The Examination of Alternative Methods for Sperm Cell Lysis and Purification of Sexual Assault Samples

a Master Thesis Project

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Abstract

Forensic sexual assault samples typically contain a mixture of a small quantity of sperm cells and a high quantity of epithelial cells. This relationship complicates the generation of a DNA profile, which prompts the need for differential lysis and extraction of the DNA. Differential extraction is a lengthy method that includes multiple tube transfers and requires a purification step. Amicon Ultra centrifugal filters are employed to purify samples; however, these filters are costly and susceptible to potential breaks in the supply chain. The objective of this project was to examine alternative methods utilizing “off-the-shelf” reagents for sperm cell lysis or sample purification, with the goal of streamlining the current differential extraction protocol.

Three methods for sperm cell lysis were studied: alkaline lysis, lysis with the nonionic detergent NP-40, and lysis with a natural decondensation assay. Isopropanol precipitation was examined as an alternative purification method. Additionally, reducing the number of washing steps in the differential extraction protocol was investigated.

The results showed that optimized alkaline lysis (25 µL NaOH (0.2 M), 25 µL Tris-HCl (0.2 M) and TE buffer) was comparable to the differential extraction protocol in terms of DNA recovery. Alkaline lysis was also comparable to the differential extraction when performed on mock sexual assault samples. Isopropanol precipitation likewise showed equal DNA yields, complete DNA profiles and peak heights compared to Amicon filtration. Lastly, it was shown that reducing the number of washing steps of the reference protocol produced comparable DNA profiles. In conclusion, the findings suggest that the differential extraction protocol can be modified by using alkaline lysis for sperm cell lysis or isopropanol for purification, as well as by performing only one washing step.

Preface

This project was performed in collaboration with the forensic group at the Division of Applied Microbiology (TMB), Lund University. The forensic group at TMB is a part of the biology section at the National Forensic Centre (NFC). These past few months have given me tremendous insight into the world of forensic DNA analysis, a subject I have always found intriguing.

I would like to sincerely thank my supervisors, Linda Jansson and Johannes Hedman, for providing great guidance and support during my time at TMB. Thank you for always being available to answer any and all questions raised, and for investing time and energy in this project, from small details to broad concepts. Thank you to my examiner Jenny Schelin for first of all putting me in touch with Linda and Johannes, as well as your feedback and support. I would also like to thank Lisa Lindahl for always being ready to assist me with various tasks in the lab.

Additionally, I would like to express my gratitude to Elin Lundkvist and Christina Forsberg at NFC for granting me the opportunity to visit NFC in Linköping, observe the differential extraction process firsthand, and perform some of the methods in their project lab. My two trips to Linköping were both immensely educative and exciting.

Finally, thank you to everyone at TMB for being so welcoming and kind during my months there.

Popular summary

Examining Methods to Streamline DNA Extraction from Sperm Cells in Sexual Assault Samples

Sperm cells are usually the most interesting biological material to analyze in forensic sexual assault samples. However, today's method for extracting DNA from sperm is both time-consuming and requires purification with expensive filters. It is therefore relevant to investigate alternative protocols that can help streamline the process.

The term "sexual offense" it the Swedish legal umbrella term for crimes of a sexual nature, with major crimes including sexual assault. Typically, the biological material of highest interest in sexual assault samples is sperm cells, as DNA can be extracted and potentially linked to a suspect. However, due to the nature of most sexual assault samples, where sperm cells are present in lower quantities in comparison to other cell types (e.g. epithelial cells), successful extraction and analysis of sperm cell DNA is difficult. Because of this, a method called differential extraction is typically performed, where the sperm cells and other cells in the mixture are treated separately.

Differential extraction is a lengthy protocol that requires multiple transfers of material between tubes, which can lead to loss of DNA and contamination. On top of this, it uses the strong reagent dithiothreitol (DTT) to successfully break open the sperm cells and release the DNA. DTT interferes with downstream analysis and DNA profiling, for which reason the samples must be purified. At the National Forensic Centre (NFC), purification of samples is done with centrifugal filters called Amicon filters. As there is only one supplier of these filters, the process is susceptible to disruptions in the supply chain, which occurred during the Covid-19 pandemic. Furthermore, Amicon filters are expensive. Therefore, it was relevant to examine alternative methods for sperm cell disruption (lysis) and sample purification that could simplify the current method, preferably while using easily accessible reagents.

This project examined three alternative methods for sperm cell lysis, and one alternative method for purification of samples treated with DTT. Also, reducing the number of times the intact sperm cells are washed was assessed, as this is a time-consuming step in the current protocol, and requires a lot of pipetting.

Alkaline lysis, a method where the sperm cells are lysed by being subjected to heat and a strong base, was seen to produce DNA yields equal to the current protocol, as well as complete DNA profiles. Likewise, precipitation with isopropanol was seen to purify samples as well as Amicon filtration. Lastly, reducing the number of washing steps resulted in equal DNA recovery in comparison with the protocol used today. Thus, incorporating these methods could simplify today's differential extraction protocol significantly.

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LIST OF ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
CE	capillary electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
IPC	internal positive control
ILS	internal lane standard
NFC	the National Forensic Centre
RFUs	relative fluorescence units
SDS	sodium dodecyl sulfate
ssDNA	single-stranded DNA
STR	short tandem repeat
UF	ultrafiltration
(q)PCR	(quantitative) polymerase chain reaction

1. Introduction

Deoxyribonucleic acid (DNA) analysis is an indispensable tool in forensic science today. Advanced methods of DNA profiling have been developed since the initial description of highly polymorphic regions in the genetic code by Alec Jeffreys in 1985, and these methods are routinely used in modern forensic laboratories (Carracedo and Sánchez-Diz, 2005). Importantly, forensic analysis of biological evidence left at crime scenes can be used to identify suspects and convict perpetrators.

The term "sexual offence" is a general descriptor that includes a range of different crimes, from minor offenses to severe crimes such as rape. According to the Swedish National Council for Crime Prevention, reports of rape accounted for the majority of reported sexual offenses in Sweden in 2021 (Brå, 2022). The most common forensic samples collected from sexual assaults that are of an evidentiary nature contain a mixture of sperm cells and other cell types, typically vaginal epithelial cells, with the other cell types often outnumbering the sperm cells. This relationship may complicate the generation of a DNA profile, which prompts the need for differential lysis and extraction of the DNA (i.e. separate lysis and extraction of sperm and epithelial cells in a mixture), as sperm cells are typically of the highest interest in the samples (Schellhammer et al., 2022).

Sperm cells have a distinct morphology which aids in egg fertilization. For example, the histones in sperm DNA are up to 85% replaced by protamines that form disulfide bridges, enabling tight packing and protection of the genetic material (Schellhammer et al, 2022). Additionally, sperm cells have a unique organelle, called the acrosome, which covers the front part of the cell head and contains important enzymes released during fertilization. Further, sperm cells have a dynamic plasma membrane with its lipids and proteins arranged at the sides of the cell head in a way that differs from somatic cell membranes (Flesch & Gadella, 2000).

The Chelex®-based method for differential lysis of sperm cells that is currently used at the National Forensic Centre (NFC) requires treatment with dithiothreitol (DTT) to reduce the cells' disulfide bonds. DTT is a strong reducing agent that can disrupt downstream processes if it is not filtered out, which is currently done using specific filter tubes (Amicon® Ultra-2 or Ultra-4, Merck Millipore) (The Swedish Police Authority, n.d.). This renders the method susceptible to possible breaks in the supply chain, something that occurred during the Covid-19 pandemic, especially since there is only one supplier, Merck Millipore, of Amicon filters. Supplier interruptions can therefore lead to delays in forensic DNA analysis. Further, the method is labor-intensive, requires multiple tube transfers, and the Amicon filters are relatively expensive, costing approximately 100 SEK per tube (Fisher Scientific, n.d.). It is therefore relevant to investigate alternative protocols that both avoid the use of Amicon filters and that are not as laborious as the current method.

In this project, three alternative protocols for differential extraction were examined: alkaline lysis, lysis with the non-ionic detergent NP-40, and lysis with a natural decondensation assay. All three protocols have previously been examined in an article by Schellhammer et al. (2022),

exhibiting promising results. Purification with isopropanol precipitation was additionally assessed as an alternative to the current Amicon filter purification step. Finally, a reduction in washing steps was investigated, to potentially shorten the current differential extraction protocol.

1.1 Aim

The aim of this thesis was to modify the current protocol for differential lysis by examining alternative protocols that could replace the lysis and/or purification step. The overall goal was to find a more user-friendly method compared to the current protocol, as well as one that is less reliant on a single supplier for delivery. Various principles for lysis were considered, including lysis using a non-ionic detergent, lysis in alkaline conditions and lysis with natural decondensation assays, as well as isopropanol sample purification. Potential simplifications of the current protocol, by reducing the washing steps, have also been considered. The following research questions were central for this thesis:

1. Do any alternative methods for differential extraction/purification produce sufficient DNA yield, both quantitatively and qualitatively?

2. Can the alternative methods be optimized to obtain better results?

3. Are the protocols compatible with NFC's method for DNA quantification and short tandem repeat (STR)-analysis?

4. Is the best-performing protocol able to produce equivalent or better results in comparison to the current Chelex-based method?

2. Background

2.1 DNA and Genetics

All cells in the human body must be able to produce proteins necessary for their function and management. To achieve this, a type of genetic instruction manual is required. This is the role of DNA, which is the genetic material present in all cells of the body. DNA stores important genetic information, and functions as a template for the replication of the genetic material in new cells (Butler, 2005).

The DNA strand architecture includes the nitrogenous nucleobases adenine (A), thymine (T), cytosine (C), and guanine (G), as well as the negatively charged sugar-phosphate backbone structure onto which these bases are linked. A full DNA molecule consists of two strands, where a base on one strand is hybridized to a base on the other (A to T, and G to C) through hydrogen bonds. This creates the arrangement of the DNA double helix, with the nucleotide sequence determining the genetic code (Berg et al., 2015).

With around three billion base pairs (bp), the human genome contains a vast collection of protein-coding and noncoding regions that all need to fit within the cell nucleus. This is achieved by coiling the double-stranded DNA (dsDNA) around histones, specific proteins that enable dense packing of the DNA. The DNA wrapped histones (referred to as nucleosomes) are the constituents of chromatin fiber, which itself is arranged into three-dimensional structures known as chromosomes (Berg et al., 2015). There are a total of 23 pairs of chromosomes in the human genome, of which 22 pairs are autosomes, and one pair consists of two sex chromosomes. Females carry two copies of the X chromosome as their sex chromosome pair, while males carry one copy of the X chromosome and one copy of the Y chromosome (Butler, 2005). The human gametes, i.e. the reproductive cells, only carry one copy of the chromosomes (haploid), while somatic cells carry both (diploid). The two chromosomes in the pairs are homologous, meaning the order of the genes are the same. However, there may be sequence variations, from one to several bp. The specific location of a gene (or other DNA sequence) in the genome is known as a locus (pl. loci), and the copies of a gene at the same locus are referred to as alleles. The two alleles in an individual are inherited from the parents, one from the mother and one from the father, and may be heterozygous (different) or homozygous (identical) (Butler, 2005).

Repeat DNA sequences, referred to as STRs, are found throughout the genome (Butler, 2005, p 85). STRs consist of repeated 2–6 bp sequences, caused by the evolutionary process of DNA strand slippage during replication (Tang et al., 2017). The markers are found at the same genetic positions (loci) in a population but are highly polymorphic, meaning the lengths of the repeats (i.e. how many times the sequences are repeated) vary among individuals. STRs at several different loci are examined when determining who the DNA belongs to (Butler, 2005).

Several thousand loci have been mapped, and selected standardized polymorphic markers are used for forensic identification. At NFC, 15 STR markers are routinely analyzed during DNA

typing, of which 12 are required by the European union, along with a marker indicating the individual's gender (The Swedish Police Authority, 2022). Analysis is carried out with multiplex polymerase chain reaction (PCR), by applying an STR kit that enables co-amplification of multiple markers during one run. The markers are identified by the locus and labelled according to the number of repeats (Butler, 2005).

2.1.1 Sperm Morphology

The spermatozoon (plural spermatozoa) is the gamete produced by male humans and other animals. Sperm cells have a distinct morphology, consisting of a head and a tail, which enable the cells to reach and fertilize the female egg cell (oocyte). The tail is made up of microtubules that grant the sperm motility, as well the middle piece, located right below the head. This section is wrapped with mitochondria, providing the cell with energy (Guraya, 1987). An outline of the sperm morphology is illustrated in Figure 1.

As mentioned in section 2.1 on DNA and Genetics, gametes only contain one copy of each autosomal chromosome, and one of the sex chromosomes (Britannica, 2023). They are located in the nucleus, which is contained in the sperm head. During formation of spermatozoa in the testes, the histones around the DNA are converted into cysteine-rich proteins called protamines, leading to the formation of disulfide bonds. This causes an extensive condensation of the DNA and nucleus. The compaction, along with the almond shape that the head assumes because of the flattening of the nucleus, most likely plays a part in oocyte penetration and fertilization (Durairajanayagam et al., 2015). Further, the nucleus is covered by a two-layered nuclear envelope that is mostly free of pores, which likely contributes to the protection of DNA against hydrolytic enzymes (Mortimer, 2018). Another protective component of the head is the acrosome, an organelle produced by the Golgi complex that is unique for the spermatozoon. Located on the apex of the sperm head, the acrosome is a vesicle that contains several enzymes that are released when the acrosome membrane fuses with the oocyte membrane during fertilization. It covers around 40–70% of the tip of the head (Durairajanayagam et al., 2015). Lastly, the sperm cell membrane is unique as the lipids and proteins are arranged at the sides of the sperm head (Flesch & Gadella, 2000). It bears mentioning that an individual who has undergone a vasectomy will produce semen that does not contain spermatozoa (azoospermic semen) (Butler, 2005).

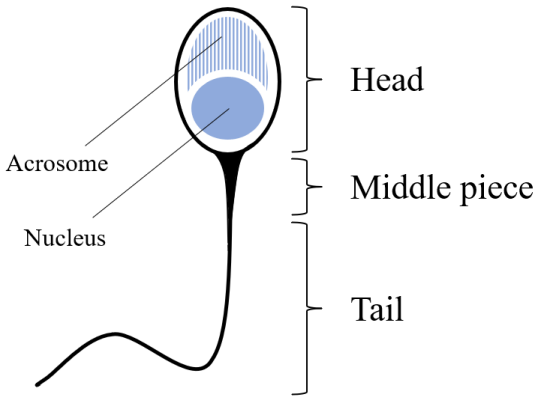

Figure 1. A basic outline of typical sperm morphology. The sperm cell head, middle piece and tail are shown, as well as the acrosome and nucleus.

2.2 Forensic DNA Extraction

Extracting genetic material from samples is an early and critical step in the forensic analysis workflow. Following collection of biological material from a crime scene, victim, or suspect, the subsequent step is to obtain potentially present DNA, separating it from other substances that may be present in the sample, and determining if the amount and quality is acceptable for further analysis. Depending on the nature of the sample, different methods for DNA extraction can be employed (Butler, 2005). This section will cover some of the methods used for DNA extraction, specifically in relation to the lysis of cells in samples containing sperms and other cells.

2.2.1 Chelex Extraction

Chelex-based DNA extraction is a common technique used in forensic laboratories today, including at NFC. This method is advantageous due to its simplicity, speed, and use of non-toxic solutions. It utilizes Chelex 100, a synthetic resin made of styrene divinylbenzene copolymers. The Chelex 100 resin is a chelate, a compound that forms highly stable bonds with charged metal ions, which is attributed to the copolymer-paired iminodiacetate ions (Walsh, Metzger, and Higuchi, 2013). Because Mg^{2+} is a cofactor of DNases that cleave DNA, the addition of Chelex helps protect the molecules from damage (Butler, 2005). The Chelex beads can be directly added to a biological sample that is subsequently subjected to a high temperature to lyse the cells and release the DNA. The alkaline conditions provided by the resin helps disrupt the cell membranes (Cattaneo, K. Gelsthorpe and Sokol, 2006) Conveniently, following centrifugation, the supernatant can directly be analyzed with PCR (Butler, 2005).

2.2.2 Differential Extraction

The most common forensic samples collected from sexual assaults (e.g. from sexual assault kits) that are of an evidentiary nature contain a mixture of sperm cells and other cell types from the victim, typically vaginal epithelial cells, with the other cell types often exceeding the sperm

in quantity. This relationship can complicate the generation of a DNA profile, which gives rise to the need for differential lysis of cells and extraction of the DNA (Schellhammer et al., 2022).

As previously mentioned, the histones in sperm DNA are largely replaced by protamines that form disulfide bridges. As a result, the nuclear material is more tightly packed than in somatic cells. The disulfide bonds, in combination with the distinctive properties of the acrosome and sperm plasma membrane, necessitate the need for strong reducing agents when lysing the cells (Schellhammer et al., 2022). Traditionally, differential extraction involves an initial lysis of the more easily lysed epithelial cells by the addition of sodium dodecyl sulfate (SDS) and proteinase K, followed by centrifugation and subsequent separation of the lysed epithelial cell fraction (supernatant) and intact sperm cells (pellet). Differential extraction is concluded with the addition of more SDS and proteinase K along with the reducing agent DTT to the sperm cells (Butler, 2005). The process of differential extraction is illustrated in Figure 2. SDS is a strong detergent that lyses cells by interacting with the cell membrane, causing lipid and membrane protein solubilization (Brown and Audet, 2008). Proteinase K, a serine protease, is added due to its ability to digest proteins in a nonspecific manner (Thermo Fisher, n.d.-b). While SDS and proteinase K break down the epithelial cells in the sample, DTT is required to reduce the strong disulfide bonds present in the sperm head and nuclei (Butler, 2005). However, SDS can degrade DNA polymerases, and DTT has been seen to disrupt downstream processes by introducing nicks in the DNA strands and by quenching fluorescence in PCR dyes (Schrader et al., 2012). Thus, it is necessary to purify the samples before further analysis, e.g. with Amicon Ultra filters (Fjelstrup et al., 2017; Boiso, Sanga, and Hedman, 2015).

