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Author(s): Jack Ballantyne, Ph.D.

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National Center for Forensic Science
University of Central Florida
P.O. Box 162367 · Orlando, FL 32826
Phone: 407.823.4041
Fax: 407.823.4042
Web site: <http://www.ncfs.org/>

Biological Evidence

DNA Profiling of the Semen Donor in Extended Interval Post-Coital Samples

FINAL REPORT

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Principal Investigator:

Jack Ballantyne, PhD
Professor
Department of Chemistry
Associate Director for Research
National Center for Forensic Science
P.O. Box 162367
Orlando, FL 32816-2366
Phone: (407) 823 4440
Fax: (407) 823 4042
e-mail: Jack.Ballantyne@ucf.edu

ABSTRACT

The ability to obtain an autosomal STR profile of the semen donor from a living victim rapidly diminishes as the post-coital interval is extended. This is of particular concern in those instances where victims of sexual assault provide vaginal samples several days after the incident. In an attempt to overcome the technological impediments of typing success with these samples, we previously employed the use of Y chromosome STR profiling which, by specifically targeting only the male DNA in the sample, can reduce or even eliminate the possibility of male profiling masking or critical PCR reagent titration due to the presence of an overwhelming amount of female DNA. Using Y-STR profiling and additional strategies such as cervical sampling and post-PCR purification, we were able to obtain Y-STR profiles from samples collected 5-6 days after intercourse. However, the reproductive biology literature demonstrates the presence of several spermatozoa in the human cervix up to 7-10 days post coitus. The question thus arises as to why, even with improved extraction and profiling techniques, we still fail to routinely recover profiles from samples collected ≥ 6 days after intercourse. Thus the aim of the current work was to develop novel strategies to permit the recovery of male donor DNA profiles from extended interval post-coital samples (≥ 6 days). Using a combination of novel methods to selectively enhance male DNA fractions, we demonstrate the ability to obtain male donor profiles in extended interval post-coital samples collected 6 to 9 days after intercourse. This represents a significant improvement in the time frame in which male profiles can be successfully recovered from post-coital samples.

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EXECUTIVE SUMMARY

1. The ability to obtain an autosomal STR profile of the semen donor from a living victim rapidly diminishes as the post-coital interval is extended. This is of particular concern in those instances where victims of sexual assault provide vaginal samples several days after the incident. In an attempt to overcome the technological impediments of typing success with these samples, we previously employed the use of Y chromosome STR profiling which, by specifically targeting only the male DNA in the sample, can reduce or even eliminate the possibility of male profiling masking or critical PCR reagent titration due to the presence of an overwhelming amount of female DNA. Using Y-STR profiling and additional strategies such as cervical sampling and post-PCR purification, we were able to obtain Y-STR profiles from samples collected 5-6 days after intercourse. However, the reproductive biology literature demonstrates the presence of several spermatozoa in the human cervix up to 7-10 days post coitus. The question thus arises as to why, even with improved extraction and profiling techniques, we still fail to routinely recover profiles from samples collected ≥ 6 days after intercourse.

2. In the work described herein we sought to refine and to extend, if possible, the post-coital time limit for which a male profile can be obtained from intimate samples. We employed a number of strategies to increase the signal to noise ratio of the DNA profiles obtained since, at the present limits of detection (4-5 days), there are indications that although the profiles are present the specific allelic signals are not strong enough to yield a profile.

3. Initially, we attempted to improve the efficiency of STR amplifications by utilizing increased amounts of DNA polymerase, alternative high fidelity polymerases, PCR additives and alternative extraction protocols. No significant improvements were observed using any of these modifications.

4. In an attempt to increase the recovered amount of input male DNA available for Y-STR amplifications, the use of DNA extraction concentration and purification was evaluated. The concentration of an entire extract into 10 – 12 μ l permitted the amplification of all of the DNA in the original extracts. Using this approach, partial Y-STR profiles were recovered from 6 -day post-coital samples. This subsequently proved to be the first component of our strategy for increasing the post-coital interval from which DNA profiles can be recovered.

