic 2 – Number of concordance and discordance sample results between the Acid-phosphatase test, the prostate-specific antigen Semiquant test and the Christmas-tree staining.

Out of the 48 real samples analysed only 22 were in concordance on all three tests (positive or negative), making a total of 45,83%. The remaining 26 results (54,17%) were in discordance. Of these 26 discordances, 15 were positive for both AP test and PSA Semiquant and negative for CT Staining, one was negative for AP test and PSA Semiquant and positive for CT Staining, four were positive for PSA Semiquant and CT Staining and negative for AP test and another four were negative for AP test and CT Staining and positive for PSA Semiquant.

2.3. Concordance and discordance between the PSA Semiquant results and CT Staining results

Graphic 3 presents the number of concordances and discordances observed when comparing the results of real samples between PSA Semiquant and CT Staining.

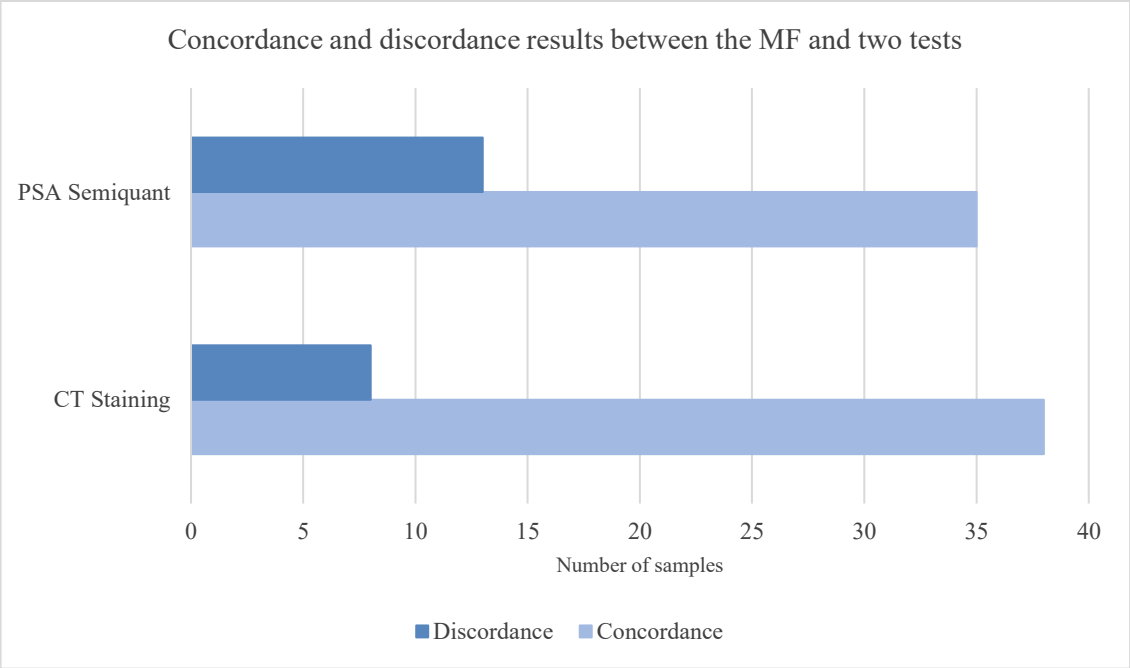


Graphic 3 - Number of concordance and discordance sample results between the prostate-specific antigen test (PSA Semiquant) and the Christmas-tree staining (CT Staining).

By analysing the graphic above it is possible to conclude that 23 out of the 46 real casework sample (analysed with CT Staining) are in discordance between both tests, making that 50% of the total.

2.4. Concordance and discordance between the Male Fraction and two tests

Graphic 4 shows the concordance and discordance of the genetic profiles obtained in the Male Fraction and the PSA Semiquant and CT Staining tests.



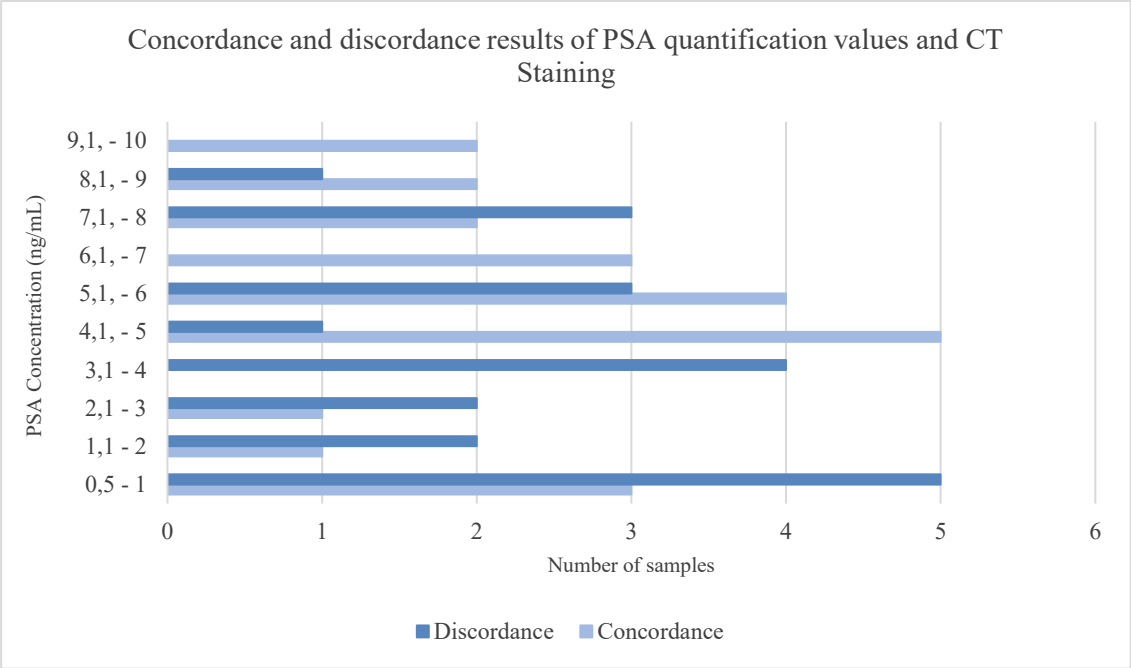
Graphic 4 – Number of concordance and discordance sample results between the prostate-specific antigen test (PSA Semiquant) and the Male fraction (MF) and between Christmas-tree staining (CT Staining) and MF.