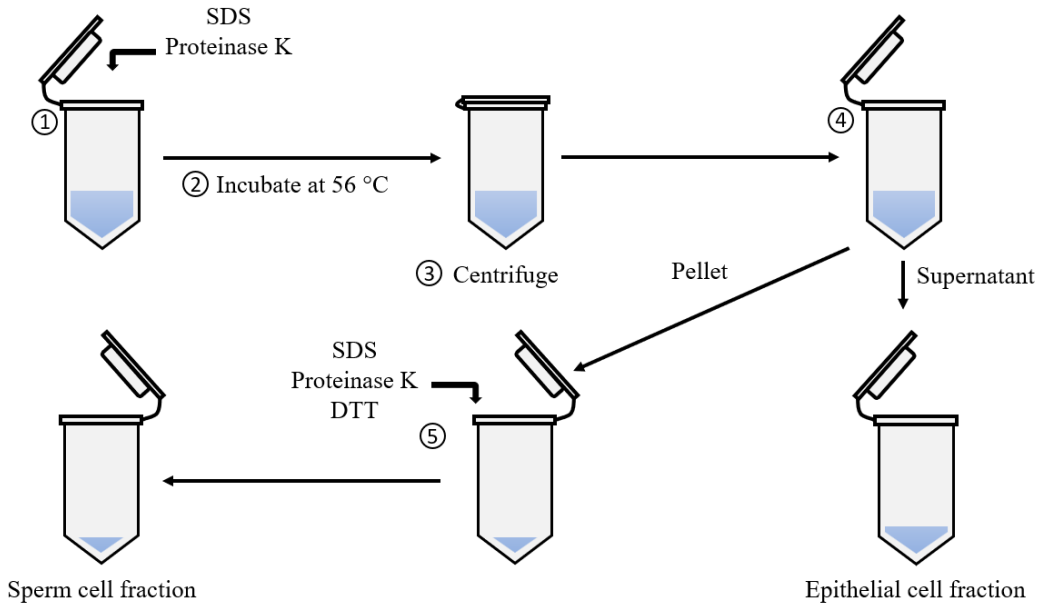

Figure 2. Illustration of the general process of traditional differential extraction.

2.2.3 Alkaline Lysis

Alkaline lysis is an alternative approach to differential extraction that bypasses the use of strong agents such as DTT. The alkaline lysis protocol was initially used to isolate plasmid DNA from

bacteria or genomic DNA from plants but has since the 1990s been investigated as a potential alternative to differential extraction of forensic samples (Klintschar and Neuhuber, 2000). This method is faster and simpler than conventional differential extraction, involves less tube transfers, and can be performed using non-proprietary reagents. During alkaline lysis, samples are exposed to an alkaline environment and incubated in heat for 2–5 minutes, depending on the nature of the sample. The alkaline solution, typically sodium hydroxide (NaOH), causes ionization of certain amino acids (cysteine, tyrosine, aspartic acid, and glutamic acid), and subsequent protein denaturation and solubilization. The high hydroxide ion (OH^-) concentration also causes the splitting of disulfide bonds as a consequence of the OH^- forming new bonds with the sulfur atoms (Ruhr-Universitaet-Bochum, 2016). Following incubation, the sample is neutralized with an acid such as Tris-HCl to halt the reaction (Rudbeck and Dissing, 1998). Figure 3 illustrates the general process of alkaline lysis.

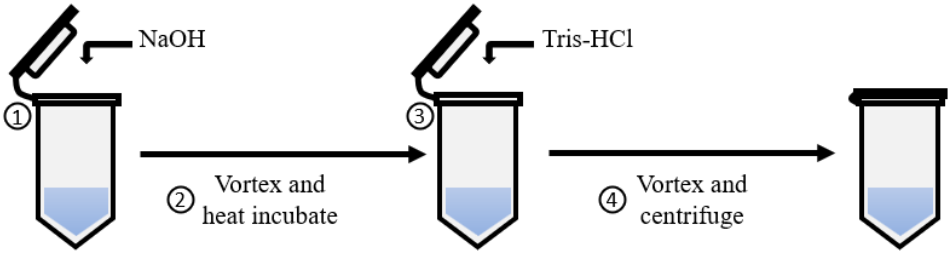

Figure 3. Illustration of the general process of alkaline lysis.

2.2.4 NP-40 Lysis Buffer

NP-40 is a non-ionic detergent that can be included in the lysis buffer to facilitate cell lysis (Schellammer et al., 2022). Detergents are classified as such due to their amphipathicity, which is derived from the presence of a hydrophobic tail and a hydrophilic head in the molecular structure. Mild detergents, like NP-40, are able to form micelles with lipids and proteins, leading to cell membrane disruption without complete denaturation of non-membrane proteins (Thermo Fisher, n.d.-c). NP-40 is commonly used in immunoassays and for preparation of cell extracts to be analyzed with various techniques (Thermo Fisher, n.d.-d).

2.2.5 Decondensation Assays

An additional approach to sperm cell lysis is using natural decondensation assays. This method is based on the changes that sperm undergo once they enter the egg cell cytoplasm, or ooplasm. Here, the disulfide bonds in the sperm nucleus are reduced and the protamines are once again replaced by histones for a so-called pronucleus to be formed. Natural decondensation assays have been studied in relation to their employment in intracytoplasmic sperm injection (ICSI). Reduced glutathione is known to play a role in this reduction in vivo, and heparin has also been seen to be important for sperm decondensation in vitro, though this process is not fully understood (Romanato, 2003). Lysis of sexual assault samples involves exposure to modified human tubal fluid (HTF), a medium formulated to mimic the human oviduct environment, containing both glutathione and heparin (Swango et al., 2006; Schellhammer et al., 2022).

2.3 DNA Purification

Following DNA extraction from the forensic sample, a purification step is sometimes necessary to concentrate the genetic material and/or to remove substances that may interfere with PCR analysis (Norén et al., 2013). As mentioned, SDS and DTT are examples of compounds that can disturb the amplification process if still present. Several approaches can be employed; filtration and precipitation will be described in this section.

2.3.1 Amicon Ultra Filters

Produced by Merck Millipore, Amicon Ultra centrifugal filters can be used in a forensic setting to concentrate nucleic acids and to purify samples. The method is based on ultrafiltration (UF), where minute particles and dissolved molecules are effectively separated from fluids. UF allows for water and salts to pass through a UF membrane, while compounds with a higher molecular weight are retained (Merck Millipore, n.d.-b). Purification of forensic samples with Amicon filters involves centrifugation (in a swing bucket or fixed angle rotor centrifuge) against the vertical regenerated cellulose membrane that retains the DNA. Amicon filters are also engineered to include a physical deadstop that protects DNA or proteins from excessive drying, and a reverse spin option to recover an optimal number of nucleic acids or proteins (see Figure 4) (Merck Millipore, n.d.-a).

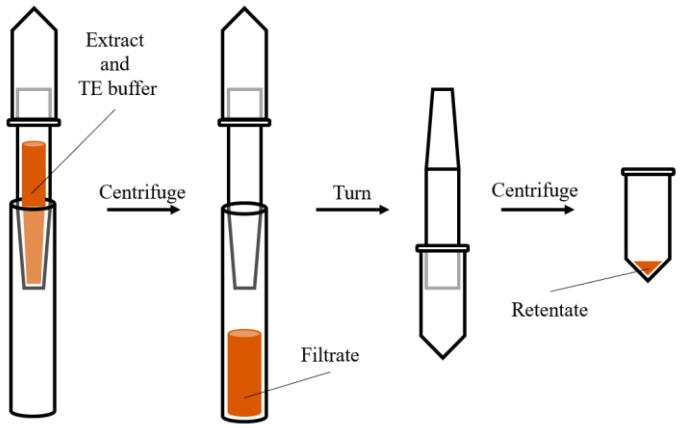

Figure 4. Illustration of Amicon Ultra 2 mL filters. Samples are centrifuged and filtered with ultrafiltration, retaining the DNA. By turning the filter, the retentate can be collected.

2.3.2 Precipitation

An additional technique for purification of DNA is through precipitation, which involves the addition of a high salt concentration followed by an alcohol such as isopropanol or ethanol. When salt is added, an electrostatic interaction occurs between the positively charged salt ions and the negatively charged phosphates of the DNA backbone which neutralizes the DNA molecule, and thus renders it less hydrophilic. When the alcohol is added, the DNA precipitates and aggregates (He et al., 2021). If the DNA quantity is very low, it is appropriate to use a carrier to ensure the recovery of nucleic acids. Carriers are substances that are inert and insoluble in e.g. ethanol. The carrier helps form a visible pellet by precipitating and

consequently trapping the DNA. Typical carriers include linear acrylamide, glycogen, and yeast tRNA (Green and Sambrook, 2016). Precipitation with isopropanol requires a smaller amount of alcohol in comparison to ethanol as the DNA is less soluble in isopropanol, which is why isopropanol may be preferred when purifying DNA. Although optimal precipitation occurs when using cooled ethanol or isopropanol, isopropanol precipitation in cold temperatures leads to increased co-precipitation of salts. Therefore, this method is performed with room temperature isopropanol (Green and Sambrook, 2017).

2.4 Polymerase Chain Reaction

PCR is the primary method used for DNA quantification in forensic DNA analysis today. Developed in 1984, PCR is a powerful technique that allows for the rapid amplification of specific sequences of DNA, even from microscopic amounts of DNA (Morling, 2009). This makes the method highly valuable for forensic investigations, as trace amounts of DNA found at a crime scene can be amplified, analyzed, and potentially linked to a specific perpetrator. Beyond forensic biology, PCR is a useful tool in areas such as anthropology, clinical diagnostics, food analysis and more (Berg et al., 2015).

The principle of PCR is based on the activity of DNA polymerases, a group of enzymes that are instrumental in the synthesis of DNA (polymerization) during the natural DNA replication process in cells. Both single strands of the DNA double helix serve as separate templates for the polymerase, thus telling the enzyme which deoxynucleoside triphosphates (dNTPs) to add to the growing complementary strand (Berg et al., 2015). However, polymerases require an oligonucleotide hybridized to the 3´-end of the template strand as they are unable to initiate replication on a single-stranded DNA strand alone. This short DNA strand is called a primer, and it provides the polymerase with a free 3´-end onto which it can attach a free nucleotide. The polymerase synthesizes a DNA strand in a 5´-to-3´ direction where the growing complementary chain binds to the template strand, producing a new dsDNA molecule (Berg et al., 2015).

Polymerase activity is utilized in vitro during PCR; by mixing the target DNA with the main components of PCR such as DNA polymerase, free dNTPs, and primer pairs, it is possible to amplify the desired DNA in an exponential manner (Berg et al., 2015). Each cycle consists of three stages in which the temperature is varied to promote a specific biochemical process. The first step is denaturation, where the temperature is raised to 94–96 °C as this is the temperature at which the hydrogen bonds within dsDNA are broken, producing two single strands (Berg et al., 2015). Reactions that use a heat-activated polymerase may require an initial denaturation at 95 °C preceding the 3-step cycling, as the enzyme will remain inactive at room temperature and requires heat to remove molecules or antibodies bound to the enzyme's active site (Butler, 2005). Once heat activation has occurred, the polymerase will remain active even in cooler temperatures. Following denaturation, the temperature is decreased to 3–5 °C below the primers' melting temperature (T_m), i.e. the temperature at which there is a dissociation between the primers and the template DNA (Brown, 2010). This allows the primers to anneal to the strands. The primer pairs consist of a forward and reverse primer that flank the desired target

sequence on each strand. When the primers have hybridized to the target sequence the added polymerase is able to initiate the synthesis of a complementary strand in the final stage, termed elongation. The temperature is once again raised, this time to the temperature right below the optimal temperature for the polymerase which is around 72–74 °C depending on the polymerase used (Brown, 2010). The PCR cycle is then repeated a number of times, with *n* cycles resulting in the synthesis of 2^n amplicons. Thus, running just 30 PCR cycles should produce more than a billion DNA fragments (Berg et al., 2015).

In addition to the main components of PCR described above, magnesium ions (Mg^{2+}) are added to the PCR reaction mixture, e.g. in the form of a $MgCl_2$-solution. Magnesium acts as a cofactor of DNA polymerase, catalyzing the addition of dNTPs and extension of the primer. Further, the positively charged magnesium ions react with the negatively charged DNA backbone, thereby neutralizing it, and promoting the primer-DNA template duplex. Consequently, the final magnesium concentration is crucial; a low Mg^{2+}-concentration will cause the lead to insufficient polymerase activity, while a too high concentration will result in nonspecific binding of primers and subsequent amplicons (Thermo Fisher, n.d.-a).

A buffer is another typical component of the PCR reaction mix. This solution helps maintaining an appropriate chemical milieu for the DNA polymerase activity and the reaction in terms of pH and salinity (Thermo Fisher, n.d.-a). Finally, bovine serum albumin (BSA) may be added to reduce the effect of PCR inhibitors that interfere with PCR by binding substances that may interfere with amplification (Kreader, 1996). Inhibitors can be biological (e.g. heme in red blood cells) or come from the environment the crime scene sample is taken from (e.g. soil, wood, and textile dyes). These substances can have different mechanisms of inhibition, for example by degrading DNA, or by inhibiting the DNA polymerase. Besides the addition of BSA or other substances, inhibition can be alleviated by diluting the template or adding more DNA polymerase. Samples containing high concentrations of substances such as DTT and SDS need to be purified prior to PCR analysis (Butler, 2005).

2.4.1 Quantitative PCR

Quantitative PCR, qPCR, also referred to as real-time PCR, is a technique that enables quantification of DNA in real time. In forensic analysis, it is a necessary method for determining whether DNA is present in the sample, and if it is of sufficient quantity to perform further analysis and typing (Martins et al., 2015). The minimum DNA concentration required for standard analyses at NFC today is 0.01 ng/µL, which roughly corresponds to 30 cells per reaction, as analysis of samples containing less than this amount will most likely be unable to produce complete DNA profiles (The Swedish Police Authority, 2022). The amount of DNA is determined by continuously measuring the fluorescent signal emitted by a so-called reporter probe or by an intercalating dye and comparing the signal to a standard curve with known concentrations (Butler, 2005). This stands in contrast to conventional PCR, where the DNA concentration can be established only after amplification, e.g. by visualizing the PCR products on a gel following electrophoresis (Brown, 2010).

A probe is a short piece of ssDNA, typically with a higher melting temperature than the primers, which is complementary to a specific sequence within the region delimited by the primers. By hybridizing at a lower temperature than the primers, the probe will bind before the polymerase begins the elongation process (Butler, 2005). A probe consists of a reporter dye attached to the 5´-end of the primer oligonucleotide, along with a quencher dye at the 3´-end that prevents fluorescence. When not hybridized to the PCR product, the quencher and reporter dye will be in proximity of each other, with the quencher suppressing the reporter signal (Brown, 2010). Once polymerization is initiated, the polymerase can cleave the oligonucleotide with its 5´-exonucleic activity. This results in the release of the reporter dye and subsequent emittance of a fluorescent signal (Butler, 2005). Fluorescent probes have a high specificity and can be used for multiplex PCR analysis, but may have a higher cost (Thermo Fisher, n.d.-e) Intercalating dyes employ an alternative approach, where the dye is embedded between the bases in dsDNA. Intercalating dyes are cheap dyes that are added in the PCR master mix and the fluorescence is measured in each cycle. Following amplification, a melting curve analysis is conducted, where the PCR products' melting points are identified as the temperature at which the fluorescent signal from the dye drastically decreases. The melting temperature is then compared with a positive control. However, as intercalating dyes bind nonspecifically, this can lead to the measurement of fluorescent signals from unwanted dsDNA, such as primer dimers. They also employ only one color, and so size separation of the DNA based on size is required. Examples of commonly used intercalating dyes are SYBR Green and EvaGreen (Butler, 2005).

The data produced from qPCR can be expressed as an amplification plot with the PCR cycle plotted against the fluorescence intensity (Figure 5a). During PCR, amplification can be divided into three distinct phases: exponential amplification, linear amplification, and plateau. As the PCR components are at an optimal concentration during the first phase, it is the most efficient amplification step where amplicons are produced exponentially. Once the component concentrations begin to decrease, the effectiveness declines, reaching linear growth. Finally, the reaction arrives at a plateau phase as the reaction components are exhausted (Butler, 2005).

The DNA concentration is calculated using both quantification cycles, C_q, of each sample, and standards. The C_q is the PCR cycle at which the fluorescent signal reaches above the signal of the background noise, which is a threshold decided by the software used. An early C_q indicates the presence of more DNA in the sample, as a higher initial concentration will result in the fluorescent signal being detectable quicker than for lower concentrations. By using standards with known DNA amounts, it is possible to obtain a relationship between the logarithm of the concentration and the C_q, which is plotted in a standard curve (Figure 5b) (Butler, 2005).

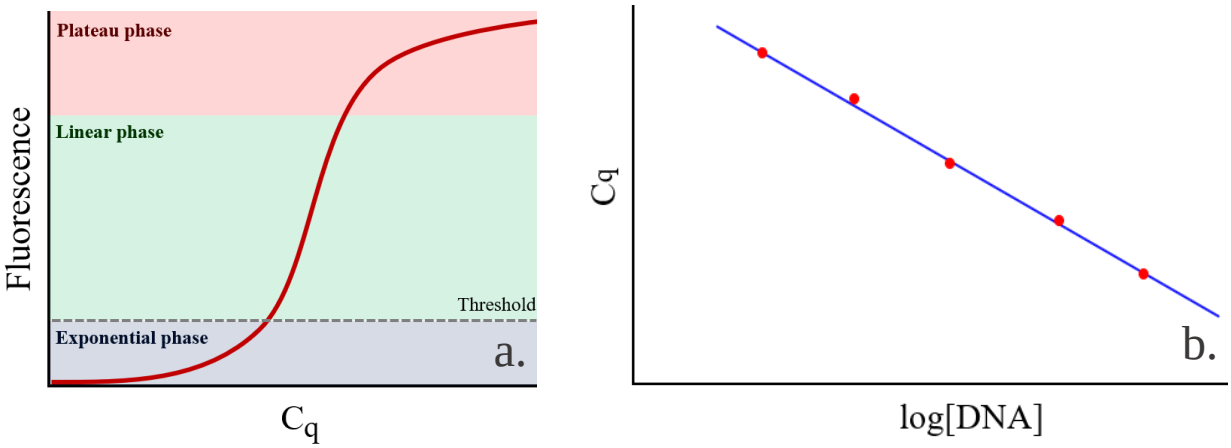

Figure 5. Example of (a) amplification plot and (b) standard curve obtained from qPCR analysis.

2.4.2 Multiplex PCR

Within forensic analysis, as well as in other fields, it is relevant to amplify more than one region of the target DNA. This can be done with multiplexing, i.e. when multiple targets are amplified in the same sample. A qPCR probe assay may include a probe that hybridizes to one specific sequence; however, it may be interesting to assess other aspects of the DNA in the sample, e.g. if it has been degraded or if a significant portion of it belongs to a male. In this case, assays containing multiple probes, all with different target sequences and attached to different fluorochromes, can be valuable. For example, degradation can be measured by analyzing two target sequences that differ in length, and the amount of male DNA can be determined by amplifying a specific sequence found only on the Y chromosome. It may also be important to determine whether the PCR is inhibited in any way, which can be done by adding a synthetic oligonucleotide and a probe that targets the short sequence. This allows for so-called internal positive control (IPC) (Swango et al., 2006; Hudlow et al., 2008). There should be no complementary sequences between the primer pairs in multiplex PCR to prevent primer-dimer formation. The elongation step may be performed for a longer period than in regular PCR to account for the extension of several regions (Butler, 2005).

