FINAL TECHNICAL REPORT

Automated Processing of Sexual Assault Cases
Using Selective Degradation

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Authors: Dr. Christian Carson (PI), Dr. Alex Garvin (co-PI), Kim Gorman

Abstract

This research addressed the problem of obtaining a sperm DNA profile from sexual assault evidence samples that also contain a large amount of epithelial cell DNA, and the ability to automate the differential extraction process. The current standard differential extraction method leaves some amount of epithelial cell DNA in the sperm fraction of the sample, which will create a mixture of epithelial and sperm cell DNA and may partially or completely mask the sperm DNA profile. This may prevent conviction or even detection of the perpetrator.

This research used a nuclease to digest the remaining epithelial DNA in the sperm fraction of mock sexual assault samples. The research demonstrated the ability to obtain clean single-source male DNA profiles from mixed stains, under a variety of sample conditions. In addition, the research optimized this procedure, including reagent concentrations and volumes, and then validated the procedure for sensitivity, reproducibility and precision. Following that, the results of this selective degradation approach were compared to the results obtained with the current standard method of differential extraction. The selective degradation methodology was demonstrated to be compatible with the DNA purification methods currently used in public crime laboratories.

This methodology was designed to be compatible with a single tube method and with a 96 well plate automated method of differential extraction. It was specifically designed to be compatible with multiple liquid handling platforms in order to utilize existing equipment in public laboratories.

This research resulted in an optimized and validated process for obtaining single source male DNA profiles from mixed stains, greatly improving the ability of the law enforcement community to process and prosecute sexual assault cases. This process eliminates sperm fraction DNA profile mixtures from almost all sexual assault evidence containing sperm cells. It often permits crime laboratories to obtain single source male DNA profiles even in situations where the standard method of differential extraction does not produce a sperm fraction male profile.

As a result, in some cases where law enforcement was unable to identify any suspect for a sexual assault, they will now be able to obtain a full DNA profile of the perpetrator. This will allow certain current and cold cases to be solved and closed. This procedure saves time for DNA criminalists in performing differential extractions. It also eliminates the time drain and uncertainty caused by mixed DNA profiles. And, it allows the differential extraction process to be easily automated, which will help reduce the backlog of sexual assault evidence.

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Executive Summary

Synopsis of Problem

This research was designed to address the problem experienced in crime laboratories of obtaining a single source sperm DNA profile from sexual assault evidence containing both sperm cell and epithelial cell DNA. The removal of epithelial cell DNA from the sperm fraction of the sample has been a time consuming step that has met with limited success. Another aspect of the problem is that traditionally this process was very difficult or impossible to successfully automate.

The method used in a crime laboratory to obtain the sperm cell and epithelial cell DNA profiles from the mixed stain is referred to as differential extraction. The method of differential extraction in use for over two decades has relied on preferential lysis of the epithelial cells using a solution that will lyse epithelial cells but not sperm cells. This allows the scientist to separate the two types of cells.

The problem arises because there is often an extreme excess of epithelial cells as compared to the number of sperm cells recovered from the sexual assault evidence. Traditionally, a mild lysis solution is introduced to the tube containing the evidentiary sample. This lysis solution will lyse the epithelial cells, but is not stringent enough to lyse the sperm cells. After epithelial cell lysis is complete the tube is centrifuged and the sperm cells create a pellet in the bottom of the tube.

As much of the lysis solution as possible, without disturbing the sperm pellet, is removed from the tube. The sperm pellet is then diluted with a buffer, mixed, and again centrifuged to create a pellet. This dilution or washing step is generally repeated 3 to 5 times. The dilutions are time consuming and tedious and ultimately it is impossible to dilute away all of the epithelial DNA. When there is an extreme excess of epithelial DNA as compared to sperm DNA it is nearly impossible to dilute the epithelial DNA to a degree that there is more DNA from the sperm cells than from the epithelial cells. When significant amounts of epithelial DNA as compared to sperm DNA remain, a DNA mixture will be obtained. In cases where there is an excess of epithelial DNA it may be impossible to deconvolute the sperm profile or no sperm profile may be obtained.

Obtaining a mixture profile causes extra work involving the statistical analysis of the profile. It also provides defense counsel with an opportunity to raise doubts in court about whether the client's profile is even represented in the mixture, and may lead to acquittal.

If no sperm profile is obtained because of an excess of epithelial DNA, then the DNA evidence is not useful in identifying the suspect even though sperm were identified by the laboratory.

Purpose of Research Grant

If the amount of epithelial cell DNA in the sperm fraction can be reduced sufficiently or eliminated, then the sperm DNA profile can be identified, even if only a small amount of male DNA is present. Many theories have been posited to solve that problem, and much research has been performed to test those theories, but the problem persists.

The purpose of this research grant was to test, develop and prove another theory for the removal of extraneous DNA from the sperm fraction of a differential extraction. In particular, this research was an attempt to develop a technique invented by Dr. Alex Garvin to avoid the repeated wash/dilution steps necessary when performing a differential extraction and, instead, to use a nuclease to digest the residual epithelial DNA in the sperm fraction.

Dr. Garvin published his initial research in the Journal of Forensic Sciences [1]. The purpose of this NIJ grant was to transform Dr. Garvin's approach from an academic bench-top experiment, using pristine samples under very controlled conditions, to proving it useful as a forensic application in crime laboratories, with samples of varying age and condition.

A further purpose was to develop a 96 well automated protocol that would function as well as, or better than, the traditional single tube method of differential extraction under a normal range of conditions.

It was posited that using selective degradation would eliminate virtually all of the epithelial cell DNA in the sperm fraction by degradation of free DNA in solution. This would be a much more efficient and effective approach than trying to rinse and dilute away the epithelial DNA remaining in the sperm fraction. It was posited that this technique could eliminate mixed DNA profiles in sexual assault evidence, and provide sperm cell DNA profiles from mixed stains even in cases where no sperm cell DNA profile can currently be identified.

By eliminating the traditional manual wash/dilution steps used to dilute extraneous DNA from the sperm fraction, the steps necessary to process a differential extraction could be automated on a liquid handling robot.

This grant was awarded to Paternity Testing Corporation (PTC) in Columbia, Missouri. The Principal Investigator is Dr. Christian Carson, who is on staff at PTC. The co-PI is Dr. Alex Garvin. The award period started on November 1, 2009 and ended on October 31, 2011.

In the process of working with Dr. Garvin's proprietary technology, PTC has also developed a kit that makes the selective degradation approach easy for a crime laboratory to duplicate. The kit includes color coded test tubes, reagents and a protocol. The kit was dubbed "E*rase*," in a naming contest won by Kathy Press in the Phoenix crime laboratory of Arizona DPS. It was a play on words that alludes to the nuclease component of this process and describes the nature of the process because it "erases" epithelial cell DNA from the sperm fraction of sexual assault

evidence. Throughout this paper the terms "E*rase*," "E*rase* kit," "selective degradation," and "the selective degradation approach to differential extraction" are used synonymously.

Research Design

In pursuing the above referenced goals the research was designed, first, to optimize the various components of the selective degradation procedure, in order to consistently obtain the best possible. Following optimization, the research design addressed validation of the procedure, including testing to establish the sensitivity, reproducibility, and precision of the selective degradation process. Research design then turned to automation.

One aspect of research design was determining the type and source of samples to test. It was determined to use noncoital vaginal swabs for certain aspects of the experiment, such as determining the amount of nuclease needed to completely digest epithelial DNA under varying conditions. Mock postcoital swabs were developed by adding semen to noncoital vaginal swabs. Actual postcoital swabs were also obtained and tested. Those swabs were collected at varying intervals following intercourse, to test the effect of the passage of time, along with the consequent reduction in harvested quantity of sperm DNA, on the results of the selective degradation process. Samples were obtained from volunteers, and from cooperating medical practices, after the patients signed the necessary disclosure and consent forms.

Soon after research began it became apparent that the research design for this grant would need to be modified. While the inventor believed that the technology was ready for forensic use, after applying the selective degradation technique to a variety of samples, it was apparent that there was a need for more extensive optimization of the originally described technique. The development of automation techniques could not begin until the procedure was optimized to perform consistently with a wide variety of samples. During the course of grant research, it was possible to complete a full range of optimization experiments.

The optimization experiments were designed to vary the incubation times and temperatures for the DNA extraction process, the variables of the nuclease treatment, and the sperm cell lysis process. Experiments were performed comparing the inclusion of a test-tube change during the procedure with leaving solutions in the same tube throughout. Experiments were also designed to determine the differences in result if the substrate material remained in the extraction throughout the process of selective degradation.

For each of those optimization experiments, the results were measured for sperm cell DNA yield and for the elimination of epithelial cell DNA, so that the optimum procedures, reagent amounts, and reagent concentrations could be determined.

The research design also included validation studies on the procedure. Experiments were designed to show the sensitivity, reproducibility and precision of results using this procedure, as well as the effect of the sample's age.

Many of the experiments were performed side by side with the traditional method of differential extraction. Results obtained by the selective degradation approach were compared with results obtained under the traditional method, focusing on situations in which relatively small numbers of sperm cells, as compared to the numbers of epithelial cells, are contained in the sample. In addition to comparing the quality and quantity of DNA profiles obtained under the two methods, the yield of sperm DNA resulting from selective degradation was compared to the yield using the traditional differential extraction method.

The results of the selective degradation approach were also compared to the results produced by Differex, a product marketed by Promega Corporation that is an alternative to the traditional method of differential extraction.

Various public crime laboratories participated in beta testing of the procedure. These laboratories have repeated many of the same experiments and for the most part have obtained consistent results. In a couple of those cases the samples were handled differently and in those cases the results varied. Following certain precautions in sample handling and adhering to the protocol should prevent those problems.

Following optimization and validation, experiments were designed to verify the compatibility of the selective degradation technique with the most popular methods of DNA purification used by crime laboratories.

Experiments were performed to demonstrate the ability to automate the differential extraction process by using the selective degradation approach.

Findings

By optimizing the variables in the selective degradation process it was possible to create a protocol that is successful with a wide range of samples. After optimization, experimental results showed the consistent ability of the selective degradation process to provide sperm fraction profiles that were either single source sperm DNA or sperm DNA profiles with traces of the epithelial cell DNA profile.

The sperm fraction DNA profiles obtained from the selective degradation process were consistently either equal to or better than the profiles obtained using the standard differential extraction method.

In situations with relatively few numbers of sperm cells as compared with the number of epithelial cells, the selective degradation process produced complete, sperm DNA profiles in some situations where the traditional method produced either a mixture profile, or only an epithelial cell DNA profile.

There were instances where the selective degradation approach appeared to result in recovery of a smaller amount of sperm DNA from samples processed side by side with the standard differential extraction method but, even then, the

selective degradation sperm DNA profile results were better because of the elimination of epithelial DNA from the sample. Ongoing research will address ways to reduce the loss of sperm cell DNA.

The selective degradation approach to differential extraction eliminated mixed DNA profiles in nearly all instances.

Comparison with the Differex kit results indicated that, with fewer sperm cells, the selective degradation approach obtains clean single source sperm DNA profiles even when the Differex kit obtains mixture profiles with the epithelial cell DNA as the major contributor.

The selective degradation approach was compatible with all of the DNA purification methods commonly used by crime laboratories. The tested purification methods included phenol chloroform isoamyl alcohol with EtOH precipitation or size filtration, Qiagen EZ1, Maxwell16, DNA IQ, and AutoMate Express.

Experiments demonstrated that, by using the selective degradation approach, the process of differential extraction can be easily automated. By eliminating the standard differential extraction washing steps, the process of differential extraction has been reduced to a series of liquid handling steps which are easily managed by the standard robots in use in crime laboratories.

The automation possibilities range from hands on moving trays in and out of incubation steps to a completely hands off approach, with the probable exception of the centrifugation step after the initial lysis. However, Hamilton is introducing a liquid handler that also incorporates the centrifugation step. The Hamilton platform's overall mechanism for handling the DNA sample appears to be tailor made for this process.

Conclusions and Implications for Policy and Practice

The implications for policy and practice are substantial. The results of this research mark the successful culmination of federal research expenditures and numerous efforts by the forensic community, researching various proposed mechanisms to eliminate the serious problem of mixed DNA profiles in sexual assault evidence.

Among the specific benefits of this technology, it will identify perpetrators of sexual offenses who could not previously be identified using DNA evidence. It will save time in the forensic laboratory and in court, and it can be expected to increase the rate of convictions of perpetrators of sexual assaults. It will also allow automation of the process of differential extraction, and consequently facilitate reduction of the backlog of sexual assault evidence.

The following is a list of some of the major impacts of this research on law enforcement practices and outcomes:

1. Mixtures in sexual assault evidence will be eliminated in almost all cases;

2. Time will be saved in the performance of differential extractions in the crime lab. Less hands on time and elimination of the rinsing steps to dilute the epithelial cells in the male fraction, as well as shorter incubation times, cut the overall processing time to roughly one third of the time needed for a standard differential extraction.

3. Time and effort previously devoted to mixtures in the laboratory involving the statistical analysis is eliminated in most cases. If the analyst would have previously obtained a mixture profile from the same sample and now obtains a single source male profile, then the hours or even days of mixture deconvolution, statistical calculation and review time for a single case may be reduced to minutes.

4. The time, effort and uncertainty resulting from mixtures at trial, possibly raising “reasonable doubt” and causing the criminal to be set free, is eliminated if the DNA profile is a single source profile.

5. In some cases, clean single-source sperm DNA profiles will now be obtained where no profile was previously available. Already, using Er*ase* on a case in Missouri for which the crime laboratory was unable to detect any discernable sperm DNA profile in the mixed stain using a traditional differential extraction resulted in a full male profile in the sperm fraction.

6. Because of the ability to successfully profile smaller amounts of sperm DNA in mixed stains than previously possible, certain current and cold cases that had no identified suspect can now be solved.

7. For case work, the resulting ability to easily automate the differential extraction process will save additional time and manpower in crime laboratories that process larger volumes of sexual assault evidence. This method of differential extraction makes it possible for a single analyst to take a 96-well tray from the initial lysis step to DNA purification with only a few minutes of hands on time.

It will be possible for crime laboratories to automate whatever portion of the process is compatible with their existing equipment, without being forced to spend funds to obtain equipment just for this purpose.

Manufacturers of the most popular robots currently in use in crime laboratories in the United States and Europe, which are Tecan, Beckman-Coulter, and Hamilton, were contacted. Collaboration with those companies to develop scripts for the robots to be able to automate differential extractions using selective degradation is in progress. The scripts will be of major assistance to those crime laboratories choosing to automate.

The Er*ase* kit is now commercially available for the single tube method, and is available for custom ordering in the 96 well format.

8. The resulting ability to easily automate the differential extraction process will be a tremendous benefit in reducing the backlog of sexual assault evidence.

Changes in Policy, Practice and Outcomes in the Forensic Laboratory

Since this technology makes it much more likely that an autosomal sperm fraction DNA profile will be produced, it changes the strategy and overall outcome of a sexual assault case in many instances. If a sperm fraction autosomal profile can be produced then the profile can be searched in CODIS. If a CODIS search reveals the identity of the assailant then many more sexual assaults may be prevented, and an overall reduction in the number of sexual assaults results in a smaller caseload.

It is the policy or practice of some crime laboratories to eliminate the differential extraction step altogether if a minimum number of sperm are not identified before DNA extraction. This leaves only the option of Y Chromosome analysis in order to identify the rapist. This results in less certain identifications and the inability to perform productive CODIS searches. With selective degradation, it will be possible to obtain an autosomal profile in many of those cases and the overall outcome may be a change in policy that allows for differential extractions to be performed whenever sperm are present.

The policies and practices employed by crime laboratories for sexual assault evidence may change as a result of this product or there may be no need to change the policies and practices because using this product may change the nature of the evidence and therefore change the overall outcome of the case. If, for instance it is the policy of the crime laboratory to perform Y Chromosome testing any time that the quantitation data indicates that there is 4 times more epithelial DNA than sperm DNA in a sample, and a case has 5 times as much epithelial DNA as sperm DNA, then Y Chromosome testing is performed. If that same case, because of selective degradation, results in the laboratory finding virtually all male DNA, and it is the policy of the laboratory to therefore perform autosomal DNA testing, then the policy did not have changed, but the selective degradation method will have caused a change in the subsequent procedures and possibly the outcome. The policy and practices of the laboratory in this instance do not need to change in order to cause a change in the outcome of a case.

The impact on policies and practices for sexual assault cases will vary from one laboratory to the next. In some instances, there will be little or no impact on policy and practices. In other laboratories there may be a very significant impact. What will show significant impact in all crime laboratories is the outcome of many cases, the overall time required to complete each case, and the likelihood that a DNA identification will be made.

The E*rase* kit has either been validated in, or is currently undergoing validation by, more than 20 U.S. crime laboratories, as well as several laboratories in Europe. Additional laboratories have expressed interest and, as others go online and report positive outcomes, it is expected that many more laboratories will also validate the kit. Several crime laboratories have completed validation of the single tube kit and

are either on line or are completing competency exams. They are pleased with the results they are witnessing, and are anxious to take advantage of this technology as soon as possible.

Completing this Final Technical Report, and publishing the developmental validation study, should give all crime laboratories the necessary confidence in this technology to begin pursuing it as soon as they have the time and resources.

This successful NIJ grant has produced a tremendous benefit to law enforcement in the United States, and around the world.

I. Introduction

1. Statement of the Problem

The problem that this research was designed to address is the problem experienced in crime laboratories of the difficulty of obtaining a good DNA profile for the male perpetrator of a crime, from sexual assault evidence that also contains the DNA of the victim. If there is much more epithelial cell DNA than sperm DNA, it may not be possible to identify the male's DNA profile at all. If the amount of epithelial cell DNA can be reduced sufficiently, then the sperm DNA profile can be identified.

The differential extraction process for obtaining sperm cell and epithelial cell DNA profiles from a mixture of cells in sexual assault evidence was first developed by Gill, Jeffreys and Werrett, in 1985 [2]. Since then this procedure has been used by most crime laboratories and the methodology has not changed significantly. The method in use for over two decades has relied on selectively lysing the epithelial cells while leaving the sperm cells intact. Centrifuging the solution of lysed epithelial cells and intact sperm cells results in a sperm pellet in bottom of the microfuge tube. As much of the lysis solution as possible, without disturbing the sperm pellet, is removed from the tube. The sperm pellet is then diluted with a buffer, mixed, and again centrifuged to create a pellet and the supernatant is removed. This dilution or washing step is generally repeated 3 to 5 times. The dilutions are time consuming and tedious and ultimately it is impossible to dilute away all of the epithelial DNA. When there is an extreme excess of epithelial DNA as compared to sperm DNA it is nearly impossible to dilute the epithelial DNA to a degree that there is more DNA from the sperm cells than from the epithelial cells. When significant amounts of epithelial DNA as compared to sperm DNA remain, a DNA mixture will be obtained. In cases where there is an excess of epithelial DNA it may be impossible to deconvolute the sperm profile or no sperm DNA profile may be obtained.

Obtaining a mixture profile causes extra work involving the statistical analysis of the profile. It also provides defense counsel with an opportunity to raise doubts in court about whether the client's profile is even represented in the mixture, and may lead to acquittal.

If no sperm DNA profile is obtained because of an excess of epithelial DNA, then the DNA evidence is not useful in identifying the suspect even though sperm cells were identified by the laboratory.

2. Literature Citations and Review

Forensic laboratories generate STR profiles of sperm cell DNA obtained from vaginal swabs taken from rape victims (and from other articles). The isolation of relatively pure sperm DNA from a vaginal swab continues to be a process that is tedious, and difficult to effectively automate. The standard differential lysis method for processing sexual assault cases (2) relies on separation of intact sperm from the DNA of digested epithelial cells by centrifugation and careful removal of supernatant, a process that remains virtually unchanged since it was first described in 1985, in spite of efforts to improve this process and to develop alternatives (3). For example, Y chromosome polymorphic markers can be amplified from unfractionated DNA (4-6). However this approach has the following disadvantages: (i) if the ratio of epithelial DNA to sperm DNA is too large, a Y Chromosome profile will not be obtained; (ii) the data provided cannot be used to probe the autosomal STR profiles in the FBI CODIS database; (iii) if the rape victim is male, a mixed profile or no suspect profile will be obtained; and (iv) males with the same paternal lineage usually have identical Y chromosome STR profiles. Since some paternal lineages contain many males with the same Y chromosome STR pattern, Y chromosome profiles have limited utility as compared to autosomal profiles because they do not provide the identity of the rapist.