From the 48 real casework PSA samples, 35 had results (72,91%) that are in concordance with the profiles obtained from the MF. Out of these 35 samples, 22 were positive for PSA and a Male Profile was obtained, nine were positive for PSA and the profile obtained was a Mixed DNA Profile and only three are negative for PSA and no profile or a Female profile was obtained in the MF.

Nonetheless, from the 46 samples analysed with CT Staining, 38 (82,61%) are in concordance with the profiles obtained in the MF. Fourteen out of the 46 were negative for the CT Staining and the profile obtained in the MF was either a Female Profile or Inconclusive and when the profile was Male or a Mixed DNA Profile positive result for CT Staining were obtained in 24 samples.

2.5. Concordance and discordance of between PSA quantification values and CT staining results

In Graphic 5 it is possible to observe the concordance and discordance between the PSA quantification sample values and the CT Staining results.

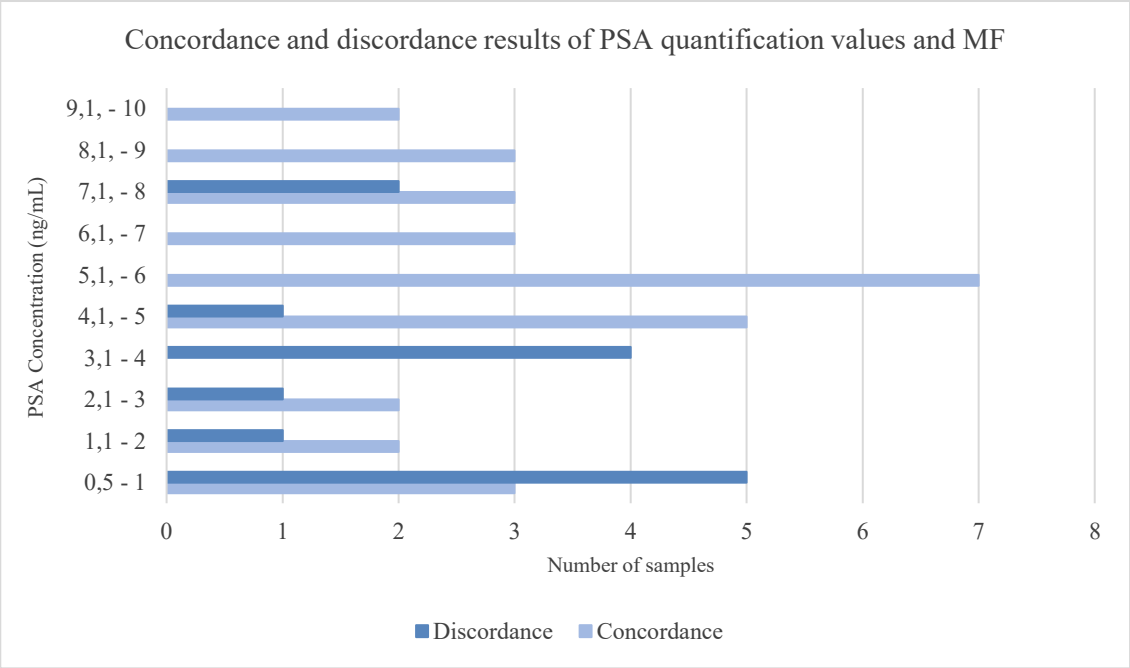


Graphic 5 – Number of concordance and discordance between the prostate-specific antigen test (PSA Semiquant) quantification sample values and the Christmas-tree staining (CT Staining) sample results.

As shown in the graphic above, 69,23% of the values with 6,1 ng/mL or above (13 values) are in concordance with the results obtained in the CT staining test (whether they are positive or negative). In the total of the 44 samples, which are above the established threshold, 52,27% of the values are in concordance with the CT staining results.

2.6. Concordance and discordance of between PSA quantification values and MF results

Graphic 6 shows the concordance and discordance between the PSA quantification sample values and the MF with results of Male or Mixed DNA Profiles.



Graphic 6 – Number of concordance and discordance between the prostate-specific antigen test (PSA Semiquant) quantification sample values and the Male fraction (MF) sample results.

Out of the 44 samples above the threshold, 68,18% of the PSA Quantification values are in concordance with the MF result. It is important to note that from the 26 values with or above a PSA concentration of 4,1 ng/mL, 23 were in concordance with the results from the MF, which correspond to 88,46% of the total.

2.7. Ratio between two concentrations

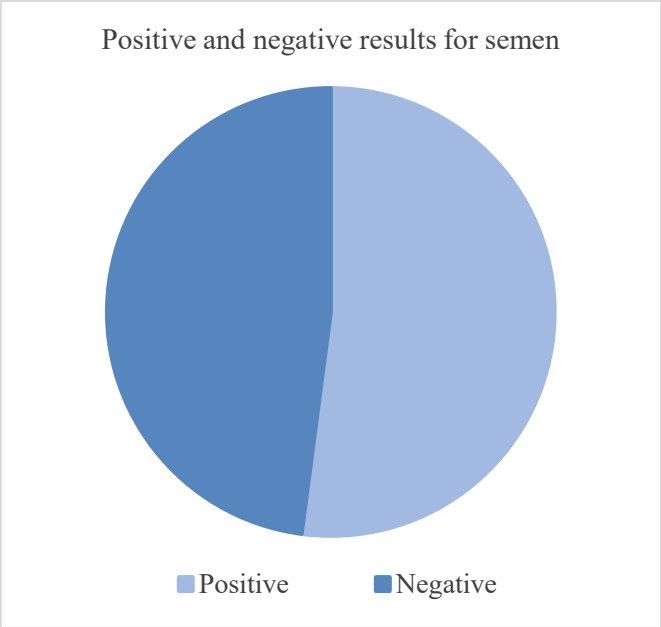
In order to see if a relation between the Total Male concentration and Total Cellular concentration existed, a ratio for these values was calculated and the results can be consulted in Table 4.

Table 4 – Real casework samples (see Appendix 3) and their respective ratio between the total concentration of the male fraction (MF) and the total concentration of the cellular fraction (CF).