5. A novel Y-chromosome specific nested PCR pre-amplification multiplex was designed to produce sufficient quantities of template male DNA prior to subsequent Y-STR amplifications. Validation of this pre-amplification multiplex demonstrated that full Y-STR profiles could be obtained from as little as 5 – 10 pg of input male DNA. The use of the pre-amplification multiplex, in combination with extract purification/concentration, resulted in a significant improvement in the time frame in which male DNA profiles could be recovered. We demonstrate the ability to obtain full and still-probative partial Y-STR profiles from samples collected up to 9 days after intercourse.

6. We developed two mini Y-STR multiplex systems (based both on the standard loci present in the Yifler[®] amplification kit and in our previously developed in-house ‘UHD multiplex’) in order

to determine if the smaller size of the mini Y-STRs would be more suitable for use with extended interval post-coital samples. Due to sensitivity limitations of the developed systems, additional strategies to improve sensitivity need to be employed before they can be evaluated for use with extended interval post-coital samples. Currently, we are attempting to develop a pre-amplification multiplex for use with the miniUHD system in order to improve sensitivity. This system could also find use in combination with the Yfiler[®] multiplex to increase the discriminatory power afforded by standard Y-STR typing.

7. We demonstrated the ability to obtain an autosomal STR profile from the male donor in cervico-vaginal samples collected up to 3 - 4 days after intercourse. We employed various strategies to attempt to isolate sperm cells from the overwhelming amount of female DNA present in extended interval post-coital samples. However, we were unable to develop efficient fraction separation techniques and were therefore unable to improve the ability to recover autosomal STR profiles in samples collected >4 days after intercourse.

8. We are currently involved in a collaborative study with the University of Tennessee to test a larger number of 4-, 7- and 9-day post-coital samples (~100 donor couples) using our enhanced profiling techniques. This study will also include an evaluation of a number of variables pertaining to the reproductive stages and health of the donor couples and how such variables correlate with DNA profile recovery.

I. INTRODUCTION

A. Statement of the Problem

For a variety of reasons, some victims of sexual assault provide vaginal samples more than 36-48 hours after the incident. In these cases, the ability to obtain an autosomal STR profile of the semen donor from the living victim diminishes rapidly as the post-coital interval is extended. Although it may be possible to obtain an autosomal STR profile of the semen donor from vaginal samples taken 24-36 hours after intercourse, it is normally not possible to do so when the post-coital interval exceeds 48 hours [1]. However, for many of these cases, the failure to detect the genetic signature of the male donor will not be due to the absence of male cells. Classical forensic serology studies have shown consistently that spermatozoa, albeit few in number, persist in the vaginal canal three days after intercourse and even longer [2-6]. Moreover the reproductive biology literature is replete with reports demonstrating the presence of several spermatozoa in the human cervix up to 7-10 days post coitus, which is consistent with the concept of the cervix as a sperm repository prior to fertilization [4,7-10]. The question thus arises as to why the sensitive methods of forensic DNA analysis routinely fail to detect these male cells. Here we try to address this problem by developing enhanced DNA typing methods to retrieve DNA profiles from the semen donor in extended interval post-coital samples.

B. Literature Review

The reasons for the inability to detect the genetic profile of the male donor in extended interval post-coital vaginal samples can be attributed to a combination of sperm loss or lysis and the technological limitations of the DNA typing systems employed [1]. Sperm loss after intercourse is due to vaginal lavage and drainage, menstruation, and the normal intra-cervico-

vaginal sperm degradative changes that occur over time. As a result of the latter process the few remaining sperm are expected to be in a structurally fragile state due to a somewhat damaged outer membrane. Loss can also occur during the multiple manipulations required of the differential extraction process used to separate the sperm from the non-sperm DNA fractions within the laboratory. The overwhelming majority of the DNA components in the non-sperm fraction comprise that from the vaginal epithelial cells from the victim. In addition to sperm loss, premature lysis of the few remaining fragile sperm during the differential extraction process will result in male DNA becoming admixed with female DNA.