Another approach toward avoiding selective lysis is to physically separate sperm from intact epithelial cells. This has been done by flow cytometry (7) however this technique is unlikely to be applied to casework due to the expense of cell sorters and the difficulty of operating them. Attempts have also been made to use anti-sperm antibody coated magnetic beads (8). Epitope stability, however, was a problem with this approach when applied to casework because detergents are required to efficiently elute sperm from the swabs and these detergents destroy the epitopes recognized by the anti-sperm antibodies. Sperm can also be physically separated from the much larger intact epithelial cells by size using a 10 micron filter (9), or from digested epithelial cells by collection on a 2 micron filter (10). However, these filtration methods still require centrifugation, and do not provide male fraction DNA from postcoital vaginal swabs that is as good as or better than that provided by the standard method. Laser dissection of sperm from a slide has also been proposed (11-12), but this method is low throughput and will most likely not be adopted for routine processing of sexual assault cases. The Differex method from Promega and a new version of Differex (13) both require manual steps in order to process a swab cutting to extraction ready male and female fractions .

3. Hypothesis/Rationale for Research

As noted above in the Statement of the Problem and in the Literature Review, the approach under traditional differential extraction of attempting to rinse the

remaining epithelial cells away from the sperm pellet never removed all of the epithelial cells. The remaining epithelial cells interfered with obtaining the sperm DNA profile. In many cases, using the traditional differential extraction method, it was only possible to obtain a mixed result, showing both the sperm cell DNA and epithelial cell DNA profiles, and sometimes only a portion of the sperm cell DNA profile. In other cases, it resulted in no sperm cell DNA profile being identified.

The hypothesis for this research is that the residual epithelial cell DNA in the sperm fraction of the evidence sample can be digested by application of a nuclease with appropriate supplemental reagents, rather than by attempting to rinse away the epithelial cell DNA.

By selectively degrading only the unwanted epithelial cell DNA, a sperm DNA profile is generated. In addition, this selective degradation differential extraction process can be effectively automated without loss of sperm cells or any other detrimental effect of the process.

The predicted result from this hypothesis is a demonstration that the hypothesis is true, and a demonstration that these procedures work.

The entire grant research involves investigations into the validity, optimization, comparative performance, and automation of the approach put forward in this hypothesis.

Dr. Alex Garvin, the inventor of the underlying technology for this selective degradation approach and the co-investigator on this grant, had submitted a research paper to the Journal of Forensic Sciences in 2008 [1] demonstrating that he had been able to accomplish this result in a research laboratory setting. A major purpose of the grant, and a major part of the work under the grant, was to apply Dr. Garvin's approach to forensic samples under a wide range of conditions, demonstrating its validity, and optimizing its performance, in a forensic laboratory setting both for individual casework and for 96-well-plate automation.

The range of conditions under which experiments were performed include testing samples containing varying amounts of sperm cells and testing postcoital swabs collected at varying intervals following intercourse, up to 72 hours later. A variety of substrates that are commonly available in sexual assault cases were tested. Samples varying in age from hours after collection to more than 11 years after collection were tested. Conditions of DNA extraction, centrifugation, nuclease treatment and sperm cell lysis were optimized for use in a forensic laboratory. Experiments that varied the incubation times and temperatures, tube types, and the salt and nuclease concentration were performed in order to achieve the optimal results. The necessity for changing tubes after the nuclease treatment was also explored and evaluated. Each area was evaluated in regard to elimination of epithelial cell DNA and in regard to sperm cell DNA yield.

This research also validated the technology for forensic use by testing sensitivity, reproducibility, precision, and sperm cell mixtures.

The original hypothesis included the supposition that if the epithelial cells could be selectively degraded, that this method would produce results that were superior to the results achieved by existing technology. The results of the selective degradation method were then compared with results obtained under the traditional method. Because both methods provide satisfactory results when there are large quantities of sperm cells, the focus was on situations in which relatively few sperm are contained in the sample. In addition to comparing the quality and quantity of DNA profiles obtained employing the two methods, sperm cell DNA yield was compared between the two processes.

After comparing the selective degradation method to the traditional method of differential extraction, selective degradation was also compared to the results of differential extraction using Differex. This work was performed in a crime laboratory that routinely uses the Differex product marketed by Promega Corporation for differential extractions. Differex separates the sperm cells and epithelial cells using a gradient.

This research also demonstrated that selective degradation is compatible with downstream DNA purification techniques commonly used by crime laboratories.

After the optimization and compatibility experiments were completed, experiments were performed to demonstrate that it is possible to automate the differential extraction process using this selective degradation approach.

II. Methods

The experimental design of this research started with the proprietary method discovered by Alex Garvin for performing differential extractions using selective degradation in order to remove epithelial DNA remaining in the sperm fraction of a sexual assault sample. A kit, called E*rase*, was designed to deliver this procedure to forensic laboratories in a format that is easy to implement.

This grant research was designed to demonstrate the viability of this approach for use in forensic laboratories across a variety of conditions. Experiments were designed to optimize and validate the procedure, compare it to current methods of differential extraction, to demonstrate the compatibility of this approach with popular downstream DNA purification methods, and to develop a system that allows for 96 well plate automation of the selective degradation process using equipment typically available in crime laboratories.

The individual experiments are described in the Results section of the paper.

This Methods section describes sample acquisition and preparation, certain protocols, and other matters related to methods used in this research.

The selective degradation approach to differential extraction has been made into a commercially available kit, called E*rase* Sperm Isolation Kit. The experimental design of this research includes repeated comparisons of results from the E*rase*

approach and the standard approach to differential extraction. To give the reader an understanding of where the differences between the two methods are found, the following table is provided.

Comparison of relative time investment for E*rase* versus the Standard differential extraction Method for single tube extractions.

	E*rase* (single tube)	**STANDARD**
1.	epithelial cell lysis (1 hr.)	epithelial cell lysis (2 hr.)
2.	centrifugation, (5 min)	centrifugation (5 min)
3.	remove female fraction by pipetting all but 50ul of supernatant (< 5 min)	remove female fraction by pipetting as much supernatant as possible without disturbing sperm pellet (< 5 min)
4.	add Solutions 1 and 2 (seconds)	wash steps, repeated 3-5 time (5+ min each)
5.	sperm cell lysis (15 min)	sperm cell lysis (minimum 2 hr)
6.	Hands on after sampling 5 to 10 minutes	Hands on after sampling 20 to 30 minutes
7.	Overall process (< 2hr)	Overall process (> 4 hr)

This table shows the overall procedures employed in differential extraction for E*rase* and the standard method. The table demonstrates that, aside from the incubation times, the two methods are virtually identical except that at step 4 the E*rase* approach adds a nuclease to digest the remaining epithelial DNA in the sperm fraction, while the traditional approach attempts to remove the epithelial DNA by diluting it with repeated wash steps.

1. Sample Acquisition and Preparation

A. Semen Preparations and Sperm Cell Concentrations

Semen samples were obtained from donors, diluted in phosphate buffered saline (PBS), sperm cell concentrations calculated and ultimately used for semen-only samples or to spike noncoital vaginal swabs to prepare mock postcoital swabs.

Fresh semen was collected for each set of experiments to be sure of the integrity of the sperm cells. Fresh samples were used because actual sexual assault forensic samples are produced from freshly ejaculated semen that ultimately forms a dry stain. Considering the normal processing of sexual assault evidence, semen

diluted in water and stored was not consistent with a forensic sample. Further, it was discovered that the sperm cells in diluted semen could not be stored for long periods of time because they became too damaged and vulnerable to the nuclease. When semen was diluted in water, or when semen was stored in water or PBS for more than a day, the yield of extracted sperm cell DNA was significantly decreased compared to the DNA yield from semen freshly diluted in PBS then spotted and dried. For the research presented in this report, semen was diluted in 1X PBS in order to provide a more isotonic condition for the sperm cells. Further, semen dilutions were used within the first day after preparation. Using these conditions, the E*rase* extractions yielded substantial sperm cell DNA from semen that had been diluted.

Freshly ejaculated semen was collected by donors and allowed to liquefy. From this a dilution series was made using 1x PBS. Within 24 hours of PBS dilution, samples of the diluted semen were pipetted into tubes or onto swabs and allowed to dry. Semen was initially diluted PBS, then diluted serially 5 times in PBS. Two of the diluted samples (1/800 and 1/1600) were selected to count. After thorough mixing, 1ul of each was removed and placed onto a glass slide. The slide was heated in an 80°C oven for 20 minutes to dry and fix the cells. The slide was washed one time with 95% ethanol and then stained using the Christmas tree staining method [14]. Once the slides were stained and dried all of the sperm cells found within the 1ul region were counted. Based on the number of cells per 1ul, the concentration of sperm cells was determined for each dilution. Only serial dilutions made from dilutions with proportionately equivalent 1/800 and 1/1600 sperm cell counts were used for these experiments.

From the calculated cell concentrations, known numbers of sperm were pipetted either into tubes or onto cotton swabs for semen only samples, or onto noncoital vaginal swabs for mock postcoital swabs. In all cases, the samples were allowed to dry a minimum of overnight at room temperature. Samples placed into tubes were dried in a vacuum oven at 40°C.

B. Noncoital Vaginal Swabs

Vaginal swabs from female donors having abstained from unprotected coitus for a time period of greater than 72 hours were used to provide a source of vaginal epithelial cells for experiments with and without the addition of sperm cells.

C. Harvesting Epithelial Cells

Vaginal epithelial cells for experiments using epithelial cells without sperm cells were obtained by eluting cells from vaginal swabs. These were used to determine the amount of nuclease necessary to remove all epithelial DNA from the sperm cell fraction. Epithelial cells were eluted from noncoital vaginal swabs in microfuge tubes by vortexing the swab substrate in TE buffer, pelleting the cells with the centrifuge with the aid of spin baskets, and discarding the supernatant. The cell pellets were suspended in 100ul of TE^{-4} and transferred into a single tube. Five noncoital vaginal swabs are needed to obtain a series of samples representing

0.5, 1, 1.5 and 2 swab amounts of vaginal epithelial cells. From the total volume of cells obtained, 10% was used to represent 0.5 swab, 20% for 1 swab, 30% for 1.5 swabs and 40% for 2 swabs. After dividing the cell mixtures into different tubes, the samples were diluted to 400ul each with TE^{-4} and 8ul detergent and 7ul proteinase K was added. From this point the samples were processed with the E*rase* procedure.

D. Preparation of Mock Postcoital Swabs

Mock postcoital swabs were prepared by pipetting known numbers of sperm cells onto non postcoital vaginal swabs. The swabs were prepared from dilutions of fresh semen, spotted on the vaginal swabs and dried immediately (see 1-A. above). The mock postcoital swabs were utilized in testing for sensitivity and precision of the differential extraction procedures.

E. Collecting and Preparing Postcoital Swabs

Postcoital swabs were obtained from anonymous donors from collections made at varying specified times after unprotected coitus. The postcoital interval was recorded for each sample to indicate the expectation for the relative abundance of sperm cells on each sample. Postcoital swabs were collected from volunteers that kept record of the number of hours after coitus that the sample was collected. A sample of each collection was processed using E*rase* extractions to obtain epithelial and sperm cell fraction profiles.

Postcoital samples were most similar to sexual assault samples. Postcoital samples were routinely processed with side by side with the Er*ase* extraction procedure and the standard differential procedure. The sperm cell DNA yields were compared and it was determined that there was no significant difference in the sperm DNA yield between the methods.

For replicated experiments testing the nuclease and the sperm cell lysis steps of the Er*ase* procedure, postcoital swabs samples were utilized in the following manner. One or more swabs were processed using the first treatment step of the E*rase* procedure, the epithelial cell lysis step. With the E*rase* procedure, up to eight 50ul samples were derived from a single swab. Following epithelial cell lysis, the samples underwent centrifugation and the substrate swab material was removed. To prepare samples for replications, the pellet material was resuspended into the supernatant instead of removing any supernatant. From this suspension, 50ul aliquots (8 aliquots) were used to perform replicated experiments to study the subsequent steps in the E*rase* procedure. For larger experiments, multiple postcoital swabs were used and combined in a larger tube, mixed well and 50ul samples distributed into tubes for Er*ase* nuclease and sperm cell lysis step testing.

2. DNA Purification

Over the course of these experiments, sperm cell DNA fractions that were obtained from differential extractions were purified and concentrated using one of

the following methods. The preferable methods were phenol/chloroform extraction followed by ethanol precipitation (more labor, lower cost), the BioRobot EZ1 Workstation (Qiagen) (less labor, higher cost) or the Maxwell 16 (less labor, higher cost). Vivacon100 (Vivaproducts) also was used extensively. Vivacon 100 is relatively easy to use, but the recovery was not as consistent as the other methods.

3. Purified DNA Quantification

Total DNA yields were measured to estimate the amount of human DNA in a sample. Total purified DNA was quantified using fluorometry with pico green technology using either Quant-It reagents (Invitrogen) or QuantiFluor reagents (Promega). However, when vaginal swabs were used, abundant bacteria were often encountered. When samples of the Er*ase* pellet were observed with a microscope, intact bacteria cells that were stained with the Christmas tree stain could be observed along with the sperm cells. This indicated that bacterial DNA probably contaminated many sperm cell DNA fractions. However, total DNA quantification values were only used to compare yields from sperm cell-only samples, to measure yields from replicated experiments from equivalently divided postcoital swabs, and to estimate the amount of DNA needed for PCR amplification. Because of variable amounts of bacterial between different postcoital or noncoital collections, total DNA measurements were not used to compare yields in such samples. Samples for fluorometry were prepared according to the manufacturer's procedures and measured using methacrylate 1.5mL disposable cuvets in a Turner Quantech Fluorometer.

For direct measurements of human DNA, the MavenQST Quantitative Sex-Typing Kit was utilized.

Semen extender

The possibility that the yields of sperm cell fraction DNA could be enhanced if the sperm cells could be shielded better from the nuclease was explored. Semen extender (a product used to protect living sperm cells from the detrimental effects of freeze-thawing) was used in an attempt to protect forensic sperm cells from nuclease. For human semen extenders, the key ingredients were egg yolk and glycerol [15]. To incorporate these into the Er*ase* extraction during nuclease treatments, Solution #1 was reformulated to include them. The composition of the reformulated Solution #1 was: 0.05M MgCl, 0.05M CaCl, 0.05M Tris, 12% glycerol, and 20% egg yolk. Egg yolk was prepared by removing the egg yolk from a fresh egg and cooking it at 56°C for 30 minutes. Solution #1 prepared this way was used in replacement of the regular Solution #1 of the Er*ase* extraction procedure to determine whether sperm cell DNA yields could be increased.

4. Er*ase* Kit

The Er*ase* Sperm Isolation Kit consists of an extraction solution, proteinase K solution, and three proprietary reagent solutions involved in the introduction of

active nuclease into the extraction, the subsequent inactivation of the nuclease, and sperm lysis. Those three solutions are simply labeled Solution #1, Solution #2 and Solution #3.

A. Development of Kit Format.

The goal was to develop a user friendly kit that was intuitive. In a forensic setting it is extremely helpful to have kits that do not require constant consulting with the directions or that are likely to result in a mistake if the directions are not continually consulted. E*rase* was developed with this in mind. Color coding makes it easy to determine which solution is needed. The same volumes are pipetted with solutions 1,2 and 3. This makes it less likely that the incorrect volume will be pipetted. The kit was designed to minimize the likelihood of analyst error. Soon after the initial optimizing experiments for the procedure, the Beta version of the kit format was established.

An extraction solution, proteinase K solution, and three reagents are supplied in the kit. All solutions are prepared according to the recipes provided and aliquots are placed into the appropriate reagent bottles and vials. Each of the 3 reagents are single use reagents. They are discarded after use. There are 10 aliquots of each reagent in each kit so there is no need for the analyst to attempt to re-use the reagent. The 3 reagents are color coded red (#1), white (#2) and blue (#3). These colors were chosen to make it very easy for the analyst to know which reagent they are using without having to read the tube each time. The colors are very intuitive for USA analysts and the analyst can clearly associate the color scheme with the number of the reagent. Aliquots of the reagents were developed to provide safety from contamination due to reuse or damage from freeze thaw cycles. From this arrangement, E*rase* extractions can be performed in a consistent manner. Furthermore, a special microfuge tube is used for the DNA extraction (a 2.0ml Dolphin microcentrifuge tube, designed for pelletizing samples). This tube allows for easier removal of the supernatant without disturbing the sperm pellet and has the advantage of a 50ul mark that allows visual removal of all except 50ul or the supernatant without measuring.

B. Extraction Procedure

Extraction buffer with sample substrate (swab)

Samples for differential extraction were first extracted in a solution of detergent, Tris buffer, EDTA and proteinase K. The substrate material was placed directly into the extraction solution in a Dolphin tube and vortexed vigorously to thoroughly saturate the swab material with the extraction solution.

Epithelial cell lysis incubation

The sample was incubated in a 56°C oven for one hour.

Centrifugation

After incubation, the tubes were pulse centrifuged briefly to remove condensation from the lid and sides. The swab substrates were removed from the extraction

solution and placed into spin baskets inserted into the tubes. The samples were centrifuged for 5 minutes at 14,000xg.

Supernatant removal F1

After centrifugation the substrate and spin basket were discarded. The supernatant was carefully removed avoiding disturbing the pellet. Using the Dolphin tube and a pipette, the supernatant is easily removed down to a visible mark on the tube that leaves 50ul of supernatant in the tube above the pellet. Because it is not necessary for the pipette tip to get close to the sperm pellet, it is unlikely that sperm cells are lost.

C. E*rase* Differential Extraction Protocol

E*rase* Beta Protocol

E*rase* differential extractions were tested throughout the grant period under numerous varied conditions. For comparison of the experiments, all experiments were performed using the E*rase* Beta version of the protocol unless noted otherwise.

Subsequent to issuance of the Beta protocol, additional improvements were made regarding incubation times for the nuclease step, and for the sperm lysis step. The protocol that incorporates these changes is the current protocol for the E*rase* kit, and is referred to in this paper as the E*rase* Sperm Isolation Kit protocol. Experiments using the E*rase* Sperm Isolation Kit protocol are noted.

The differences in the E*rase* Beta protocol and the current E*rase* Sperm Isolation Kit protocol are as follows:

1) Nuclease incubations are for 15 minutes (Beta protocol 1 hour) at 37°C, and

2) Sperm cell lysis incubations are for 15 minutes (Beta protocol 5 minutes) at 56°C.

E*rase* Sperm Isolation Kit

1. For each sample to be extracted, add 400µL Extraction Buffer and 7µL Proteinase K solution (PK) to a 15mL conical tube and mix gently to create a master mix.
Example:
8 samples x 400µL lysis = 3200µL Extraction Buffer, and
8 samples x 7µL PK = 56µL PK added to 15mL conical tube

2. Place solid substrate (e.g. cutting, swab, etc.) into 2mL Dolphin Tube (Tube A). (When using either a half or complete sexual assault swab, it is recommended to use only the outer layer of the swab. Fewer sperm are trapped in the swab when only the outer layer is used.)

3. Pipette 400µL of the master mix from step #1 into each tube and vortex for 20 seconds. Pulse centrifuge for 2 seconds to remove liquid from the sides and cap of the tube.

4. Incubate at 56ºC for 1 hour to complete the PK digestion.

5. Pulse centrifuge samples to remove condensation from caps.

6. Using sterile forceps, place the substrate in a spin basket, place the basket back in the SAME tube, cap and centrifuge tube at maximum speed for 5 minutes.

7. Remove spin basket and discard substrate.

8. BE VERY CAREFUL NOT TO DISTURB THE SPERM PELLET.

9. Using the lower line on Tube A as a guide, slowly pipette approximately 330-350µL of liquid from Tube A into Tube B until approximately 50µL remains in Tube A. (LEAVE APPROXIMATELY 50µL OF EXTRACTION BUFFER OVER SPERM PELLET.)

10. Tube B is the non-sperm fraction and is ready for DNA purification.

11. If desired, the sperm pellet in Tube A may be mixed and a smear made to confirm the presence of spermatozoa.

The order in which solutions are added in the following steps is critical.

12. Add 10 µl of Solution #1 to Tube A.

13. Add 10 µl of Solution #2 to Tube A.
If transferring sample to Tube C, mix well by pipetting 50 µl up and down 10 times and be sure that the sperm pellet has been fully re-suspended. Transfer the liquid to Tube C. (DO NOT VORTEX. Solution #2 is sensitive to vortexing, but can be pipette mixed.)
If using Tube A for subsequent steps, mix by pipetting with Solution #2 tip (10ul) 30 times. Then with that tip wash the inside of Tube A with the sample mixture.

14. Incubate samples for 15 minutes at 37ºC.

15. Add 10µL of Solution #3 to Tube C to inhibit the nuclease and to lyse the sperm cells. Mix briefly with a vortex.

16. Incubate samples at 56 ºC for 15 minutes.

17. Tube C (or Tube A if no tube transfer) contains the sperm cell fraction and is ready for DNA purification using a manual phenol:chloroform extraction.