Sample Number	Ratio MF/CF	Sample Number	Ratio MF/CF	Sample Number	Ratio MF/CF	Sample Number	Ratio MF/CF
1	7/3	**13**	1/7	**25**	0/1	**37**	0/1
2	5/2	**14**	1/1	**26**	9/5	**38**	0/1
3	26/3	**15**	16/5	**27**	0/1	**39**	1/9
4	0/1	**16**	0/1	**28**	0/1	**40**	0/1
5	0/1	**17**	-	**29**	44/7	**41**	0/1
6	0/1	**18**	0/1	**30**	0/1	**42**	0/1
7	0/1	**19**	8/9	**31**	0/1	**43**	3/1
8	11/9	**20**	1/9	**32**	0/1	**44**	1/1
9	0/1	**21**	0/1	**33**	0/1	**45**	16/9
10	27/2	**22**	0/1	**34**	0/1	**46**	1/2
11	2/9	**23**	0/1	**35**	0/1	**47**	0/1
12	0/1	**24**	0/1	**36**	0/1	**48**	0/1

2.8. Final result

The final result implies the certainty of the trace analysed being semen. This step was based on the experts' conclusions in their forensic report and the results are represented in Graphic 7. When the trace is positive for semen it means that spermatozoa were observed in the microscope with CT Staining.



Graphic 7 - Positives and negatives semen results (based on the expert is final reports).

Out of the 48 analysed real casework samples, 25 were positive for semen and 23 were negative, corresponding to 52,08% and 47,92% respectively.

From the 23 negative samples (where spermatozoa were not observed), 20 have positive PSA Semiquant values and one negative PSA value was positive for semen.

2.9. Irregular samples results

The comparison between the two preliminary tests, the confirmatory test and the profile obtained in the MF showed some irregular results. For this reason, it is important to examine and compare the parameters that were analysed in irregular samples. Table 5 shows the comparison of the parameters - note that a positive in the MF column means a Male of Mixed DAN profile.

Table 5 – Comparison of the results obtained in the 9 irregular results (Acid-phosphatase test – AP test; Prostate-specific antigen test – PSA Semiquant; Christmas-tree staining – CT Staining; Male Fraction – MF).

Sample Number	AP Test	PSA Semiquant	CT staining	MF
1	Positive	Positive	Negative	Positive
2	Positive	Positive	Negative	Positive
3	Positive	Positive	Negative	Positive
20	Positive	Positive	Negative	Positive
24	-	Positive	Negative	Positive
25	-	Positive	Negative	Positive
27	Positive	Positive	Negative	Positive
29	Negative	Negative	Positive	Positive
48	Positive	Positive	Negative	Positive

3. Internal validation

The validation parameters were performed with the dilutions already made in the calibration curve. The insertion of the PSA cards and the expected values of the concentrations (Table 6) were according to the manufacturers protocol.

Table 6 - Expected value per Prostate-specific antigen (PSA) concentration.

PSA Concentration (ng/mL)	0,25	0,5	1	2	4	10
Expected value (ng/mL)	0,2	0,25	0,3	0,5	1	4

3.1. Sensitivity

Three drops of all dilutions were added to the PSA cards and after 10-12 minutes they were read in the PSA Semiquant Reader. The results obtained are shown in Table 7.

Table 7 – Sensitivity test results, with the two repetitions of the Prostate-specific antigen (PSA) concentration quantified in the SeraQuant Reader and their respective percentage.

Samples	PSA Concentration (ng/mL)	PSA quantification values		Percentage (%)	
		1st repetition	2nd repetition	1st repetition	2nd repetition
1	0,25	0,14	0,18	70	90
2	0,5	0,18	0,16	72	64
3	1	0,26	0,32	87	107
4	2	0,5	0,44	100	88
5	4	0,97	1,12	97	112
6	10	3,93	4,08	98	102

The SERATEC® PSA Semiquant Reader was able to read quantities above and below the expected value on all PSA concentrations.

3.2. Specificity

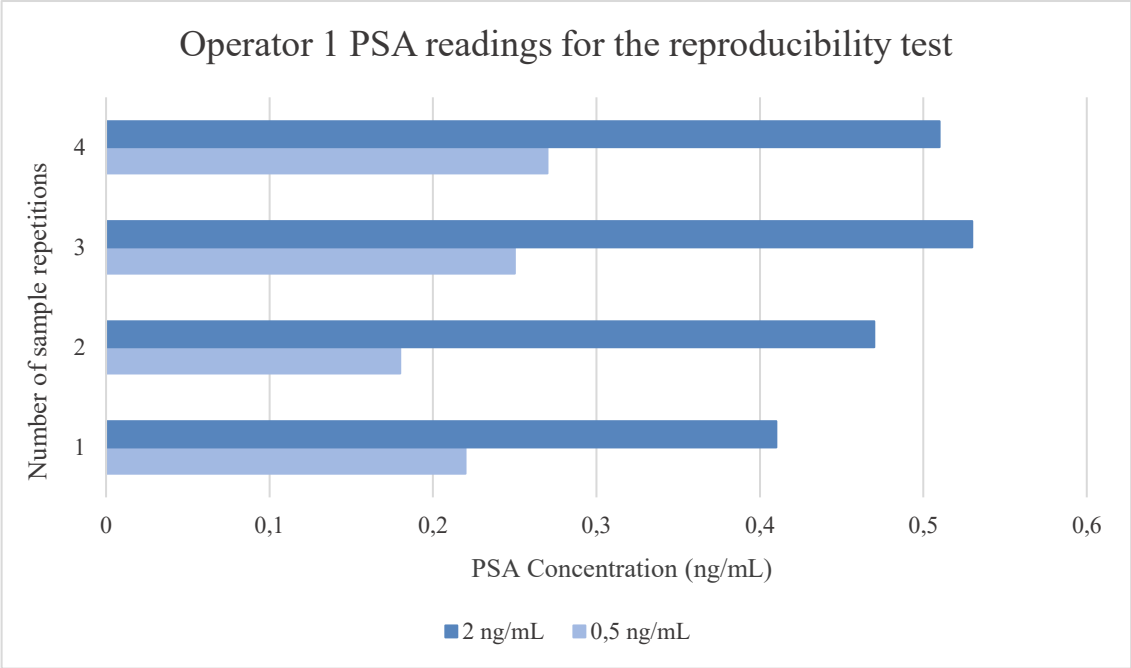
Three drops of the 2 ng/mL dilution and from SERATEC® PSA buffer, were added to PSA cards in triplicate, the results of the specificity parameter are presented in Table 8.

Table 8 – Specificity test results, with the three repetitions of the Prostate-specific antigen (PSA) concentration quantified in the SeraQuant Reader.