The technological impediments to typing success with extended interval post-coital samples pertain to the low copy numbers of DNA templates present as well as the detection sensitivity of the autosomal STR systems employed. Standard protocols permit the detection of as little as 50-100 pg of DNA [11-16], which is roughly equivalent to 17-33 haploid (sperm) cells, but the sperm fraction in such samples may contain fewer cells (<10) and hence may be below the analytical detection limit of the system. Moreover, DNA from sperm cells that have prematurely lysed into the non-sperm (or female epithelial cells) fraction may be undetectable due to the kinetics of the PCR process itself. In those instances the male/female DNA ratio would be $\ll 1/100$ and the minor component (male) would not be detectable since the major contributor (female) would out-compete for, and titrate out, the critical PCR reagents required for male DNA amplification [17-20]. An obvious solution to this problem would be to substitute Y chromosome markers for the standard set of autosomal markers currently employed. The demonstrated efficacy and high sensitivity of Y-STRs for discerning the genetic profile of the male donor in admixtures of body fluids has resulted in the increasing use of these markers in sexual assault cases [15,17-25].

In our previous work, we have used a number of carefully selected Y-STR loci in a variety of multiplex or singleplex formats to extend the post-coital interval from which a genetic profile of the semen donor can be obtained [1]. The developed Y-STR typing strategies enable the routine detection of the male donor Y-STR haplotype in cervico-vaginal samples recovered up to 4 days post coitus [1]. The success was attributed to a number of factors that significantly improve the sensitivity and specificity of the analysis. Firstly, we utilized a subset of Y-STR loci that were carefully selected for their superior performance under stressed conditions in both multiplex and singleplex formats. Specifically these loci are detectable with low copy number templates in the presence of a vast excess of potentially confounding female DNA. Secondly, sperm and non-sperm DNA is co-extracted without a differential extraction process to prevent the unnecessary loss of the small number of structurally fragile sperm remaining in the cervico-vaginal tract several days after intercourse. Thirdly, low copy number detection was facilitated by increasing the cycle number to 34-35 cycles and by the ability to input up to 450 ng of co-extracted sperm/non-sperm DNA into the PCR reaction without the appearance of confounding female artifacts. Lastly, the proper collection of post-coital cervico-vaginal samples, instead of the lower or mid-vaginal tract samples often taken, was required for optimal recovery of sperm for analysis.

While we had obtained success using specific sub-sets of the core Y-STR loci (designated MPA and MPB) to detect the male haplotype, it was also important to test whether the commonly used commercial Y-STR kits, namely the Applied Biosystems AmpF ℓ STR $^{\circledR}$ Yfiler $^{\text{TM}}$ PCR Amplification Kit [24] and the Promega PowerPlex $^{\circledR}$ Y System [14], could also provide DNA profiles from extended interval post-coital samples as these are the systems that operational crime laboratories would likely use [26]. In order to be suitable for direct application

to crime laboratories, all experiments in this early work were performed in accordance with the manufacturer's recommended conditions. During the course of this evaluation, several post-coital samples were evaluated (12 hr, 24 hr, 48 hr and 72 hr) [26]. Using both differential and non-differential extractions, profiles were recovered up to and including 72 hours [26]. Subsequent to these initial evaluations, a number of DNA profile enhancement strategies (e.g. sampling by cervical brushing and post-PCR purification) were employed in an attempt to extend the interval in which Y-STR profiles could be obtained from extended interval post-coital samples (≥ 3 days). Using these strategies, full Y-STR profiles were routinely recovered 3-4 days after intercourse [27]. Profiles were also obtainable 5-6 days post-coitus although mainly partial profiles were obtained at this stage [27]. The use of post-PCR purification increased the ability to obtain Y-STR profiles, particularly from 5-6 days [27]. Remarkably, an 8-locus Y-STR profile was obtained from a 7-day post-coital sample [27].

