Document Title:	**Rapid and Selective Extraction of Male DNA from Rape Kits and Other Forensic Evidence Using Pressure Cycling**
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FINAL SUMMARY OVERVIEW

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SUMMARY OVERVIEW

The processing and interpretation of mixed DNA samples consisting of sperm and epithelial cells has long been recognized as a bottleneck in forensic DNA analysis [1, 2]. The examination of physical evidence submitted in such cases can be tedious and time-consuming. As a result, subsequent DNA analysis and interpretation can be challenging especially if the evidence left behind by a male suspect is overwhelmed by the female component.

A two-step organic differential extraction is one of the most popular methods to separate different sources of DNA encountered in a rape kit [3]. In the first step of digestion, vaginal epithelial cells in the mixture are lysed with proteinase K and sodium dodecyl sulfate (SDS) and a detergent and the sperm cells remaining in the solution are collected by centrifugation. In the second step of digestion, the sperm cells are lysed with a buffer containing proteinase K, SDS and dithiothreitol (DTT) as reducing agent. Both fractions are purified separately with phenol/chloroform/isoamyl alcohol. This method is employed to achieve a complete separation of two different cell types present in the mixture and to obtain a clean male DNA profile that is not obscured by the female DNA. In addition, the process of removal of cells from cotton swabs can be very poor. Often the mixtures contain a trace level of sperm cells and a high level of expertise is required to ensure adequate recovery of male DNA. Overall, the method is time-consuming, technique-dependent and difficult to automate, and can result in relatively inefficient separations of female DNA from the male sample components. The goal of the current study is to provide a rapid, reliable and efficient method to selectively recover sperm DNA from sexual assault evidence.

This study involves the application of pressure cycling technology in the selective digestion of sperm cells from evidence mixtures with an emphasis on the role of buffer composition on sperm DNA yields and increase in selectivity of extraction. The cells were extracted into 1X PBS buffer (pH 7.4) with varying buffer compositions and subjected to pressures up to 45000 psi to determine the effect of pressure on sperm and vaginal epithelial DNA recovery. Another goal of this study was to enhance sample recovery from cotton swabs. Cotton swabs are often used to collect evidence in a crime scene but the inefficient sample recovery from this substrate has been the subject of numerous studies in the past. In order to enhance DNA recovery and hence improve downstream genetic analysis, the effect of alkaline lysis on sample recovery from cotton swabs was studied. Cotton swabs containing sample were incubated in different concentrations of sodium hydroxide under varying temperature and incubation times. Following extraction, all the samples were purified using phenol chloroform isoamyl alcohol purification and quantified with Promega Plexor® HY system followed by an STR analysis using Promega PowerPlex® 16 HS system. The results indicate that the use of pressure cycling technology (PCT) and optimized alkaline conditions selectively lysed female epithelial cells whereas exposing the sample to a higher temperature and a short incubation time in a water bath and in the presence of alkaline conditions produced lysis of sperm cells. The amalgamation of these two methods in a sequential manner resulted in the separation of female and male fractions from a mixture.

Pressure Cycling Technology (PCT) uses Barocycler® NEP 2320, a commercially available instrument from Pressure BioSciences Inc. (South Easton, MA), equipped with a hydrostatic pressure chamber that generates alternating cycles of ambient and high

pressures with a range of 5- 45 kpsi. Samples such as cotton swabs or cuttings of cloth can be directly extracted using this technique by simply placing them in a PULSE™ tube, a specially designed tube to withstand high pressures, along with an appropriate buffer. Different PCT parameters such as pressure and number of cycles were tested to obtain optimum recoveries and selective extraction of a single cell type. The data indicates less than 5% DNA recovery occurs from both male and female cells when pressure cycling technology was used as a stand-alone treatment for cell lysis. In the presence of reducing agents such as DTT and TCEP, almost 60-70% sperm DNA was recovered using a pressure treatment at 45000 psi for 60 cycles.