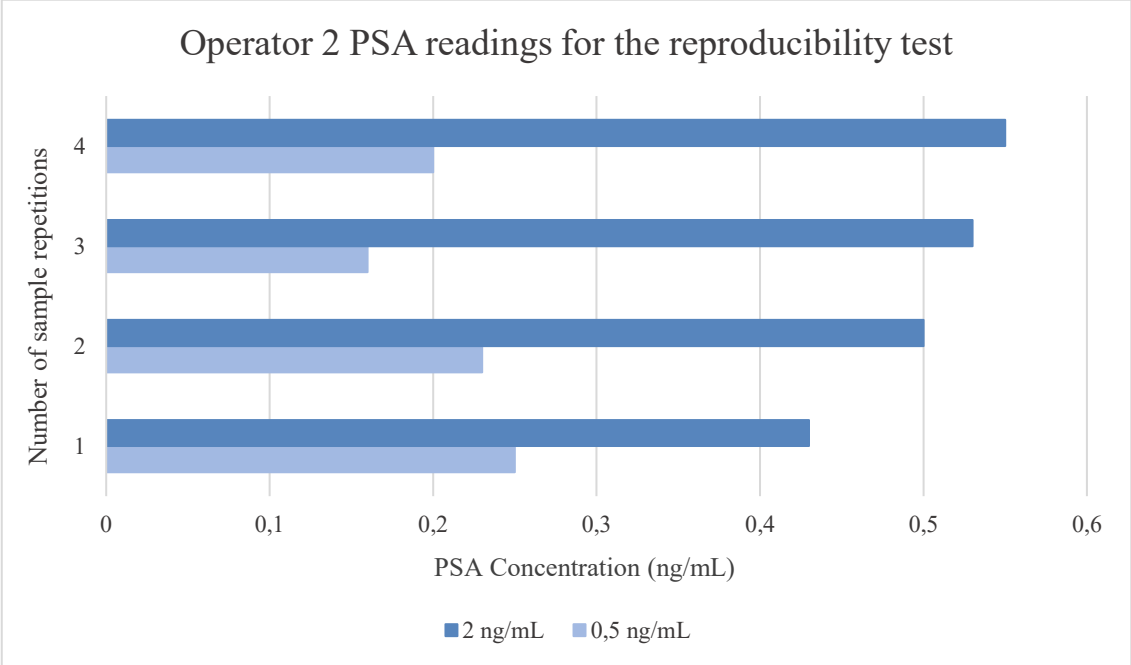
Samples	PSA Concentration (ng/mL)	PSA quantification values		
		1st repetition	2nd repetition	3rd repetition
1	0,00	0,01	0,01	0,00
2	2	0,43	0,52	0,46

3.3. Reproducibility and Repeatability

As the previous parameters for validation three drops of a 0,5 and 2 ng/mL PSA concentration dilution were added to PSA cards. Operator 1 has no experience in forensic genetics but has knowledge of the instrument and Operator 2 is a forensic biology expert with no knowledge of the instrument. The reproducibility test results are shown in Graphic 8 for Operator 1 and Graphic 9 for Operator 2.



Graphic 8 – Operator 1 reproducibility test results for four repetitions of samples with a prostate-specific antigen test (PSA) concentration of 0,5 and 2 ng/mL.



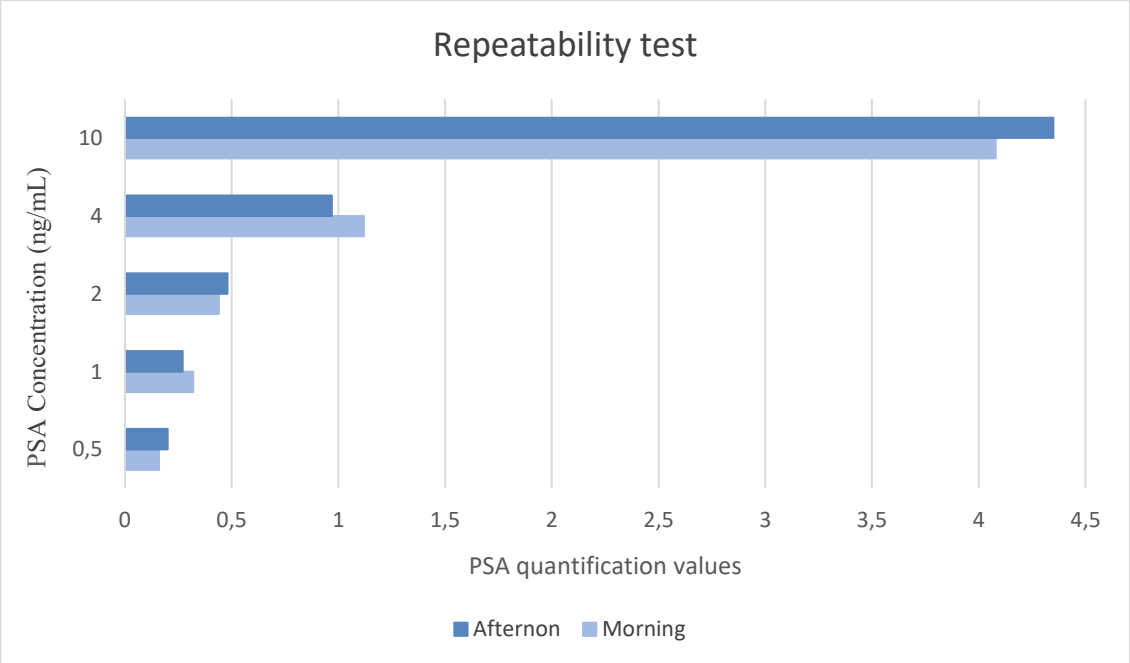
Graphic 9 - Operator 2 reproducibility test results for four repetitions of samples with a prostate-specific antigen test (PSA) concentration of 0,5 and 2 ng/mL.

Table 9 shows the results from both operators in percentage. For both operator 1 and 2 the total percentage obtained was similar in both concentrations. The PSA concentration of 2 ng/mL has the best percentage in both operators.

Table 9 – Percentage per repetition and total percentage, for Operator 1 and 2 and for 0,5 and 2 ng/mL of Prostate-specific antigen (PSA) concentration readings.

Operator	Samples	PSA Concentration (ng/mL)	Percentage (%)				Total %
			1st repetition	2nd repetition	3rd repetition	4th repetition	
1	1	0,5	88	72	100	108	92
	2	2	82	94	106	102	96
2	1	0,5	100	92	64	80	84
	2	2	86	100	106	110	101

As for the repeatability test, two PSA quantification readings were done one in the morning and one in the afternoon. Three drops of a series of five dilutions were added to the PSA Semiquant cards. Graphic 10 shows the results for this parameter.



Graphic 10 – Prostate-specific antigen (PSA) quantification values in two different times of the day (morning and afternoon), for a series of five dilutions with PSA concentrations of 0,5, 1, 2, 4 and 10 ng/mL.

Table 10 shows the results from reading times in percentage. The total percentage of variations between the measurements performed is 95% in the morning and when measured five hours later (in the afternoon) the variation percentage was 94%.