2.5 DNA Typing

At the heart of forensic biology lies DNA typing, or DNA profiling. Since its introduction in the 1980s, typing has become a groundbreaking tool for law enforcement in the chase for perpetrators of different offences (Butler, 2005). This final section describes the technology used to obtain a DNA profile that can match even a small number of cells with an individual.

As discussed earlier, STRs are markers of interest in DNA profiling; as the repeats are located at separate loci, the PCR-technique must be able to amplify multiple sequences in a single run. As the fragments will go on to be separated based on size using capillary electrophoresis (CE), the PCR products must be of different sizes (Brown, 2010). During amplification of repeated sequences such as STRs, the DNA polymerase may undergo strand slippage, resulting in either a reduced or increased number of repeats in the newly formed amplicon. Such amplicons where the number of repeats differ from the “true” alleles are called stutter products and are seen in

the electropherograms as minor peaks adjacent to the true allele peaks (Butler, 2005). In mixed profiles, it can be difficult to distinguish stutter products from alleles of a minority profile.

2.5.1 Capillary Electrophoresis

Once the STR markers have been amplified and fluorescently labeled, the fragments in the mixture must be separated to identify the allele lengths. Capillary electrophoresis (CE) is a typical method utilized for this purpose. The principle of CE is based on electrophoresis, a technique in which a charge is applied, causing the molecules to migrate. As the DNA backbone has a negative charge, and the nucleic acids will be negatively charged in buffer systems, these molecules will move from a cathode towards an anode that together establish an electric field. The DNA must pass through a medium, a gel or a polymer, which causes size-based separation (Butler, 2005).

CE employs narrow capillaries that are filled with a fresh batch of polymer solution ahead of each run. The polymer helps separate the DNA based on size; flexible and linear chains of the polymer retain larger molecules while smaller ones can more easily pass through. A high voltage (around 15 000 V) can be applied in CE which causes fast separation. An electrophoresis buffer is used to provide a milieu in the capillary that a current can be applied to (Butler, 2005).

Before injection, the amplified and labeled samples are mixed with formamide and an internal lane standard (ILS), and briefly heated at approximately 95 °C. The formamide functions as a denaturant, breaking the hydrogen bonds in the hybridized DNA strands, while the ILS is added to provide accurate assessment of fragment size. The heating step is necessary to fully denature the dsDNA into ssDNA. An allelic ladder is also typically injected into the CE instrument to provide a size reference during DNA typing; the allelic ladder contains regularly occurring alleles for an STR marker among humans that have been artificially produced for the purpose of providing a correct sizing standard (Butler, 2005).

The data produced from CE is presented as peaks in an electropherogram, with the fragment size on the x-axis and the fluorescent signal intensity on the y-axis. Heterozygous alleles, i.e. alleles at the same locus of different lengths, are shown as two peaks while homozygous alleles, alleles of the same length, are shown as one peak (Butler, 2005). An example of an electropherogram with detected allele peaks is shown in Figure 6.

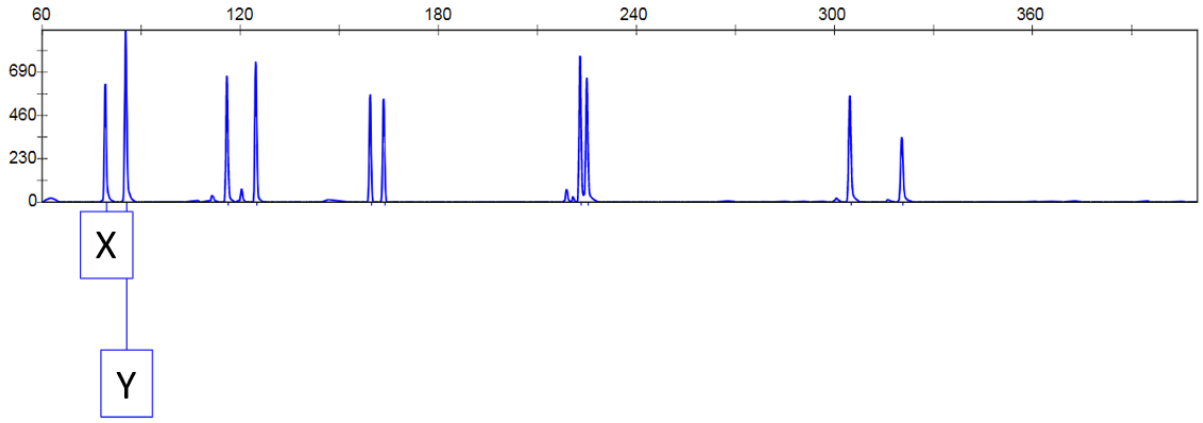

Figure 6. Example of electropherogram produced from CE analysis. Four STR markers are shown, with two close peaks representing the alleles for one marker. The marker for the X and Y chromosome is also shown in the figure.

3. Materials and Methods

3.1 Experimental overview

Chelex differential lysis followed by Amicon purification was used as the reference method when evaluating the alternative differential extraction and purification protocols. Figure 7 illustrates the broad organization of the protocols assessed.

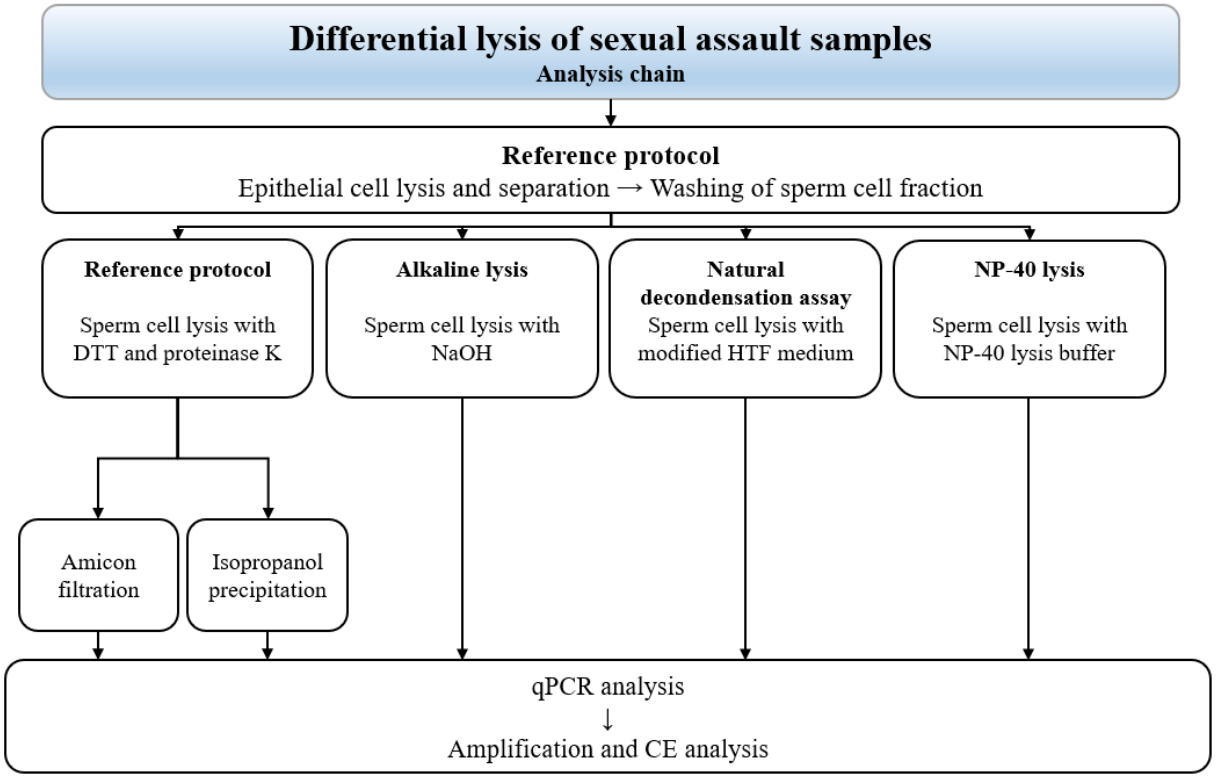

Figure 7. Structure of the differential extraction analysis chain with the examined alternative protocols. Three alternative direct-to-amplification sperm cell lysis methods were assessed, along with one method for sample purification.

The study of the alternative protocols can be generally divided into four phases:

1. **Initial screening of alternative protocols**
   In the screening phase, the performance of selected protocols on a 10 µL 1:10 sperm dilution was evaluated. The cell lysis protocols at this stage were performed on liquid sperm cell samples that had not been separated from potentially present epithelial cells (non-isolated sperm samples). The protocols that showed an equivalent or greater DNA concentration compared to the reference method were selected for further initial optimization. No DNA typing was performed during this stage.

2. **Optimization of selected protocols**
   The optimized protocols from the initial phase were applied on isolated sperm cell fractions that were obtained by performing the initial part of the reference method protocol, including the removal of epithelial cells, and washing of sperm fraction. In this phase, 10 µL 1:300

sperm dilutions were processed to assess the protocol performance on low DNA quantities (yielding approximately 0.05 ng/µL). The protocols that showed an equivalent or higher DNA concentration compared to the reference method were selected for DNA typing in the next phase.

3. **Evaluation of compatibility with forensic DNA profiling**
   In the third phase, optimized protocols were evaluated regarding their compatibility with CE analysis. Total peak height was taken into consideration, as well as the number of correctly detected alleles. The protocols were further optimized starting with 10 µL 1:100 or 1:300 sperm dilutions to assess the protocol performance on low DNA quantities.

4. **Execution of optimized protocol on mock sexual assault samples**
   Evaluation of the best performing protocols from stage three on cotton swab cuttings. Swab cuttings, with excess epithelial cells from a female donor and small volume sperm cells, were prepared. The aim of this phase was to assess the protocol performance on simulated real-life samples.

3.2 Sample collection and preparation

Raw semen samples and female saliva samples were collected upon informed consent prior to the project start. Semen samples were diluted to various dilutions throughout the study (1:10, 1:100, 1:300) with nuclease-free Ambion™ water.

The cotton swab cutting samples were prepared by cutting cotton swabs in equal sizes into separate microfuge tubes. 150 µL saliva was added to the swab cuttings, followed by 2 µL or 0.2 µL of the 1:100 liquid sperm dilution. The swab cuttings were then airdried overnight.

3.3 DNA extraction

3.3.1 Differential lysis with Chelex extraction

Differential lysis using Chelex 100 resin followed by Amicon filtration was used as the reference method in this project (744-06, NFC). A mixture of 300 µL 0.5% digest buffer (prepared with 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, Milli-Q water with 0.5% SDS) and 60 µL proteinase K was added to a microfuge tube containing 10 µL liquid semen or swab cuttings. The tubes were vortexed and incubated at 56 °C for 30 minutes. If the sample contained swab cuttings, the cuttings were swirled around with a wooden stick for two minutes to dislodge any cells from the material. The swab cuttings were then removed. Next, the samples were briefly vortexed and centrifuged for five minutes at 11 000 x g (RCF). Approximately 300 µL of the supernatant containing the epithelial cell fraction was then transferred to a new tube, with about 50 µL of the pellet containing the sperm fraction remaining. 100 µL 20% Chelex was added to the epithelial cell fraction. The Chelex was placed on a magnetic stirrer during transfer to the epithelial cell fraction. 0.5 mL 2% digest buffer (prepared with 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, Milli-Q water with 2% SDS) was added to the sperm fraction, which was then vortexed and centrifuged for five minutes at 11 000 x g (RCF). Following centrifugation, the supernatant

was removed and discarded, with 30–50 µL of the pellet being saved. This washing step was repeated two additional times. Next, 1 mL nuclease-free water was added to the sperm fraction, which again was vortexed and centrifuged at 11 000 x g (RCF) for five minutes. The supernatant was removed and discarded, saving approximately 30–50 µL pellet. This step was followed by the addition of 170 µL 5% Chelex to the sperm fraction, and 16 µL equal parts proteinase K and DTT. The sperm fraction was vortexed, and, along with the epithelial cell fraction, incubated at 56 °C for 45 minutes. All samples were vortexed for 10 seconds following incubation, and then incubated at 100 °C for 30 minutes. After the final incubation step, all samples were once again vortexed for 5–10 seconds, and finally centrifuged at 11 00 x g (RCF) for maximum 1 minute. Purification was carried out with Amicon Ultra 2 mL filters according to section 3.4.1.

3.3.1.1 Reduced washing steps

The Chelex extraction method was performed with reduced washing steps. Experiments were conducted with one and two washing steps with 1 mL 2% digest buffer.

3.3.2 Alkaline lysis

Alkaline lysis was executed according to the protocol described by Schellhammer et al. (2022) with some modifications. 1 M NaOH and 1 M Tris-HCl were prepared by separately dissolving NaOH and Tris-HCl in deionized water. NaOH was added to the sample, vortexed, and incubated. The reaction was then neutralized by the addition of Tris-HCl to the microfuge tube. Finally, samples were vortexed and centrifuged at 11 000 x g (RCF) for 1 minute.

To obtain optimal conditions for DNA recovery, 1 M NaOH and 1 M Tris-HCl were diluted with TE buffer to 0.2, 0.3, 0.4, 0.6 M and 0.2, 0.3, 0.4, 0.6, 0.8, 1 M, respectively. Experiments with equimolar NaOH and Tris-HCl concentrations were performed, along with additional experiments where 50% higher Tris-HCl concentration was added (i.e. 0.3 M Tris-HCl to 0.2 M NaOH, etc.). Different incubation temperatures (75, 80 and 85 °C) and times (2, 5, 10 min) were also studied. Finally, the effect of various NaOH and Tris-HCl volumes (25, 50, 60, 75 µL) was examined. All samples were diluted with TE buffer to reach a final volume of 200 µL.

3.3.3 NP-40 lysis

NP-40 lysis was executed according to the protocol described by Schellhammer et al. (2022). 10% NP-40 was diluted to 1%, 0.75% and 0.5% with nuclease-free water. 10 µL liquid sperm was added to three replicates containing 190 µL NP-40 buffer. The samples were then incubated on ice for 30 minutes and vortexed every 10 minutes. Samples were then centrifuged at 11 00 x g (RCF) for 1 minute.

3.3.4 Natural decondensation assay

Lysis with the natural decondensation assay was executed according to the protocol described by Schellhammer et al. (2022). A modified HTF medium containing 10 mM glutathione and 26

µM heparin was prepared. 10 µL of liquid sperm samples were added to the medium to obtain a final volume of 200 µL, vortexed, and incubated at 37 °C for 15, 30 and 60 minutes. Following incubation, samples were vortexed and centrifuged at 11 000 x g (RCF) for maximum 1 minute.

3.4 Purification protocols

3.4.1 Amicon filtration

Purification using Amicon Ultra 2 mL filters was performed as outlined in protocol 744-07 (NFC). The Amicon filters were assembled, and the filter section marked according to the sample. The sample to be purified and TE buffer (up to 2 mL) was added to the filter section. The filter units were centrifuged in a swing bucket centrifuge at 3225 x g (RCF) for 15 minutes. The filtrate was then discarded, circa 2 mL TE buffer was added to the filter section, and the filter units were centrifuged once again at 3225 x g (RCF) for 15 minutes. Next, the filtrate and filtrate section were discarded, and the units were turned upside down with the retentate section at the bottom. The units were centrifuged at 1000 x g (RCF) for two minutes. The volume of DNA extract in the retentate section was measured with a pipette and documented, and then transferred to a new microfuge tube. The extract was diluted with TE buffer to 200 µL.

3.4.2 DNA precipitation with isopropanol

Purification with isopropanol was performed in place of the purification step with Amicon Ultra 2 mL filters in the reference protocol. 1/10 volume 3 M sodium acetate (NaOAc) was added to the DNA sample (e.g. if the total DNA sample volume was 100 µL, 10 µL NaOAc was added). 1 µL glycogen per 20 µL of the total DNA sample and NaOAc volume was then added. In later stages, 1 µL linear acrylamide was used as a carrier as it has been shown to improve the DNA yield in other in-house studies. The solution was mixed by pipetting up and down gently. Next, an equal volume of room temperature isopropanol was added, and the solution was once again mixed gently. The mixture was centrifuged at 14 000 x g (RCF) for 30 minutes. The supernatant was then discarded, and 1 mL room temperature 70% ethanol was added to the microfuge tube. The mixture was centrifuged at 14 000 x g (RCF) for 15 minutes with the same tube orientation as the previous centrifugation run. The supernatant was discarded, and the pellet was airdried for 5–20 minutes. Lastly, the pellet was dissolved in 200 µL TE buffer.

3.4.3 DNA precipitation with ethanol

1/10 volume 3 M NaOAc was added to the DNA sample followed by 1 µL linear acrylamide. The solution was mixed by pipetting up and down. Next, cold ethanol (99%) was added in a 2.5-fold excess, and the solution was mixed gently. The mixture was incubated at -20 °C for 60 minutes, then centrifuged at 14 000 x g (RCF) for 30 minutes. Following centrifugation, the supernatant was discarded and 1 mL cold 70% ethanol was added. The mixture was then centrifuged at 14 000 x g (RCF) for 15 minutes with the same tube orientation as the previous centrifugation run. The supernatant was discarded, and the pellet was airdried for 5-20 minutes. Lastly, the pellet was dissolved in 200 µL TE buffer.

3.5 DNA quantification

3.5.1 LightCycler Nano Real-Time PCR

The LightCycler® Nano Real-Time PCR instrument was used for qPCR analysis during the initial screening phase. A master mix containing IMMOLASE™ DNA polymerase and an assay targeting the RB1-gene was prepared according to Table 1.

Table 1. qPCR master mix components, containing IMMOLASE DNA polymerase.

Reagent	Stock Concentration	Per Reaction	Volume per Reaction (µL)
Ambion Water			5.5
10x ImmoBuffer	10x	1x	2
dNTP	2 mM	0.2 mM	2
$MgCl_2$	25 mM	4 mM	3.2
Primer RB1_80F	10 µM	0.3 µM	0.6
Primer RB1_235R	10 µM	0.3 µM	0.6
Probe RB1 FAM	10 µM	0.2 µM	0.4
BSA	20 mg/mL	10 µg	0.5
IMMOLASE DNA Polymerase	5 U/µL	0.05 U/µL	0.2

The master mix was vortexed and 15 µL was added to the relevant wells of ThermoFisher™ Low Profile Tubes and Ultra Clear Caps 8-well strips, followed by the addition of 5 µL of the DNA sample to the same well. One replicate per sample was analyzed, along with a standard curve with known concentrations of DNA (2800M) in the range 0.001–10 ng/µL. Two replicates per standard were analyzed. 5 µL Ambion water was added to one well for negative control. Table 2 outlines the method settings used for qPCR analysis.

Table 2. Method settings for LightCycler Nano qPCR analysis.

Temperature (°C)	Time (seconds)	Cycles (n)
95	600	1
95	10	45
60	20	
72	30	

3.5.2 QuantStudio 5 Real-Time PCR

The QuantStudio™ 5 Real-time PCR instrument was also used for qPCR analysis during the later phases of this project, using the PowerQuant® five dye system assay. The PowerQuant 2X Master Mix and PowerQuant 20X Primer/Probe/IPC Mix were taken from refrigerated storage and vortexed thoroughly. A reaction mixture was prepared according to Table 3, and vortexed for 10 seconds.