5. Standard Differential Extraction Protocol

Place portion of stain in a 1.5 ml tube.
Leave some of the specimen for possible further testing.
Include a reagent blank with each set of extractions.

Add: 400ul 1X 0.01M Tris, 0.0025M EDTA, 0.05M NaCl (TNE)
25 µl 20% sarkosyl
75 µl H2O
5 µl Proteinase K
505ul total

Mix and incubate the tube at 37°C for 2 hours.

Place the specimen into the basket and centrifuge at maximum speed for 5 minutes.

Decant the supernatant fluid and save (non-sperm fraction). Save the specimen for possible PCR analysis.

Wash the pellet 3 times with 50 µl TE^{-4} by mixing the pellet then centrifuging at maximum speed for 5 minutes. Remove as much as possible of the supernatant without disturbing the pellet.

To make a smear of the pellet, mix and remove 1 or 2 ul, place on glass slide, dry and stain.

To the pellet in the original tube add extraction buffer and incubate for two hours to overnight at 56°C.

Purify DNA.

6. DNA Purification Protocols

Ethanol precipitation protocol using NH4OAc for Er*ase*.

1) Dilute sample to 450ul with TE^{-4} (add 370ul TE-4)
2) Add 600ul phenol/chloroform mixture
3) Mix well, centrifuge 10 minutes 12k x g
4) Remove 420ul of the aqueous into a 2ml tube
5) Add 233ul 7M NH4OAc, mix
6) Add 1310ul 100% ethanol, mix well
7) Freeze (30 minutes, -75°C), thaw
8) Centrifuge 60 minutes, 14k x g
9) Remove supernatant
10) Add 300ul 70% ethanol
11) Centrifuge 10 minutes, 12k x g
12) Decant supernatant

13) Dry pellet (vacuum oven)
14) Add appropriate amount of water or TE^{-4} for quantification and amplification.

EZ1 protocol for Er*ase*.

1) Label EZ1 sample tubes and elution tubes appropriately.
2) In EZ1 sample tubes, dilute the male fraction samples to 200ul by adding 120ul G2 Buffer.
3) Add 1ul polyA RNA.
4) Place reagent trays, tubes and tips into proper EZ1 positions.
5) Set EZ1 for trace protocol, and select the volume and type of elution (we typically use 50ul of water for elution).
6) Start the EZ1 machine.
7) The eluted DNA is now in 50ul. If the concentration of DNA is high enough proceed to quantification and amplification.
8) If the DNA concentration is low, speed vacuum centrifuge the eluted samples until the desired volume is reached or the sample is completely dry.
9) Add an appropriate amount of TE^{-4} or water for quantification and amplification.

Vivacon 100 protocol for Er*ase*.

1) Dilute male fraction sample to 500ul with TE^{-4}.
2) Add 600ul phenol/chloroform mixture.
3) Mix well, centrifuge 10 minutes, 12k x g.
4) Remove upper aqueous phase (470ul) and place into a vivacon100.
5) Spin sample in vivacon100 for 10-15 minutes at 2000 x g.
6) Discard liquid that passed through the column.
7) Add TE-4 to fill the vivacon100 and centrifuge: 10-15 min., 2000 x g.
8) Discard liquid that passed through the column.
9) Add sterile water to fill the vivacon100 and centrifuge: 10-15 min., 2000 x g.
10) Discard liquid that passed through the column.
11) Add sterile water to fill the vivacon100 and centrifuge: 10-15 min., 2000xg.
12) Discard liquid that passed through the column.
13) Flip vivacon100 into a fresh tube, spin 2 minutes, 5000 x g.
14) Dry samples completely with speed vacuum centrifuge.
15) Add appropriate amount of TE^{-4} or water for quantification and amplification
16) Mix pellet with TE^{-4}, heat at 56°C for 30 minutes, centrifuge liquid back into the bottom of the tube, mix again.

Protocol for Er*ase* samples using the Maxwell 16 LEV Instrument with the DNA IQ Casework Pro Kit.

1) Dilute Er*ase* extraction samples to 400ul with TE^{-4} (add 320µl TE-4 to the sperm fraction or 50µl TE-4 to the epithelial fraction).
2) Add 200ul of the Lysis Buffer provided with the Maxwell kit to each sample.
3) Consult the Maxwell 16 Casework Pro protocol. Place reagent cartridges, LEV plungers, and Elution Tubes with 50µl of Elution Buffer± into their proper

positions on the Maxwell Cartridge Rack (±for LCN samples that may need further concentration, use water instead of Elution Buffer).
4) Add each sample to the first well of each Maxwell cartridge.
5) Place the Maxwell Cartridge Rack into the Maxwell 16 instrument.
6) Start the Maxwell 16.
7) The eluted DNA is now in 50ul. If the concentration of DNA is high enough proceed to quantification and amplification.
8) If the DNA concentration is low, speed vacuum centrifuge the eluted samples until the desired volume is reached or the sample is completely dry.
9) Add an appropriate amount of TE^{-4} or water for quantification and amplification.

7. PCR Amplification

The following PCR primer kits were used for DNA analysis:

Primer set	Company
PowerPlex16	Promega
PowerPlex Amelogenin	Promega
PowerPlex CTTV	Promega
SGM Plus	ABI
MavenQST Quantitative Sex-Typing Kit	Maven Analytical

8. Analysis of Amplified DNA.

PCR DNA amplifications were resolved either:

a. Using 5.75% polyacrylamide gels using vertical electrophoresis systems. The gels were scanned on FMBIOIII laser scanners to detect the fluorescent amplicons; or
b. Using capillary electrophoresis performed using an ABI 310 genetic analyzer.

9. Automation Method for E*rase*

To determine the efficacy of automating the E*rase* procedure, experiments were performed using 96-well SlicPrep plates. These are 96-well plates that allowed sexual assault sample substrates to be placed in a 96-well basket within a 96-well plate. When the basket was lowered into the plate the samples are exposed to the extraction buffer. After extraction, the entire basket was lifted with a collar to centrifuge the samples and remove liquid from the substrates. For the experiments presented here, a multichannel pipette was used for removing the supernatant and adding the reagents during the protocol.

Samples used for the experiment, postcoital swabs, noncoital vaginal swabs and blank swabs, were placed into the eight wells of column 1 of a SlicPrep plate. E*rase* extraction buffer with proteinase K (600ul total) was added to each sample well. A foil seal was placed over the top of the plate to seal the wells and prevent cross contamination between them. The plate was agitated with a vortex to thoroughly wet the swabs. The plate was incubated at 56°C for one hour. The plate was removed from the oven and the SlicPrep collar was used to raise the swab substrates in the SlicPrep basket above the level of the extraction buffer. The plate was then centrifuged for 30 minutes at 3000rpm (2050xg). The basket insert containing the treated swab substrates was then carefully removed.

Using an eight-channel pipette, the supernatants from the wells were carefully removed, except for 50ul, and transferred to a different plate for storage. Then 10ul of E*rase* Solution 1 was added to each well of the original plate, followed by 10ul of Solution 2. The samples were mixed by pipetting up and down several times with the eight-channel pipette, and transferred to a new 96 well plate. A foil lid was used to seal the plate and the plate was incubated at 37°C for 15 minutes. After incubation, the lid was removed and 10ul of Solution 3 was added to the samples. The plate was re-sealed and vortexed. The plate was then incubated at 56°C for 15 minutes.

Because the test was designed to examine automation of the E*rase* procedure only (as opposed to DNA purification and following steps), purification of the E*rase* generated extracts was performed by removing the extracts from the 96-well plate and transferring them to individual tubes for phenol/chloroform extraction and ethanol precipitation. This experiment was designed to test the E*rase* procedure. The ability to perform DNA purification in a 96 well format has already been established. Ethanol precipitant pellets were resuspended in 10ul of TE-4 and 1ul was taken for quantifying total DNA with QuantiFluor dye in a fluorometer. Approximately 500pg -1ng of each sample was amplified with AmpFl STR® SGM Plus® and analyzed on a ABI Prism 310 Genetic analyzer.

Automation Protocol

The procedure below can be modified to work with any liquid handler:

1. Insert the basket into the 96 deep well plate of the Slicprep™ 96 Device and place sample substrate into the wells.
2. Add 600µl of E*rase* Extraction Buffer including proteinase K to each well with a liquid handler.
3. Seal the top of the plate with foil tape and vortex gently but throughly.
4. Incubate at 56°C for 1 hour.
5. Raise the bottom of the baskets above the Extraction Buffer and add the Slicprep collar.
6. Centrifuge at 2050xg for 30 minutes.
7. Remove the collar and the basket to remove the substrate material.
8. Place the extraction plate and necessary reagents on a liquid handler.

9. Slowly remove the supernatant until ~50µl is left in the bottom of the wells using a fixed tip height above the sperm pellet.
10. Deposit the supernatant in a storage plate. This is the epithelial cell DNA fraction.
11. Add 10µl of Solution #1 to each column of the extraction plate.
12. Add 10µl of Solution #2 to each column of the extraction plate with a mixing step and transfer to sperm fraction storage plate on a 37°C heat block.
13. Carefully time the transfers so that each column has 15-20 minutes on the heat block before proceeding to the next step.
14. Add 10µl of Solution #3 with mixing to the sperm fraction storage plate when each 15 minute time interval is finished.
15. Increase the temperature of the heat block to 56°C or seal the sperm fraction storage plate with foil tape and transfer to a 56°C oven.
16. Incubate sperm fraction storage plate at 56°C for 15 minutes.
17. The sperm and epithelial storage plates should proceed directly into DNA purification or be sealed with tape and stored frozen for DNA purification at a later time.
18. Discard extraction plate.

III. Results

1. Statement of Results

This description of the results of the grant research takes the reader first through the experiments performed to optimize the selective degradation procedure, then through experiments to validate selective degradation, then through some of the results obtained by other laboratories that volunteered to Beta test the E*rase* kit or help with validation, then through automation, and then compares selective degradation with other methods of differential extraction.

A. Optimizing Selective Degradation

As part of this research, optimization experiments were performed to make selective degradation as consistent and effective as possible under a full range of conditions in the forensic laboratory. The conditions considered include the preferred form of microfuge tube, the extraction and centrifugation processes, nuclease treatment, sperm cell lysis, and the effect of leaving the substrate in the extraction.

(i) Using Dolphin Tubes

The preferable microfuge tube required careful consideration. The reasons for the ultimate choice are explained here. The most difficult step to reproduce precisely in the standard differential extraction was removing as much of the supernatant as possible after centrifugation, without disturbing the sperm cell pellet. Some of the supernatant must be left behind with the pellet to avoid disturbing the sperm pellet and possibly causing sperm cell loss. The counterpart step in the Er*ase*

procedure purposely left 50ul of the supernatant atop the pellet for subsequent steps in the procedure, and to ultimately facilitate robotic processing.

Performing this step with a regular 2ml or 1.5ml microfuge tube proved difficult for two reasons. First, the problem of human hands being imprecise while trying not to disturb the pellet was the same for both procedures. Second, for the E*rase* method, it was difficult to determine when 50ul of the supernatant was left. To resolve both of these problems, a unique microfuge tube, the Dolphin tube (see Figure 1) designed to improve the pelletizing of samples, was selected to perform the E*rase* extraction procedure. The Dolphin tube's shape improved pelleting, leaving the pellet more intact and, more importantly to E*rase*, the design also provided a delineation at approximately 50ul and left the pellet further removed from the pipette tip, making it ideal for removing the supernatant to that mark without disturbing the pellet. See also the section on "Centrifugation and supernatant removal" below.

Figure 1, Dolphin Tube

(ii) Extraction and Lysis of Vaginal Epithelial Cells

The first step in the process of differential extraction using selective degradation involved epithelial cell lysis using proteinase K and a proprietary extraction buffer reagent. The published selective degradation method incubated the sample with proteinase K for 4 hours at 56°C. Since shorter incubation times had not been explored, decreased times were tested in order to determine the amount of time necessary to successfully lyse the epithelial cells. Additionally, experiments were performed to establish the optimal incubation temperature and proteinase K concentration. Several experiments indicated that the incubation period could be reduced without affecting the quality of the sperm cell DNA fraction profiles (data not shown).

Figures 2 through 5, presented in the following discussion, show the progress in optimizing the extraction and lysis procedures for selective degradation.

The concentration of proteinase K in the extraction buffer of the original selective degradation method was determined to be sufficient for epithelial cell lysis (data not shown). This amount of proteinase K was higher than the amount used in the PTC standard differential extraction. Experiments were performed in order to determine if the increased concentration of Proteinase K effected the sperm cell DNA yields. Figure 2 shows the results from testing proteinase K concentration and the incubation periods and temperatures on sperm DNA yield. Reactions were performed in triplicate with semen-only samples having 50,000 sperm cells each. The samples were extracted using the Er*ase* protocol except that during the first incubation they were treated with either 0.2mg/ml or 0.35mg/ml proteinase K, at varying temperatures and times. Total DNA from the sperm cell fractions was extracted by phenol/chloroform and ethanol precipitation, and the samples were quantified using picogreen fluorometry.

Figure 2 shows that none of the treatments significantly reduced the amount of sperm cell DNA recovered and that there were insignificant differences between the tested incubation temperatures and times. The differences were well within the variations expected from DNA purification.

Figure 2

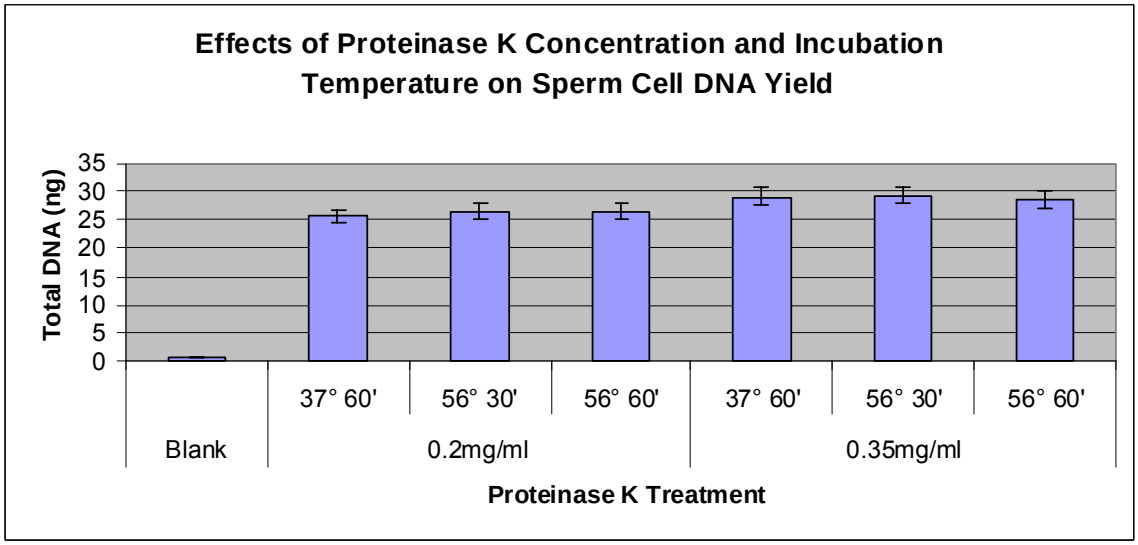


Figure 2 indicates the total DNA yield from the sperm cell DNA fractions of Er*ase* extractions performed on semen-only samples under the conditions indicated. Each sample used approximately 25,000 sperm cells. Different samples were treated with either 0.2mg/ml or 0.35mg/ml proteinase K for 60 minutes at 37°C, 30 minutes at 56°C and 60 minutes at 56°C. After the proteinase K step, the samples were further processed using the standard Er*ase* protocol. The chart shows the average values and standard deviation for three replications.

The next set of experiments were performed in order to determine the most effective conditions for eliminating as much epithelial cell DNA as possible from the sperm fraction.

(iii) Centrifugation and Supernatant Removal

The centrifugation time and speed used for selective degradation was the same (5 minutes at 10,000xg) as that used for the standard differential method. For both methods a spin basket was used to hold the substrate material out of the extraction solution during centrifugation. Removing the supernatant in both procedures involved careful pipetting to avoid disturbing the pellet. As described above, the E*rase* kit used a Dolphin tube that aided pelleting and removal of the supernatant. Using this tube to remove the supernatant to 50ul eliminated the problems otherwise associated with this step. Optimum centrifugation spin times and speeds for pelleting sperm are well established in the industry, and nothing about the process of selective degradation suggested that any benefit could be derived from experiments in this regard.

As described above, supernatant removal was optimized by implementation of the Dolphin microfuge tube.

(iv) Nuclease Treatment

This section on nuclease treatment presents data on the following subjects:

(iv)(a) Optimizing Magnesium and Calcium Concentrations

(iv)(b) Effect of DN*ase* Concentration on Epithelial Cell Elimination

(iv)(c) Effect of Incubation Time on Epithelial Cell Elimination

(iv)(d) Unique Capability of Selective Degradation with Lower Numbers of of Sperm Cells

(iv)(e) Sensitivity of Selective Degradation on Samples with High Ratios of Epithelial to Sperm DNA

(iv)(f) Effect of Source of Nuclease on Epithelial DNA Elimination

(iv)(g) Effect of Source of Nuclease on Sperm DNA Yield

(iv)(h) Effect of Nuclease Concentration on Sperm DNA Yield

(iv)(i) Effect of Incubation Temperatures on Sperm DNA Yield

(iv)(j) Effect of Incubation Times on Sperm DNA Yield

(iv)(k) Additional Nuclease Variables Considered

After removal of the epithelial DNA fraction, in place of the wash steps traditionally undertaken to remove extraneous DNA from the sperm cell fraction, the selective degradation approach introduces a nuclease to the sample containing the sperm

cell fraction. The exact compositions of the solutions used for this purpose are proprietary. However, the primary reagents are identified, and the optimization of the source, concentration and ratio of reagents, as well as the procedures for combining them, are discussed.

The E*rase* Sperm Isolation Kit procedure established a new arrangement and new formulations of the reagents that allows the selective degradation procedure to be presented in a user friendly manner. As identified in the E*rase* Sperm Isolation Kit protocol in the Methods section of this paper, the degradation of the extraneous DNA in the male fraction involves the use of "Solution #1" and "Solution #2." A subsequent step, which deactivates the nuclease and lyses the sperm cells, involves the addition of "Solution #3."

The experiments described below involved using the selective degradation method in this kit format. As individual reagents were tested, other steps in the procedure were held constant until that step was studied. For experiments involving the nuclease, the procedure upon adding 10ul of Solution #3 was to consistently incubate the tube for 5 minutes at 56°C. When the conditions for Solution #3 were tested, optimal nuclease treatments had been established and were applied consistently.

(iv)(a) Optimizing Magnesium and Calcium Concentrations

Solution #1

Solution #1 in the Er*ase* kit provided the Mg^{2+} and Ca^{2+} ions for nuclease activity. The original selective degradation protocol called for the addition of the nuclease before the addition of this solution. For better ease of mixing, the protocol for the Er*ase* kit added these reagents first.

The concentrations of $MgCl_2$ and $CaCl_2$ were tested to establish optimal amounts, but the original concentrations of 7.14mM $MgCl_2$ and 7.14mM $CaCl_2$, were found to be optimal for full activity of the nuclease. Molar concentrations of $MgCl_2$ were tested at 0.36mM to 7.14mM. $CaCl_2$ was tested at 0.07mM to 7.14mM. Only when the $MgCl_2$ and $CaCl_2$ concentrations were both in the 7.14mM range was the nuclease activity able to sufficiently digest epithelial cell DNA (data not shown).

Solution #2

Solution #2 contains the nuclease. The selective degradation method used nuclease to remove epithelial cell DNA from the pelleted sperm cells. The stock nuclease reagent was inactive because it was stored in a solution that contained EDTA. EDTA bound and sequestered any Mg^{2+} or Ca^{2+} ions that were required for the nuclease to exhibit activity. The $MgCl_2$ and $CaCl_2$ already added to the sample from the addition of Solution #1 served to activate the nuclease once it was added.

The following experiments identified the limitations with using nuclease. Samples used to test the nuclease step included vaginal epithelial cells eluted from vaginal noncoital swabs, mock postcoital swabs and postcoital swabs. Postcoital swabs were prepared as described in the Methods section. Optimal conditions were established to obtain maximum sperm cell DNA yield along with maximum reduction of epithelial cell DNA in the sperm fraction. The following experiments were directed to determine the conditions needed to both eliminate epithelial cell DNA sufficiently and to protect sperm cell DNA to maximize yield.

Epithelial Cell DNA Elimination

(iv)(b) Effect of Nuclease Concentration on Epithelial Cell DNA Elimination

Experiments for monitoring the elimination of vaginal epithelial cell DNA were performed using isolated noncoital vaginal swabs or using mock postcoital swabs and real postcoital swabs with sperm cells present. Initial experiments clearly demonstrated that the amount of nuclease used in the originally published selective degradation procedure was insufficient to remove the amounts of epithelial DNA present in many vaginal and postcoital vaginal swabs.