Despite the improvement in profile recovery in sexual assault evidence we were able to previously achieve, successful profile recovery from samples > 5 days was still challenging. It was evident from the limited partial profiles obtained for the 6- and 7- day samples that biological material was indeed present after this length of time. For samples collected ≥ 6 days, the difficulty, and often complete inability, to obtain genetic profiles was likely not due to an absence of semen but rather as a result of the inability to detect the minute amount of sperm or male epithelial cells due to the analytical detection limits of the typing systems used. Therefore the goal of the current work was to develop novel strategies for improved recovery, purification and analysis of the small amount of male DNA present in extended interval post-coital samples. Our initial efforts were focused on an evaluation of various extraction and purification techniques in order to ensure that we were isolating as much male DNA as possible. We

additionally evaluated the use of various amplification enhancements such as the use of alternative polymerases, PCR-additives, and post-PCR purification. While we were still able to routinely recover profiles from samples collected up to 5 days after intercourse, none provided a significant improvement.

Next we reasoned that a more viable approach to overcoming the limitations of current analytical detection limits would be to pre-amplify the male or ‘Y chromosomal’ DNA prior to specific Y STR loci analysis. Theoretically, if the Y-chromosomal DNA could be specifically targeted using a loci-specific enrichment strategy, an increase in the signal to noise ratio of the Y chromosomal DNA compared with the epithelial DNA could be obtained. The result should be the attainment of good quality Y-STR profiles from the semen donor. In addition, this approach would remove the need for differential DNA extraction protocols, thereby increasing the speed and simplicity of analysis. Unlike whole genome amplification strategies for pre-amplification of the entire genome, we have uniquely developed a chromosome-specific nested PCR-based pre-amplification multiplex system that specifically targets the Y chromosome. This nested PCR-based pre-amplification reaction utilizes Y-chromosome specific primers in the first round amplification located in the regions flanking commonly used Y-STR loci. This results in the production of increased amounts of starting templates prior to subsequent Y-STR analysis. The primers were designed to accommodate the various primer sets used by all commercially available Y-STR amplification kits. While the development and validation of this pre-amplification system will be presented in full detail in separate publications (currently in preparation), the results of the validation demonstrated that full Y-STR profiles could be obtained from as little as 5 – 10 pg of input male DNA. Therefore, it was reasonable to assume

that the use of this Y-chromosome specific pre-amplification multiplex would be ideal for the analysis of extended interval post-coital samples.

In the present work, we demonstrate that the use of a Y-chromosome specific nested PCR pre-amplification multiplex, in combination with other sample enhancements such as extract concentration and purification, permit the recovery of potentially probative Y-STR profiles from cervico-vaginal samples collected up to and including 9 days after intercourse. This represents a significant improvement in the time frame in which male DNA profiles should be recoverable from sexual assault evidence.

C. Statement of Hypothesis or Rationale for the Research

Based on previous work conducted in our laboratory, it was evident that biological material from the semen donor, even if only trace amounts, was present in the cervico-vaginal tract 6 – 7 days after intercourse. However as a result of the analytical detection limits of current analytical methods, we were unable to successfully analyze the biological material present. It is a responsibility of the forensic community to provide suitable methodologies for the identification of the donor of biological evidence, even if present in trace amounts, in order to assist in the resolution of criminal investigations. Therefore in the present work, we sought to determine if enhanced sample processing and STR analysis methods could be employed in order to extend the interval in which DNA profiles of the semen donor in extended interval cervico-vaginal post-coital samples could be obtained.

II. METHODS

Sample Preparation

All body fluid samples were collected in accordance with procedures approved by the University's Institutional Review Board. Buccal swabs were collected from male and female volunteers by swabbing the inside of the cheek in order to obtain reference profiles for each donor. All profiles obtained throughout this study were compared to the donor reference profiles in order to verify the accuracy of the obtained profiles. All profiles reported in this work were verified and matched the donors' reference profiles. Post-coital cervico-vaginal swabs (x2) were collected by each of four female volunteers who recovered the samples after separate acts of sexual intercourse at various time points (1, 2, 2.5, 3, 4, 5, 6, 7, 8 and 9 days) using sterile cotton tipped applicators (Lynn Peavey, Lenexa, KS). The volunteers were instructed to take the samples from in and around the cervix by swabbing multiple times for 20-30 sec at each specific time interval. All volunteers were asked to abstain from intercourse at least 7 days after intercourse to ensure a sufficient period between collections. Only one set (i.e. pair) of swabs was taken subsequent to each separate act of sexual intercourse to preclude a progressive and unnatural loss of semen due to the sampling process itself. In some cases, a pre-coital cervico-vaginal swab was also obtained before intercourse to determine whether any male DNA was present from prior sexual intercourse. All samples were dried overnight and then stored at -20°C until analysis.