Table 3. PowerQuant reaction mix components.

Reagent	Volume per Reaction (µL)
Water, Amplification Grade	7
PowerQuant 2X Master Mix	10
PowerQuant 20X Primer/Probe/IPC Mix	1

18 µL of the reaction mixture was then added to the relevant wells of an MicroAmp™ Optical 96-Well Plate or a MicroAmp Optical 8-Cap Strip, followed by the addition of 2 µL of the DNA sample to the same wells, and 2 µL amplification grade water to one well for negative control. One replicate per sample was analyzed, along with a standard curve with known concentrations of DNA (2800M) in the range 0.001–10 ng/µL. Two replicates per standard were analyzed. Table 4 outlines the method settings used for qPCR analysis.

Table 4. Method settings for QuantStudio qPCR analysis.

Temperature (°C)	Time (seconds)	Cycles (n)
98	120	1
98	15	39
62	35	

3.6 DNA profiling

DNA typing involved amplification and analysis of the samples in the ABI GeneMapper™ ID-X genotyping software.

Samples containing less than 0.05 ng/µL DNA (as determined by DNA quantification) were deemed acceptable for subsequent amplification and STR analysis. Dilution of the samples was undesired as it was important to assess the compatibility of undiluted samples with CE analysis. However, samples containing a DNA concentration substantially higher than 0.05 ng/µL were diluted further.

3.6.1 Amplification

Amplification was performed in a VeritiPro™ Thermal Cycler using the PowerPlex® ESX 16 Fast kit. A reaction mix was prepared according to Table 5, and vortexed thoroughly.

Table 5. PowerPlex ESX 16 Fast kit reaction mix components.

Reagent	Volume per Reaction (µL)
PowerPlex ESI/ESX Fast 5X Master Mix	5
PowerPlex ESX 16 Fast 10X Primer Pair Mix	2.5

7.5 µL of the reaction mixture was added to the appropriate wells of either MicroAmp 8-Tube Strips or a MicroAmp Optical 96-well plate, followed by 17.5 µL of the DNA sample. 17.5 µL amplification grade water was added to one well for negative control, and

7.5 µL 2800M DNA diluted to 0.05 ng/µL was added to one well for positive control. The thermal cycler was set to run the cycling program outlined in Table 6.

Table 6. PowerPlex ESX 16 Fast thermal cycling program for amplification.

Temperature (°C)	Time (seconds)	Cycles (n)
96	60	1
96	5	30
60	35	
72	5	
60	120	1
4	∞	∞

3.6.2 Capillary electrophoresis

An Applied Biosystems™ (ABI) 3500 Series Genetic Analyzer was used to carry out CE. Prior to analysis, the plate layout was prepared and imported into the 3500 Data Collection software. The instrument oven was pre-heated at 60 °C for at least 30 minutes before the CE analysis. A solution containing a 24:1 proportion of thawed formamide and the internal standard WEN ILS 500 ESS was prepared. The solutions were vortexed thoroughly before and after mixing, and 10 µL of the mix was added to the appropriate wells of a new ABI microtiter plate. 1 µL of the samples from the PowerPlex ESX 16 amplification was added to the plate. 1 µL of the PowerPlex 16 Allelic Ladder Mix was added to at least two wells, and 1 µL amplification grade water to one well for negative control.

The plate was sealed with a plastic film and placed in an ABI GeneAmp 9700 thermal cycler (pre-heated to 94 °C) for three minutes. Following heating, the plate was placed in an ice block for a minimum of two minutes. The film was then removed, and the plate was loaded in the Genetic Analyzer, with a septa mat placed on top, for CE analysis. Following analysis, the results were analyzed using the ABI GeneMapper™ ID-X software. Evaluation of the protocols included the analysis of the internal lane standard (ILS), total peak heights and number of correctly detected alleles compared to the reference method.

3.7 Data analysis

To perform statistical analysis of the results and ensure reproducibility, experiments were conducted with three biological replicates (n = 3) unless specified otherwise. Data analysis was carried out in Microsoft Excel. For comparison of the means of two groups, a t-test was performed. When comparing more than two groups, one-way ANOVA was performed. In all cases, the significance level α was set to 0.05. If the one-way ANOVA indicated a significant difference between two or more groups, a post hoc test was performed using Bonferroni correction with the new significance level was set to α/m, where m is the number of comparisons.

For qPCR analysis with the PowerQuant assay, degradation was analyzed by calculating the degradation index (DI), i.e. the ratio of the concentration of short target DNA (autosomal) and

the concentration of long target DNA (degradation). A DI much higher than 1 indicates DNA degradation.

For analysis of the CE results, total peak heights were calculated as the sum of the peak heights of all correctly detected alleles (32 in total), including the alleles detected on the X and Y chromosomes. For samples with mixed profiles (female saliva donor and male sperm donor), overlapping alleles were excluded when calculating total peak heights. A ratio between the average total peak height for the detected STR peaks belonging to the female donor and male donor was calculated according to Equation 1, where a ratio of ≤ 1 indicates a greater amount of DNA belonging to the sperm donor.

$$average\ total\ peak\ height_{female\ donor} \Big/ average\ total\ peak\ height_{male\ donor} \quad (1)$$

4. Results and Discussion

Differential extraction with SDS, proteinase K and DTT, and subsequent Amicon purification was initially performed on a raw semen sample as well as 1:10 and 1:100 dilutions to obtain a reference quantity. Table 7 lists the DNA concentrations provided by qPCR, as well as the concentration of the epithelial cell fraction separated from the raw semen sample. However, it is important to note that this reference quantity is not used for exact comparison throughout the study, as some inherent variability will always be present. This can be due to factors such as the limited number of replicates and samples being repeatedly frozen and thawed. Additionally, during the screening and optimization phases, fresh sperm dilutions would be prepared from the same stock dilution, which could introduce variability across the results. Nevertheless, the same sperm dilutions were used when performing analysis of several protocols in one run.

Table 7. DNA concentrations obtained from qPCR analysis of reference protocol performed on 10 µL raw semen sample, 1:10 and 1:100 sperm dilutions, as well as the epithelial cell fraction.

Sperm sample	**Average DNA concentration (ng/µL ± SD)**
Raw semen sample (sperm cell fraction)	2.61 ± 0.23
Raw semen sample (epithelial cell fraction)	0.22 ± 0.12
1:10 dilution (sperm cell fraction)	0.20 ± 0.02
1:100 dilution (sperm cell fraction)	0.03 ± 0.004

4.1 Initial screening of alternative protocols

During the initial screening phase, an examination of the alkaline lysis protocol on a non-isolated sperm sample with varied NaOH/Tris-HCl concentrations of equal molarity (0.2, 0.4 and 0.6 M) at 75 °C for 5 minutes was carried out to determine the effectiveness of the method. The results show a similar DNA yield between 0.2 and 0.4 M NaOH/Tris-HCl, but with a higher variance in the attempt using 0.2 M (Figure 8). The findings also showed that 0.6 M NaOH/Tris-HCl produced a negligeable DNA yield, and therefore this concentration was excluded from further optimization procedures. The results are consistent with various literature on the use of alkaline lysis, both within and outside of forensic settings, where the NaOH concentration used is typically 0.2 M (Rudbeck and Dissing, 1998; Klintschar and Neuhuber, 2000; Schellhammer, et al., 2022). While a high NaOH molarity may lead to more effective lysis of sperm cells, too high concentrations in combination with high temperatures likely lead to DNA damage through depurination, a process in which a purine is lost. This is commonly caused by acidic conditions, but high alkaline conditions may also lead to depurination (AAT Bioquest, 2021). Thus, it was crucial to find the correct balance of effective lysis conditions that did not damage the DNA.

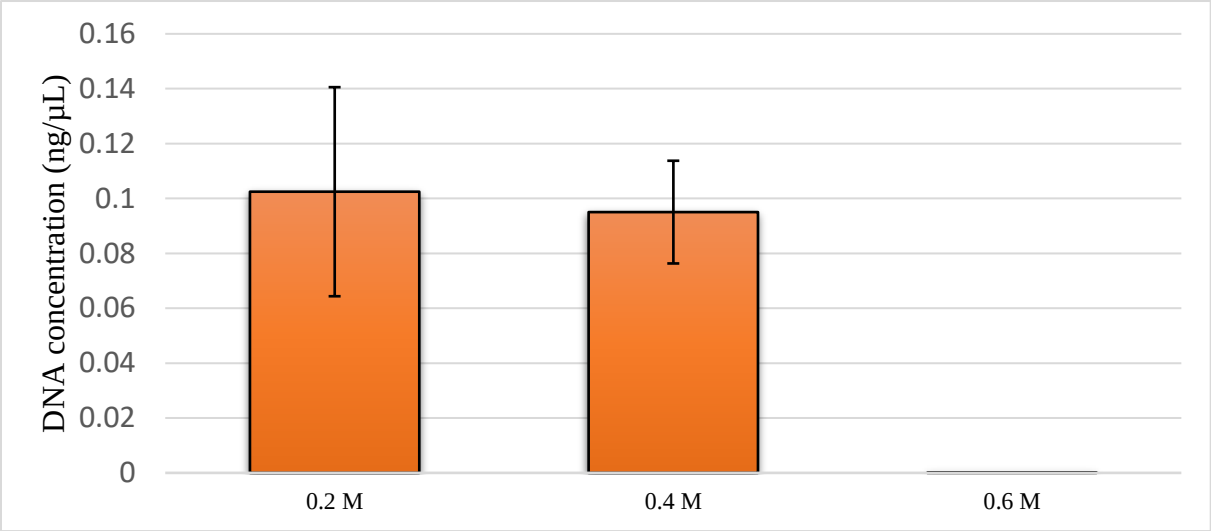

Figure 8. DNA concentrations obtained from qPCR analysis with RB1 assay of alkaline lysis performed on 10 µL 1:10 sperm dilution with varying NaOH/Tris-HCl concentrations. The use of 0.2 and 0.4 M NaOH and Tris-HCl produced favorable DNA recovery in comparison with 0.6 M NaOH and Tris-HCl.

Because less variance in DNA yield was observed in the attempt using 0.4 M NaOH/Tris-HCl, further experiments were performed applying 0.4 M. The next step was to evaluate optimal parameters (incubation time and incubation temperature) for better DNA recovery. Temperatures 75, 80 and 85 °C were assessed in three separate experiments, as well as 2-, 5- and 10-minute incubation times. The results showed an optimal recovery at 75 °C (Figure 9). Similar DNA yield was obtained between 2- and 5-minute incubations, whereas 10-minute incubation resulted in a slight decrease in DNA recovery. The findings are expected as DNA stability decreases as the temperature increases, and so prolonged exposure to high temperatures expectedly will lead to increased DNA damage (Purschke et al., 2010). Moving forward into the optimization phase, incubation at 75 °C for 5 minutes was used for the alkaline lysis protocol. It is worth noting that while all three experiments presented in Figure 9 were conducted on the same sperm sample and sperm dilutions, it is possible for variation to be introduced due to them being performed separately. Nevertheless, the results provided insight into the effect of the incubation time and temperature.

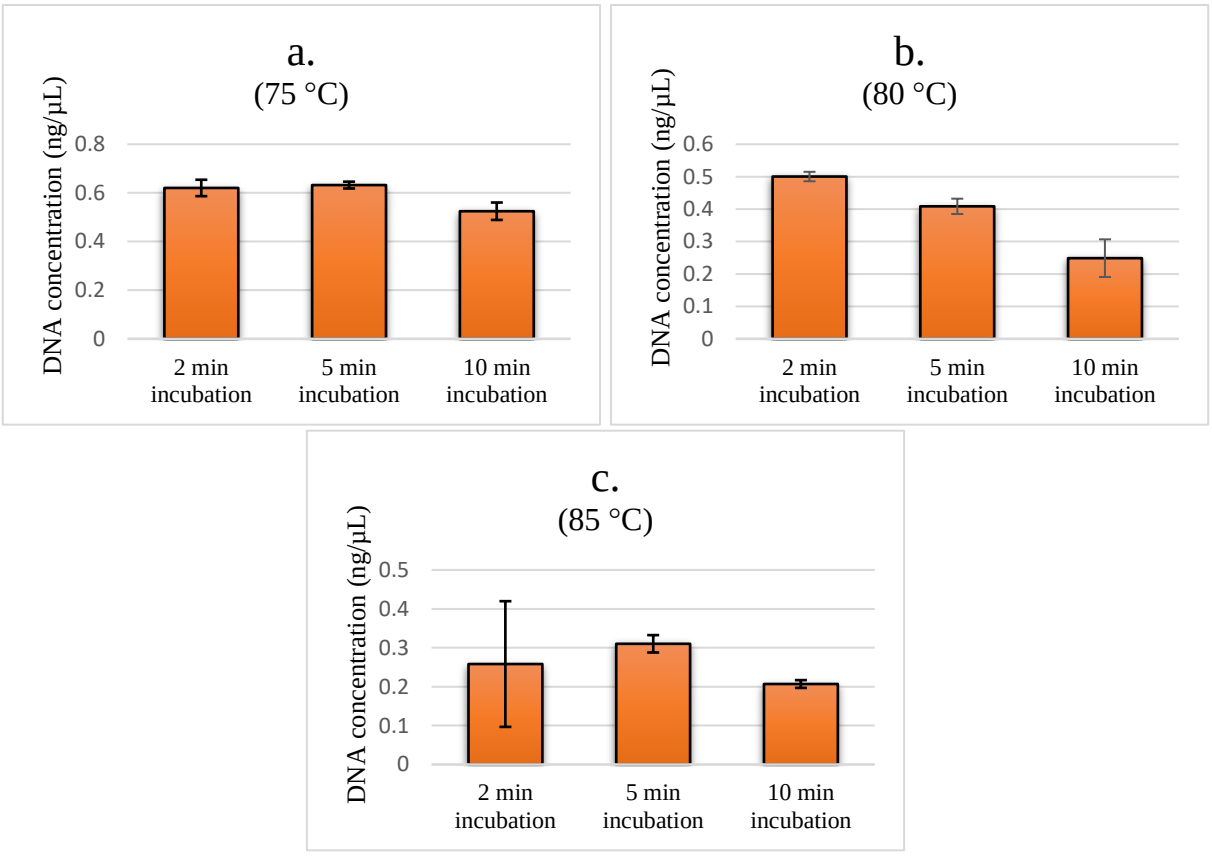

Figure 9. DNA concentrations obtained from qPCR analysis with RB1 assay on samples processed by alkaline lysis. The lysis process was performed on 10 µL 1:10 sperm dilution with varying incubation times (2, 5 and 10 minutes) and temperature: (a) 75 °C, (b) 80 °C and (c) 85 °C. Optimal DNA recovery was seen with 2- and 5-minute incubation at 75 °C.

Next, an initial screening of the NP-40 lysis protocol with different concentrations of NP-40 (0.5%, 0.75% and 1%) was conducted. This method produced DNA yields approximately 1/100 of the yield obtained from the reference method across all three NP-40 concentrations. Thus, no further optimization of the protocol was conducted. The low DNA recovery may be due to the nature of the lysis buffer; as mentioned, mild detergents can disrupt cell membranes without denaturing proteins, which may lead to insufficient cleavage of the disulfide bonds present in sperm cells. NP-40 may also cause PCR inhibition at high concentrations (Schrader et al., 2012). However, this lysis buffer was found to be efficient for sperm cell lysis when studied by Schellhammer et al. (2022) and has previously been observed as effective in direct amplification of crude blood samples (Zhang, Kermekchiev and Barnes, 2010). It is possible that difference in sample preparation, where Schellhammer et al. used 1/12th of a cotton swab dipped in 1:10 sperm dilution as samples, could explain this discrepancy. Thus, though no further optimization was conducted in this project, the method may not be completely ruled out as an alternative.

Continuing with natural decondensation assay lysis, all incubation times (15, 30 and 60 min) produced high DNA concentrations (1.15 ± 0.90, 1.04 ± 0.05 and 1.06 ± 0.08 ng/µL, respectively) during the initial screening phase. These yields were high in comparison to the reference method presented earlier, but as the reference method was not executed concurrent with the natural decondensation assay, no statistical comparison could be made. Additionally, the results from qPCR with the PowerQuant five dye system showed delayed IPC C_q values

(21.52 ± 0.08, 21.28 ± 0.03 and 21.27 ± 0.18, respectively) compared with the standards used as reference (20.57 ± 0.02, n = 10), suggesting mild inhibition. As heparin has previously been seen to be inhibitory in the amplification process due to it binding to DNA, this result was not unexpected (Yokota et al., 1999). Consequently, as with the NP-40 lysis protocol, no further optimization was performed.

Lastly, precipitation with isopropanol was evaluated. Initially performed on samples containing 10 µL 1:10 sperm dilution in concurrence with the reference protocol, isopropanol purification was performed on both sperm cell fractions and epithelial cell fractions. The results suggested an equal DNA recovery following isopropanol purification in comparison with the reference protocol in both the sperm cell fraction and epithelial cell fraction (0.230 ± 0.05 and 0.187 ± 0.02 ng/µL, respectively, p = 0.334 for the sperm cell fraction, 0.020 ± 0.002 and 0.016 ± 0.001 ng/µL, p = 0.060 for the epithelial cell fraction, Figure 10).

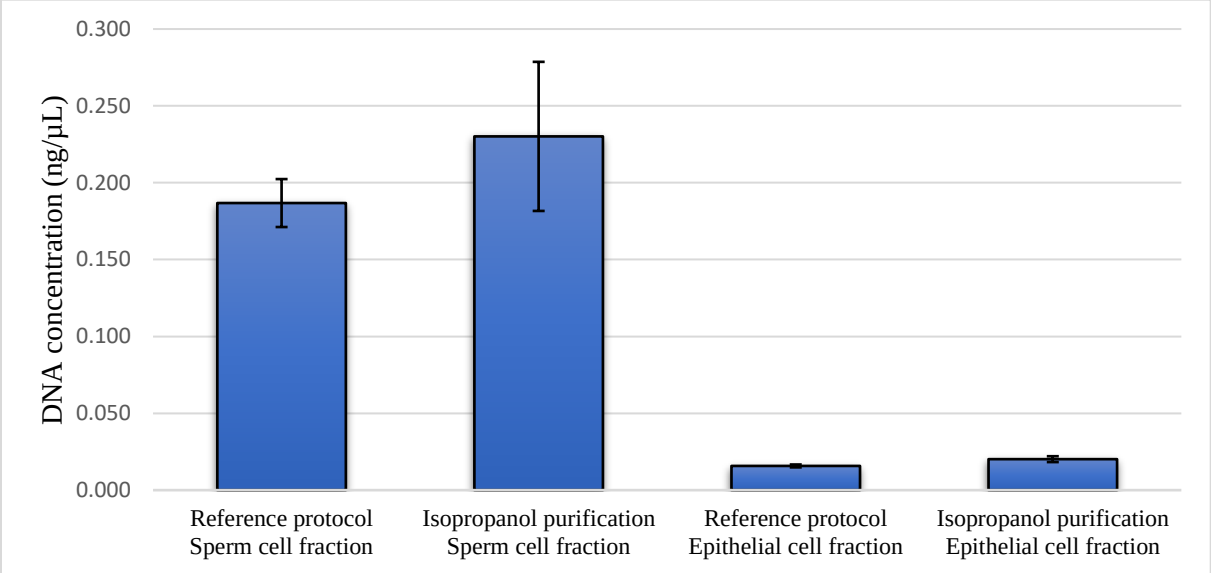

Figure 10. DNA concentrations obtained from qPCR analysis with PowerQuant assay of Amicon filtration and isopropanol precipitation performed on isolated sperm cell fraction, showing comparable DNA recovery of both sperm cells and epithelial cells between the methods.