Table 10 – Percentage values per prostate-specific antigen (PSA) concentration in the morning and afternoon times.

Samples	PSA Concentration (ng/mL)	Percentage (%)	
		Morning	Afternoon
1	0,5	64	80
2	1	107	90
3	2	88	96
4	4	112	97
5	10	102	109

IV. Discussion

1. Calibration Testing

Reliability refers to the consistency of a measurement, that is, the ability to reproduce results under the same conditions.

As shown in Figure 15, the calibration curve was obtained by using 38 points (showed in Figure 14) and has a reliability of 92%, due to the outlier in the 10 ng/mL concentration

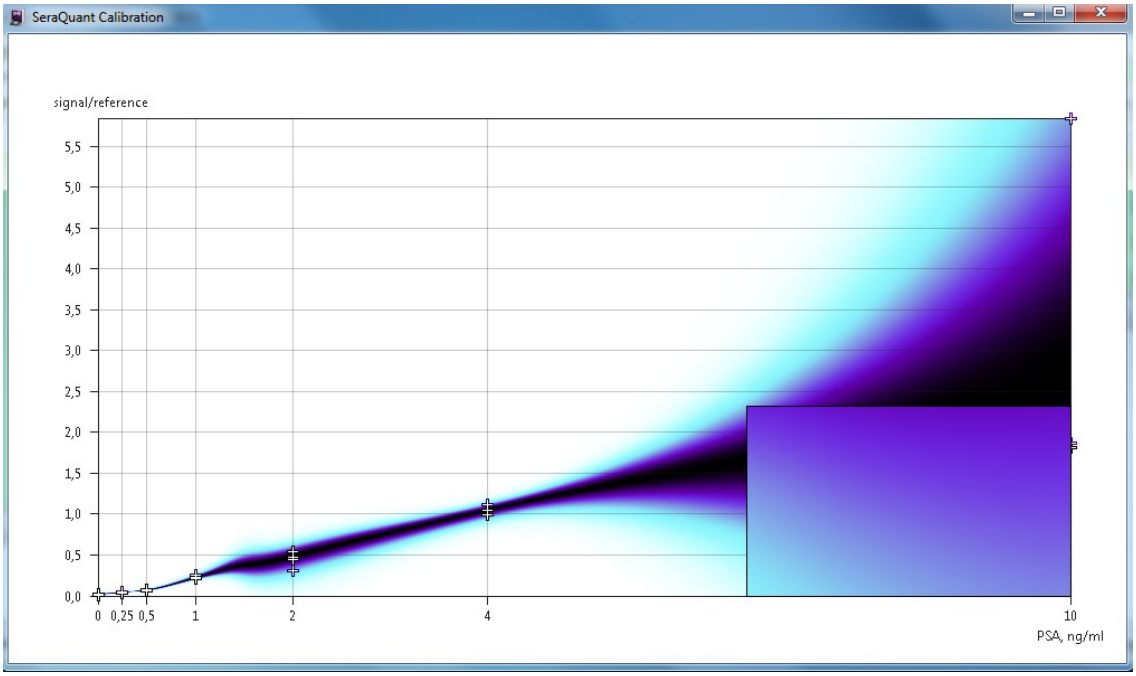


Figure 15 - Calibration curve using 38 SERATEC® prostate-specific antigen (PSA) Semiquant cards.

Although a reliability of 92% seems low compared to other scientific accuracy standards, the manufacturer highlighted that the highest recorded calibration is around 96%. This value is the result of the statistical treatment used in the software, which considers the combined probability of reaching the same concentration column (i.e., 0,25 ng/mL or 0,5 ng/mL) when, on average, 2/3 of the results are removed at random. This means that the likelihood of the final result still being the same, even if 36% of the measurements were to be randomly deleted, is 92%. An outlier in the 10 ng/mL concentration was the reason for the presented reliability.

To further increase the reliability, more PSA cards with a 10 ng/mL concentration were read, but this only decreased it even more. Ten PSA cards with this concentration were needed in order to increase the reliability to 94%. Taking this into account the only viable option was to remove the outlier, however the SeraQuant Reader software only allows for the last card read to be eliminated and the outlier was one of the first cards to be read. Nevertheless, given the manufacturer is observation on the reliability, no changes were made, the outlier was kept, and the reliability of the calibration curve was considered acceptable.

2. Real Casework Samples

It is important to remember that the AP test can detect other body fluids beyond seminal fluid and the PSA Semiquant detects PSA in the seminal fluid and not semen.

2.1. Results from the AP tests, PSA Semiquant and CT Staining on real casework samples

Evaluating the percentages from the 46 samples that have the three tests in common, the PSA Semiquant has the higher percentage of positive results and the CT Staining the lowest. As for the negative results the confirmatory test has the highest percentage compared to the preliminary tests.

2.2. Concordance and discordance between two preliminary tests and the confirmatory test

The 26 discordances obtained between the AP test, the PSA Semiquant and the CT Staining real casework samples were different in the following aspects:

- 15 samples were positive for the preliminary tests and negative for the confirmatory test. These results may be due to one of two reasons, first the individual could be azoospermic or have had a vasectomy meaning that there wouldn't be spermatozoa or very few. Secondly, there could be too many debris, making the microscopy observation difficult or the CT Staining was not performed properly.

- The one sample where both preliminary tests were negative, but the CT Staining test was positive, can be attributable to the fact that this item was badly packed when it arrived in the BBG.
- Four samples where the PSA Semiquant was positive and the other two tests were negative. What may have led to these results is the fact that the PSA Semiquant could have identified PSA from another body fluid other than seminal fluid, since the profiles obtained in these samples were either female or inconclusive.
- The other four samples were there was discordance, was between the AP test being negative and the PSA Semiquant and the CT Staining both positive. This can be explained through the fact that the phosphatase reagent may have been "expired", or the expert didn't do the test correctly and not enough trace was transferred to the filter paper.

2.3. Concordance and discordance between the PSA Semiquant results and CT Staining results

Out of the 50% of discordances between the PSA Semiquant results and CT Staining results, the one sample where the PSA was negative, and the CT Staining positive was due to the way it was packed when it arrived at the BBG. The other 45 analysed samples were all positive for PSA Semiquant and negative for CT Staining. meaning that either a) the PSA Semiquant probably identified PSA in another type of body fluid and not in seminal fluid or b) the spermatozoa were difficult to observe, or the individual doesn't produce them.