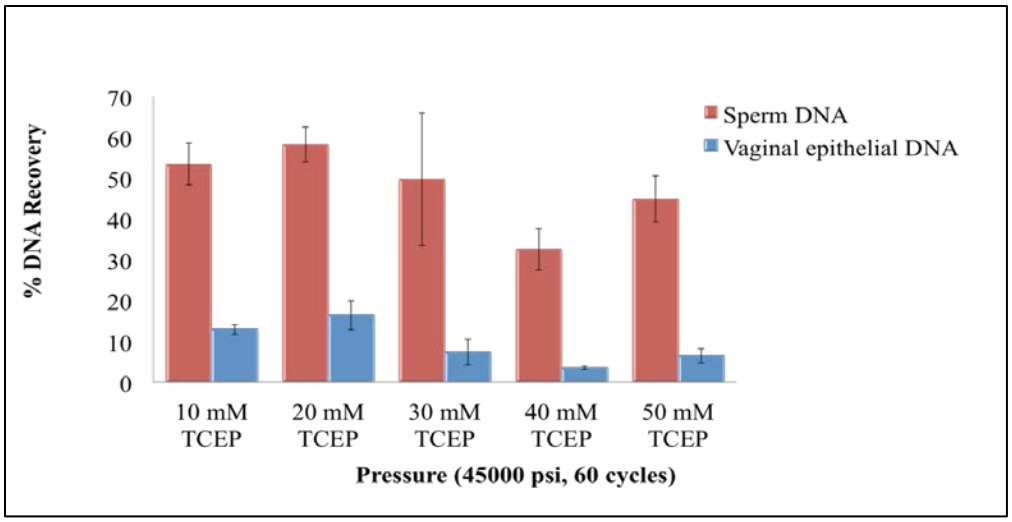


Figure 1. The effect of a stronger reducing agent (Tris (2-carboxyethyl) phosphine) on DNA recovery, with pressure treatment at 45000 psi for 60 cycles, indicates a significant increase in sperm DNA yields with minimal digestion of vaginal epithelial cells. (n=3 ± standard error)

Treatment with TCEP further improved selectivity with nine times more sperm DNA recovered compared to epithelial DNA, Figure 1. These results demonstrate the potential of this technology in analyzing samples from sexual assault casework that often contain mixtures of sperm and vaginal epithelial cells.

When the study was conducted on samples deposited on a cotton swab, the overall recoveries dropped to less than 5% with TCEP treatment at high pressure of 45000 psi. This is a significant loss in recoveries that may be attributed to the inefficient sample elution from the cotton matrix. Many buffer compositions including the use of detergents, cellulase enzyme, reducing agents, and temperature were studied in the process to determine their effect on cell recovery from a swab. The best yields from cotton swabs were obtained when the swabs were incubated in a DTT solution, but the overall recoveries were still low at 14%±4 and selectivity was compromised. The study demonstrates that pressure cycling technology improves DNA yields from liquid samples but requires additional treatments to obtain optimal yields when the sample is present on a cotton substrate. Since cotton swabs are commonly used for evidence collection in a crime scene, studies were done to improve recoveries from a swab while maintaining selectivity at the same time.

Hudlow et al. demonstrated differential recovery of sperm DNA using alkaline lysis treatment coupled with DNase digestion [4]. Application of the protocol described by Hudlow et al. to mixed stains indicated significant sample loss leading to poor DNA recoveries. Determination of the amount of DNA recovered at every step indicated that alkaline lysis results in lysis of both cell types. This study was extrapolated to observe the effects of alkaline lysis at different temperatures at different concentrations of sodium hydroxide and at different incubation times without the use of DNase treatment that was mentioned in the paper. The results indicated that alkaline lysis without DNase treatment resulted in significant amount of DNA recovery from both sperm and epithelial cells depending on temperature and concentration of sodium hydroxide. Once the sodium

hydroxide concentration was optimized, the effect of PCT on sperm cells and epithelial cells in the presence of optimized concentration of sodium hydroxide was studied.