4.1.1 Discussion of initial screening results

Figure 11 illustrates a general overview of the results obtained in the screening phase, where the green markings signify that the protocols produced sufficient concentrations in comparison with the reference method. The protocols that produced inadequate results are marked in white. The natural decondensation assay is marked orange to indicate that the protocol was evaluated in later phases.

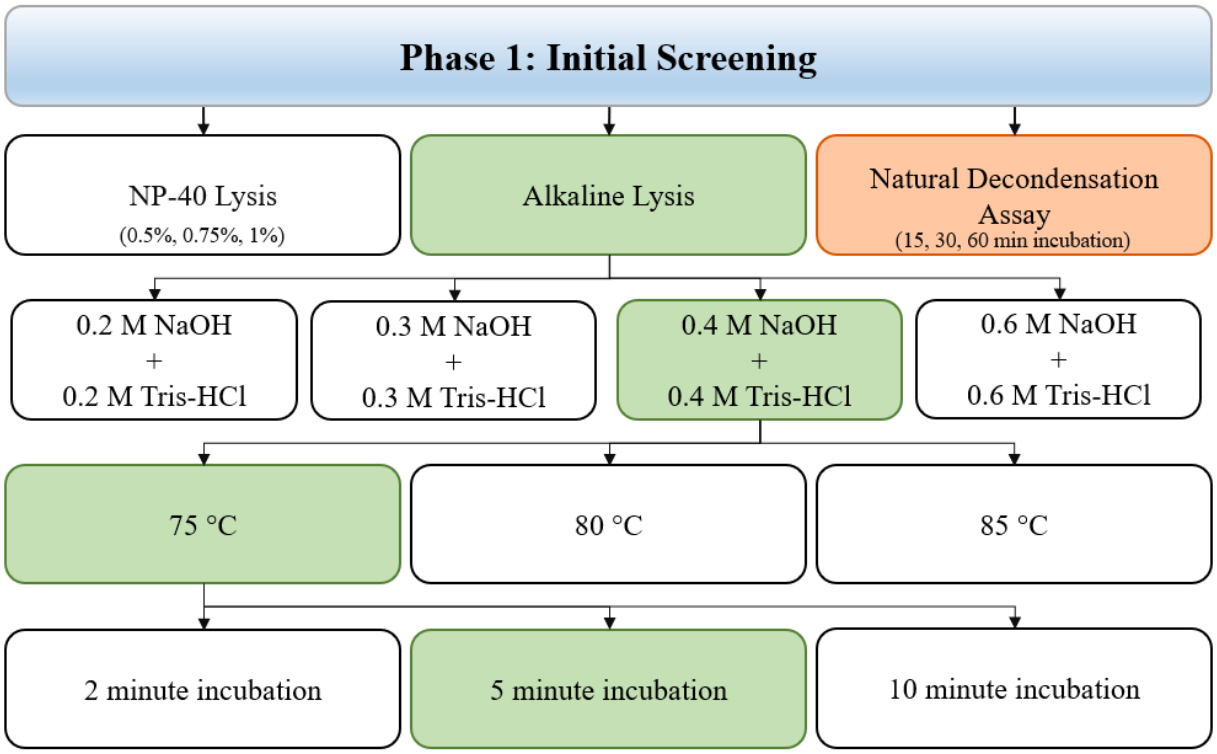

Figure 11. General overview of results obtained in the initial screening phase. Green markings signify the protocols and parameters that produced the highest concentrations. Protocols and parameters marked in white did not produce adequate results. The natural decondensation assay is marked orange, to indicate that the protocol was further assessed in later phases.

Considering the results from the screening of alternative protocols, alkaline lysis as a replacement of the cell lysis step in the reference protocol was considered the most promising method. The protocol had initially been of interest due to its simple execution, its bypassing of both tube transfers and purification step, as well as the positive findings reported in literature (Rudbeck and Dissing, 1998; Klintschar and Neuhuber, 2000; Schellhammer, et al., 2022). These features are shared with the NP-40 lysis protocol and natural decondensation assay protocol; however, besides the evident limitations of these methods in terms of recovery or inhibition, alkaline lysis has the advantage of using readily accessible reagents, as well as having a relatively shorter execution time. The duration is a notably compelling aspect of the alkaline lysis protocol when comparing it with the reference protocol, as differential lysis with proteinase K and DTT requires two longer incubation times, followed by the Amicon purification protocol which adds to the length of the process.

It is important to mention that the DNA concentrations obtained by the alkaline lysis protocol fluctuates somewhat between the different experiments during the initial screening stage. This may be attributed to early variations in the method execution, and more consistent results were achieved with increased familiarity with the protocol. However, because of the protocol's favorable characteristics mentioned above, it was relevant to conduct further assessment of the performance and optimization of the alkaline lysis method. Moving forward, the reference protocol was performed in concurrence with alkaline lysis and other methods to examine if these fluctuations persisted.

Additionally, though clear signs of inhibition were observed in the natural decondensation assay results, the high DNA recovery obtained by the method provided justification for moving forward with performing the protocol on lower sperm dilutions. This was done to confirm both if recovery remained consistently high and if inhibition remained an issue. No further optimization, such as possible dilution of the assay, was performed due to time limitations.

Finally, as the isopropanol purification protocol showed comparable results in the first screening phase in terms of DNA yield of both sperm cells and epithelial cells in comparison to the reference protocol, it was highly relevant to move forward with this method in the following phases. The protocol calls for two longer centrifugation steps (30 minutes and 15 minutes), hence there is no significant reduction in the overall duration of the purification step. However, as with the alkaline lysis protocol, isopropanol purification utilizes non-proprietary reagents such as NaOAc, isopropanol and ethanol, and so the implementation of this protocol could eliminate the need for costly purification filters.

4.2 Optimization of selected protocols

Based on the results from the screening, the modified alkaline lysis protocol, utilizing 0.4 M NaOH/Tris-HCl with 5-minute incubation at 75 °C, was performed and quantified using the RB1 assay concurrently with the reference method in the same qPCR run. Given the significantly higher average DNA yield obtained by the reference protocol compared to the alkaline lysis protocol (Figure 12a), it was relevant to investigate whether PCR inhibition was a factor for alkaline lysis. Re-quantification of the same samples was consequently performed in the QuantStudio instrument with the PowerQuant assay as this kit provides IPC values, that indicate PCR inhibition if elevated. The samples were also diluted 10 times to further assess potential inhibition. The results showed an increased DNA recovery of the undiluted samples in the alkaline lysis protocol compared to the reference protocol (0.216 ± 0.03 and 0.142 ± 0.05 ng/µL, respectively, $p = 0.16$, Figure 12b). Quantification of the samples diluted 10 times yielded an average concentration that was approximately $1/10^{th}$ of the undiluted samples (0.019 ± 0.002 ng/µL), indicating that the alkaline lysis protocol gave no PCR inhibition with the PowerQuant kit. Furthermore, no substantial delay in the IPC C_q value was detected (21.00 ± 0.070, compared with the standard, 20.33 ± 0.20). A potential explanation for the discrepancy in results from the analysis of the same samples using the RB1 assay is that the PowerQuant assay is less sensitive to inhibition, and more sample is added in the RB1 assay than in the PowerQuant assay (5 µL vs. 2 µL); consequently, the PowerQuant assay was used for further analyses to monitor potential PCR inhibition.

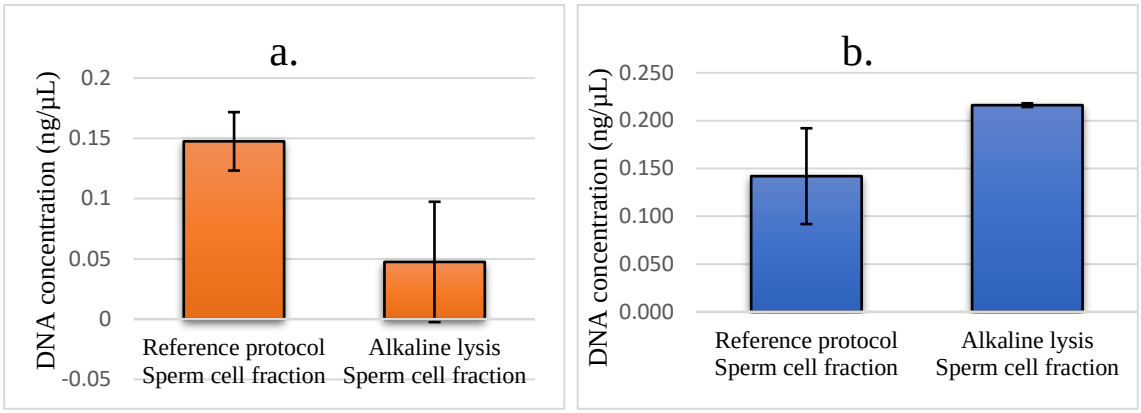

Figure 12. DNA concentrations obtained from qPCR analysis with (a) RB1 assay and (b) PowerQuant assay of reference protocol and alkaline lysis performed on isolated sperm cell fractions. Results indicate that the RB1 assay was more susceptible to inhibition by the alkaline lysis samples.

Alkaline lysis was performed on 10 µL 1:10 sperm dilutions with 0.2, 0.3 and 0.4 M NaOH/Tris-HCl, and quantified with the PowerQuant assay to further establish if previous analysis with the RB1 assay was affected by inhibition, as well as to assess the yield produced by 0.3 M NaOH/Tris-HCl. The results pointed to 0.4 M NaOH/Tris-HCl yielding the highest DNA recovery (Figure 13). There was no difference in the degradation index or average IPC C_q values between the four groups. Further, 10-time dilutions of the alkaline lysis samples yielded average concentrations approximately $1/10^{th}$ of the undiluted samples across all concentrations. It can therefore be concluded that there was no inhibition of these samples. One-way ANOVA indicated a statistical difference between at least two of the groups. Post-hoc analysis did not show a significant difference between alkaline lysis protocol with 0.4 M NaOH/Tris-HCl and the reference protocol ($p = 0.443 > 0.05/4 = 0.0125$), suggesting that the protocols are comparable.

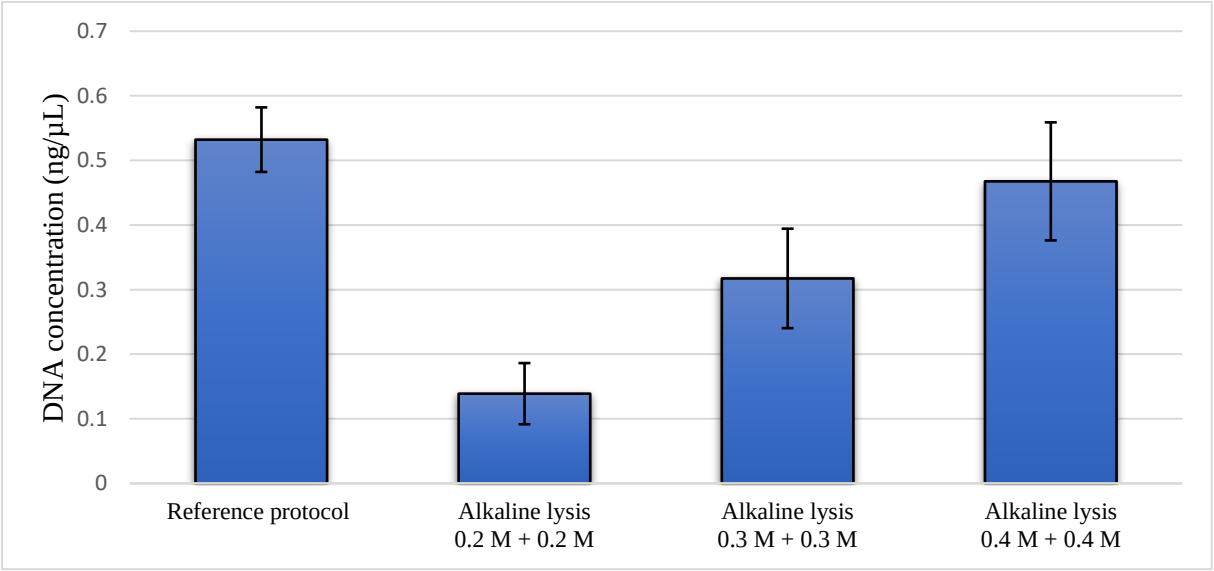

Figure 13. DNA concentrations obtained from qPCR analysis with PowerQuant assay of reference protocol and alkaline lysis (0.2, 0.3 and 0.4 M NaOH and Tris-HCl) performed on isolated sperm cell fractions. Optimal DNA recovery was found when performing alkaline lysis with 0.4 M NaOH and Tris-HCl.

To investigate the possible effect of pH on the DNA recovery and subsequent DNA stability, experiments were conducted with increased Tris-HCl concentrations in relation to the NaOH concentrations. A general measurement of the pH with a pH meter of equal volumes of 0.4 M NaOH + 0.4, 0.6, 0.8 and 1 M Tris-HCl, without any biological material present, showed a slight decrease in pH as the Tris-HCl concentration increased above 0.4 M (pH 9.67, 8.35, 8 and 7.88 for 0.4, 0.6, 0.8 and 1 M Tris-HCl, respectively). This was considered favorable as DNA is stable at pH 8 (AAT Bioquest, 2021). When performing alkaline lysis with these parameters, results from qPCR analysis showed that DNA recovery was similar across all parameters, except for when 1 M Tris-HCl was added where the DNA yield increased (Figure 13). However, DNA degradation was observed with the addition of 0.8 M and 1 M Tris-HCl (DI > 1 and > 2, respectively), as well as delayed IPC C_q values when 0.6, 0.8 and 1 M Tris-HCl was added (22.52 ± 0.14 for 0.6 M Tris-HCl compared to 20.57 ± 0.19 of standard reference. 0.8 and 1 M produced "undetermined" C_q-values, indicating significant delay).

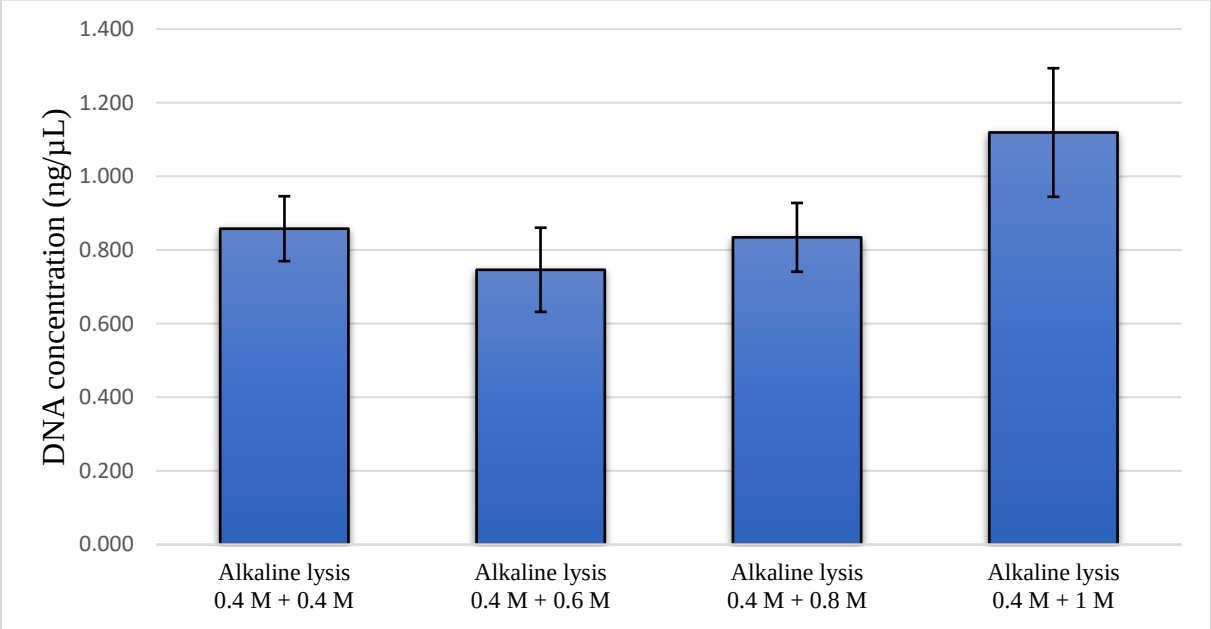

Figure 14. DNA concentrations obtained from qPCR analysis with PowerQuant assay of samples processed with alkaline lysis (0.4 M NaOH and varying Tris-HCl concentrations). Increased Tris-HCl concentrations showed high DNA recovery, but with high degradation and delayed IPC values.

Based on these results, the reference protocol, the alkaline lysis protocol with 0.4 M NaOH + 0.4 M Tris-HCl as well as 0.4 M NaOH + 0.6 M Tris-HCl was performed on a 1:300 sperm dilution to assess the effectiveness of the methods on DNA concentrations close to the lower limit for standard CE analysis (0.01 ng/µL). The findings suggest a higher overall yield obtained from the alkaline lysis protocol using 0.4 M NaOH + 0.6 M Tris-HCl, followed by alkaline lysis with 0.4 M NaOH + 0.4 M NaOH (Figure 15). The results from one-way ANOVA showed a difference in means between at least two methods, while post hoc analysis of the reference protocol and the alternative protocols suggested that the protocols are comparable ($p > 0.05/3 = 0.0167$). Due to an error during the final dilution step in the reference protocol, one replicate was removed. A delay in the IPC C_q values was detected for the experiment using 0.4 M NaOH + 0.6 M Tris-HCl (21.58 ± 0.61) compared to the standard reference (20.28 ± 0.19).