2.4. Concordance and discordance between the Male Fraction and two tests

A high percentage of PSA Semiquant and CT Staining tests were in concordance with the profile obtained in the MF, meaning that there is evidence of a connection between them. The disagreements obtained between the MF and the two tests analysed here, could be due to one out of two reasons:

- Firstly, and for cases where the genetic profile obtained in the MF was either female or inconclusive and the PSA Semiquant was positive, this preliminary test could have detected PSA from other body fluids and not from the seminal fluid.

- In the samples where the PSA Semiquant was negative, but the profiles obtained were either male or mixed could mean that the sample were the preliminary test was performed hadn't enough trace for card to identify.
- For the eight real casework samples in discordance between the MF and the CT Staining tests all of them had a male on mixed DNA profile was obtained in the MF and the CT Staining was negative. This can be attributable to the fact that the individual may have health problems or a vasectomy where there is none or very low spermatozoa counting or on the other hand the sample submitted to the CT Staining could have a low number of spermatozoa, and for those reasons no sperm cells could be observed in the microscopy.

2.5. Concordance and discordance of between PSA quantification values and CT staining results

For the 44 samples that were quantified for PSA in the SERATEC® SeraQuant Reader and where the CT Staining was performed, 52,27% were in concordance which makes it almost half of all the samples analysed. Nevertheless, when PSA concentration values were above 6,1 ng/mL almost 70% was in concordance, making this a reliable method only for high concentrations of PSA.

2.6. Concordance and discordance of between PSA quantification values and MF results

Contrary to the point above, the concordance between the PSA quantification values and the profiles obtained in the MF was high, making it a total of 68% of total 44 samples. In the same way, nearly 89% of the samples with a PSA concentration of 4,1 ng/mL or over, were in concordance with the profiles obtained. However, between the values of 0,5 and 4 ng/m 11 out of 18 values were in discordance, making this method reliable for high concentrations of PSA.

By analysing point 2.5 and 2.6 it is safe to say that the most probable reason for the high percentage of discordance in lower values with both CT Staining and MF results

may be due to the fact that the PSA Semiquant is identifying PSA from other body fluids other than seminal fluid.

2.7. Ratio between two concentrations

Regrettably, no certain ratio was found between both concentrations in order to find a relation between them and the PSA concentration values.

However, it is a possibility that PSA Semiquant is capable of identifying PSA from other body fluids, since some samples have a good value of PSA (approx. 4 ng/mL) and a high Total Cellular concentration, but a low Total Male concentration and an inconclusive profile in the MF (see sample 22, for example, from Appendix 3).

2.8. Final result

As said in point 2.8 in the Results section, 52% of the 48 samples analysed for this study obtained a positive result for semen. Out of this percentage 22 samples were positive for PSA. On the other hand, from the 23 samples that obtained a negative result for semen 20 were positive for PSA making it 86,96% of the negative samples. For such a high percentage of negative semen results have positive PSA, some reasons were considered:

- First, and for samples were the profile obtained in the MF was inconclusive or female, the PSA Semiquant most likely identifies PSA in other body fluids, and not exceptionally on seminal fluid.
- Second, and for samples where the profile obtained in the MF was either male or mixed, the CT Staining may have not been executed properly or the individual may have health problems or may have had a vasectomy.
- Another reason that was considered, is the fact that for real casework samples the threshold may need to be on a higher value, but for this an intensive study on these kinds of samples needs to be performed.
- Finally, it is possible that the material and colour of the fabric affects the PSA results. In order to analyse this possibility a throughout study needs to be done.

2.9. Irregular samples results

All the samples considered to be irregular had the MF with a male or mixed DNA profile and one or more tests negative. For samples 1, 2, 3, 20, 24, 25, 27 and 48 the AP tests and the PSA Semiquant were more sensitive than the CT Staining. The main reason for this irregularity is the fact that the CT Staining test was either performed in an incorrect way or the individual was oligospermic, azoospermic or has had a vasectomy done. Sample number 29 was the only one where this type of discordance, between all the tests performed and the profile obtained, occurred. This sample had the most irregular results and can only be explained by the way the sample was packed before it arrived in the BBG-LPC.

*

It is important to note that while the AP test is more affordable than the PSA Semiquant, its chemical reagent is toxic, which implies a more careful handling when using it and it is more susceptible to contaminations since it involves the experts direct use in the sample. On the other hand, the PSA Semiquant even though it is more expensive, does not require the same care as the AP test and the contamination risk is lower. Nevertheless, when comparing these two preliminary tests is important to know that they do not substitute one another. The AP test is designed to detect suggestive stains while the PSA Semiquant detects the type of biological trace, therefore the BBG uses these preliminary tests as a complement of each other.

3. Internal validation

Comparing the percentage of each repetition in the sensitivity test in this validation parameter, the greater the concentration the greater the respective percentage, with the PSA concentration of 4ng/mL being the one with the hight average percentage. These percentages also confirm the results obtained in the Results section, point 2 (Real casework samples), where the greater the concentration the greater the concordance between the tests. Meaning that the SeraQuant Reader is more sensitive for higher PSA concentrations and there are less false positives. [115]

By analysing the results from Table 8, it is possible to conclude that the instrument is specific for PSA detection, since the values read when the concentration is 2 ng/mL are close to the expected value of 0,5. Seeing that the samples used for the validation protocol, were controlled, there are no false positives. [115]

The percentages obtained for both operators in each repetition in the reproducibility test are similar and high, as well as the quantification values. For this reasons it can be said the method is reproducible, especially in higher PSA concentrations. [115]

As for the results obtained in the repeatability test in both day times (morning and afternoon), they can also be considered similar since their average percentage are 95% and 94%. As the others validation parameters results, the percentages were higher the higher the PSA concentration. Overall, the method is repeatable. [115]

V. Conclusion

Over the years, more and more crimes of sexual nature are committed, which makes the identification of the perpetrators of these types of crimes crucial. For this, new methods and instruments are developed every year to improve the identification of semen traces in items submitted to forensic exams.

Alternative methods for the semen confirmatory test (microscopy observation) can be helpful since it eases the expert in the visual search of spermatozoa. The present study aimed to evaluate and internally validate the SeraQuant Reader instrument in order to assess the viability of the PSA Semiquant test in becoming a confirmatory test.