The original recommended nuclease preparation had a final concentration of 0.9U/ul nuclease from Sigma (1). This amount was chosen for processing ½ of a sexual assault kit vaginal swab so that the other half could be saved for future testing. Many laboratories routinely use a whole swab from a sexual assault for differential extraction or they may add more than a single swab portion to the extraction to obtain greater numbers of sperm cells in the extraction. However, this also increased the amount of epithelial cell DNA in the sample that must be eliminated in order to obtain an epithelial-DNA-free sperm fraction DNA profile. In order to verify that the Er*ase* extraction could be used successfully for a wide range of sample possibilities, experiments were performed to determine the amount of epithelial cell DNA that could be eliminated from a sample.

Initial experiments with various postcoital and mock postcoital swabs indicated that 0.9U/ul of nuclease was insufficient to remove all epithelial cell DNA in numerous examples (data not shown). New experiments were performed using 1.43U/ul of Sigma DN*ase* I. At this concentration, some half swab samples worked well, but when whole swabs were used for extractions, the sperm fractions still had a detectible mixture of epithelial cell DNA present.

Experiments were designed to test nuclease conditions (the proteinase K concentration, and the incubation temperatures and times) using samples of only epithelial cells representing 0.5, 1, 1.5, and 2 vaginal swabs.

To determine the optimum amount of nuclease needed to remove epithelial cell DNA from the sperm fraction, vaginal epithelial cell samples harvested from noncoital swabs were processed with the selective degradation procedure using different amounts of nuclease. Various nuclease concentrations were tested to

determine the amount of nuclease needed to successfully degrade the vaginal epithelial cell DNA. Figure 3 shows results from a typical experiment using vaginal epithelial cells in the proportions indicated. Although some remaining epithelial DNA was detected in these experiments for samples treated with nuclease, total epithelial DNA quantification values of less than 50pg would not be expected to be sufficient for amplification. But, higher concentrations of DN*ase* proved beneficial in further reducing extraneous DNA from the sperm fraction, as discussed next.

Figure 3

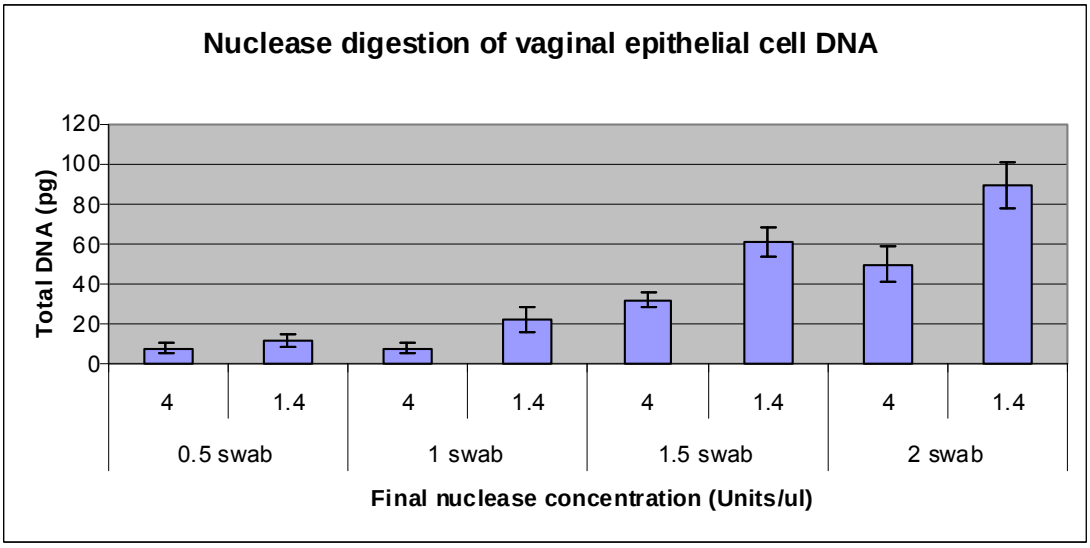


Figure 3 indicates the total DNA recovered in the sperm cell DNA fraction from E*rase* extractions using only vaginal epithelial cells for the sample. Equivalently divided noncoital vaginal swabs representing 0.5, 1, 1.5 and 2 swabs were extracted using the selective degradation procedure at two different concentrations of nuclease (4 and 1.4 units/ul) in order to determine the amount of nuclease needed to remove the epithelial DNA from the sperm cell fraction. Total DNA was measured from the sperm cell fraction and the yields of DNA recovered in the sperm cell fraction for each amount of epithelial cells added. The chart shows the average and standard deviations of three replications.

Mock postcoital swabs and postcoital swabs were used to measure epithelial cell elimination in the presence of sperm cells. This permitted detection of any observable epithelial cell profile along with the sperm cell profile in the sperm fraction, as nuclease concentration increased. Numerous extractions were performed using increasing amounts of nuclease. When sufficient amounts of nuclease was used, the epithelial cell DNA profile could not be detected in the sperm fraction, except in cases where vaginal swabs were collected with the intent of introducing extreme amounts of epithelial DNA.

The nuclease concentration was adjusted so that the resulting sperm cell DNA profiles, from swabs with high ratios of epithelial cells to sperm cells, were free of epithelial cell DNA in the sperm fraction profile. Supplemental Figure 4 shows an example of an experiment comparing nuclease concentrations. Mock postcoital swabs with 5,000 sperm cells added were processed using the E*rase* kit format of the selective degradation process. For the experimental data shown in Figure 4, samples were treated at 5, 5.7, and 6.4 Units/ul nuclease. In all cases the sperm

cell profile was prominent. When 6.4 Units/ul nuclease were used, no epithelial cell DNA profile was detected. In other experiments, 6.4Units/ul final concentration of nuclease was determined to be sufficient for eliminating nearly all or all epithelial cell DNA in the cases studied.

See Supplemental Figure 4, Optimum Nuclease Concentration for Epithelial Digestion.

This concentration of nuclease (6.4Units/ul) has been used for E*rase* extractions since. Further demonstration that this amount was effective can be seen in figures later in this paper, showing the sperm cell fraction DNA profiles from mock postcoital swabs with as few as 200 sperm cells (Figure 7). For comparison, in those experiments duplicate swabs were also processed with the standard differential extraction method.

(iv)(c) Effect of nuclease Incubation Time on Epithelial Cell Elimination

Initially experiments were designed in order to determine if longer nuclease incubation periods could help decrease the amount of epithelial cell DNA in the sperm cell fraction. Incubation periods of 30, 60, 90 and 120 minutes at 37°C were tested with different amounts of epithelial cell DNA (Figure 5). Results indicated that longer incubation periods did not remove significantly greater amounts of the epithelial cell DNA. The data demonstrated that epithelial cell DNA was effectively eliminated in the 30 minute incubation. Further adjustments to the nuclease incubation time were made during consideration of sperm cell DNA yield testing below.

Figure 5

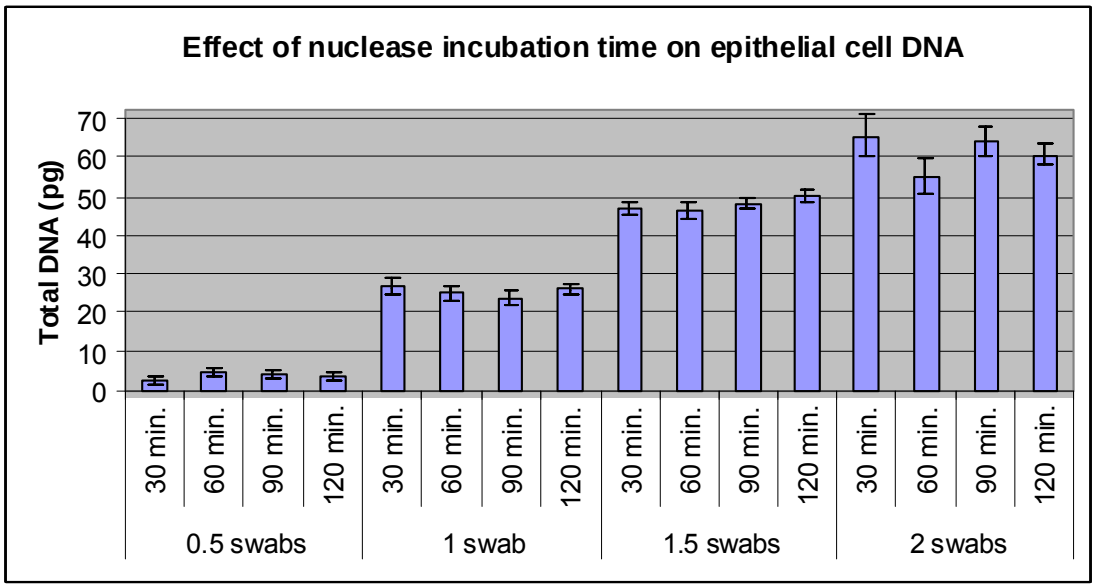


Figure 5 represents the total epithelial DNA recovered from the sperm fraction of samples of non coital vaginal swabs incubated 30, 60, 90, and 120 minutes at 37ºC during the nuclease treatment step. The chart presents the average total DNA recovered and the standard deviations for three replications of the experiments.

(iv)(d) Unique Capability of Selective Degradation with Small Number of Sperm

After the Er*ase* Beta protocol was established, experiments were performed that compared it to the standard differential extraction procedure under conditions of limited sperm cell quantity. Supplemental Figure 6 shows the results from extracting a mock postcoital swab spiked with 500 sperm cells. The figure shows the epithelial cell fraction (female) and the sperm cell fraction (male) profiles from PP16 amplification of standard differential extraction compared to selective degradation extraction of mock postcoital swabs spiked with 500 sperm cells. No male profile was obtained from the standard differential extraction. Selective degradation obtained a clean single source male profile.

See Supplemental Figure 6, The Performance of Selective Degradation with Lower Numbers of Sperm Cells.

This data also identifies the significance of the results of this research regarding incomplete digestion of epithelial DNA in the male fraction when using selective degradation. By eliminating nearly all epithelial cell DNA, the sperm DNA can successfully compete with the epithelial DNA during amplification. The overall result is that either only a trace epithelial cell profile is present or no detectable epithelial cell DNA is present in nearly all cases. The data represented by Figure 6 demonstrates that selective degradation is more effective at eliminating epithelial DNA than the standard differential extraction method, to the extent that selective degradation often produces a clean single source male profile even in situations where the traditional method cannot.

(iv)(e) Sensitivity of Selective Degradation on Samples with High Ratios of Epithelial to Sperm DNA

Testing was performed to determine whether the selective degradation procedure would be capable of eliminating epithelial DNA and producing a sperm DNA profile, even in situations in which there were extremely sperm cells as compared to numbers of epithelial cells.

Evidence demonstrating the sensitivity of the Er*ase* Beta procedure is shown in Supplemental Figure 7. Mock postcoital swabs having 200, 500, 1000, and 2000 sperm cells on noncoital swabs were extracted with the Er*ase* Beta protocol and the fractions were amplified using PP16 primers. Er*ase* extractions were able to eliminate a sufficient amount of the epithelial DNA that only the sperm DNA profile was obtain in the sperm cell fraction of the Er*ase* extraction.

See Supplemental Figure 7, Sensitivity of Selective Degradation on Samples with High Ratios of Epithelial to Sperm DNA.

(iv)(f) Effect of Nuclease Source on Epithelial DNA Elimination

Different sources of nuclease were obtained and tested for their ability to function properly in the selective degradation extraction. Suppliers of Nuclease included Sigma, Pierce, USB, and Worthington. The original source of nuclease was Sigma. Nuclease from this source exhibited high quality, but the cost was high, the supply was inconsistent and only small lots of nuclease were available. Other sources were tested to determine if cost could be reduced and availability could be improved without reducing the quality.

All of the sources provided nuclease that functioned similarly in side by side selective degradation extraction experiments (data not shown). Worthington offered the best pricing and three grades of nuclease from Worthington were tested. Experiments were performed using the Worthington enzymes compared to the Sigma enzyme on vaginal epithelial cells alone, sperm cells alone, and on equally divided postcoital swabs. Considering pricing, availability, continuity of supply and lot sizes available, the Worthington products offered the best product. Samples of three grades of Worthington nuclease were obtained to determine their ability to sufficiently degrade epithelial cell DNA and to produce a sufficient yield of sperm cell DNA.

The three grades of Worthington nuclease were tested, DPFF and D were a chromatographically purified nuclease, and DP was a partially purified form of the enzyme. Vaginal epithelial cells were prepared from noncoital swabs and subjected to the selective degradation procedure using the Sigma and Worthington nuclease sources. Sperm cell fractions were extracted and purified. The DNA was quantified and the entire volume was amplified with PP16 primers. DNA quantification indicated that all of samples were degraded. This was demonstrated by the DNA quantitation data that indicates that all samples contained less that 20pg of epithelial cell DNA.

Images of the amplification products resolved on a polyacrylamide gel are shown in Figure 9. A control that was not treated with the nuclease shows amplification of all loci. The nuclease treated samples are typical of very low copy number amplifications. There are traces of the profile at the smaller loci but very few alleles are detected.

See Supplemental Figure 9, Testing Source of DN*ase* for Epithelial Degradation, using Noncoital Vaginal Swabs.

Sperm only samples were also tested with the selective degradation procedure using the different sources of nuclease to determine if any of these nucleases lowered the yield of sperm cell DNA. Figure 8 shows the results of triplicate experiments using 20,000 sperm cells per extraction. All three grades of Worthington nuclease performed equally and all performed as well as Sigma nuclease.

Figure 8

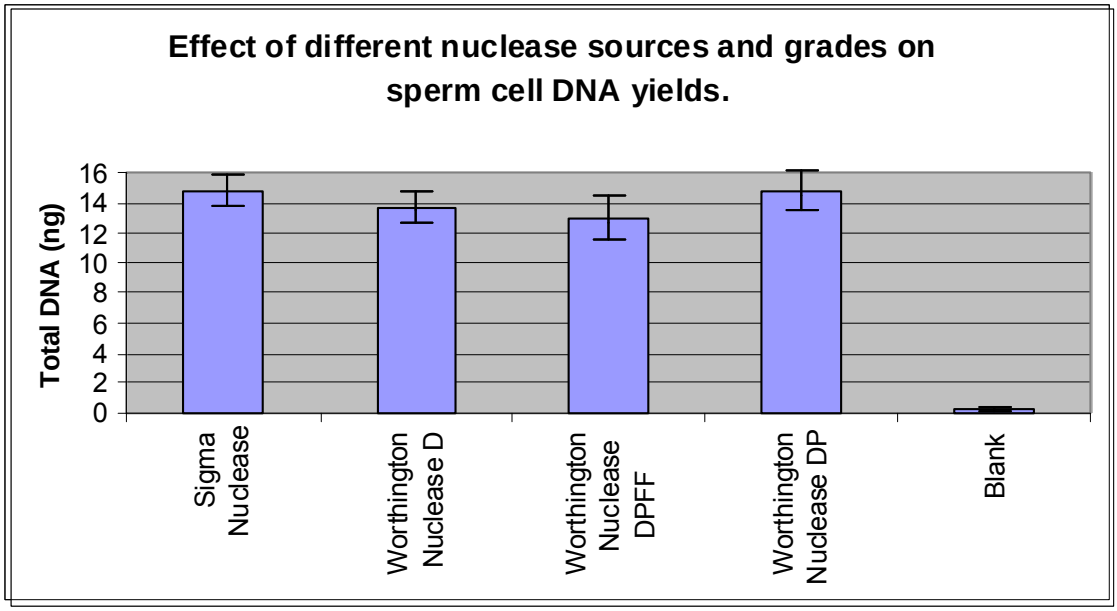


Figure 8 presents the total DNA recovered in the sperm cell DNA fractions from selective degradation extractions using different nucleases from Sigma and Worthington. Semen only samples of 20,000 sperm cells were used. The chart represents the average DNA yields and standard deviations for three replications each. No significant difference was found between different sources of nuclease.

Postcoital swabs were also processed with the selective degradation procedure, using these different grades of nuclease, as shown in Figure 10. All three grades of nuclease from Worthington performed well. No epithelial cell DNA was detected in the sperm cell DNA profiles obtained from the different grades of nuclease.

See Supplemental Figure 10, Testing Source of DN*ase* for Epithelial Degradation, using Postcoital Swabs.

Optimizing Sperm DNA Yield

(iv)(g) Effect of Nuclease on Sperm DNA Yield

Further analysis using only sperm cells indicated that sometime during or after nuclease incubation some sperm cell DNA was lost. Experiments were designed in order to establish the nuclease conditions that most effectively eliminated epithelial cell DNA while maintaining a high yield of sperm cell DNA.

In order to compare sperm cell DNA losses between the standard differential extraction method and E*rase* Beta version, equivalent numbers of sperm cells (100,000 cells in 20ul) were subjected to each procedure in triplicate and yields were determined before and after the critical step in each procedure (Figure 11). A control of 100,000 sperm cells was lysed directly in sperm lysis buffer with DTT to estimate the yield without differential extraction. Six samples were extracted with proteinase K according to the standard differential extraction method. Three of these were treated with DTT directly to lyse the sperm cells. The other three PK

treated samples were processed following the standard differential extraction protocol. Similarly, six samples were extracted with proteinase K according to the Er*ase* Beta protocol. Three samples were treated with DTT directly to lyse the sperm cells without introduction of the nuclease. The other three samples were processed according to the Er*ase* Beta protocol.

Figure 11

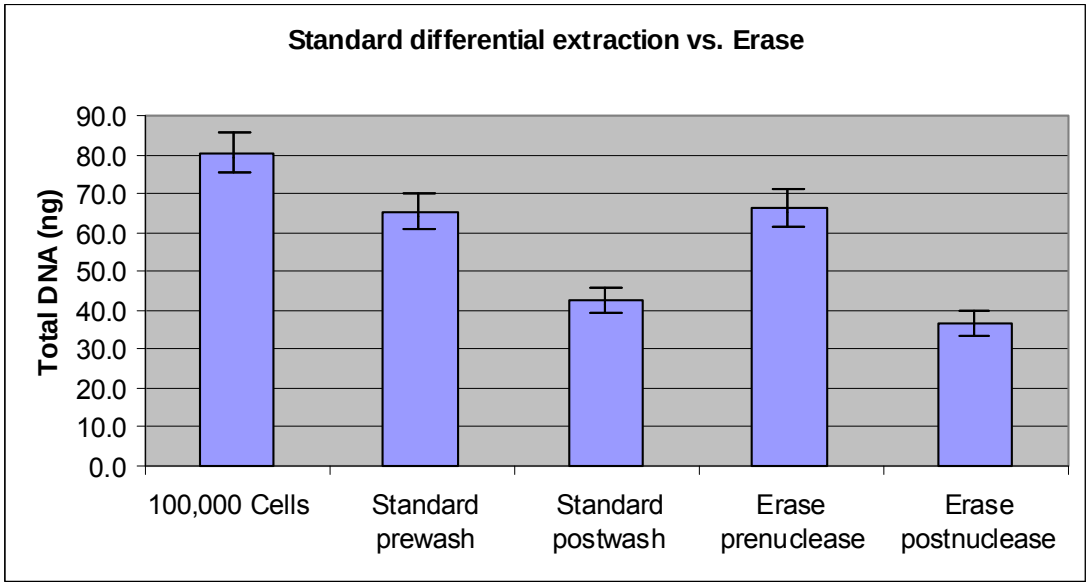


Figure 11 represents the total DNA recovered from semen-only samples with 100,000 sperm cells using either the standard differential extraction or the Er*ase* extraction procedures. The sample labeled 100,000 cells was lysed in a DNA extraction solution containing proteinase K, SDS and DTT to lyse sperm cells without going through the differential extraction process. For samples treated with the standard differential extraction procedure, total DNA was determined before and after three centrifugation and pellet wash steps. Samples treated with the Er*ase* procedure were processed and collected both before and after the nuclease treatment step. Total DNA was measured using picogreen fluorescence. The average values for three replications and the standard deviations are shown.

Some of the difference in DNA recovery between samples taken before further extraction versus those that proceeded through differential extraction was due to the fact that semen contains epithelial cells [17]. Semen donor epithelial cells in the semen add to the total amount of DNA recovered in the prenuclease treatment. During the Er*ase* procedure, the epithelial cell DNA in the postnuclease samples is degraded by the nuclease. For the standard differential samples, the prewash samples also have some unknown amount of sperm donor epithelial cells that accounts for some of the quantified DNA. Using the standard differential extraction, washing the pellet reduces epithelial cell DNA.