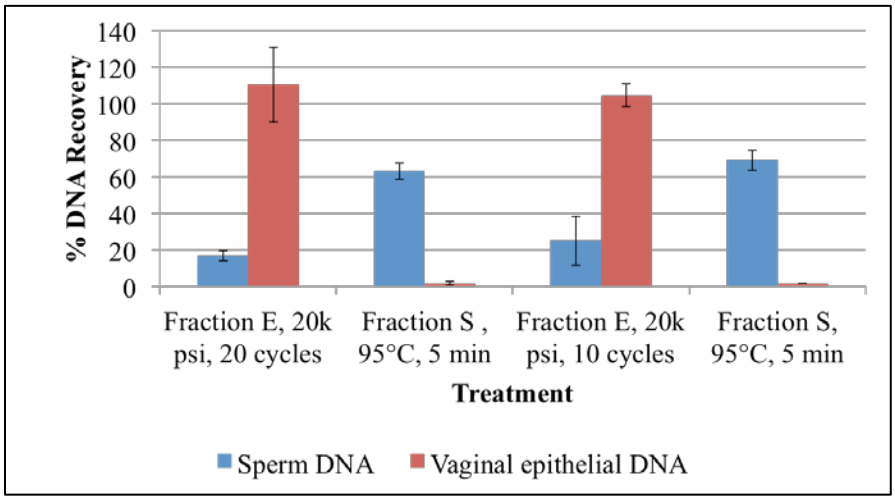
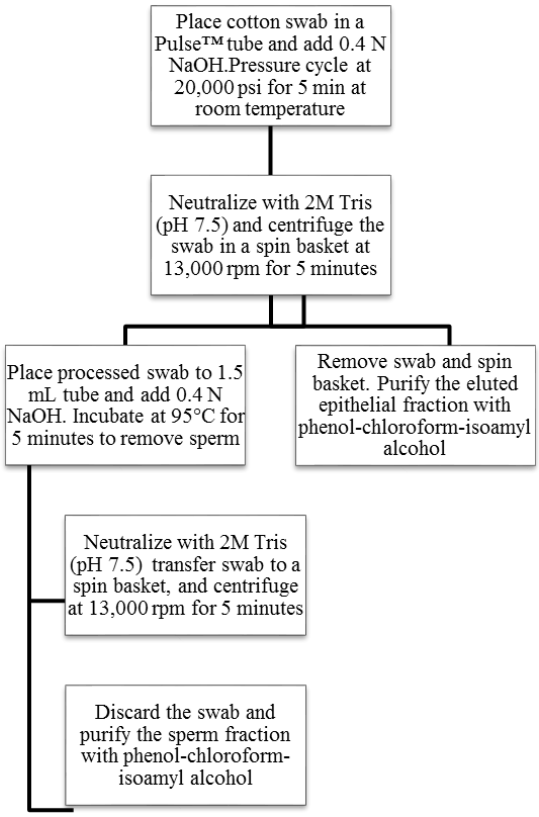


Figure 2. The effect of alkaline lysis and pressure cycling technology on DNA recovery from mixtures. The results indicate the recovery of female DNA (fraction E) with minimal sperm lysis after pressure treatment. More sperm DNA was recovered in the second step following exposure to high temperature under alkaline conditions (fraction S). (n=3 ± standard error)

For alkaline conditions, commercially available sodium hydroxide was dissolved in HPLC grade water in concentrations ranging from 0.2 N NaOH to 1.0 N NaOH to determine the effect of alkalinity on lysis of sperm cells and epithelial cells. Apart from this, the effect of temperature and incubation time at different temperatures, under varying concentrations of sodium hydroxide was also studied to determine the optimum parameters for differential lysis. By varying pressure and number of cycles, it was observed that under alkaline conditions and at 20,000 psi pressure for 10-20 cycles, epithelial cells were completely lysed with minimal lysis of sperm cells whereas more than 70% of sperm DNA was recovered when the sample was incubated at 95° C for 5 minutes, Figure 2. Based on

these results, a two-step protocol was developed using pressure cycling technology (PCT) and alkaline lysis for differential extraction of mixtures, Figure 3.