Based on the results obtained from the AP test, the PSA Semiquant quantification, the CT Staining test and the genetic profiles obtained in the MF, comparative studies were performed in order to evaluate the equipment effectiveness in identifying PSA from seminal fluid. These results allowed to determine that the PSA Semiquant was the test with the highest number of positive results and the CT Staining the lowest. When analysing the concordance tests, the reasons for the discordance between tests were down to four: the health of the individual who committed the crime, the proper performance of the CT Staining test, the AP test not being done correctly (or the quality of the phosphatase reagent) and the PSA test could be identifying PSA from other body fluids other than seminal fluid, has its supposed to. Nevertheless, when looking at the concordance test between the PSA quantification values and the MF profiles, nearly 89% of the samples with high PSA concentration (above 4,1 ng/mL) were in concordance with the male or mixed DNA profile that was obtained in the MF. On the other hand, almost 87% of the samples that were negative for semen had positive results for PSA. The important samples to analyse, from the semen negative ones, are those where the profile obtained in the MF was inconclusive or female and the PSA positive, this could mean that contrary to what is promised of the test (identifying exclusively PSA from seminal fluid) the PSA Semiquant could be identifying PSA from other body fluids, which means that this preliminary test has a high percentage of false positives.

To internally validate this instrument, four validation parameters were evaluated: sensitivity, specificity, reproducibility and repeatability. The sensitivity of the SeraQuant Reader was measured through a serial dilution with six different PSA concentrations, the

results obtained confirmed the instrument sensitivity for lower as well as high PSA concentrations, although its more sensitive in higher concentrations. For the specificity test a 0,00 and 2 ng/mL concentration of PSA dilution was used, and the results proved that the equipment (in this study) is specific for PSA identification since the values quantified in the 2 ng/mL concentration were approximated to the expected value of 0,5.

For the reproducibility and repeatability parameters, a PSA concentration was studied in two dilutions, a 0,5 and a 2 ng/mL. In the reproducibility test two operators carried out the quantification and the results confirmed that the method is reproducible, especially in higher PSA concentrations. As for the repeatability test, it was assessed in two different times of the day (in the morning and in the afternoon), with an interval of five hours between them, and the consistency of the results obtained proved the method repeatability, mainly in high PSA concentrations.

Overall, the SeraQuant Reader was internally validated and ready to be implemented in laboratory. All things considered, the PSA Semiquant test does identify PSA and was considered adequate as a preliminary method. Regrettably it cannot substitute CT Staining as a confirmatory method for the identification of semen, in the BBG-LPC. In real casework samples there is a great variability in the results and sometimes irregular samples results are obtained, adding this to the fact that the PSA Semiquant test, in this study, identified PSA from other body fluids, on the real casework samples, and had high percentage of false positives, makes it and unsuitable confirmatory method for semen traces.

To further improve the results obtained, a new study can be conducted, aiming to investigate the influence of the material and colour of the fabrics in the identification of seminal fluid in real casework samples, since the semen may have a greater adhesion to certain materials and the colorimetric process may affect this.

VI. References

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Appendices

Appendix 1 - Automatic document with the immunochromatographic rapid test result.

SERAQUANT® Measurement Report

Measurement time: 2024-07-22 13:33:02

Case #:

Exhibit:

Operator:

Measurement result

Positive: PSA = $\mathbf{4.68}^{+\infty}_{-0.352}$ ng/ml @ 95% confidence

Error (false pos.) probability < 0.01%

Resolution*

Traces of seminal fluid detected.

* Resolutions are specified by the user when preparing a calibration curve. SERATEC cannot be hold liable for the resolution content.

Traceability Details

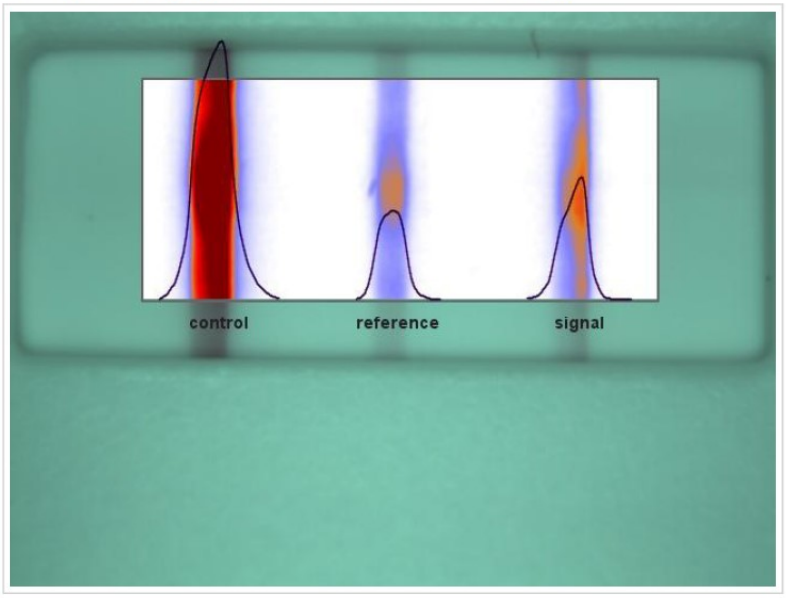
Lot number: 230019, **expires** 2025-12, **est. sensitivity** = 0.16 ng/ml

Calibration: Curva de Calibracao 04, **reliability** = 94,3%

by: Raquel LPC

Application-specific threshold: 0.50 ng/ml

Sample #1 (signal/reference = 1.28321)



Appendix 2 – BBG dilution protocol for the PSA Calibration Curve #4.

SERATEC®
Gesellschaft für Biotechnologie mbH

Protocolo: Diluição do PSA standard

para a preparação das curvas de calibração 04 de PSA

Material standard:	Int. PSA Free Standard	Lote	
tampão de diluição	Tampão de diluição do PSA	Val.	
concentração do PSA standard	500 ng/ml		
quantidade requerida do PSA standard para esta curva de calibração	40,00 µL		

ponto de medição	Volume	número de repetições	Data de validade (3 meses a 2-8°C)
10 ng/mL	2 mL	3	
4.0 ng/mL	1 mL	5	
2.0 ng/mL	1 mL	5	
1.0 ng/mL	1 mL	5	
0.5 ng/mL	1 mL	5	
0.25 ng/mL	1 mL	5	
0.00 ng/mL	1 mL	10	

Descrição do processo de diluição

preparação da solução de 10 ng/mL:

40 µL do PSA standard + 1960 µL do tampão do PSA

preparação da solução de 4 ng/mL:

400 µL da solução de 10 ng/mL + 600 µL do tampão do PSA

preparação da solução de 2 ng/mL:

200 µL da solução de 10 ng/mL + 800 µL do tampão do PSA

preparação da solução de 1 ng/mL:

100 µL da solução de 10 ng/mL + 900 µL do tampão do PSA

preparação da solução de 0.5 ng/mL:

51 µL da solução de 10 ng/mL + 949 µL do tampão do PSA

preparação da solução de 0.25 ng/mL:

24 µL da solução de 10 ng/mL + 976 µL do tampão do PSA

preparação da solução de 0.00 ng/mL:

0,0 mL da solução de 10 ng/mL + 1000 µL do tampão do PSA

NOTA: Colocar 80µL da solução em cada teste

Nome:_____ Data:_____

Appendix 3 – Real casework samples tests and DNA analysis results.