Experiments to determine how much epithelial cell DNA was present in the samples tested were not conducted, but previously published data on semen DNA pre and post vasectomy indicates that the amount of epithelial DNA in semen varies greatly (6). The variation was estimated to be between 1% and 80% epithelial cell DNA for different individuals. The majority of cases fell between

15% and 40% epithelial cell DNA of the total DNA. Thus, the possible range of sperm DNA loss using Er*ase* could be between 0% and 55%, and most likely between 20% and 40%. This experiment did not take into account the amount of semen donor epithelial cells in the semen. While the difference between pretreatment and post-treatment was skewed, the loss of DNA between the standard differential extraction and Er*ase* was similar.

It was likely that some DNA was lost during the Er*ase* procedure. Experiments were designed to determine the basis for sperm cell DNA loss during the Er*ase* procedure.

The most likely steps in the Er*ase* procedure responsible for this loss of sperm cell DNA are either the nuclease treatment or the sperm cell lysis step. Using a microscope on high power (1000X), Christmas tree stained sperm cells observed immediately after nuclease digestion did not appear to be damaged physically by nuclease treatment and it was not evident that the loss of yield occurred in this step (personal observations). The sperm cells did not show signs of enlargement, odd shapes or discoloration that has often been seen when damage has occurred to the cell membrane. There was no physical evidence that the nuclease had infiltrated the cell. Experiments were designed in order to determine the minimum amount of time necessary for epithelial cell DNA digestion and the minimum amount of time necessary to complete lysis of the sperm cells. Adjustments to the protocol were made in concordance with the data. It was determined that both the nuclease incubation time and the sperm cell lysis time could be shortened to 15 minutes.

During this period of optimization, comparisons of sperm cell fraction profiles and DNA yield between the standard differential extraction and selective degradation extraction were made to determine whether improvements, beyond shortening the incubation times, resulted. These comparisons were made with postcoital swabs at various postcoital intervals and with mock postcoital swabs where very few sperm cells were spiked onto noncoital vaginal swabs. (data not shown)

Optimizing Sperm Cell DNA Yield

(iv)(h) Effect of Nuclease Concentration on Sperm DNA Yield

Visual observation of the sperm cells under a microscope did not indicate membrane damage, although experimental data suggest that there is a significant loss of DNA after the nuclease treatment. (see Figure 11). After the minimum amount of nuclease needed in order to eliminate epithelial cell DNA from a sample was established, sperm cell DNA yields were studied to determine the effects of nuclease treatment conditions on sperm cell DNA yield.

Based on epithelial cell DNA digestion studies reported above, the final nuclease concentration used in selective degradation extractions was most effective at 6.4Units/ul. This concentration of nuclease was tested to determine the effect on sperm cell DNA yield. Repeated experiments using the selective degradation method with and without nuclease were performed side by side. The results

verified that there was an approximately 50% loss of DNA between the non-nuclease treated cells and the nuclease treated cells. Part of that loss was due to loss of epithelial cell DNA from the sperm donor, but it is presumed that some of the loss is due to the nuclease treatment. In order to determine if less nuclease would result in better sperm cell DNA yields, nuclease concentrations of 2.6, 3.6, and 5 Units/ul final concentration were tested under the Beta conditions (Figure 12). The chart shows the results from this experiment. No detectable difference in quantity of sperm cell DNA was seen with varying concentrations of nuclease.

Figure 12

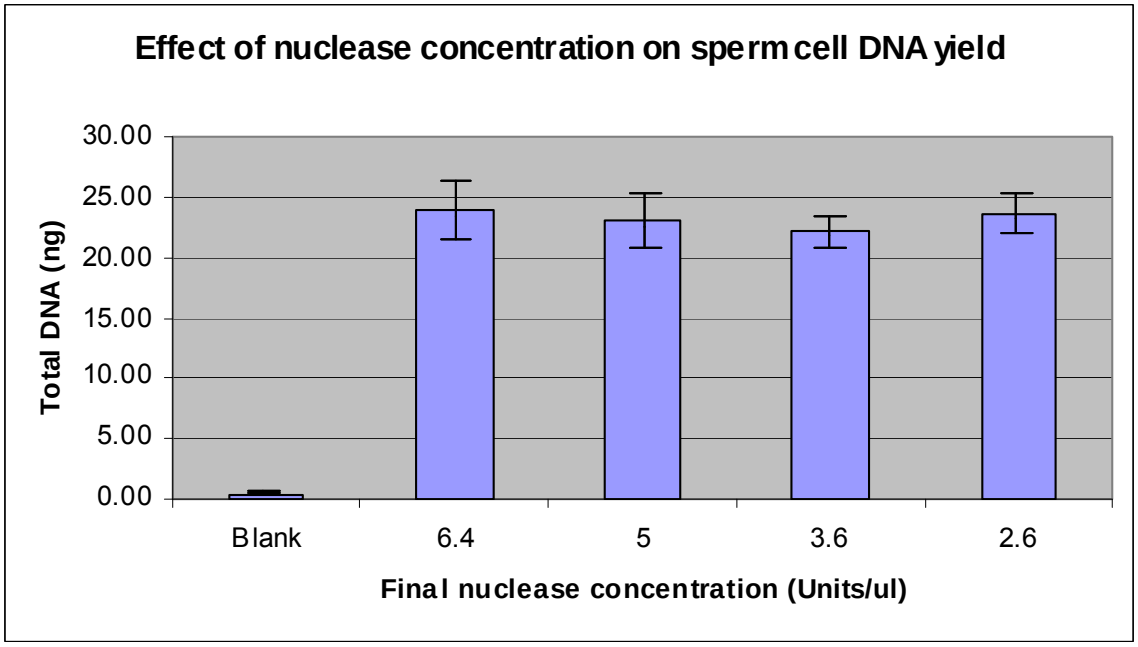


Figure 12 represents the total DNA yielded from 50,000 sperm cells in semen only samples using nuclease concentrations of 6.4, 5, 3.6, and 2.6 Units/ul. The average yield of three replications for each experiment is shown along with bars indicating the standard deviation. Changes in nuclease concentration caused no significant change in sperm DNA yield.

(iv)(i) Effect of Incubation Temperatures on Sperm DNA Yield

Experiments to determine the optimal nuclease incubation temperature were performed on 100,000 sperm cells in order to identify any negative effects on sperm cell DNA yield. The temperatures studied were room temperature, 37°C, and 56°C. A temperature of 56°C for nuclease incubation did not perform as well as those at the lower temperatures and that temperature was abandoned (data not shown). Experiments comparing the effects of 37°C and room temperature nuclease incubations were performed. Many of these experiments did not have significantly different results (Figure 13).

Figure 13

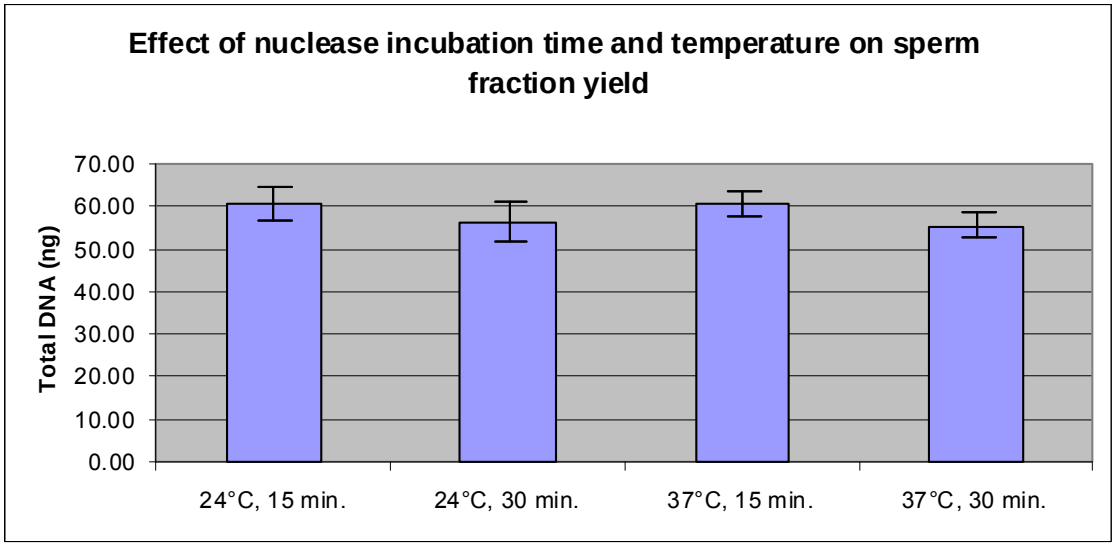


Figure 13 presents the total DNA recovered from 100,000 sperm cells in semen only samples extracted using nuclease incubations at 24°C for 15 and 30 minutes and 37°C for 15 and 30 minutes. The chart shows the average and standard deviations for three replications.

Similarly, treated postcoital vaginal swabs were prepared for amplification. In this example a 25-30 hour postcoital swab was placed in Er*ase* extraction buffer, the resulting supernatant and sperm pellet were mixed and divided equally into 4 tubes. The temperature and time of the nuclease incubation period was varied. Supplemental Figure 14 shows the DNA profiles for the D3S1358 locus from PP16. The data indicates elimination of all detectable epithelial cell DNA profile when using a 15 minute 37°C nuclease incubation. Additional experiments verified that incubation for 15 minute at 37°C was sufficient to eliminate virtually all epithelial cell DNA from the sperm fraction.

See Supplemental Figure 14, Optimal Nuclease Incubation Time and Temperature.

(iv)(j) Effect of Incubation Times on Sperm DNA Yield

Incubation time experiments were established to determine if the nuclease incubation period should be adjusted to maximize sperm cell DNA yields. While an extensive incubation time was fine for eliminating epithelial cell DNA, there was concern that longer periods of nuclease treatment were affecting the yields of sperm cell DNA. Triplicate samples of 50,000 sperm cells were extracted with the Er*ase* procedure (Figure 15). During nuclease incubation, the samples were subjected to different incubation periods (1, 5, 15, 30, 45 and 60 minutes). Control samples for each incubation period were processed without nuclease. The yield

for each incubation period was compared. Although no specific time period interval had a significant drop in the amount of recovered DNA the overall experiment shows a very clear relationship between the amount of time the sperm cells are exposed to the nuclease and the amount of DNA recovered.

Figure 15

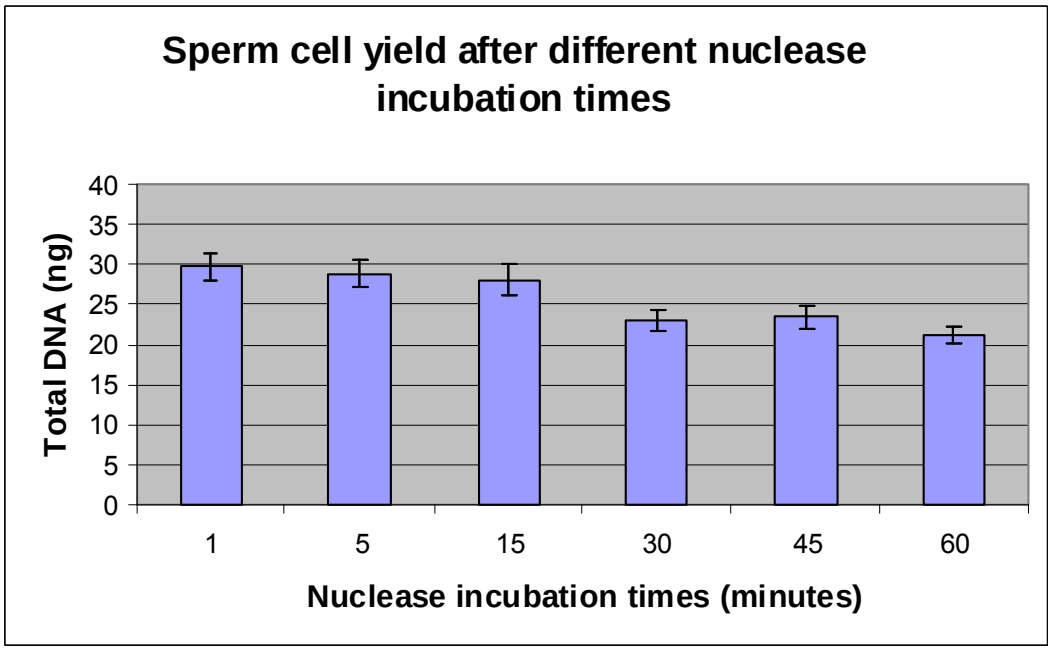


Figure 15 represents the total DNA recovered in the sperm cell DNA fraction from 50,000 sperm cells in semen only samples comparing nuclease incubations of 1, 5, 15, 30, 45 and 60 minutes. The results are presented as the averages of three replications with standard deviation bars.

After establishing that the amount of nuclease used for Er*ase* extractions was not contributing to additional degradation of sperm cell DNA, further tests were conducted to establish the optimal nuclease incubation period. For each experiment, postcoital swabs were processed using the Er*ase* extraction buffer and divided equally for nuclease treatments. Samples were treated with nuclease for 0, 5, 15, 30, or 45 minutes. The subsequent sperm fraction DNA samples were amplified using the MavenQST Quantitative Sex-Typing Kit for Amelogenin and Sex-determining region Y (SRY) loci. The amplification products were analyzed using capillary electrophoresis. Quantitative assessment of nuclease incubation time was determined from the ratio of Y Amelogenin to X Amelogenin peak values (See Table 1). The Y-locus to X-locus ratio of the peak values for the Amelogenin locus reflect the respective amounts of X and Y DNA. When female epithelial cell DNA was present in the sperm cell DNA fraction, the X peak values were greater that Y peak values, which indicated that nuclease activity was insufficient. When epithelial cell DNA was sufficiently degraded, the Y Amelogenin peak value was approximately equal to the X Amelogenin peak value, and the sperm cell DNA fraction consisted entirely or almost entirely of male DNA. Incubation times in

excess of 5 minutes generally gave balanced X and Y Amelogenin amplification. However, some postcoital swabs did not give balanced Y and X Amelogenin peaks until 15 minutes of nuclease incubation time. Earlier experiments indicated that at 0 minutes of nuclease incubation time there was a significant amount of epithelial DNA remaining in the sperm fraction.

This experiment indicates that a 15 minute incubation time is optimal for elimination of the epithelial cell DNA from the sperm fraction.

Table 1, Summary of Incubation Time Data

Swab	PC16		PC22		PC49		PC75		PC77		PC80		PC67	
Hours postcoital	0-6		0-6		25-30		31-36		31-36		37-42		43-48	
Incubation Time (minutes)	5	15	5	15	5	15	5	15	5	15	5	15	5	15
Percent Y Amelogenin	49%	50%	45%	49%	49%	50%	28%	50%	32%	50%	41%	47%	34%	49%
Improvement	1%		4%		1%		22%		21%		6%		15%	

Summary of data comparing nuclease incubation times of 5 minutes and 15 minutes. Percent Y Amelogenin values were determined by dividing the Y Amelogenin peak value by the sum of X and Y Amelogenin peak values. Thus, 50% Y Amelogenin values indicate equal X and Y peaks and that therefore the sperm cell DNA fraction consisted of only male DNA. The data indicate that while the epithelial cell DNA was eliminated from some samples in 5 minutes, others required a 15 minute incubation before Y Amelogenin peak values were equal to the X Amelogenin peak values.

(iv)(k) Additional Nuclease Variables Considered

Spiking with Extra Nuclease

During the developmental optimization stages, there were occurrences of apparent incomplete digestion of epithelial cells. Experiments were designed to determine if nuclease added sequentially with additional incubation time periods would eliminate excess epithelial DNA. Spiking with extra nuclease for longer incubation times did not help to remove excess epithelial cell DNA (data not shown). It is not know if the remaining epithelial DNA is due to incomplete epithelial cell lysis or the inability of the nuclease to degrade all of the DNA.

Semen Extender to Protect Sperm from Nuclease

As noted previously, sperm cells that had been hydrated from dried samples were intact morphologically. However, there were indications that the nuclease was digesting some of the sperm DNA. This experiment was designed to determine if protecting the sperm cells using semen extender would shield the sperm DNA from the nucleae. Semen extender has been used to protect living sperm cells from freezing and thawing damage and subsequent chromatin damage (4). For the purpose of protecting forensic sperm cells from damage during the selective degradation extraction process, semen extender was prepared in Solution #1 of the Er*ase* extraction kit using two of the main ingredients found in human semen extenders. Er*ase* extractions were run in triplicate comparing Solution #1 with and

without extender reagents. While some experiments that used only sperm cells indicated that the semen extender helped to increase sperm fraction DNA yield (data not shown), subsequent experiments using postcoital samples indicated otherwise. Figure 21 (in Section III-1-A-(v), Sperm Cell Lysis) reveals no significant differences between Er*ase* reactions with or without Solution #1 semen extender. Extender was also added to the Er*ase* extraction buffer, but did not increase sperm DNA yield (data not shown).

Performance of Differential Extraction Methods with Menstrual Blood.

Postcoital swabs were collected from a donor during menses 12 hours postcoitus. The swabs were obviously stained with blood. The swabs were processed using both the selective degradation method and the standard differential extraction method. The samples were purified using the BioRobot EZ1 Workstation. The samples were eluted in 50ul of water and concentrated to 10ul with a speed vacuum. The samples were quantified using QuantIt and 2ng DNA was amplified using PP16. Supplemental Figure 18 shows the epithelial cell (♀) and sperm cell (♂) fraction profiles from these postcoital swabs.

Both differential extraction methods produced single source epithelial cell fractions. The selective degradation process produced a single source male profile in the sperm cell fraction. The standard differential extraction produced mixed profiles in the sperm fraction.

See Figure 18 (in Supplement), Comparison of Methods on Vaginal Swab with Menstrual Blood.

(v) Sperm Cell Lysis --- Solution #3

This step in the procedure was designed to stop nuclease activity and to lyse the sperm cells. This step in the originally published selective degradation procedure (1) consisted of the addition of a solution of EDTA and DTT, and incubation for 5 minutes at 56°C. EDTA inactivates the nuclease remaining in the sample and the DTT lyses the sperm cells. Initial experiments testing this step in the procedure determined that a 15 minute incubation period at room temperature was just as effective as 5 minutes at 56°C (data not shown). Microscopic observations of samples after this step were made and sperm lysis always appeared complete. Fifteen minutes at 56°C was chosen for the final protocol because it allowed for variables in heating the test tube. Even if the sample is not exposed to the proper heat the sperm cells will be fully lysed in 15 minutes.

EGTA Treatments During Sperm Cell Lysis

Experiments were designed in order to determine if the nuclease remained active after introduction of solution #3. If the nuclease remained active after sperm lysis the sperm cell DNA would be digested as it was released from the lysed sperm cells, effectively reducing sperm cell DNA yield. The selective degradation

procedure used EDTA to chelate Mg^{2+} in the sample to prevent its availability for activating the nuclease. Er*ase* already included an excess amount of EDTA so that there was a sufficient amount to bind all of the Mg^{2+}.

But Ca^{2+} was also included in Solution #1 and was needed for nuclease activity. EDTA does not bind Ca^{2+} as well as Mg^{2+}. EGTA binds Ca^{2+} ions better than EDTA. EGTA was tested in the sperm cell lysis step of Er*ase* to determine if there was residual Ca^{2+}-dependent nuclease activity. Equivalent numbers of sperm cells were extracted with Er*ase* and comparisons were made with and without EGTA added before or included with Solution #3.

Figure 19 shows the results of these experiments. No significant differences in Er*ase*-extracted sperm cell DNA yield were found between the treatments as indicated on the chart. No Ca^{2+}-dependent nuclease activity was detected and the amount of EDTA used in the Er*ase* extraction was found to be sufficient for inactivating nuclease.

Figure 19

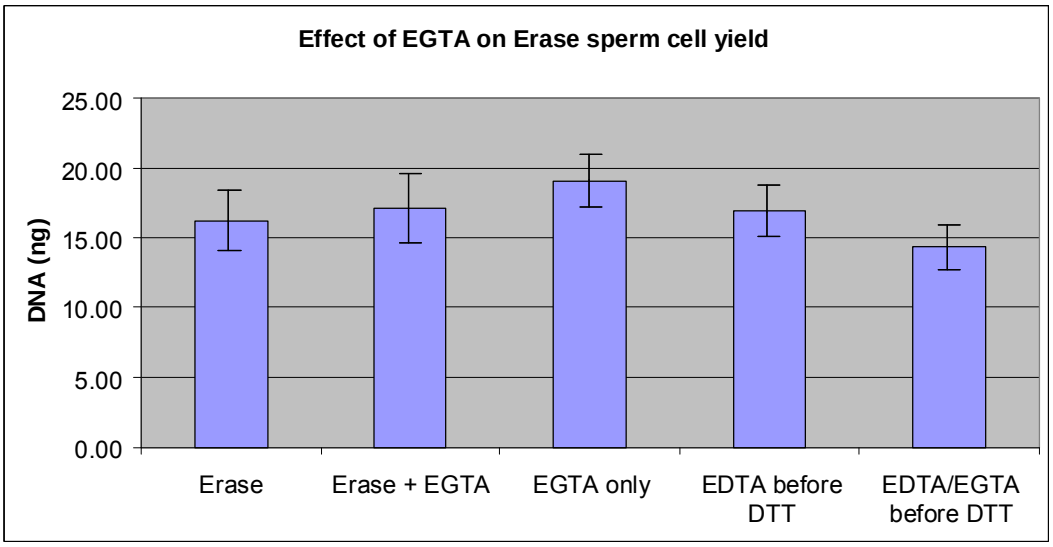


Figure 19 shows the total DNA recovered from Er*ase* reactions on 50,000 sperm cells from semen only samples with and without EGTA added before or during incubation with Solution #3. The chart shows the averages and standard deviations of three replications of each treatment.