Figure 3. Flowchart depicting the protocol for differential extraction of mixtures using alkaline lysis and pressure cycling technology



In the first step, the swab containing the mixture was transferred to a PULSE™ tube containing 0.4N NaOH and was subjected to 20,000 psi pressure for 10 cycles. After pressure treatment, the swab was transferred to a 1.5 mL microcentrifuge tube and the remaining solution in the PULSE™ tube was subjected to phenol chloroform isoamyl alcohol (PCIA) purification to remove cellular debris and purify DNA for downstream analysis. In the second step, 0.4N NaOH was added to the swab from step one and

incubated at 95°C for 5 minutes in a water bath. Following incubation, the swab was transferred to a spin basket and centrifuged at 13000 rpm for 5 minutes and the DNA from the eluate was recovered with PCIA purification. A comparison of the genotypes of the purified fractions with sperm and epithelial controls indicated that this method successfully separated male and female fractions from mixture. The result of the treatment is a rapid and effective separation of a mixture of sperm and epithelial cells such as might be collected following a sexual assault. Figure 4 provides an example of the resulting isolation of sperm and epithelial cells from a post coital sample in which the new procedure is compared with a standard differential extraction carried out at the Broward Sheriff's Office DNA laboratory.

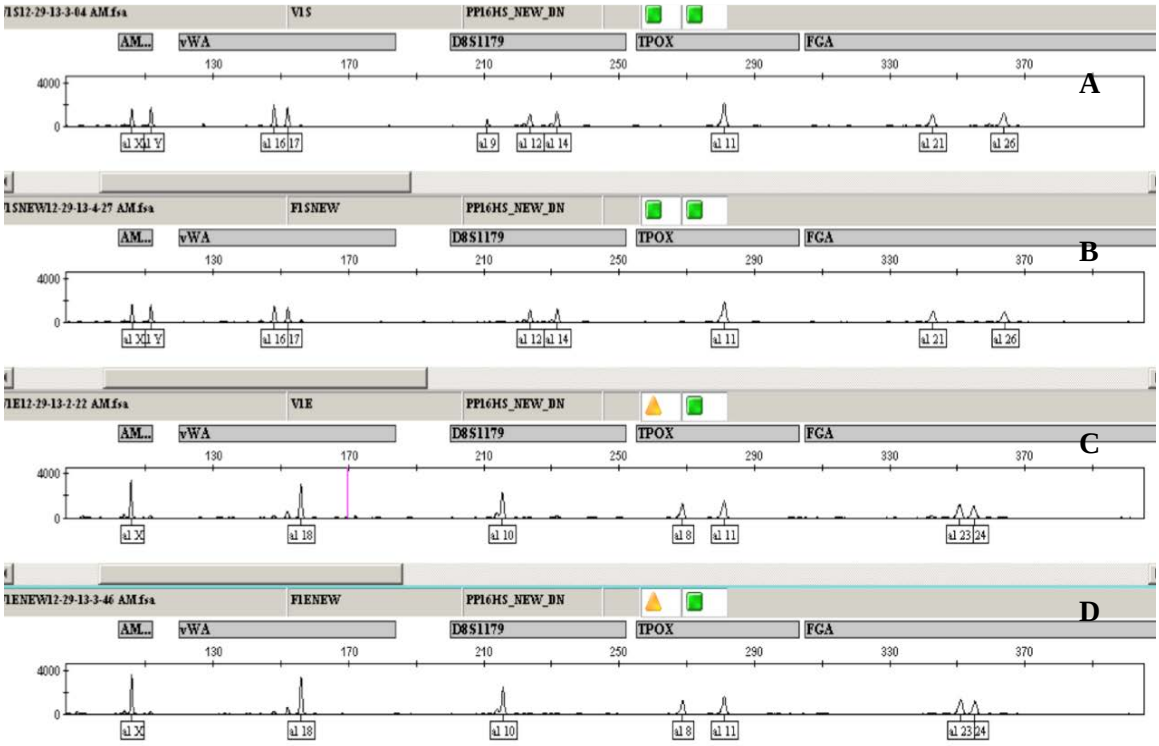


Figure 4. STR profiles of sperm and epithelial fractions extracted from a post-coital swab. One swab was processed using Broward sheriff's office (BSO) crime lab method and another swab from the same volunteer was processed using alkaline lysis and pressure cycling technology. A) Sperm fraction recovered using BSO protocol B) Sperm fraction