Sample Number	Phosphatase test	PSA ng/mL	Microscopy	Total Male [ ] ng/µL	Total Y [ ] ng/µL	Total Cellular [ ] ng/µL	Obtained Profile (Differential Extraction)	
							Cellular Fraction	Male Fraction
1	Positive	7.25	Negative	0.2191	0.117	0.0952	Mixed DNA Profile	Male Profile
2	Positive	2.22	Negative	0.7031	0.4334	0.2819	Mixed DNA Profile	Male Profile
3	Positive	5.42	Negative	2.2877	1.3266	0.2639	Mixed DNA Profile	Male Profile
4	Positive	4.62	Negative	0.0007	-	1.9948	Female Profile	Inconclusive
5	Positive	2.87	Negative	0.525	0.0005	12.5738	Female Profile	Female Profile
6	Negative	1.35	Positive	0.153	0.1107	1.8713	Mixed DNA Profile	Male Profile
7	Negative	0.64	Negative	0.0004	0.0003	0.017	Female Profile	Inconclusive
8	Negative	5.09	Positive	0.0044	0.0016	0.0036	Female Profile	Mixed DNA Profile
9	Negative	4.39	Positive	0.0309	0.017	25.3591	Female Profile	Mixed DNA Profile
10	Negative	4.27	Positive	0.0027	0.0024	0.0002	Inconclusive	Male Profile
11	Positive	6.6	Positive	0.3277	0.2294	1.5249	Mixed DNA Profile	Male Profile
12	Positive	3.68	Negative	0.0002	0.0006	0.1152	Mixed DNA Profile	Inconclusive
13	Positive	0.6	Positive	0.0261	0.0223	0.1762	Male Profile	Male Profile

Sample Number	Phosphatase test	PSA ng/mL	Microscopy	Total Male [ ] ng/µL	Total Y [ ] ng/µL	Total Cellular [ ] ng/µL	Obtained Profile (Differential Extraction)	
							Cellular Fraction	Male Fraction
14	Positive	9.32	Positive	0.3457	0.303	0.3468	Mixed DNA Profile	Male Profile
15	Positive	0.17	Negative	0.0541	-	0.0169	Inconclusive	Female Profile
16	Negative	1.06	Negative	0.0004	0.0007	0.07161	Female Profile	Inconclusive
17	Negative	0.20	Negative	0.0003	0	-	Female Profile	Inconclusive
18	Positive	3.45	-	0.0001	-	0.3568	Mixed DNA Profile	Inconclusive
19	Positive	6.77	Positive	0.6521	0.4884	0.7362	Mixed DNA Profile	Male Profile
20	Positive	1.89	Negative	0.0045	0.0051	0.0416	Mixed DNA Profile	Male Profile
21	Positive	5.43	Positive	0.0172	0.0079	17.6623	Female Profile	Mixed DNA Profile
22	Positive	3.51	Negative	0.0013	0.0019	0.1430	Female Profile	Inconclusive
23	Positive	2.95	Positive	0.014	0.0106	1.432	Mixed DNA Profile	Male Profile
24	-	5.65	Negative	0.0203	0.0094	37.4566	Female Profile	Mixed DNA Profile
25	-	8.27	Negative	0.0202	0.0169	15.4691	Female Profile	Mixed DNA Profile
26	Positive	5.41	Positive	0.4942	0.6774	0.2729	Male Profile	Male Profile
27	Positive	5.39	Negative	0.122	0.0151	42.6087	Female Profile	Mixed DNA Profile

Sample Number	Phosphatase test	PSA ng/mL	Microscopy	Total Male [ ] ng/µL	Total Y [ ] ng/µL	Total Cellular [ ] ng/µL	Obtained Profile (Differential Extraction)	
							Cellular Fraction	Male Fraction
28	Positive	8.70	Positive	0.8021	0.5730	12.1544	Female Profile	Male Profile
29	Negative	0.27	Positive	0.0251	0.1440	0.004	Female Profile	Male Profile
30	Positive	5.94	Positive	0.1417	0.1189	3.182	Mixed DNA Profile	Mixed DNA Profile
31	Positive	8.26	Positive	0.0746	0.0843	2.7931	Mixed DNA Profile	Male Profile
32	Positive	0.87	Negative	0.0051	0.0002	20.2274	Female Profile	Female Profile
33	Negative	0.15	-	0.0030	0.0001	12.7625	Female Profile	Female Profile
34	Positive	4.77	Positive	0.0821	0.0567	10.655	Female Profile	Mixed DNA Profile
35	Positive	0.59	Negative	0.0004	0.0004	0.0527	Male Profile	Inconclusive
36	Negative	3.76	Negative	0.0111	0.0008	13.3521	Female Profile	Female Profile
37	Negative	0.67	Negative	0.0003	-	0.0989	Female Profile	Inconclusive
38	Positive	9.68	Positive	0.0198	0.0130	16.4224	Mixed DNA Profile	Male Profile
39	Positive	4.73	Positive	0.1200	0.0880	1.0460	Female Profile	Mixed DNA Profile
40	Positive	6.81	Positive	2.5025	3.0342	32.1171	Female Profile	Male Profile
41	Positive	7.2	Negative	0.0005	0.0006	0.1042	Male Profile	Inconclusive
42	Positive	7.35	Negative	0.0002	0.0007	0.3771	Mixed DNA Profile	Inconclusive

Sample Number	Phosphatase test	PSA ng/mL	Microscopy	Total Male [ ] ng/µL	Total Y [ ] ng/µL	Total Cellular [ ] ng/µL	Obtained Profile (Differential Extraction)	
							Cellular Fraction	Male Fraction
43	Positive	0.79	Positive	0.1003	0.1868	0.0342	Male Profile	Male Profile
44	Positive	7.7	Positive	0.0186	0.0293	0.0175	Mixed DNA Profile	Male Profile
45	Positive	4.68	Positive	0.1127	0.2178	0.0632	Male Profile	Male Profile
46	Positive	0.79	Positive	0.0787	0.0692	0.1504	Mixed DNA Profile	Male Profile
47	Positive	7.27	Positive	0.9495	0.8404	20.1815	Female Profile	Male Profile
48	Positive	0.5	Negative	0.0091	0.0177	0.8945	Male Profile	Male Profile