Heat Treatments during Sperm Cell Lysis

Ultimately, heat inactivation has been shown to physically destroy nuclease activity [16]. Heat treatments of 65°C, 85°C and 100°C were applied before adding Solution #3 (see Figure 20). Because the yield of sperm cell DNA did not increase with heat treatment, it can be assumed that there must be no residual nuclease activity during the sperm lysis step in the selective degradation extraction.

Figure 20

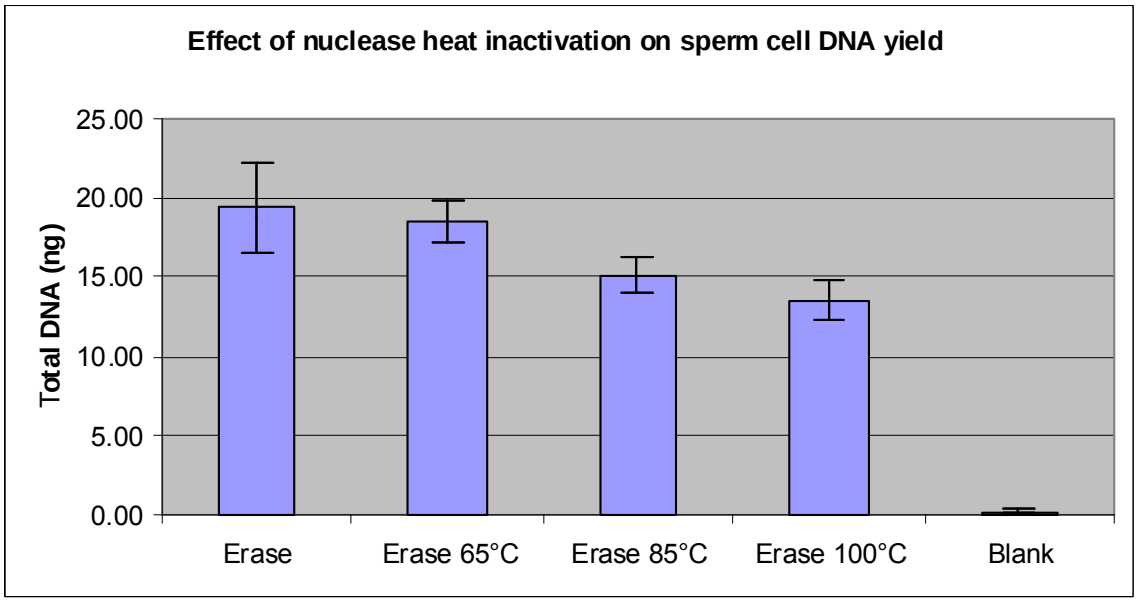


Figure 20 presents the results from E*rase* extraction experiments using 50,000 sperm cells from semen only samples where the samples were treated with 65°C, 85°C or 100°C heat after nuclease incubation and before sperm cell fraction lysis. The average of three replications with the standard deviations are shown.

Including SDS during Sperm Cell Lysis

The standard differential extraction used SDS in the sperm lysis step and E*rase* did not. Detergent from the initial extraction buffer remained in the E*rase* extraction during the sperm lysis step, but the concentration was lower than in the initial epithelial cell lysis and the detergent may be less effective than SDS. Because SDS is known to be an efficient detergent for sperm cell lysis, SDS was added to Solution #3 to determine if any additional sperm cell DNA could be recovered. Figure 21 shows the results from experiments comparing E*rase* extractions with and without 2% SDS (final concentration). There was no increase in sperm cell DNA yield when SDS was added to Solution #3. This experiment supported previous microscopic data that indicated sperm cell lysis was complete after addition of solution #3 and incubation.

Figure 21

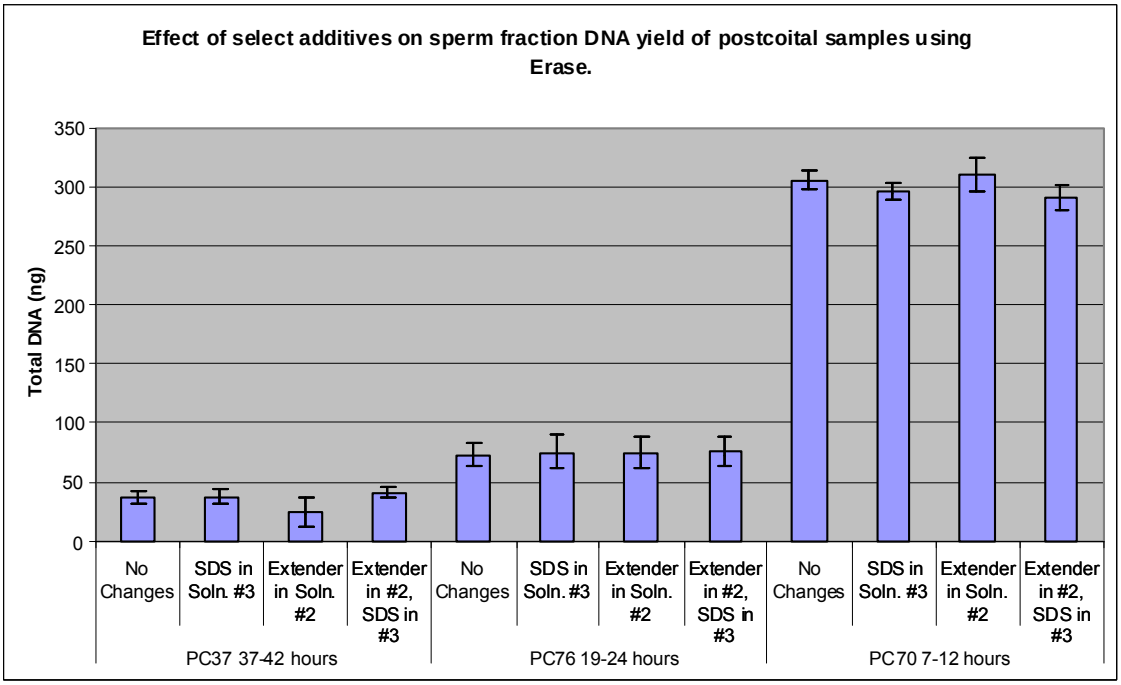


Figure 21 represents the averaged results for total DNA yield from the male fraction of E*rase* extractions of postcoital samples using various additives. Each postcoital swab sample was treated with Erase *proteinase* K extraction buffer and then divided equally for the step shown. The basic E*rase* procedure was compared with and without the addition of SDS, with egg yolk extender added to solution #2 and with both extender and SDS added. The results represent the average total DNA yield and their standard deviations for three replications.

(vi) Retaining Substrate Material in Extraction throughout Selective Degradation Procedure

The Erase procedure calls for removing the substrate after the sperm pelleting centrifugation step. The centrifugation step pelleted most sperm cells, but it is assumed that some of the sperm cells remained in the swab after centrifugation. It is common knowledge in the forensic DNA community that if a sexual assault substrate is re-extracted after the initial extraction process it is often possible to obtain sperm cells that remained on the substrate after the initial extraction process. Experiments were designed in order to determine whether introducing the substrate back into the sperm fraction tube after the sperm pelletting centrifugation step would increase the sperm cell DNA yield. The experimental data varied in significance, but the overall trend was that the addition of the substrate tended to lower the sperm fraction DNA yield. It is surmised that some of the sperm cell DNA may be trapped in the substrate after final centrifugation.

Figure 22

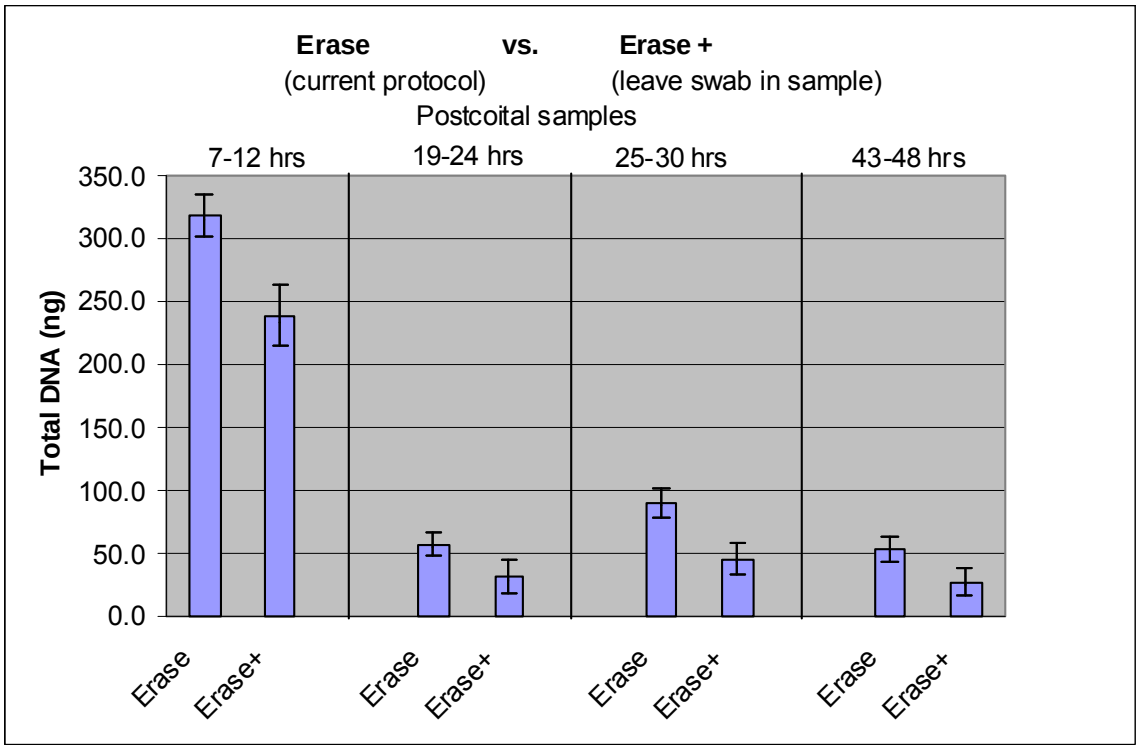


Figure 22 presents the results from Er*ase* extractions where the substrate material (swab) was either removed from the extraction (Er*ase*) or was added back after centrifugation (Er*ase+*). The chart shows the average total DNA yields and standard deviations for three replications each using four different postcoital swabs. Leaving the substrate in the extraction tended to decrease the yield of sperm DNA.

B. Validation

PTC also performed experiments to establish the developmental validation of the Erase kit. The developmental validation consists of experiments designed to determine the efficacy and reliability of this method for forensic casework analysis, including the determination of the conditions and limitations of operation of the new method.

The developmental validation studies include sensitivity, substrate storage stability, reproducibility, comparison to current methods with case type samples, mixtures, and precision and accuracy.

The Developmental Validation Study is contained in a separate attachment. We apologize for any redundancy that may have been caused by the fact that the Developmental Validation Study was drafted as a separate, stand-alone document, with its own recitation of methods, etc.

Please note also that Figures and Tables are numbered in their own, self-contained sequence in the Validation paper, without reference to the figure and table numbering in this Technical Report.

The significance of the developmental validation studies are discussed in this Technical Report, both in Section III-1-A regarding the comparison of performance of selective degradation to other methods, and in the three subsections of the Conclusion, Section IV-1, 2, and 3.

C. Beta Test Site Results

(i) Validation by Crime Laboratories

Erase has been validated by or will undergo validation in many crime laboratories around the country. Laboratories in Missouri, Illinois, Arizona, California, Louisiana, Maryland, Michigan, Texas, Florida, Virginia, Idaho, New York, Pennsylvania, Wisconsin and the FBI are currently validating the Erase method of differential extraction, along with several laboratories in Europe.

Several laboratories have either completed validation and are online with the method or are awaiting publication of the developmental validation of this process to go online.

With only one exception, every laboratory has reported positive findings with the Erase validation kit. In one instance an intern was allowed to proceed with testing the Erase kit. After speaking with her she stated that she had made several mistakes in the procedure during the testing. In addition, she did not have a clear understanding of how to make dilutions and of how many sperm were necessary to obtain a profile. Some of her work apparently involved dilutions to only10 sperm on a vaginal swab. It would not be expected that a male profile would be obtained under those conditions.

(ii) Difficult Case, St. Louis County Police Crime Laboratory

The St. Louis County Police Crime Laboratory (SLCPCL) worked a case in which they were unable to obtain a sperm DNA profile using traditional differential extraction methods, even though sperm were present on the slide for the sample. After two attempts using the standard differential extraction method, only a female profile with a trace of male DNA had been obtained by the crime laboratory from the sperm fraction.

At the time this case was in progress, E*rase* kits were not commercially available. The crime laboratory delivered a portion of the sample to PTC. PTC performed the differential extraction process on one half of a vaginal swab, using the Er*ase* method. PTC then returned purified DNA from the sperm and epithelial fractions to the St. Louis County Crime Laboratory.

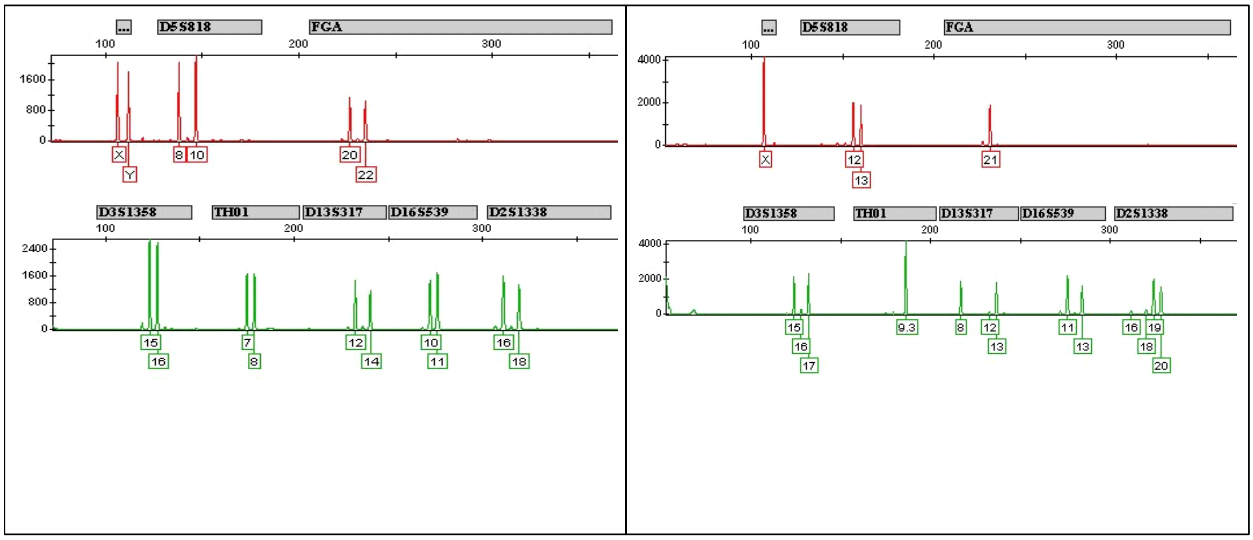
Using the purified DNA from the E*rase* extraction, the crime laboratory successfully produced a full 16 loci sperm DNA profile.

The electropherograms for some of the tested loci from the sperm fraction, representing both the traditional and selective degradation methods, are included in Figure 24. The full profile is not made available for privacy reasons.

Figure 24: E*rase* vs. Standard Method on a Difficult Sample

The sperm fraction of a sexual assault sample was processed by a crime laboratory using a standard differential extraction method. The profile obtained by the crime laboratory was female with a trace of male contributor. Subsequent attempts by the laboratory produced the same result. The sample was sent to PTC for processing with E*rase* and then the extract was returned to the crime laboratory. A full, mixture free male profile was obtained by the crime laboratory using the E*rase* extract.

E*rase* Profile **Standard Method Profile**



The profile produced by Erase had no above threshold alleles from the victim and only a trace of the victim below threshold. The sperm fraction profile was a full male profile and the statistics were straight forward.

The St. Louis County analyst was Ms. Margaret Walsh. Ms. Walsh is available to discuss the details of this case as they pertain to the use of E*rase*. In addition, the St. Louis County Police Crime Laboratory is now using Erase routinely, and both Ms. Walsh and Kyra Lienhop, DNA Technical Leader, would be happy to discuss the laboratory's experience with Erase generally.

In this instance, Erase made the difference in a DNA identification of the suspect as opposed to a trace profile where he could not be excluded.

(iii) Substrates other than Swabs

Several of the crime laboratories introduced various substrates for internal validation of E*rase*. The E*rase* method worked well with every substrate that the

crime laboratories tested. A partial list of substrates tested include those substrates most likely to be encountered in a forensic case, as follows: condom, tampon, sanitary napkin, denim, black cotton, nylons, and mesh underwear.

The Missouri State Highway Patrol Crime Laboratory (MSHP) tested the Erase kit and a number of substrates. Table 2, below, shows the substrates tested, and the results.

Table 2, Substrates Tested by Missouri State Highway Patrol Crime Laboratory

Differential Description	Sample type	Quantity (ng/uL)	Profile Description	Gender Characteristics
Post-coital	Swab of condom (inside) (NS)	0.0317	No profile	N/A
Post-coital	Swab of condom (inside) (S)	1.41	Single source	Male
Post-coital	Swab of condom (outside) (NS)	9.52	Single source	Female
Post-coital	Swab of condom (outside) (S)	0.0053	Partial, single source	Male
Post-coital (7 hrs.)	Tampon (NS)	0.0672	Partial, single source (one additional allele)	Female
Post-coital (7hrs.)	Tampon (S)	10.55	Single Source	Male
Postcoital	Sanitary napkin (NS)	19.16	Mixture	Male/Female
Postcoital	Sanitary napkin (S)	0.566	Single Source	Male
Neat semen	Denim (NS)	0.235	Single Source (one additional allele)	Male
Neat semen	Denim (S)	0.00933	Single Source	Male
1:1 female blood:semen	Black cotton (NS)	0.366	Partial, mixture	Male/Female
1:1 female blood:semen	Black cotton (S)	3.61	Single Source	Male
1:1 female blood:semen	blue mesh underwear (NS)	0.223	Mixture	Male/Female
1:1 female blood:semen	blue mesh underwear (S)	2.43	Single Source	Male
1:1 female blood:semen	white nylons (NS)	0.809	Mixture	Male/Female
1:1 female blood:semen	white nylons (S)	1.2	Single Source	Male
Reagent Blank	BLANK (NS)	0	No profile	N/A
Reagent Blank	BLANK (S)	0	No profile	N/A

S – Sperm Fraction NS – Non-Sperm Fraction

The Missouri State Highway Patrol Crime Laboratory tested Er*ase* with various substrates that are typical of those from sexual assault cases. Table 2 is a compilation of the results of those tests. The expected results were obtained on all substrates.

(iv) Differex Comparison

Differex is a commercially available product sold by Promega Corporation, and it is used by some crime laboratories as an alternative to the standard method of differential extraction. Selective degradation differential extraction results were compared to Differex results by the crime laboratory in Lausanne, Switzerland. It is an attachment to this report entitled Differex Comparison Data. The data from this comparison is available in an attachment labeled Differex Comparison Data.

PTC was not familiar with the Differex product and it was necessary to verify that the differences in results were not due to PTC's inexperience with Differex. The advantage of this crime laboratory study was that both Differex and Er*a*se could be tested by a laboratory with extensive experience using Differex.

The results of the Differex study are discussed below in Section E., Performance of Selective Degradation Compared to Other Methods.

D. Automation of Selective Degradation

The standard method of differential extraction that has been used in crime labs for many years is not amenable to automation. Using standard methods, a substrate is introduced to a mild lysis buffer that is stringent enough to lyse epithelial cells, but will not lyse sperm cells. After the extraction incubation period, the substrate is placed in a basket and the tube is centrifuged in order to remove the liquid from the substrate and to pellet the sperm cells. Then the analyst attempts to pipette as much of the supernatant (epithelial cell fraction) as possible from the tube with the sperm pellet without disturbing the sperm pellet.