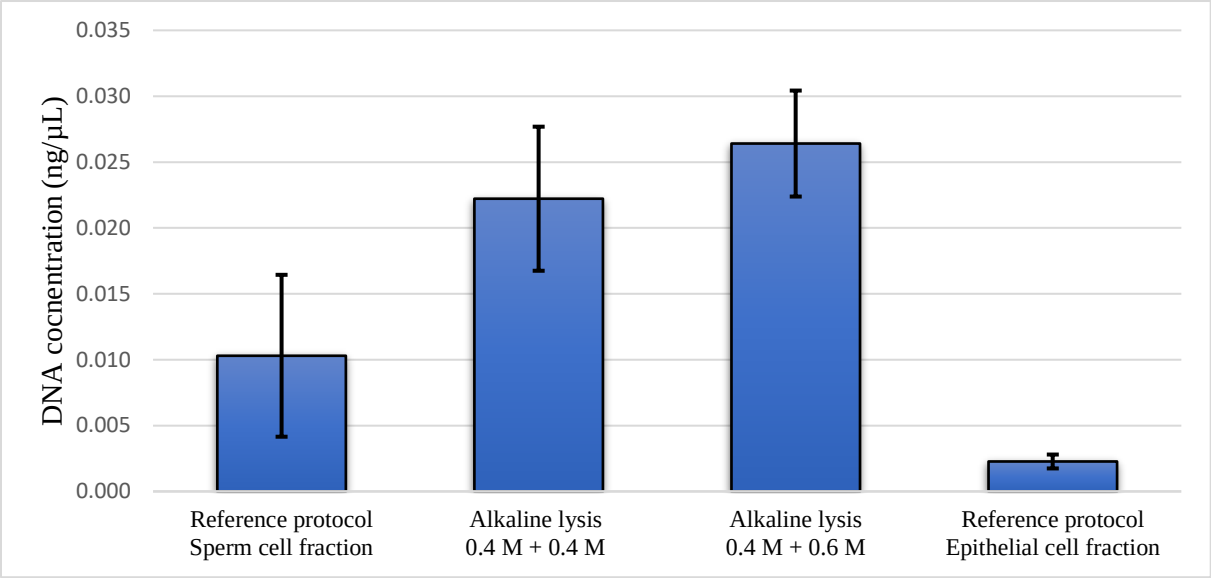

Figure 15. DNA concentrations obtained from qPCR analysis with PowerQuant assay of reference protocol, alkaline lysis (0.4 M NaOH + 0.4 M Tris-HCl and 0.4 M NaOH + 0.6 M Tris-HCl) performed on isolated sperm cell fractions (1:300 sperm dilution), as well as reference protocol performed on epithelial cell fraction. Comparable DNA recovery was observed between the reference protocol and alkaline lysis.

As the natural decondensation assay protocol had shown high DNA recovery during the initial screening, it was performed again during this later phase. This time, the natural decondensation assay was performed on a 1:300 sperm dilution (15-minute incubation), in concurrence with the reference protocol, to assess the effectiveness of the method close to the lower limit of standard CE analysis (0.01 ng/µL) and to confirm whether inhibition was a factor. The average DNA yield was similar for natural decondensation assay and reference protocol (0.012 ± 0.003 and 0.010 ± 0.006 ng/µL respectively, $p = 0.852$). In contrast to the screening phase, no substantial delay in the IPC C_q values was detected (20.96 ± 0.02) compared to the standard reference (20.39 ± 0.20, $n = 10$). Due to an error during the final dilution step in the reference protocol, one replicate was removed.

Finally, showing promising results in the screening phase, isopropanol purification was also performed on the sperm cell fraction from a 1:300 sperm dilution. Once again, this resulted in an equal DNA yield in comparison with the reference protocol (0.013 ± 0.002 and 0.010 ± 0.006 ng/µL, $p = 0.773$, Fig 16). Due to an error during the final dilution step, one replicate from the reference protocol was removed, leaving only two replicates, and thus reduced reliability.

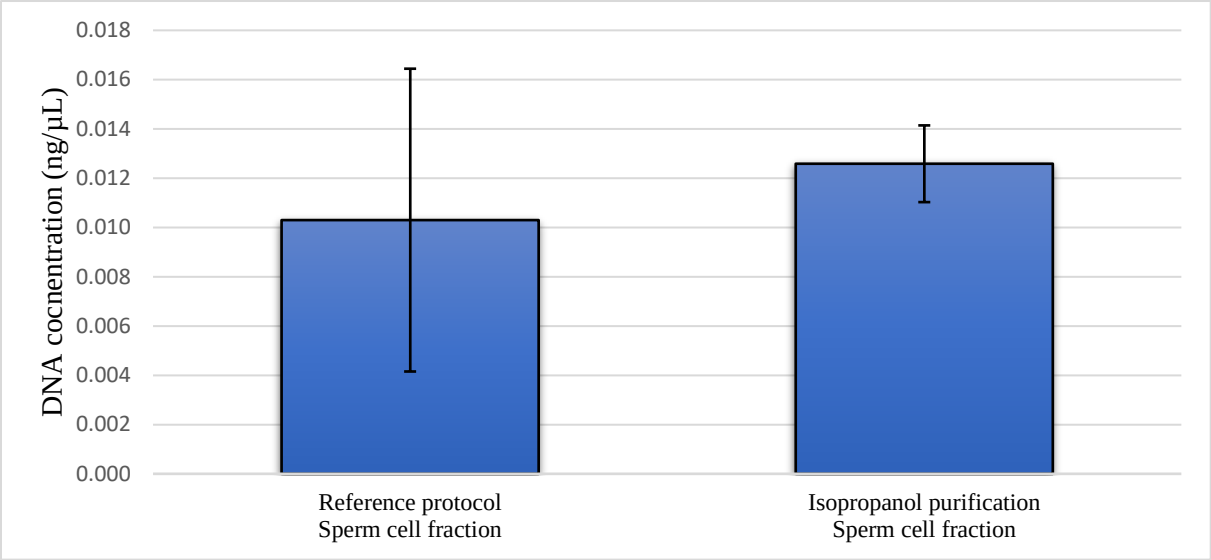

Figure 16. DNA concentrations obtained from qPCR analysis with PowerQuant assay of reference protocol (n = 2) and isopropanol purification (n = 3) performed on isolated sperm cell fractions (1:300 sperm dilution). Equal DNA recovery could be observed for both methods.

4.2.1 Discussion of optimization of selected protocols

Figure 17 illustrates a general overview of the results obtained in the optimization phase, where the green markings signify that the parameters produced adequate concentrations in comparison with the reference method. The parameters that showed inhibition in qPCR analysis are marked in white. Isopropanol precipitation is marked in orange to indicate that the protocol was evaluated in later phases, but not optimized.

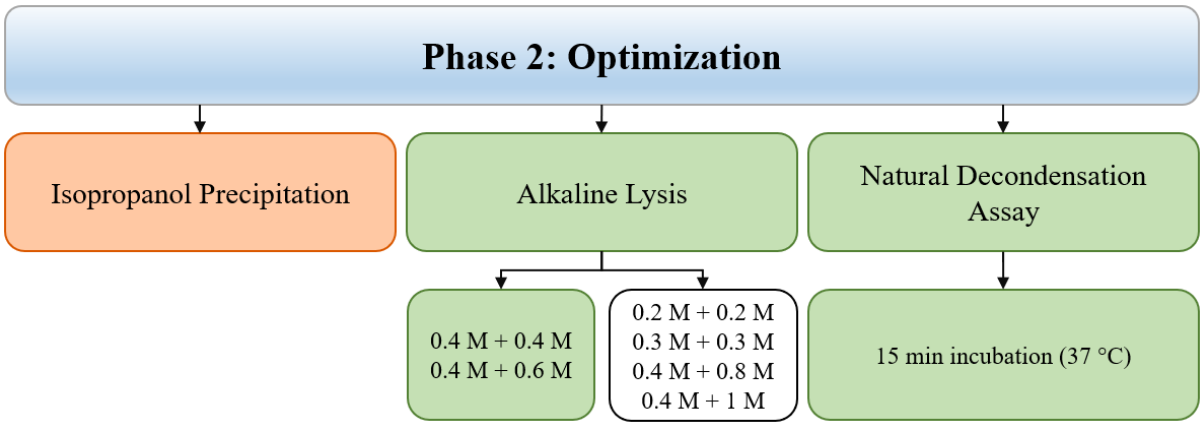

Figure 17. General overview of results obtained in the optimization phase. Green markings signify the protocols and parameters that produced adequate concentrations. Parameters marked in white did not produce adequate results. Isopropanol precipitation is marked in orange to signify that no optimization was conducted but the protocol was evaluated in later phases.

The main goal of the optimization phase was to examine if it was possible to improve the alkaline lysis protocol that had been shown to be the best performing cell lysis method from the screening phase. Optimization experiments were performed on isolated sperm cell fractions to evaluate the method's effectiveness when the epithelial cell fraction had been separated from the sperm cell fraction, to mimic how the protocol is performed on real casework samples.

From the early results in the section above, it was clear that the potential inhibition of qPCR by the alkaline lysis protocol was a concern; results from the RB1 assay showed a comparatively lower yield with regard to the reference protocol (Figure 12a). Additionally, a high variance was observed (Figure 12a) which was not seen in the PowerQuant analysis of the same samples, further suggesting that amplification was not optimally performed with the RB1 assay. While a more sensitive assay may not be as prone to inhibition, it was important to examine and confirm the potential effect on PCR amplification. As subsequent analysis with the PowerQuant assay showed both normal IPC values and expected values in the dilutions, it can be concluded that inhibition for the PowerQuant assay was not a factor, and the quantitative results were reliable. Analysis of alkaline lysis using 0.4 M NaOH/Tris-HCl, as well as lowered NaOH/Tris-HCl concentrations (0.2 M and 0.3 M) with the PowerQuant assay pointed to a similar trend seen in the previous experiments, with increased recovery with higher NaOH/Tris-HCl concentration and no apparent inhibition. Even higher DNA yields were obtained in the optimization experiments where the Tris-HCl molarity was increased in relation to the NaOH concentration, possibly due to the stabilization of the DNA molecule with the lowered pH. On the other hand, this increase led to both PCR inhibition as seen by the delayed IPC values, and increased DNA degradation in the case of 1 M Tris-HCl added. The contrast between the high recovery and high degradation is a notable aspect of the results; possibly, some degradation is favorable as this cleaves the DNA into pieces, making it easier for primers to bind. However, as inhibition and degradation are generally undesirable, these parameters were studied further.

These findings indicated that 0.4 M NaOH/Tris-HCl was still the optimal concentration to proceed with in terms of the alkaline lysis protocol. The results were confirmed in the experiment using a 1:300 sperm dilution, where the 0.4 M + 0.4 M protocol produced a high DNA recovery without showing substantial inhibition by elevated IPC values. Thus, these parameters were chosen for the next phase where compatibility with DNA profiling was conducted. However, despite the apparent PCR inhibition, it was also interesting to investigate the compatibility of the 0.4 M + 0.6 M alkaline lysis protocol with CE, so these samples likewise went on to be analyzed in the next phase.

The natural decondensation assay also showed positive results in comparison with the reference protocol when performed on a more diluted sperm sample. However, in contrast to the experiment during the screening phase, the IPC values were not as significantly delayed when performing the protocol on a 1:300 sperm dilution. This may be due to the difference in the sperm volumes that the medium was added to; as approximately 160 µL modified medium was added to the isolated sperm cell fractions containing around 40 µL of the sperm cell fraction during the optimization phase, this may have led to a slight dilution of the medium. In contrast, 190 µL of the modified medium was added to 10 µL non-isolated sperm samples during the screening phase. As no apparent complications in terms of inhibition or degradation could be observed during the optimization phase, the natural decondensation assay was selected for further evaluation of compatibility with DNA profiling.

Lastly, the effectiveness of isopropanol purification of the samples with lower sperm concentrations proved to be consistent with the results from the screening phase. Therefore, it

was decided that no further optimization was necessary, and the protocol was deemed suitable to evaluate in the next phase.

4.3 Evaluation of compatibility with forensic DNA profiling

All samples from the experiment comparing the reference protocol with alkaline lysis (0.4 M + 0.4 M and 0.4 M + 0.6 M NaOH/Tris-HCl) (Figure 15) were amplified and analyzed using CE. In the samples processed by the alkaline lysis protocol, the ILS signal was negatively affected and too low to reach the lowest threshold set for an applicable ILS signal (50 RFUs). As the ILS signal is crucial to determine the DNA amplicon sizes in the sample, no DNA profiling results could be obtained. It was concluded that the alkaline lysis protocol negatively affected either the PowerPlex ESX 16 Fast amplification or the CE analysis.

The protocols were performed once more on a 1:300 sperm dilution with varying NaOH/Tris-HCl concentrations (0.2, 0.3 and 0.4 M NaOH; 0.2, 0.3, 0.4, 0.45, 0.6 and 0.8 M Tris-HCl) in the alkaline lysis protocol to investigate whether different compositions could yield an equivalent DNA recovery while being compatible with the DNA profiling process. The results obtained from DNA quantification with PowerQuant assay suggest that the DNA concentration increases with the NaOH concentration, with a highest recovery from the 0.4 M + 0.6 M protocol (Table 8). The results from one-way ANOVA indicate a significant difference between two or more of the groups, while the Bonferroni post hoc t-test ($\alpha = 0.005$) only showed a significant difference between the reference protocol and the 0.4 M + 0.6 M protocol (0.008 ± 0.001 and 0.020 ± 0.002 ng/µL, respectively, $p = 0.0037$), but no difference between the reference protocol and the 0.4 M + 0.4 M protocol (0.018 ± 0.004 ng/µL, $p = 0.0483$). Furthermore, delayed IPC C_q values ($C_q > 21$) were detected in all optimization experiments except 0.2 M + 0.2 M and 0.2 M + 0.3 M, in comparison with the standard reference (20.51 ± 0.13, $n = 10$). One replicate from the 0.3 M + 0.3 M experiment was removed due to the incorrect NaOH concentration being added.

The CE results showed that the ILS signal was lower than normal, but high enough to provide accurate amplicon sizing (>50 RFUs). Although equal DNA yields are generated with the alkaline protocol, the results suggest that higher concentrations of NaOH and Tris-HCl lead to a suppression of the ILS and a subsequent decrease in both number of STR alleles and peak heights (Table 8). One explanation for this result may be sample stacking, a phenomenon that involves an increase in the sample conductivity due to higher levels of salt. This affects the CE analysis as the salt ions and DNA molecules compete for injection onto the capillary (Butler, 2005).

Table 8. DNA concentrations obtained from qPCR analysis of reference protocol and alkaline lysis (with varying NaOH and Tris-HCl concentrations), number of correctly detected alleles, and total peak height in CE analysis. The protocols that produced measurable peaks after the ILS limit was reduced to 50 RFUs are italicized.

Protocol	**Average DNA concentration (ng/µL ± SD)**	**No. of correctly detected STR alleles (n)**	**Total peak height (RFUs)**
Reference protocol	*0.008 ± 0.001*	*32* *32* *32*	*18850 ± 1840*
Alkaline lysis (0.2 M + 0.2 M)	*0.014 ± 0.004*	*27* *30* *27*	*14310 ± 3410*
Alkaline lysis (0.2 M + 0.3 M)	*0.012 ± 0.004*	*18* *18* *18*	*6730 ± 2710*
Alkaline lysis (0.2 M + 0.4 M)	0.07 ± 0.003	0	N/A
Alkaline lysis (0.3 M + 0.3 M)	*0.016 ± 0.002*	*6* *6*	*2700 ± 96*
Alkaline lysis (0.3 M + 0.45 M)	0.012 ± 0.002	0	N/A
Alkaline lysis (0.3 M + 0.6 M)	0.016 ± 0.002	0	N/A
Alkaline lysis (0.4 M + 0.4 M)	0.018 ± 0.004	0	N/A
Alkaline lysis (0.4 M + 0.6 M)	0.020 ± 0.002	0	N/A
Alkaline lysis (0.4 M + 0.8 M)	0.010 ± 0.004	0	N/A

To investigate the potential effect of sample stacking, ethanol purification of three samples from the previous experiment (0.3 M + 0.3 M, 0.4 M + 0.4 M, and 0.4 M + 0.6 M) was performed. The samples selected showed a high DNA yield in qPCR analysis but produced few or no peaks during CE analysis. Purification was performed on only one of the three replicates. The corresponding non-purified samples were analyzed in the same CE run as the purified samples. An increased number of correct alleles could be detected following purification as well as higher peak heights, across all samples tested (Table 9, and Figure 18).

Table 9. Comparison of the number of correctly detected alleles in non-purified and samples purified with ethanol precipitation from Figure 15 (0.3 M + 0.3 M, 0.4 M + 0.4 M, and 0.4 M + 0.6 M) (n = 1).

Protocol	**No. of correctly detected STR alleles (non-purified)**	**No. of correctly detected STR alleles (purified)**	**Total peak height (RFUs) (non-purified)**	**Total peak height (RFUs) (purified)**
Alkaline lysis (0.3 M + 0.3 M)	6	32	3389	42400
Alkaline lysis (0.4 M + 0.4 M)	0	32	N/A	92400
Alkaline lysis (0.4 M + 0.6 M)	0	32	N/A	27400

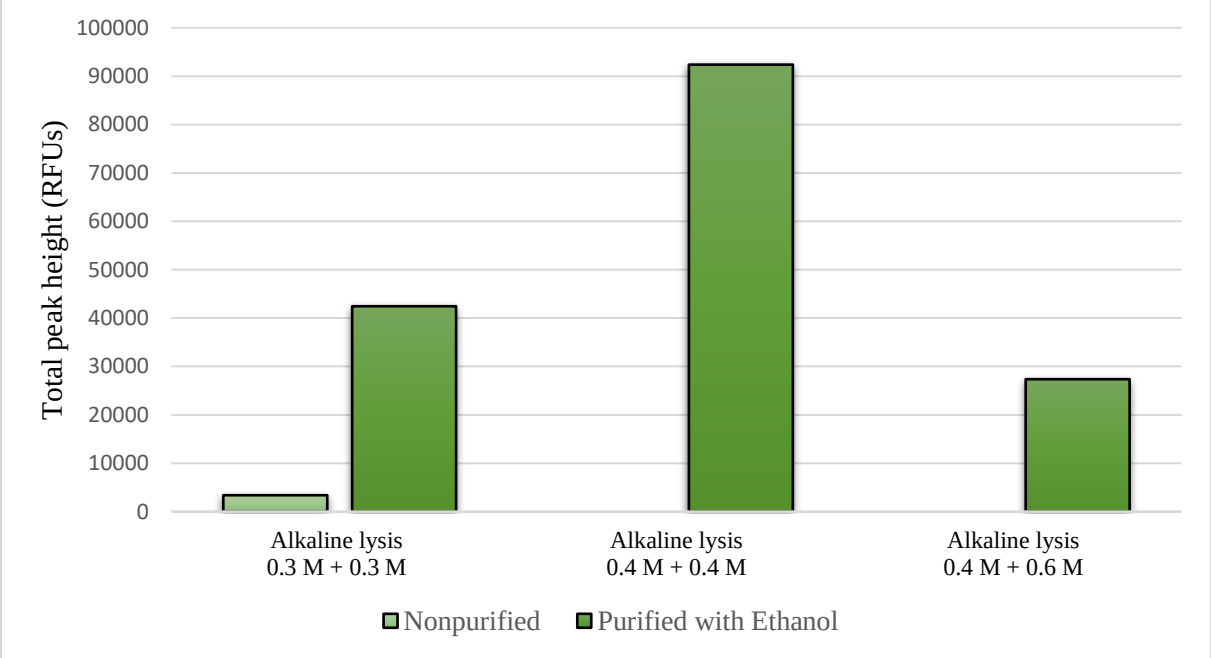

Figure 18. Total peak heights (RFUs) obtained from CE analysis of non-purified samples that had previously shown little or no peaks, and the same samples purified with ethanol precipitation (n = 1). All samples showed higher total peak height and complete DNA profiles following purification.