recovered using alkaline lysis and pressure cycling technology C) Epithelial fraction recovered using BSO protocol D) Epithelial fraction recovered using alkaline lysis and pressure cycling technology. The profiles indicate identical genotypes were obtained using both the protocols.

Different variables such as environmental conditions, inhibitors, and sample substrate affect the final outcome of analysis. Following the SWGDAM guidelines, the efficiency of the new lysis method was evaluated in the presence of PCR inhibitors, after exposure of the samples to environmental insults, and when the sample was present on different substrates. The sensitivity of the method was evaluated to determine the effect of variable mixture ratios and quantity of sample on the ability to generate a conclusive autosomal STR profile. A reproducibility study to gauge the consistency of this method and a correlation study to evaluate how this method compares to existing protocols were also done.

An inhibitor mix was prepared consisting of 12.5 mM indigo, 0.5 mM hematin and 2.5 mg/mL humic acid. Semen samples were diluted to 1:50 and 50 µL of the diluted semen was added to a vaginal swab. The swab containing the mixture was spotted with 5 µL of inhibitor mix. A small cutting of the air-dried swab was extracted. The results from stability studies indicate that alkaline lysis and PCT successfully recovered DNA from samples exposed to an inhibitor mix containing humic acid, hematin and indigo. Complete male autosomal profiles were obtained from all samples including swabs that were exposed to the outside environment for one week. Two kinds of studies were performed to determine the sensitivity of this extraction method. In the first study, variable ratios of mixture of sperm cells and epithelial cells were extracted to evaluate how this method performs in

enriching sperm fraction and generating a conclusive male DNA profile when the sample is overwhelmed with vaginal epithelial cells. The results from sensitivity studies indicate that a clean male autosomal STR profile can be obtained with samples containing a 1:1 mixture. Above this level, increasingly larger amounts of female DNA are present, Figure 5.

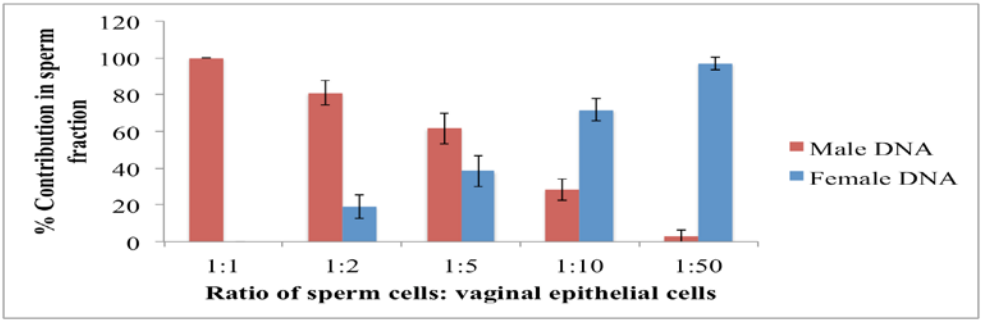


Figure 5. Sensitivity studies. Sperm fraction enrichment is observed in samples overwhelmed by almost ten times more female cells. More male DNA is recovered from samples containing up to five times more female cells.