The sperm fraction is diluted/washed several times with buffer in order to dilute the remaining epithelial cell DNA. Using this method, it can be very difficult to remove enough of the epithelial cell DNA so that the resulting DNA profile will be entirely from the sperm cell DNA. When the amount of epithelial cell DNA is very large and there are relatively few sperm cells it is often impossible to remove enough of the epithelial DNA that a sperm DNA profile is obtained. A mixed DNA profile often results.

The dilution/washing steps along with the need for very carefully avoiding the sperm pellet while removing as much of the supernatant as possible make the standard differential extraction process very difficult or impossible to automate.

The protocol for the selective degradation method as compared with other methods is extraordinarily simple to automate. There are no dilution and precise

pipetting steps. There is no need to attempt to wash away the epithelial cell DNA remaining in the sperm fraction. There is no need for additional centrifugation steps. After the initial epithelial lysis buffer is introduced, there is one centrifugation step that will allow for the removal of liquid from the substrate and will simultaneously pellet the sperm cells. After that, the process of elimination of epithelial DNA from the sperm fraction can be completely hands off. The robot performs a transfer of a portion of the epithelial fraction to another plate. Basic liquid handlers can be programmed to remove liquid to a designated height leaving the sperm pellet undisturbed. The robot introduces solutions #1 and #2 into the sperm fraction, mixes and incubates the samples, then transfers the samples to a new plate. The robot then adds solution #3 buffer to the sperm fraction and incubates the sample. At that point, both the epithelial and sperm DNA fractions are ready for DNA purification.

A wide variety of automated DNA purification method can be used from that point forward.

During this research, DNA extracted using the selective degradation process was successfully purified with the following purification systems:

InVitrogen Charge/Switch
Promega DNA IQ
Qiagen Mini-Amp
Qiagen EZ-1 Automated System
Maxwell 16 LEV System
Phenol/chloroform, ethanol precipitation
Vivacon100

The E*rase* Sperm Isolation Kit was tested using a simulated automation experiment using an 8-channel pipette and whole postcoital swabs to determine the suitability of selective degradation for automation. Similar experiments were also performed using a Tecan MiniPrep 75 liquid handler.

The JOE labeled electropherograms of the AmpFl STR® SGM Plus® amplifications from one column of a 96 well plate extracted using simulated automation and the E*rase* Sperm Isolation Kit are found in Supplemental Figure 23. Postcoital swabs, collected at various intervals following coitus, were placed into wells 1A, 1C, 1E, 1G, and 1H. DNA-free swabs were placed into wells 1D and 1F. A noncoital vaginal swab was placed into well 1B.

See Figure 23 (in Supplement), Profiles from Selective Degradation Extractions Performed in Column 1 of a 96 Well Plate

Expected results were obtained from every tested sample and all profiles were consistent with previous manual testing of similar samples.

The resulting sperm and epithelial cell fractions are single source male and female profiles, respectively, for all postcoital sample intervals tested (ranging from 0 - 36 hours). In addition, the DNA-free amplifications are not contaminated by the

neighboring wells. The noncoital sample in 1B demonstrates the efficiency E*rase*'s removal of the epithelial DNA from the sperm cell DNA fraction even when no sperm are present.

Forensic laboratories have numerous liquid handling devices available to choose for automation of case work. Scripts will need to be written and validations performed on each model of robot, but the Erase validation for automation has been developed to support a general application that can be adapted to any liquid handling device.

In house, automation was performed using a very basic Tecan model. External laboratories have been recruited for validation on other instruments, but those experiments are not complete. The key steps to optimize the procedure are 1) removal of the supernatant to leave 50ul over the pelleted sperm cells, 2) addition of 10ul each of Solution 1 and Solution 2, thoroughly mixing, then transferring to a new 96 well plate, and 3) the addition of 10ul of Solution 3, and then mixing. There are incubation steps along the way that may or may not be handled by the robot, depending on the robot model available to the laboratory. The procedure is straightforward.

Different liquid handling devices use different programs for operation. To set up the Erase method for automating differential extractions, three robotics companies have been contacted and asked to prepare scripts for their devices that can be used for Erase automation. Those companies are Hamilton, Tecan, and Beckman-Coulter.

For further information, including the Erase Sperm Isolation Kit protocol and references to Solutions 1, 2, and 3, see the Methods section of this Final Technical Report.

E. Performance of Selective Degradation Compared to Other Methods

In demonstrating the effectiveness (or ineffectiveness) of the selective degradation technology, one important aspect of this research was a head to head comparison with existing technologies.

The research performed at PTC included such comparisons, as did testing performed by other laboratories.

The comparisons performed in various laboratories repeatedly show the same results obtained by PTC. Because of the ability to remove more epithelial DNA from the male fraction, the selective degradation method of differential extraction provides substantially better results. On samples with larger amounts of sperm, it provides clean single source male profiles rather than mixed sperm and epithelial profiles. On samples containing fewer sperm, selective degradation allows the recovery of a male profile even when other current methods of differential extraction provide only a partial male profile or no male profile at all.

(i) Research Performed at PTC

There were three different sets of experiments at PTC that directly compare the performance of selective degradation to the performance of the traditional method of differential extraction.

(a) Comparison with Samples with Five Hundred Sperm -- After completing the optimization experiments for selective degradation, PTC performed a test on noncoital vaginal swabs, with 500 sperm cells added to each, as discussed above in Section III-1-A-(iv)(d). The experiment used the selective degradation method and the traditional method for differential extraction.

As shown in Figure 6, the traditional method primarily produced an epithelial cell DNA profile in the sperm fraction, although traces of the male profile could be seen at some loci. But no sperm DNA alleles could be called In this case, the traditional method of differential extraction was not capable of removing enough epithelial DNA to isolate the sperm cells from the epithelial DNA, and as a result no sperm profile was identified.

In this same experiment, the selective degradation method produced a clean male profile. Sufficient epithelial DNA was removed from the sperm fraction, by degradation, to allow the male profile to be easily identified.

(b) Comparison with Samples containing Menstrual Blood -- In another experiment, described in Section III-1-A-(iv)(k), a comparison between the selective degradation and traditional methods was conducted on samples that contained menstrual blood. The samples were vaginal swabs, collected at 12 hours postcoitus.

As can be seen in Figure 18, the standard differential extraction method produced sperm cell fraction samples that were mixed with DNA from both the epithelial cell donor and the semen donor. The sperm fraction under the selective degradation method provided a clean male DNA profile, with no need for mixture interpretation.

(c) Sensitivity Study – In the Developmental Validation Study, PTC performed a sensitivity study, comparing the traditional method of differential extraction with selective degradation. These experiments were devised to determine the minimum number of sperm cells necessary to obtain a single source sperm DNA profile using selective degradation. These experiments are more fully described in the Validation Study.

Duplicate mock forensic samples were prepared using noncoital vaginal swabs spiked with varying amounts of sperm. Samples were prepared using 2660, 1330, 665, 333, and 166 sperm cells. Traditional and selective degradation differential extractions were performed. The DNA was amplified using the PowerPlex16 system and separated using polyacrylamide gel electrophoresis. The results at the D21S11 locus are displayed in Figure 1 of the Validation Study, and Table 1 of that study summarizes the data.

Using the traditional method, the sperm fraction showed a mixed profile for all samples. The 2,660 sperm sample was a mixture with the sperm donor as the major contributor. At both 1,330 and 665 sperm, the contributions from the sperm and epithelial donors were approximately equal. At 333 and 166 sperm, the standard method produced on the epithelial donor's DNA profile. No sperm DNA profile could be identified.

The selective degradation method gave a clean male profile, with no significant epithelial cell DNA, at 2,660, 1,330, 665, and 333 sperm. For the sample with 166 sperm, the selective degradation method produced a mixed DNA profile, with approximately equal contributions from the sperm cell and epithelial cell donors.

(ii) St. Louis County Police Crime Laboratory

The St. Louis County Police Crime Laboratory has had experience with the selective degradation method of differential extraction both in actual case work, and in performing some of the experiments for the developmental validation of E*rase*.

(a) Difficult Case -- The St. Louis County Police Crime Laboratory (SLCPCL) worked a case in which they were unable to obtain a sperm DNA profile using traditional differential extraction methods, even though sperm were present on the slide for the sample. After two attempts using the standard differential extraction method, only a female profile with a trace of male DNA had been obtained by the crime laboratory from the sperm fraction.

Because E*rase* kits were not yet available, SLCPCL delivered a portion of the sample to PTC. PTC performed the E*rase* differential extraction process on one half of a vaginal swab. PTC then returned purified DNA from the sperm and epithelial fractions to the St. Louis County Crime Laboratory.

Using the purified DNA from the E*rase* extraction, the crime laboratory successfully produced a full 16 loci sperm DNA profile. That profile had no above threshold alleles from the victim and only a trace of the victim below threshold. The statistics were straightforward, and the case went to court.

Electropherograms for a portion of the sperm fraction profile under both methods are shown in Figure 24, above.

As this actual case indicates, E*rase* has the potential to obtain male profiles for many cases that could not previously be solved using autosomal DNA from a sexual assault.

Ms. Walsh (the St. Louis County Police Crime Laboratory analyst on this case) also mentioned that E*rase* has been very helpful with male-male profiles in sexual assault cases where the victim has had relations with two men (either two rapists, or the rapist and a consensual partner). Because E*rase* removes so much

epithelial DNA, the crime lab obtains a mixed profile of just the two males, and not all three parties. She stated that this allows her to deconvolute the two male profiles. Using their traditional method in the past, the epithelial profile also appeared in the male fraction, and she was unable to deconfolute the profiles. Her conclusions are consistent with the results of the Mixture Studies performed at PTC for the developmental validation study.

(b) Comparison with Case Type Samples -- The St. Louis County Police Crime Laboratory performed some of the experiments for the E*rase* developmental validation study, including the comparison to current methods with case type samples. In this portion of the study, SLCPCL compared their traditional method of differential extraction with the selective degradation method, on postcoital swabs that were collected at two different intervals following coitus. These experiments are described in more detail in the attached Developmental Validation Study. The electropherograms for the sperm fractions in these comparisons are shown in Figure 2 of the Validation Study.

The 13-18 hour postcoital sample processed with the E*rase* Sperm Isolation Kit produced a single sperm fraction DNA contributor, with a well balanced male profile, ideal for identity matching. The sample processed with the standard differential extraction method produced a mixed donor profile in the sperm fraction, with the male as the major contributor. The sperm DNA profile is suitable for searching and matching.

The 31-36 hour postcoital sample processed with the E*rase* Sperm Isolation Kit produced a single contributor, well balanced male profile. The sample processed with the standard differential extraction method produced a mixed donor profile with the major profile being from a female contributor. Some alleles from the male donor are identified, but searching for a matching DNA profile would be difficult and may be impossible. In the event a potential match is identified, the statistical calculations necessary would be tedious and significantly less powerful than a single source DNA profile match.

The sensitivity studies that show how few sperm the selective degradation require in order to obtain a sufficient sperm DNA profile, are somewhat theoretical in the sense that in case work the crime lab doesn't know how many sperm they are starting with. However, comparisons with vaginal swabs collected at different intervals postcoitus are representative of actual casework situations. It is assumed that in general, the longer the interval before collection of the swab, the fewer sperm cells obtained. This study of case type samples is consistent with the findings of the sensitivity studies, and shows some of the advantages of the selective degradation method in situations routinely encountered in the forensic laboratory setting

(iii) Los Angeles County Sheriff's Department Crime Laboratory

The Los Angeles County Sheriff's Department Crime Laboratory performed experiments comparing their traditional method of differential extraction to E*rase*.

Side by side comparisons by the Los Angeles County Sheriff's Department produced data typical of standard differential/E*rase* comparisons.

Typical data from one of several experiments is shown. Two sets of swabs, one 12 hour postcoital swabs and the other a greater than 24 hour postcoital swabs were processed for a standard differential/E*rase* comparison. The data indicates that the 12 hour postcoital swab produced a single source sperm DNA profile with both methods, although there was some X/Y peak height imbalance (26%) noted for the standard differential extraction method. The standard differential method produced a mixed DNA profile for the >24 hour postcoital swab, while the E*rase* method produced a single source sperm DNA profile, with no indication epithelial DNA, but with some X/Y peak height (8%) imbalance noted.

The results for loci from the longer than 24 hour postcoital swab are shown in Figure 25.

Figure 25 Comparison of Methods at L.A. County Sheriff's Department

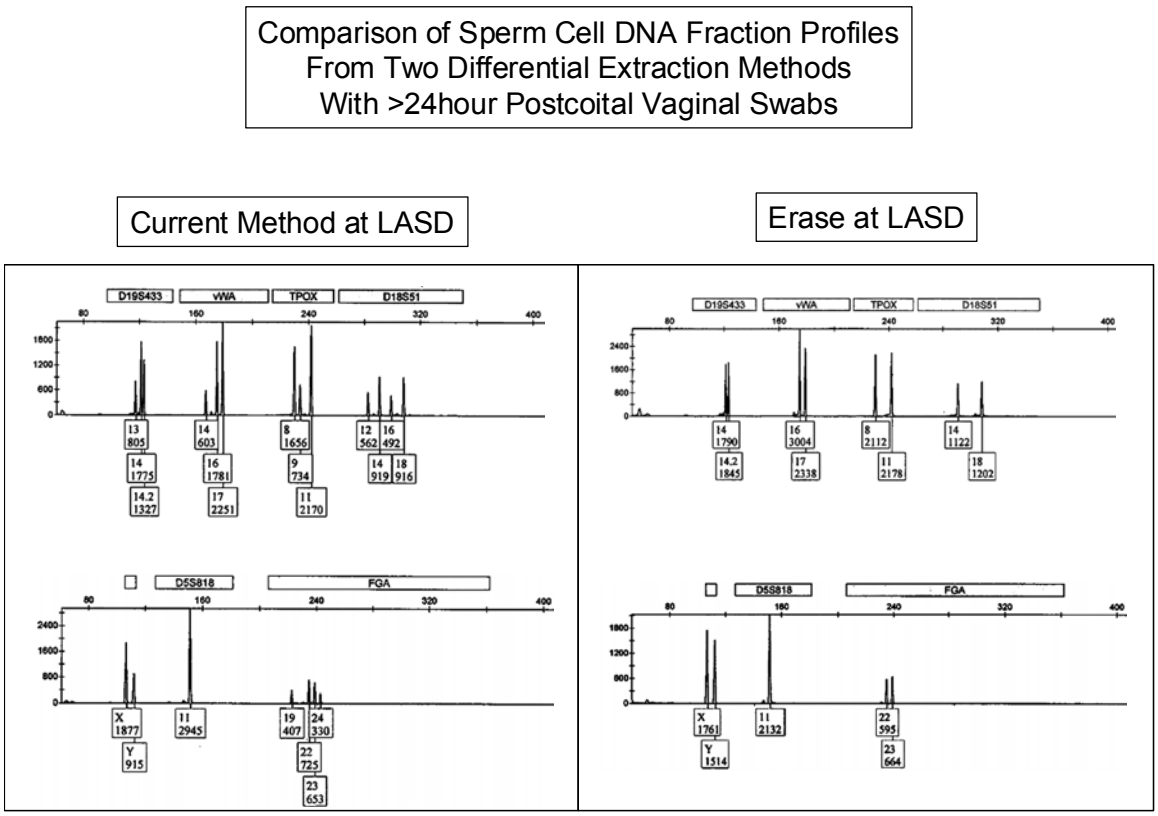


Figure 25 shows the results at a few loci in the sperm fraction from a 24 hour swab, under both methods, at the L.A. County Sheriff's Department Crime Laboratory.

Comparisons of another set of 12 and 24 hour postcoital swabs gave similar data, although there was less epithelial DNA in the subsequent >24 hour swab processed with the standard differential method than in the mixture in figure 25.

(iv) Differex Comparison

Differex is a product marketed by Promega Corporation as an alternative to the traditional method of differential extraction. It is likely the alternative to the standard differential extraction method that is most used by crime laboratories to process sexual assault casework.

A direct comparison of Erase with Differex was performed by a crime laboratory in Lausanne, Switzerland that has been using Differex on casework for a number of years. At the time of this study, Differex was the only differential extraction method used by the Lausanne crime lab for processing sexual assault cases. PTC was not familiar with the Differex product, and it was necessary to verify that the differences in results obtained in house were not due to inexperience with Differex. The advantage of this crime laboratory study was that both Differex and E*rase* could be tested by a laboratory with extensive experience using Differex.

Other details of the comparison are addressed in the separately attached study. In this section the comparisons between the Differex method and selective degradation method of differential extraction were made on samples containing varying numbers of sperm cells. The number of sperm for the four different sets of samples were 50,000 sperm; 16,700 sperm; 5,000 sperm and 1,670 sperm.

The CE data for these experiments is reflected in electropherograms in the attached Differex Comparison. The remainder of the discussion in this Section addresses the comparative results shown by that data.

The Differex results for samples containing 50,000 sperm have epithelial DNA profiles above the peak height threshold in the male fraction at several loci. However, the sperm DNA is clearly the major contributor and there appears to be much more sperm DNA than epithelial DNA in the sperm fraction. The Differex results with 50,000 sperm show a mixed profile, that profile will be easily deconvoluted, and will require little interpretation by the analyst.

The selective degradation method produced clean single-source sperm profile, with no indication of epithelial DNA.

The samples with 16,700 sperm have similar results to the samples with 50,000 sperm. Differex produced a mixture profile with more sperm DNA than epithelial DNA. Selective degradation produced a clean sperm profile with no indication of epithelial DNA.

The samples with 5,000 sperm cells demonstrate a greater difference between the two methods. Using 5,000 sperm, the male fraction of the Differex extraction generated a mixed profile, with the epithelial DNA profile as the major contributor and the selective degradation method produced a clean male profile, with no indication of epithelial DNA.

The comparison of the samples with 1,670 sperm cells are dramatically different. The Differex male fraction is mostly X at the Amelogenin locus and the Differex

male fraction profile is a mixed profile with the female as the major contributor. The selective degradation method continued to produce a clean male profile with no indication of epithelial DNA. The vWA locus has above peak height stutter, but those alleles are not consistent in size with the epithelial cell DNA profile.

The overall conclusion of the Differex comparison is that selective degradation provided a cleaner sperm profile than Differex in all differential extractions tested. With decreasing numbers of sperm cells the Differex method demonstrated an inability to isolate the sperm cells from the epithelial DNA producing mixed and/or partial sperm DNA profiles from the sperm fraction, while the selective degradation method continued to provide a clean and full sperm DNA profile.

The comparisons between the selective degradation method of differential extraction and other commonly used methods of differential extraction consistently provide similar results, whether performed by PTC or by public crime laboratories. The method of selective degradation effectively removes epithelial DNA from the sperm fraction of the differential extraction therefore providing better sperm DNA profiles than the methods compared. Selective degradation often results in single source sperm DNA profiles when other method produce mixed profiles.

2. Tables

The body of this Final Technical Report contains two tables, and the attached Developmental Validation Study contains three tables, as follows:

Table 1, Summary of Incubation Time Data, Section III-1-A-(iv)(j).

Table 2, Substrates Tested by Missouri State Highway Patrol Crime Laboratory

Validation Table 1, Sensitivity Study, in Tables section of Validation Study.

Validation Table 2, Mixtures, in Tables section of Validation Study.

Validation Table 3, Precision and Accuracy, in Tables section of Validation Study.

3. Figures

Certain figures are included in the body of this document, located where their content is being discussed in the text.

Figures that take a lot of memory, and could make it impossible to e-mail this document, are in separate attachments. The figures in the separate attachments include Figures 4, 6, 7, 9, 10, 14, 16, 17, 18 and 23. References to these figures in text also make the notation “supplemental” to indicate that they are in an attachment rather than in the text. The remaining figures are located in the text above.

Figure List

This list identifies figures in this report by number, title and location.

Figure 17, replaced by Table 1, Summary of Incubation Time Data, Section III-1-A-(iv)(j)

Figure 18 (in Supplement), Comparison of Methods on Vaginal Swab with Menstrual Blood, Section III-1-A-(iv)(k)

Figure 19, Effect of EGTA on Selective Degradation Sperm DNA Yield, Section III-1-A-(v)

Figure 20, Effect of Nuclease Heat Inactivation on Sperm Cell DNA Yield, Section III-1-A-(v)

Figure 21, Effect of Select Additives to Sperm Lysis Step on Sperm DNA Yield, Section III-1-A-(v)

Figure 22, Effect of Leaving the Substrate in the Extraction on Sperm DNA Yield, Section III-1-A-(vi)

Figure 23 (in Supplement), Profiles from Selective Degradation Extractions Performed in Column 1 of a 96 Well Plate.