Because not all alleles were detected in the experiment using 0.2 M + 0.2 M (Table 8), inhibition of DNA profile generation was still a factor. Because of this, the NaOH/Tris-HCl volume was lowered to allow for subsequent dilution of the samples, up to 200 µL with TE buffer. This was done to investigate whether a full DNA profile could be obtained without performing an additional purification step, as this would reinforce the simplicity of the protocol. Alkaline lysis was performed with varying NaOH/Tris-HCl volumes (50 µL, 60 µL and 75 µL NaOH/Tris-HCl). 0.2 M was selected as the concentration for NaOH and Tris-HCl as this had earlier proved to produce the highest DNA recovery with the most detected allele peaks following CE analysis. As the DNA yields obtained when performing the protocols on 1:300 sperm dilutions were

close to the lower limit of standard CE analysis (0.01 ng/µL), optimization was performed on 1:100 sperm dilutions to reduce the stochastic effects of such low concentrations.

The addition of 50 µL + 50 µL, 60 µL + 60 µL and 75 µL + 75 µL NaOH and Tris-HCl followed by dilution up to 200 µL resulted in a similar DNA recovery for all volumes (0.092 ± 0.01 ng/µL, 0.082 ± 0.002 ng/µL and 0.081 ± 0.005 ng/µL, respectively). As the DNA concentrations exceeded the 0.05 ng/µL limit for CE, the samples were diluted 1:2. Two replicates from the 60 µL + 60 µL experiment, and one replicate from the 75 µL + 75 µL experiment, failed to produce any CE results due to a too low ILS signal (< 50 RFUs). In the other samples, all 32 alleles were detected, except one replicate from the 75 µL + 75 µL experiment where 31 alleles were detected (Figure 19).

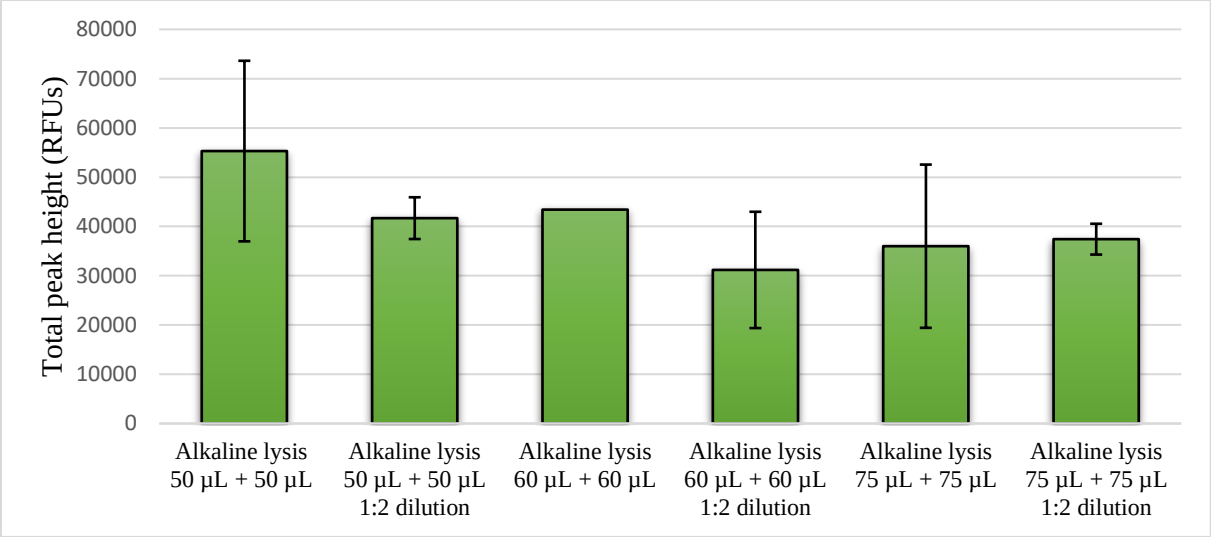

Figure 19. Total peak heights (RFUs) obtained from CE analysis of alkaline lysis protocol with various NaOH/Tris-HCl (0.2 M) volumes added, and their 1:2 dilutions.

Alkaline lysis with even lower volumes of NaOH and Tris-HCl, 25 µL + 25 µL followed by dilution to 200 µL was performed concurrent with the reference protocol on a 1:100 sperm dilution (n = 6). The results from qPCR analysis showed similar DNA recoveries with both protocols (0.073 ± 0.03 ng/µL for the reference protocol and 0.072 ± 0.03 ng/µL for alkaline lysis, p = 0.978, Figure 20a).

CE analysis showed a comparable total peak height for both protocols (55100 ± 21700 RFUs for the reference protocol and 47400 ± 9500 RFUs for the alkaline lysis protocol, p = 0.731, Figure 20b). All 32 alleles were detected in every sample, apart from one sample of the alkaline lysis protocol where 31 alleles were detected.

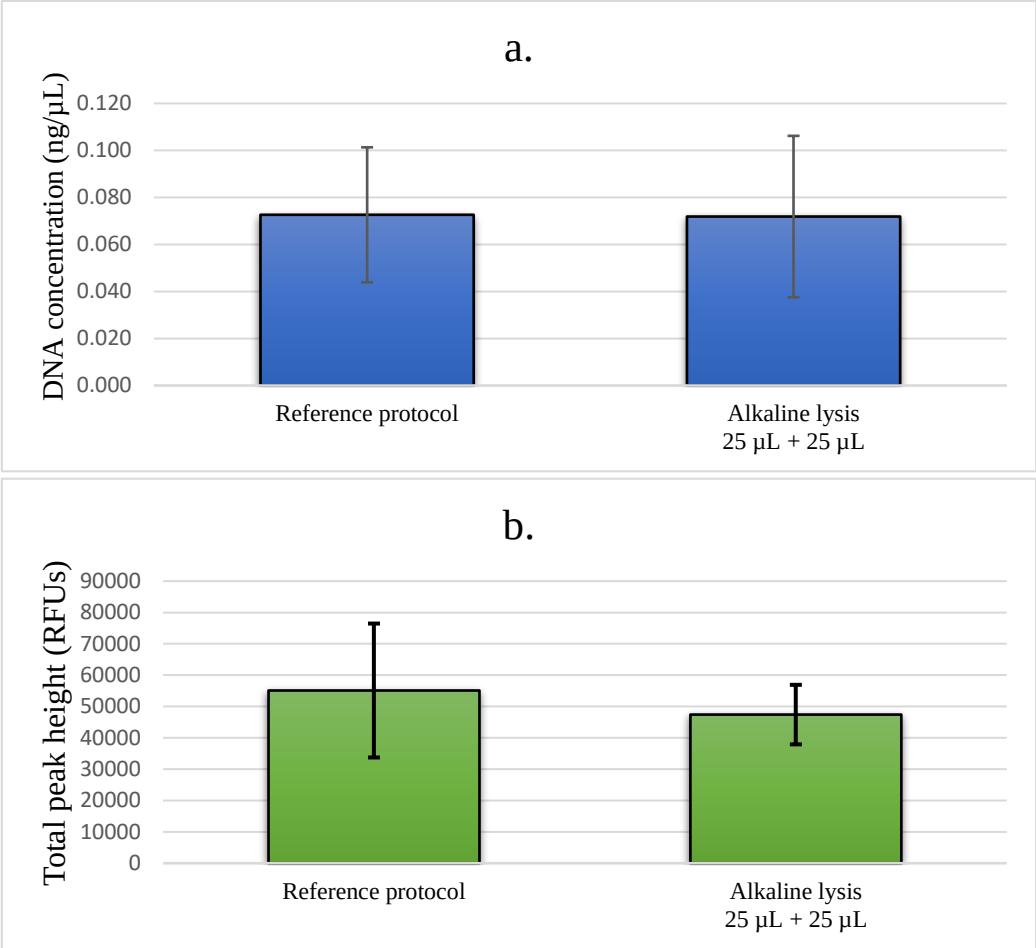

Figure 20. (a) DNA concentrations obtained from qPCR analysis with PowerQuant assay and (b) total peak heights (RFUs) obtained from CE analysis of reference protocol and alkaline lysis (25 µL + 25 µL 0.2 M NaOH/Tris-HCl) performed on isolated sperm cell fractions (1:100 dilution) (n = 6). Equal DNA recovery was observed for both methods.

Continuing with the natural decondensation assay, as the DNA recovery was comparable to the reference protocol when performed on low sperm concentrations during the optimization phase, and there was no significant delay in IPC C_q values, the samples were then analyzed with CE. Partial DNA profiles were detected in all three replicates (Table 10). Furthermore, the average total peak height was roughly $1/10^{th}$ of the average total peak height of the reference protocol. As heparin is a known inhibitor of PCR, it is possible that the amplification process was affected by the heparin present. The sample volume used in the PowerPlex ESX 16 assay is significantly greater than in the PowerQuant assay (17.5 vs 2 µL, respectively); consequently, the impact of heparin on inhibition may be more pronounced when larger quantities are added.

Table 10. DNA concentrations obtained from qPCR analysis with PowerQuant assay of the reference protocol, the natural decondensation assay, and isopropanol purification. Number of correctly detected alleles, and total peak height in CE analysis

Protocol	**Average DNA concentration (ng/µL ± SD)**	**No. of correctly detected STR alleles (n)**	**Total peak height (RFUs)**
Reference protocol	0.010 ± 0.006	32	26400
		0	
Natural decondensation assay	0.012 ± 0.003	9	3460 ± 775
		13	
		13	
Isopropanol purification	0.013 ± 0.002	32	18410 ± 718
		32	
		32	

Finally, in CE analysis of the samples purified with isopropanol from the optimization phase, all 32 alleles were detected in the samples where isopropanol purification had been performed (Table 10). Alleles peaks could only be detected in one of the two replicates of the reference protocol. Consequently, no exact statistical comparison could be made between the two protocols.

4.3.1 Discussion of compatibility with forensic DNA profiling

Figure 21 illustrates a general overview of the results obtained in the evaluation of compatibility with forensic DNA phase, where the green markings signify that the parameters produced sufficient concentrations in comparison with the reference method regarding amplification and CE analysis. The parameters that were not compatible with the DNA profiling process are marked in white. Isopropanol precipitation is marked in orange to indicate that the protocol was evaluated in later phases, but not optimized.

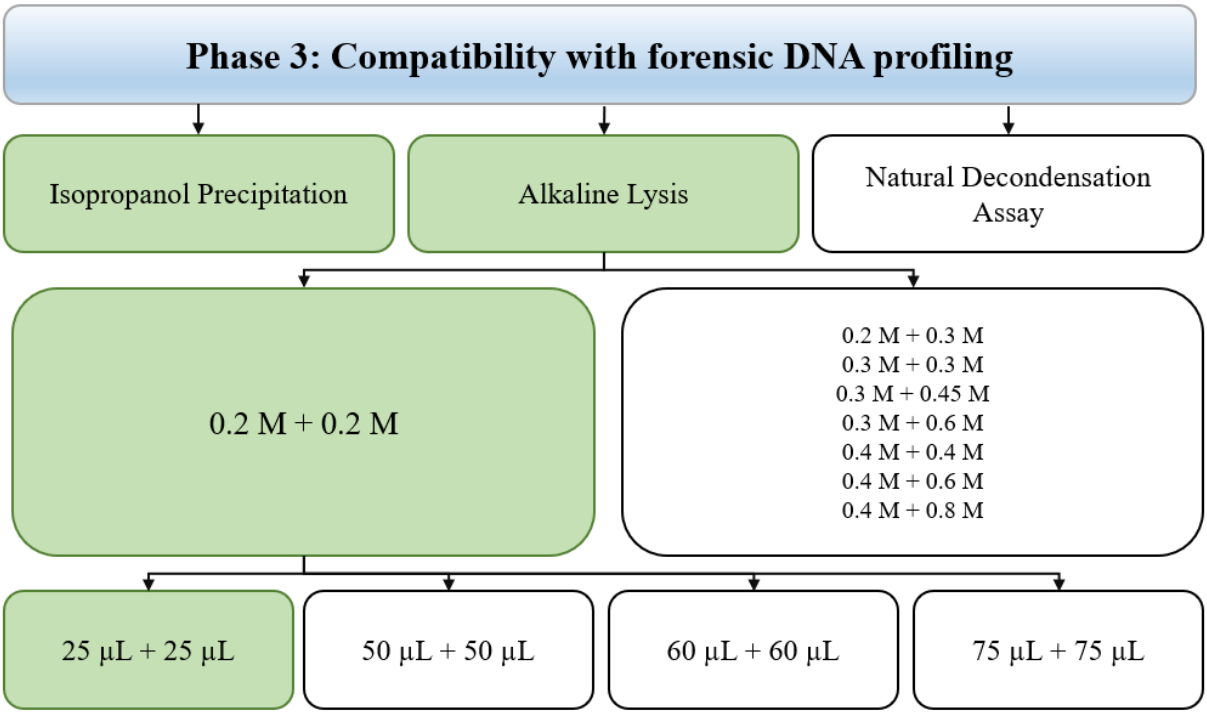

Figure 21. General overview of results obtained in the evaluation of forensic DNA profiling compatibility phase. Green markings signify the protocols and parameters that produced adequate concentrations. Parameters marked in white did not produce adequate results.

At this stage, selected and optimized protocols had been proven to be successful in terms of both lysing low sperm cell concentrations and purifying samples treated with DTT, while also showing favorable characteristics in terms of qPCR inhibition and degradation. The aim of this phase was to evaluate methods' effectiveness in terms of DNA profiling.

The alkaline lysis protocol displayed early problems in the DNA profiling process, with little or no alleles being detected in the samples that had previously shown to give the highest DNA recovery. Subsequently, it was imperative to revisit the optimization process and adjust the parameters that possibly affected the analysis. As presented in Table 8, decreasing the NaOH and Tris-HCl molarity relieved the issue of suppressed peaks, while maintaining a sufficiently high DNA recovery during quantification. However, the lowest concentration composition (0.2 M + 0.2 M) still did not produce peaks for all expected alleles.

The results from the ethanol purification of the samples confirmed that some components of the alkaline lysis samples were interfering with CE analysis. As discussed earlier, this was most likely due to the salt content in the samples hindering capillary injection (sample stacking), and ethanol precipitation was specifically selected over isopropanol precipitation as ethanol is less efficient in precipitating salts. These findings suggested that alkaline lysis, while an effective method from sperm cell lysis, had to be adjusted further to minimize the interference of salts.

The experiments using 25, 50, 60 and 75 µL NaOH/Tris-HCl followed by a dilution up to 200 µL were performed to assess if reduced reagent volumes could yield similar DNA concentrations while avoiding sample stacking. The results indicated that the reference protocol

and even the lowest volume tested, 25 µL, exhibited comparable effectiveness in lysing low sperm dilutions. When diluted up to 200 µL with TE buffer, all allele peaks were detected in the 25 µL + 25 µL alkaline lysis samples. It was thus concluded that this optimized protocol was suitable to perform on mock sexual assault samples in the final phase.

Regarding isopropanol purification of samples treated with DTT, the protocol likewise showed compatibility with amplification and CE analysis, producing 32 STR peaks in all samples. This protocol was consequently determined to be a valid alternative protocol for the purification of samples that had undergone differential lysis with DTT. However, the protocol was not attempted on the mock sexual assault samples due to time limitations.

Lastly, the natural decondensation assay was unsuccessful in producing enough detectable STR peaks in CE analysis. Despite the effectiveness in the PowerQuant quantification step, inhibition could possibly be a factor in the PowerPlex ESX 16 amplification due to the difference in volume sample added (17.5 µL for PowerPlex, 2 µL for PowerQuant). However, the possible PCR inhibition was not investigated further and for the purpose of this project, evaluation of this protocol was completed at this stage.

4.4 Execution of optimized protocol on mock sexual assault samples

During the final phase, the optimized alkaline lysis protocol was performed on swab cuttings that had been airdried following the addition of 150 µL saliva and 0.2 or 2 µL 1:100 sperm dilution. The purpose was to replicate samples obtained from real-life sexual assault samples while staying in the concentration range of approximately 0.05 ng/µL sperm DNA. The results obtained from quantitative analysis of the reference protocol and the alkaline lysis protocol with 25 µL + 25 µL 0.2 M NaOH and Tris-HCl showed a slightly higher DNA recovery from the alkaline lysis protocol in comparison to the reference protocol (Figure 22). The total DNA concentrations were equal between protocols when 2 µL (0.052 ± 0.01 ng/µL for alkaline lysis, 0.044 ± 0.03 for the reference protocol, $p = 0.42$) and 0.2 µL (0.020 ± 0.01 ng/µL for alkaline lysis, 0.012 ± 0.005 for the reference protocol, $p = 0.40$) sperm was added.

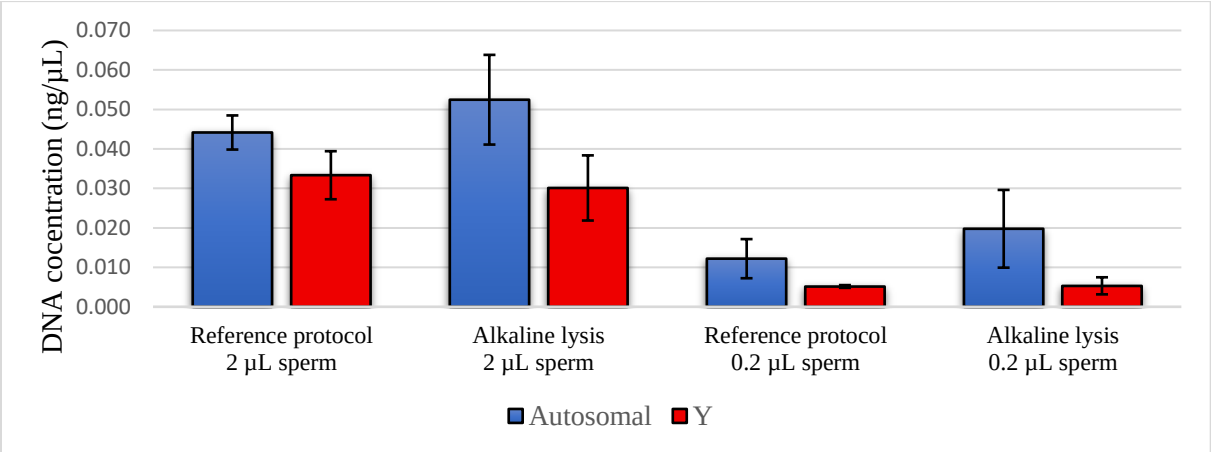

Figure 22. DNA concentrations obtained from qPCR analysis with PowerQuant assay of reference protocol and alkaline lysis performed on mock sexual assault samples. Autosomal DNA concentration is marked in blue and Y DNA concentration is marked in red.