A complete male autosomal STR profile was obtained from samples with ten times more female cells. Loss of male alleles were observed when samples had female component in excess of fifty times to that of male cells. For the second sensitivity study, the total number of cells was reduced while maintaining a mixture ratio of 1:5 sperm to epithelial cells. This was done to determine the effect of alkaline lysis and PCT on recovering sufficient DNA yields to generate an autosomal STR profile from low samples levels. Post-coital samples were extracted using alkaline lysis coupled with pressure cycling technology, organic differential extraction, and a selective digestion method used by the Broward sheriff's office (BSO) crime lab. This was done to compare the DNA yields and STR profiles recovered using the new protocol with established practices. Most of the practicing labs

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use some modification of the organic extraction protocol. This comparison study was therefore done to evaluate the performance of the new alkaline method by comparing the DNA yields in the final sperm fraction. The data indicates that alkaline-based pressure cycling lysis recovered more male DNA from all post-coital samples compared to organic differential extraction. These yields were either comparable or better than the samples extracted using Broward sheriff's office (BSO) method but female DNA carryover and loss of male profile is observed when the sample is overwhelmed by fifty times female tissue.

In summary, alkaline lysis and PCT method uses inexpensive buffers, has a very short extraction time, and more importantly, can recover most of the DNA from the matrix, which is a significant improvement to the methods in existence.

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2) Vuichard S., Borer U., Bottinelli M., Cossu C., Malik N., Meier V., Gehrig C., Sulzer A, Morerod M., Castella V. Differential DNA extraction of challenging simulated sexual assault samples: a Swiss collaborative study. *Investigative Genet*ics **2011**, *2* (1), 11.
3) Gill P., Jeffreys A. J., Werrett D. J. Forensic Application of DNA "fingerprints". *Nature.* **1985**, *318* (12), 577-579.
4) Hudlow W. R., Buoncristiani M. R. Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence. *Forensic Science International Genetics* **2012**, *6 (1)*, 1-16.

Appendix: Dissemination of Research Findings

1) Nori, D. V., McCord, B. R., A novel method to achieve differential lysis of mixtures with the aid of alkaline lysis and pressure cycling technology (PCT), *Forensic Science International: Genetics* 2014 (submitted)

2) Dimsoski P., Martinez V., Nori D., McCord B. The application of immunomagnetic capturing of epithelial cells for forensic differential extractions, *Journal of Forensic Sciences* 2014 (submitted)

3) Nori D., McCord B., 2014. "The role of alkaline lysis and pressure cycling technology in DNA recovery from mixtures" (Oral presentation) American Academy of Forensic Sciences (AAFS) 66th annual scientific meeting, Seattle, WA.

4) Nori D., McCord B., 2013. "Application of pressure cycling technology (PCT) in differential extraction" (Poster presentation) American Academy of Forensic Sciences (AAFS) 65th annual scientific meeting, Washington, DC.

5) Nori D., McCord B., 2012. "Differential extraction of mixtures in sexual assault casework using pressure cycling technology (PCT)" (Poster presentation) American Academy of Forensic Sciences (AAFS) 64th annual scientific meeting, Atlanta, GA.

6) Nori D., McCord B., 2011. "Application of pressure cycling technology (PCT) in differential extraction" (Oral presentation) American Academy of Forensic Sciences (AAFS) 63rd annual scientific meeting, Chicago, IL, USA. (Update)

7) Nori D., McCord B., 2011. "Application of pressure cycling technology (PCT) in differential extraction" (Poster presentation) American Academy of Forensic Sciences (AAFS) 63rd annual scientific meeting, Chicago, IL, USA. (Update)

8) Nori D., McCord B., 2010. "Application of pressure cycling technology (PCT) in differential extraction" (Poster presentation) 21st International Symposium on Human Identification (ISHI), San Antonio, TX, USA.

Publications

Nori, D. and McCord, B. A novel method to achieve differential lysis of mixtures with the aid of alkaline lysis and pressure cycling technology (PCT), Analytical and Bioanalytical chemistry, in preparation.

Nori, D. A novel method for rapid and selective extraction of male DNA from rape kits using alkaline lysis and pressure cycling technology. PhD Thesis, Florida International University, 2014.

Patents

Invention disclosure, Bruce McCord, Deepthi Nori, Richard Schumacher, Nate Lawrence, Application of alkaline lysis and pressure cycling technology in differential extraction of mixtures, Provisional patent filed February 18, 2014.