Figure 24, Erase vs. Standard Method on a Difficult Sample, Section III-1-C-(ii).

Figure 25, Comparison of Methods by the L.A. County Sheriff's Department, Section III-1-E.

Validation Figure 1, Sensitivity Study, in the Figures section of Validation Study.

Validation Figure 2, Case Type Samples, in the Figures section of Validation.

Validation Figure 3, Precision and Accuracy, in the Figures section of Validation.

IV. Conclusions

1. Discussion of Findings

The major goals of this research were: (i) to optimize the technique of performing differential extractions using selective degradation to remove epithelial DNA from the sperm fraction, as originally published by Garvin [1]; so that it will work on most samples encountered in a forensic sexual assault case (ii) demonstrate that the selective degradation method is a valid method for use in forensic laboratories; (iii) demonstrate that by using the selective degradation method, the process of differential extraction may be automated, and design the process to be able to coordinate with whatever liquid handling equipment a crime laboratory is currently using; and (iv) compare the performance of the selective degradation method to other methods of differential extraction currently in use.

The following is a discussion of findings in the Results section of this paper, for each category of experiments (optimization, validation, etc.). It ties the findings and conclusions to the goals of the research.

A. Optimization of Selective Degradation

During the optimization experiments, even the shape of the microfuge tube that would best facilitate differential extraction was determined.

Experiments varying proteinase K concentration, incubation temperature and incubation time established optimal settings for lysing epithelial DNA, without reducing the yield of sperm DNA.

The findings regarding the elimination of epithelial DNA demonstrated that even after optimization of the selective degradation method, small amounts of epithelial DNA remain in the sperm fraction. But findings regarding the ability of the selective degradation method to obtain male profiles from samples with low amounts of sperm, when other methods cannot, suggests that the reduction in epithelial DNA is effective.

Experiments to optimize the nuclease treatment began with finding the optimal concentrations of Magnesium and Calcium necessary to activate the nuclease. Concentrations and sources of nuclease, as well as incubation times and temperatures, were optimized for maximum sperm DNA yield.

In a number of areas, the original protocol published by Garvin [1] was improved upon. As a result of these experiments, the selective degradation procedure for differential extraction has been optimized for use in the forensic laboratory, and is shown to be an improvement on current technology. This meets the first goal of this NIJ research grant.

B. Validation of the Selective Degradation Method

The developmental validation consisted of experiments designed to determine the efficacy and reliability of this method for forensic casework analysis, including the determination of the conditions and limitations of the new method.

The developmental validation studies included determinations of sensitivity, substrate storage stability, reproducibility, comparison to current methods with case type samples, mixtures, and precision and accuracy.

This research found that the selective degradation method was sensitive enough to identify a male profile from samples with as few as 166 sperm. Selective degradation was able to produce single source sperm DNA profiles after postcoital mock forensic samples had been stored at room temperature for nine months, and even on historical samples stored at room temperature for more than eleven years old.

Both the quantity of sperm DNA recovered from the selective degradation method, and the DNA profiles themselves, were found to be reliably reproducible in the hands of different analysts and when processed by different laboratories. It was also found that the selective degradation method worked well with samples that included a mixture of DNA from two sperm donors. The absence of DNA from the victim made deconvolution of the DNA profiles much easier than it would have been if the victims DNA was present.

The comparison to current methods using case type samples found that, as the interval between coitus and collection of the vaginal swab increased, the selective degradation method performed increasingly better than the traditional method. These findings were consistent with the sensitivity study findings showing the superior performance of the selective degradation method on samples with lower amounts of sperm cells.

The findings from the developmental validation study demonstrate that the selective degradation method of differential extraction is a robust method, with certain advantages over other current methods, and is capable of producing results reliably and repeatedly by multiple analysts and laboratories. This method is shown to be useful and usable for forensic casework analysis. This conclusion regarding the validity of the method meets one of the goals of this NIJ research.

C. Automation of Differential Extraction

The experiments regarding the ability to automate differential extractions by using the selective degradation method found that the method works well in a 96 well format, and can be automated using existing automation equipment in crime laboratories.

As each step of the selective degradation process developed, it was designed so that a robot could perform the same steps that would be performed by the analyst at the bench. This allowed for a very smooth transition from the single tube method to a 96 well plate format. All of the steps involved in automation of this process are steps that can easily be performed and programmed for very basic liquid handlers.

The more sophisticated robots may be able to handle a few more of the incubation steps than the most basic liquid handler, but all of the hands-on steps that would require more than a simple transfer of a plate by the analyst can be handled by the vast majority, if not all, of the liquid handling robots currently available in public crime laboratories.

The possibilities range from hands on moving of trays in and out of incubation steps, to a completely hands off approach with the probable exception of the centrifugation step after the initial lysis. However, Hamilton is introducing a liquid handler that also incorporates the centrifugation step, and that robot's overall mechanism for handling the DNA samples appears to be tailor made for the selective degradation process.

In most instances, the crime laboratories will be able to implement this methodology using existing equipment. Because most if not all of the standard liquid handlers currently employed by crime laboratories can be programmed to the parameters necessary for this differential extraction method, public crime laboratories will not have to invest in new equipment in order to use this methodology. A few hours of programming or executing a pre-prepared script and they will be ready to validate this method.

Demonstration of the ability to automate differential extractions using the method of selective degradation has accomplished another major goal of this NIJ research.

D. Comparison of Selective Degradation to other Current Methods

This section involves comparisons between the selective degradation method and the traditional method of differential extraction, as well as the Differex alternative. It draws together the results of experiments from various other portions of the study, to focus attention on the comparison between methods.

Comparisons between methods are documented here as performed by PTC, the St. Louis Count Police Department Crime Laboratory, the Los Angeles County Sherriff's Department Crime Laboratory, and the crime laboratory of Lausanne, Switzerland (for the Differex comparison).

These experiments consistently find that, no matter who performs the differential extraction, the selective degradation method of differential extraction consistently has significant performance advantages over other current methods.

Those advantages include:

(i) a clean, single source sperm DNA profile is developed in most cases, and mixtures with the epithelial DNA profile are generally avoided;

(ii) a single source sperm DNA profile is generally obtained even if the sample has so few sperm that the traditional method produces either a minor contributor partial sperm DNA profile, or no sperm profile at all. One practical application of the sensitivity of selective degradation is its superior performance on vaginal swabs collected at longer intervals following coitus;

(iii) comparison of selective degradation method to the Differex method of differential extraction produced results similar to the comparisons of selective degradation to the traditional method of differential extraction, with selective degradation producing far more instances of single source sperm profiles;

(iv) selective degradation's increased sensitivity also produced superior results when samples contained menstrual blood, because of the ability to eliminate the extraneous DNA contributor from the sperm fraction;

(v) the elimination of more epithelial DNA from the male fraction facilitates the deconvolution of the DNA profiles of multiple sperm donors;

(vi) the amount of analyst hands-on time to perform the differential extraction, as well as the overall time from start to finish of the differential extraction procedure, is much reduced using selective degradation (see chart at beginning of Methods section showing time savings for individual steps and overall); and

(vii) finally, there is the advantage of automation.

The comparisons performed in various laboratories repeatedly duplicate the same results obtained by PTC. Because of the ability to remove more epithelial DNA from the sperm fraction of the differential extraction, the selective degradation method of differential extraction provides substantially better results. Determining the relative performance of the selective degradation method compared to other current methods fulfills another goal of this grant.

Overall, this discussion of findings demonstrates that this research has accomplished all of the goals of the research grant, and provided a substantial benefit to law enforcement.

2. Implications for Policy and Practice

The implications for policy and practice are substantial. The results of this research mark the successful culmination of federal research expenditures and numerous efforts by the forensic community, researching various proposed mechanisms to eliminate the serious problem of mixed DNA profiles in sexual assault evidence.

Among the specific benefits of this technology, it will identify perpetrators of sexual offenses who could not previously be identified using DNA evidence. It will save time in the forensic laboratory and in court, and it can be expected to increase the rate of convictions of perpetrators of sexual assaults. It will also allow automation of the process of differential extraction, and consequently facilitate reduction of the backlog of sexual assault evidence.

The following is a list of some of the major impacts of this research on law enforcement practices and outcomes:

1. Mixtures in sexual assault evidence will be eliminated in almost all cases;

2. Time will be saved in the performance of differential extractions in the crime lab. Less hands on time and elimination of the rinsing steps to dilute the epithelial cells in the male fraction, as well as shorter incubation times, cut the overall processing time to roughly one third of the time needed for a standard differential extraction.

3. All of the time and effort previously devoted to mixtures in the laboratory involving the statistical analysis is eliminated in most cases. If the analyst would have previously obtained a mixture profile from the same sample and now obtains a single source male profile, then the hours or even days of mixture deconvolution, statistical calculation and review time for a single case may be reduced to minutes.

4. All of the time, effort and uncertainty resulting from mixtures at trial, possibly raising "reasonable doubt" and causing the criminal to be set free, is eliminated if the DNA profile is a single source profile.

5. In some cases, clean single-source male profiles will now be obtained where no profile was previously available. Already, using Er*ase* on a case in Missouri for which the crime laboratory was unable to detect any discernable sperm DNA profile in the mixed stain using a traditional differential extraction resulted in a full male profile in the sperm fraction.

6. Because of the ability to successfully profile smaller amounts of sperm DNA in mixed stains than previously possible, certain current and cold cases that had no identified suspect can now be solved.

7. For case work, the resulting ability to easily automate the differential extraction process will save additional time and manpower in crime laboratories that process larger volumes of sexual assault evidence. This method of differential extraction makes it possible for a single analyst to take a 96-well tray from the initial lysis step to DNA purification with only a few minutes of hands on time.

It will be possible for crime laboratories to automate whatever portion of the process is compatible with their existing equipment, without being forced to spend funds to obtain equipment just for this purpose.

Manufacturers of the most popular robots currently in use in crime laboratories in the United States and Europe, which are Tecan, Beckman-Coulter, and Hamilton, were contacted. Collaboration with those companies to develop scripts for the robots to be able to automate differential extractions using selective degradation is in progress. The scripts will be of major assistance to those crime laboratories choosing to automate.

The Er*ase* kit is now commercially available for the single tube method, and is available for custom ordering in the 96 well format.

8. The resulting ability to easily automate the differential extraction process will also be a tremendous benefit in reducing the backlog of sexual assault evidence.

Changes in Policy, Practice and Outcomes in the Forensic Laboratory

Since this technology makes it much more likely that an autosomal sperm fraction DNA profile will be produced, it changes the strategy and overall outcome of a

sexual assault case in many instances. If a sperm fraction autosomal profile can be produced then the profile can be searched in CODIS. If a CODIS search reveals the identity of the assailant then many more sexual assaults may be prevented, and an overall reduction in the number of sexual assaults results in a smaller caseload.

It is the policy or practice of some crime laboratories to eliminate the differential extraction step altogether if a minimum number of sperm are not identified before DNA extraction. This leaves only the option of Y Chromosome analysis in order to identify the rapist. This results in less certain identifications and the inability to perform productive CODIS searches. With selective degradation, it will be possible to obtain an autosomal profile in many or most of those cases and the overall outcome may be a change in policy that allows for differential extractions to be performed whenever sperm are present.

The policies and practices employed by crime laboratories for sexual assault evidence may change as a result of this product or there may be no need to change the policies and practices because using this product may change the nature of the evidence and therefore change the overall outcome of the case. If, for instance it is the policy of the crime laboratory to perform Y Chromosome testing any time that the quantitation data indicates that there is 4 times more epithelial DNA than sperm DNA in a sample, and a case has 5 times as much epithelial DNA as sperm DNA, then Y Chromosome testing is performed. If that same case, because of selective degradation, results in the laboratory finding virtually all male DNA, and it is the policy of the laboratory to therefore perform autosomal DNA testing, then the policy did not have changed, but the selective degradation method will have caused a change in the subsequent procedures and possibly the outcome. The policy and practices of the laboratory in this instance do not need to change in order to cause a change in the outcome of a case.

The impact on policies and practices for sexual assault cases will vary from one laboratory to the next. In some instances, there will be little or no impact on policy and practices. In other laboratories there may be a very significant impact. What will show significant impact in all crime laboratories is the outcome many cases, the overall time required to complete each case, and the likelihood that a DNA identification will be made.

The E*rase* kit has either been validated in, or is currently undergoing validation by, more than 20 U.S. crime laboratories, as well as several laboratories in Europe. Additional laboratories have expressed interest and, as others go online and report positive outcomes, it is expected that many more laboratories will also validate the kit. Several crime laboratories have completed validation of the single tube kit and are either on line or are completing competency exams. They are pleased with the results they are witnessing, and are anxious to take advantage of this technology as soon as possible.

Completing this Final Technical Report, and publishing the developmental validation study, should give all crime laboratories the necessary confidence in this technology to begin pursuing it as soon as they have the time and resources.

This successful NIJ grant has produced a tremendous benefit to law enforcement in the United States, and around the world.

3. Implications for Further Research

A. Time Sensitivity of Nuclease Step

Although this product is very reliable and easy to use there are areas that could benefit from improvement. The nuclease step is very time sensitive. Forgetting or leaving a sample in nuclease for too long could result in significant loss of sperm DNA. A product that has a very flexible incubation time would be beneficial. The analyst would not have to worry that if for some reason they could not get back to the extraction for a significant period of time that their DNA would be lost. More flexibility is always better.

B. Compromised Sperm Membrane

In cases where the sperm membrane is compromised the nuclease appears to penetrate the membrane readily and causes loss of sperm DNA. Therefore, in cases where microscopically the sperm membrane appears to be significantly damaged it is not advisable to use this product. Developing a product that can be used in all cases would be a significant value for crime laboratories.

Even in cases with no apparent damage to the sperm cell membrane there may be DNA loss using this method. Further research revolves around ways to employ this method without damaging DNA within the sperm cell. If it is possible to utilize this method without fear of damage to sperm DNA then the product becomes more useful to the DNA analyst. Several methods are being explored with one method giving very promising results. Future funding opportunities may be sought to develop this method further, and to explore other possibilities.

C. Sperm DNA Loss

Both the traditional method of differential extraction and the selective degradation method incur loss of some amount of sperm cell DNA during the differential extraction process.

Side by side comparison with the traditional method of differential extraction indicates no consistent difference in loss of sperm cell DNA between the traditional method and the E*rase* method. But differences in side by side comparisons of E*rase* samples processed with and without nuclease indicate that selective degradation causes a loss of sperm DNA during the nuclease step. Although selective degradation may not result in greater sperm DNA loss than the traditional method of differential extraction, improvements to the selective degradation method could result in a greater recovery of sperm DNA from forensic samples.

If the sperm DNA loss during the nuclease step of selective degradation can be eliminated then that would be another major improvement to the differential extraction process.

Comprehensive investigation into the causes of DNA loss would be beneficial. The amount of DNA evidence available from the perpetrator of the crime is often very small. Some minimum amount of DNA is necessary in order to obtain a DNA profile. Additional research may lead to improved methods for differential extraction that retain a larger portion of this critical evidence, leading to more criminal identifications and convictions.

D. Direct Amplification

Another area to be explored is direct amplification of the selective degradation sperm and epithelial cell DNA fractions. There are several advantages that would be realized from direct amplification. The cost and processing time associated with DNA purification would be virtually eliminated. The loss of DNA associated with DNA purification would be eliminated. There would be less chance of sample contamination because the sample would be handled less, and direct amplification would require less analyst hands-on time to complete.

PTC is exploring a number of possible ways to accomplish this goal. Ongoing research relating to direct amplification of differential extracts from selective degradation is needed.

V. References

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VI. Dissemination of Research Findings

This research has resulted in the refinement and validation of a more effective method for performing differential extraction of sexual assault evidence. PTC and the co-investigators under this grant have been very active in publicizing the resulting method, along with examples and explanations of the improved results from this method. Some of the publicity was structured into the grant, and much of it was provided voluntarily.

Seminars

The selective degradation method of differential extraction was presented at the following seminars, either as a presentation to attendees, a poster or a vendor booth, or some combination of the three. Vendor booths made literature and additional information about selective degradation available to all attendees, in a setting that allowed all interested parties to ask questions and be given a full explanation, as well as to request a validation kit. For those unfamiliar with it, the Mid-America Forensic DNA Conference is an annual conference sponsored by PTC. It is attended by approximately one hundred DNA Criminalists each year, primarily from the Midwest.

1. 8th Annual Mid-America Forensic DNA Conference
   April, 2010 in Columbia, Missouri
   A. Presentation by Dr. Alex Garvin (co-PI)
      "Purification of Sperm DNA from Vaginal Swabs using DN*ase*"
   B. Vendor Booth by PTC

2. National Institute of Justice (NIJ) Conference 2010
   June, 2010 in Arlington Virginia
   A. Poster Presentation by Dr. Christian Carson (PI)
      "Purification of Sperm DNA from Vaginal Swabs using the Erase Sperm Isolation Kit"

3. 21st International Symposium on Human Identification by Promega
   October, 2010 in San Antonio, Texas
   A. Poster presentation by Dr. Alex Garvin (co-PI) and Kim Gorman
      "Purification of Sperm DNA from Vaginal Swabs using the Erase Sperm Isolation Kit"
   B. Vendor Booth by PTC

4. 9th Annual Mid-America Forensic DNA Conference
   April, 2011 in Columbia, Missouri
   A. Presentation by Kim Gorman, PTC
      "Erase: Better Differential Extractions"
   B. Validation Presentation by Kathy Press, Arizona Department of Public Service, Central Regional Crime Laboratory, Phoenix
      "Laboratory Validation of Erase for Differential Extractions"

C. Vendor Booth by PTC

5. National Institute of Justice (NIJ) Conference 2011
June, 2011 in Arlington Virginia
A. Poster Presentation by Kim Gorman, PTC
"Purification of Sperm DNA from Vaginal Swabs using the Erase Sperm Isolation Kit"

6. Green Mountain DNA Conference 2011
July, 2011 in Burlington, Vermont
A. Presentation by Michelle Beckwith, PTC
"Erase: Better Differential Extractions"

7. Midwestern Association of Forensic Scientists (MAFS), 2011 Meeting
September, 2011 in Lombard (Chicago), Illinois
A. Vendor Booth by PTC
(an Erase poster was also displayed, but not on the program)

8. American Society of Crime Laboratory Directors (ASCLD), 2011 Sympos.
September, 2011 in Denver, Colorado
A. Poster presentation by Michelle Beckwith
"Using Selective Degradation to Improve Differential Extraction Quality and Throughput"

9. 22nd International Symposium on Human Identification by Promega
October, 2011 in National Harbor, Maryland
A. Poster presentation by Kim Gorman
"Using Selective Degradation to Improve Differential Extraction Quality and Throughput"
B. Vendor Booth by PTC

10. California Association of Criminalists, Fall 2011 Meeting
October, 2011 in Sacramento, California
A. Presentation by Michelle Beckwith, PTC
"Using Selective Degradation to Improve Differential Extraction Quality and Throughput"
B. Vendor booth by PTC

11. Promega Users Working Group
October, 2011 in Vancouver, Canada
A. Presentation by Kim Gorman, PTC
"Erase: Better Differential Extractions"

12. Promega Users Working Group
November, 2011 in Ottawa, Canada
A. Presentation by Michelle Beckwith, PTC
"Erase: Better Differential Extractions"

13. 10th Annual Mid-America Forensic DNA Conference
    April, 2012 in Columbia, Missouri
    A. Presentation by Ruth Montgomery, Missouri State Highway Patrol, & Kyra Lienhop, St. Louis County Police Department
    "Erase: Validation and Casework"
    B. Vendor Booth by PTC

14. Louisiana Association of Forensic Scientists (LAFS)
    April, 2012 in Baton Rouge, Louisiana
    A. Presentation by Winnie Kurowski, Arcadiana Crime Laboratory, and Kim Gorman, PTC
    "Automation of Erase for Sexual Assault Cases"
    B. Poster
    C. Vendor Booth by PTC

Beta Testing by Crime Laboratories and Marshall University

PTC has provided Erase kits with tubes and reagents, as well as the complete protocol for this selective degradation method of differential extraction, to many crime laboratories, and to Marshall University. All of those institutions expressed interest in beta testing and ultimately using this method. Certain results from beta testing crime laboratories were reported above. A number of those laboratories are ready to go on-line with this method as their permanent method of differential extraction as soon as the developmental validation study is published.

Through the combination of publications, seminar presentations, posters and vendor booths, and bringing kits and instructions and support to crime laboratories and academic institutions in the United States and Europe, we have made a substantial start in introducing the results of this research to the entire forensic community. The hope is that this will accelerate the adoption of this method of differential extraction, and the value generated by this research, for the benefit of the entire law enforcement community and all Americans.

Thank you very much for the opportunity to perform this research and to present this Final Technical Report.