CE analysis showed a similar trend, with comparable total peak heights between the alkaline lysis and reference protocol for both 2 µl and 0.2 µl sperm added. All 32 alleles from the sperm donor's DNA profile were detected in the samples where 150 µL saliva and 2 µL sperm was added to the cotton swabs, with the exception of one sample from the reference protocol, where no CE results could be obtained. Fewer alleles, including the ones from the female saliva donor's DNA profile, were detected in the samples containing 0.2 µL sperm (Figure 23). The ratio between the average total peak height from the correctly detected STR allele peaks for the female donor and male donor (Equation 1 in section 3.7 Data analysis) suggested that approximately 2.4x more sperm DNA was extracted from the mock sexual assault samples with 2 µL sperm added (ratio 0.423) for alkaline lysis, and 2.9x more for the reference protocol (ratio 0.34). The ratio in the samples with 0.2 µL sperm suggested a 3.3x higher epithelial DNA concentration for alkaline lysis and 2.75x higher for reference protocol.

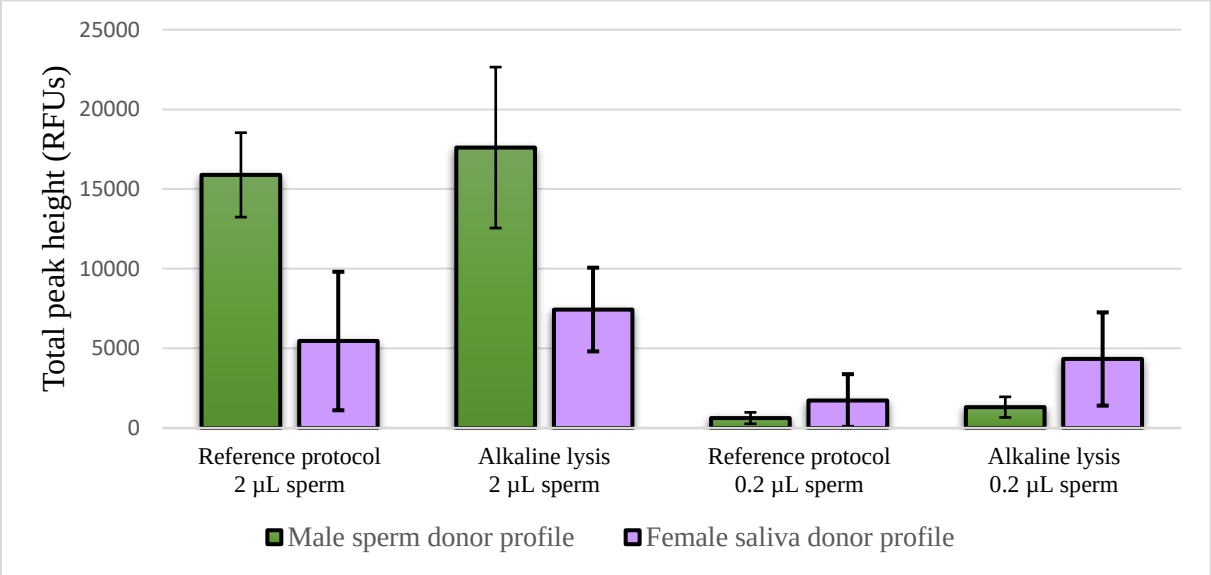

Figure 23. Total peak heights (RFUs) obtained from CE analysis of reference protocol and alkaline lysis performed on mock sexual assault samples. The total peak heights for the male sperm donor profile are marked in green, total peak height for the female saliva donor profile are marked in purple.

An experiment was conducted on the mock sexual assault samples (2 µL sperm, 1:100) to examine the results of reduced washing steps in the reference protocol. The experiment was conducted with five replicates, with two outliers being removed (one from the test with two washing steps and one from the test with one washing step) after being identified by a Grubbs's test ($p < 0.01$). The results from PowerQuant qPCR analysis indicated an equal DNA yield was obtained with reduced washing steps (Figure 24a), and CE analysis showed a higher average total peak height for the correctly detected STR allele peaks from the male donor profile (Figure 24b).

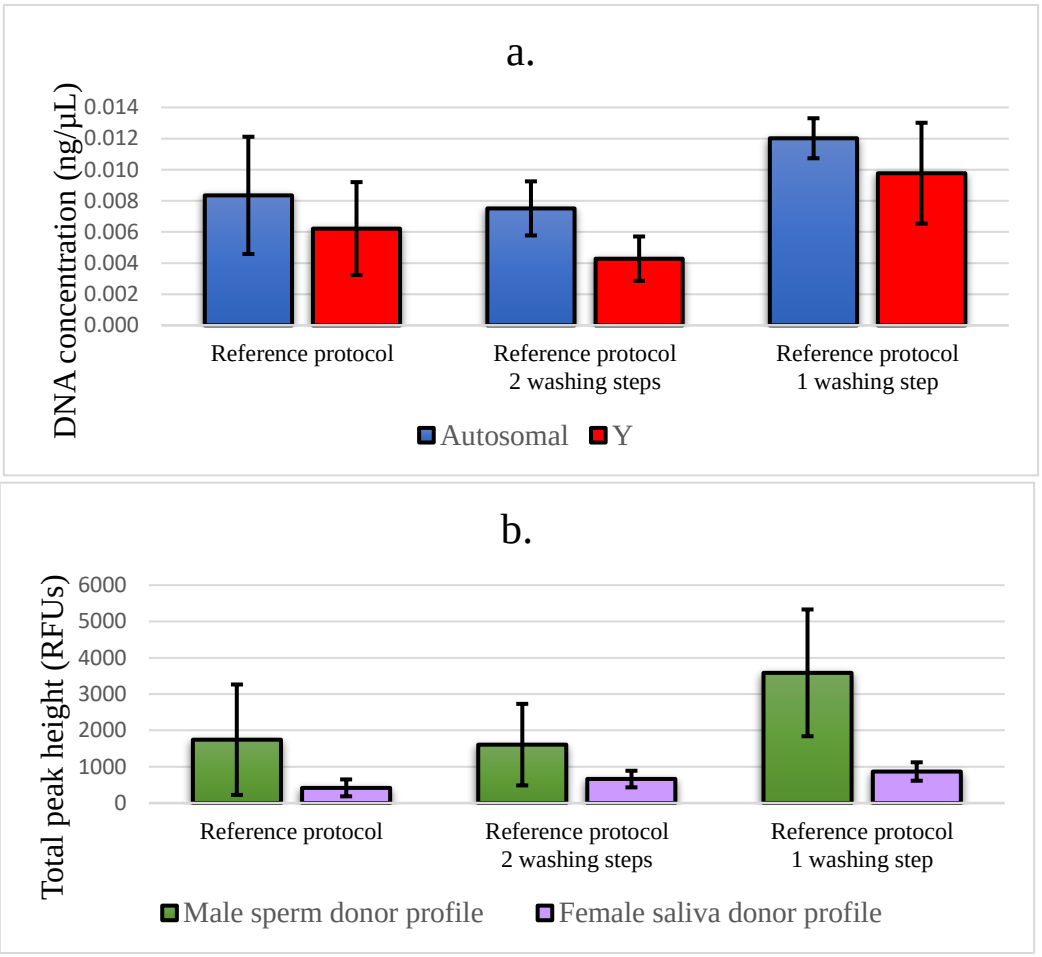

Figure 24. (a) DNA concentrations obtained from qPCR analysis with PowerQuant assay and (b) Total peak heights (RFUs) obtained from CE analysis of reference protocol with reduced washing steps performed on mock sexual assault samples (n = 5). Autosomal DNA concentration is marked in blue and Y DNA concentration is marked in red. The total peak heights for the male sperm donor profile are marked in green, total peak height for the female saliva donor profile are marked in purple.

4.4.1 Discussion of execution of protocols on mock sexual assault samples

The final phase was essential to confirm that the optimized alkaline lysis protocol could be a suitable alternative to the existing sperm cell lysis method which utilizes DTT. Thus far, the protocol has proven to be comparable to the reference protocol in terms of quantification and in the DNA profiling process. Moreover, it was possible to correct issues that had emerged during earlier phases, such as potential qPCR inhibition and sample stacking, while not compromising the effectiveness of the method. Thus, it was imperative to assess the reliability and robustness of the method when an excess volume of epithelial cells and a small volume of sperm cells had been added to cotton swabs, as these proportions are typically observed in real-life sexual assault samples. Real sexual assault samples will naturally contain varying concentrations of different cell types, including sperm cells and vaginal epithelial cells, and it is crucial that the cells of interest can be accurately analyzed and identified. It is therefore important that the lysis method used efficiently targets a small amount of sperm cells, if they are present.

The results observed during the final phase suggest that the optimized alkaline lysis protocol could be used to obtain both DNA concentrations and DNA profiles from the mock sexual assault samples that are comparable to the reference protocol. Importantly, all expected STR allele peaks for the sperm donor were detected during the final stage, and while mixed with the DNA profile from the saliva donor, higher total peak heights were observed for the sperm donor's profile despite the low initial volume. The sperm donor's STR allele peaks were also detected in the samples with 0.2 µL sperm added; however, the total STR peak heights from the saliva donor's profile were higher (Figure 23). Although this would likely complicate the interpretation of the DNA profile in a real-life scenario, it was shown that the reference protocol produced similar results. An aspect that may have affected the findings is sample preparation; as 0.2 µL is a considerably small volume, it was difficult to accurately add this volume of sperm to the cotton swab with a pipette. A more accurate way of preparing the samples would be to dilute the sperm dilution 10 times and add 2 µL of the new dilution.

Finally, results also showed that decreasing the washing steps can generate DNA profiles that are comparable with the profiles obtained when all three washing steps in the reference protocol are performed. By removing one or more washing steps, the reference protocol can be shortened and the risk of cell loss during the removal of the supernatant can be mitigated.

4.5 General discussion

In consideration of the results presented, analyzed, and discussed above, this section will provide an exploration into the general conclusions that can be drawn from the experiments, the practical applications of the methods discussed, and the consequences for future forensic analysis of sexual assault samples.

Alkaline lysis of sperm cells in forensic samples had shown promising potential in previous studies, such as Schellhammer et al. (2022), and was interesting due to it being simple, quick, and using off-the-shelf reagents. With the final optimized alkaline lysis protocol (25 µL 0.2 M NaOH + 25 µL 0.2 M Tris-HCl and TE buffer up to 200 µL), the method could be performed with high sperm DNA recovery and accurate DNA profiling, without being inhibited in any way. Replacing traditional differential lysis using DTT with alkaline lysis would be advantageous from both a time perspective and performance perspective. The sperm lysis step can be executed in under 10 minutes within one microfuge tube and produces lysates that are direct-to-amplification ready, bypassing the use of an additional lengthy purification step (Figure 25). Thus, alkaline lysis would significantly preserve both time and material costs.

One drawback with alkaline lysis may be the final dilution step, where TE buffer is added to a total volume of 200 µL. This is the current standard final volume for forensic samples that have been extracted with differential extraction, but as the DNA concentrations from real crime samples can be very low, there may be a benefit to minimizing the dilution. It is possible that the dilution with TE buffer contributes to the reduction of sample stacking during CE injection, and that the addition of 25 µL + 25 µL NaOH and Tris-HCl without further dilution would interfere with the CE analysis. However, there may be some margin regarding minimizing the

final volume as the addition of 50 µL + 50 µL NaOH and Tris-HCl also resulted in all STR peaks being detected.

Further, alkaline lysis had demonstrated high efficiency in DNA recovery when using reagents with higher compared with the use of 0.2 M NaOH/Tris-HCl. While lowering the concentration was shown to produce similar DNA yields as the reference protocol, it is possible that the use of 0.4 M NaOH could be even more successful in this respect. However, the possible effect of sample stacking prevents these samples from being properly analyzed without being purified. The ethanol precipitation that was subsequently performed is a straight-forward technique utilizing easily accessible reagents, but the addition of an extra purification step following alkaline lysis runs counter to the aim of employing a simpler and more user-friendly approach. Nonetheless, in the event of future crises or unexpected Amicon filter supply chain disruptions, the results have shown that alkaline lysis is a viable alternative to traditional differential lysis. This applies to both the final optimized protocol, and with the use of more concentrated reagents followed by ethanol precipitation.

Like alkaline lysis, isopropanol precipitation has been shown to be a feasible alternative purification method in place of Amicon filtration. While not as timesaving as direct-to-amplification lysis options, this protocol likewise uses reagents that are widely accessible for laboratories and is convenient in that it can be performed easily at one laboratory bench. Amicon filtration, in contrast, involves slightly more laborious steps (e.g. the discarding of the filtrate and addition of TE buffer) as well as an extra tube transfer step which can introduce risks of contamination and potential DNA loss. However, isopropanol precipitation includes two steps where the supernatant is removed which may be a delicate process if the pellet is small, which can potentially lead to DNA loss.

Regarding the other protocols tested, the NP-40 lysis and the natural decondensation assay protocols were unable to exhibit successful performance in terms of cell lysis and DNA profiling, respectively. Optimization of the natural decondensation assay could be conducted to evaluate if the compatibility with the DNA profiling process could be improved. However, the reagents used in NP-40 lysis buffers and natural decondensation assays are likely more susceptible to potential breaks in supply, compared with the reagents in alkaline lysis.

In summation, possible improvements of the reference protocol can be made by replacing certain methods with easier or faster options that negate the need for costly filtration units. Besides alkaline lysis working as an alternative to sperm cell lysis with DTT and isopropanol precipitation as an alternative to Amicon filtration, additional improvements such as the reduction of washing steps can be made to simplify the existing protocol. While there are no bottlenecks in the DNA extraction process at NFC currently, reducing the duration of the differential lysis protocol can have a positive effect on resource usage and the work environment (Figure 25). Further, the alternative protocols can be employed in the event of future crises.

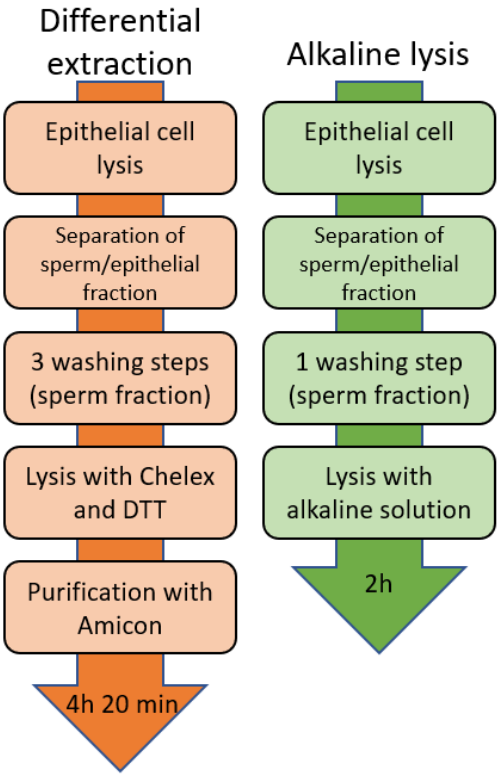

Figure 25. Comparison of the execution time of the reference protocol and alkaline lysis protocol.

4.6 Future prospects

Moving forward, further testing and method validation is necessary to determine whether the proposed protocols are appropriate alternatives for real-life handling of sexual assault samples. Validation should be performed to establish the method's accuracy, precision, and specificity. The limit of detection, limit of quantification and robustness should also be examined. Validation should include testing with more biological replicates, as the use of three replicates was a limitation of this study. Validation should preferably be conducted at NFC's lab to establish compatibility with kits and instruments used to analyze real-life samples.

The stability of the sample over a long period of time must also be assessed, to ensure that DNA does not degrade over time in the final sample conditions. Naturally, this is a crucial aspect of forensic DNA extraction, as samples must be able to be analyzed years down the line if necessary.

5. Conclusion

The aim of this thesis was to examine alternative protocols that could replace either the sperm cell lysis or purification steps in the reference protocol, to obtain a more streamlined method that is less reliant on a single supplier for delivery than the current protocol. This also included examining if reducing the washing steps could produce clear DNA profiles. Four research questions were listed in the aim of this project, and they will be briefly answered in this section.

1. *Do any alternative methods for differential extraction/purification produce sufficient DNA yield, both quantitatively and qualitatively?*

   Optimized alkaline lysis and isopropanol precipitation are two alternative methods for sperm cell lysis and purification, respectively, that produce equal DNA recovery quantitatively and qualitatively. Additionally, the differential extraction protocol can be streamlined by removing two washing steps.

2. *Can the alternative methods be optimized to obtain better results?*

   The alkaline lysis protocol was optimized to produce optimal DNA recovery both quantitatively and qualitatively.

3. *Are the protocols compatible with NFC's method for DNA quantification and short tandem repeat (STR)-analysis?*

   Optimized alkaline lysis and isopropanol precipitation are both compatible with qPCR analysis and CE analysis.

4. *Is the best-performing protocol able to produce equivalent or better results in comparison to the current Chelex-based method?*

   Optimized alkaline lysis and isopropanol precipitation both produce equal DNA recovery in comparison with the Chelex-based method. Performing alkaline lysis and one washing step on mock sexual assault samples resulted in higher recovery of sperm DNA in comparison with DNA from a saliva donor, which was comparable to the reference protocol.

The optimized alkaline lysis protocol has demonstrated an ability to successfully extract DNA from sperm cells at volumes as low as 0.002 µL when mixed with 150 µL saliva. These extracts were also seen to generate accurate DNA profiles when performing subsequent forensic DNA typing. Throughout the project, the reference protocol and alkaline lysis method continuously produced comparable results, suggesting that these methods are equal in their effectiveness. Moving forward, further validation of the method and study of the sample stability over an extended period must be conducted. Should these aspects be verified, it is possible that alkaline lysis can be a valid alternative to cell lysis with DTT and Amicon purification.

Likewise, isopropanol precipitation has been shown to be a promising substitute for Amicon purification. This protocol should go on to be tested on mock sexual assault samples to confirm its efficiency in more real-life scenarios, followed by further validation. As isopropanol purification and Amicon filtration are similar in terms of execution time, these methods should be compared in terms of ease of execution by laboratory personnel.

Additionally, these protocols can be useful alternatives to the current differential extraction protocol in the event of an emergency or crisis, where access to Amicon filters or other reagents may be limited. This is beneficial from a contingency perspective, where government agencies such as NFC can be prepared with substitute methods that will not halt important routine operations.

In conclusion, this project has presented methods for sperm cell lysis and sample purification that are feasible alternatives to the approaches used today. Other protocol adjustments have also shown promise in shortening the execution time of today's lengthy protocol. With more validation conducted, these methods can hopefully be implemented in the differential extraction analysis chain.

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