DNA Isolation

DNA was isolated from the samples using both a standard organic extraction and a differential organic extraction protocol as previously described [19]. Additionally, various extraction kits were evaluated throughout the work and included the DNA Investigator kit (QIAGEN, QIACube protocol), the Erase Sperm Isolation kit (Paternity Testing Corporation), the PrepFiler™ Forensic DNA Extraction kit (Applied Biosystems (AB) by Life Technologies, and the Differex™ System (Promega). All extractions were initially performed in accordance with the manufacturer's recommended conditions. Any modifications to the standard protocol are described in the Results section. An extraction blank (all reagents used in the extraction in the absence of any biological material) was included in each extraction performed to ensure that no contamination was encountered during the extraction process. The extraction blanks were subjected to the same analysis (see below) as the samples. Contamination in extraction blanks was not observed at any point during the course of the current work.

Purification

The MinElute PCR Purification kit (QIAGEN) was utilized for DNA extract purification and concentration, as well as post-PCR purification of the Y chromosome-specific nested PCR pre-amplification samples (see enhanced profiling method section in Results) [28]. The semi-automated QIACube (QIAGEN) protocol was used for all MinElute reactions in accordance with the manufacturer's instructions. All samples were eluted using nuclease free water (12 – 25 μ l elution volumes).

Quantitation

Quantification of DNA samples (2 μ l) was performed using the following real time PCR quantification kits in accordance with the manufacturer's instructions: Quantifiler[®] Y Male DNA Quantification kit, Quantifiler[®] Human DNA Quantification kit, Quantifiler[®] Duo DNA Quantification kit. Quantitation analyses were performed on an ABI 7000 or 7500 real-time PCR instrument.

Polymerase Chain Reaction (PCR)

Y Chromosome STR Amplifications

Y-chromosome STR analysis was performed using the AmpFISTR[®] Yfiler[®] PCR Amplification kit (AB by Life Technologies), the PowerPlex[®] Y Amplification kit (Promega), and the Y-plex 12[™] (Reliagene) kits. All amplifications were performed in accordance with the manufacturer's instructions using ABI 9700 thermal cyclers (AB by Life Technologies). Positive and negative controls were included with each amplification (positive controls consisted of male DNA provided with the kit; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted. During the current work, modifications to the commercial Y-STR kits were made including the use of the reduced volume reaction volumes, alternative polymerases and additives, and increased cycle number. Details of these modifications are described in the Results section.

Autosomal STR Amplification

Autosomal STR analysis was performed using the AmpFISTR[®] Profiler Plus[®] PCR Amplification kit (AB by Life Technologies) and the AmpFISTR[®] Identifier[®] PCR

Amplification kit (AB by Life Technologies). All amplifications were performed in accordance with the manufacturer's instructions. Positive and negative controls were included with each amplification (positive controls consisted of male DNA provided with the kit; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted. During the current work, modifications to the commercial autosomal STR kits were made including the use of the reduced volume reaction volumes, alternative polymerases and additives, and increased cycle number. Details of these modifications are described in the Results section.

Y Chromosome-Specific Nested PCR Pre-Amplification

Amplification took place in a 25 μ l reaction mix which utilized the Type-It Microsatellite kit (QIAGEN) and consisted of the following: 1X Type-It Multiplex PCR master mix, 0.5X Q-solution, and 2.5 μ l of a proprietary primer mix (15 primer sets to amplify 17 Y-STR loci: DYS19, DYS385 a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, Y-GATA-H4). The cycling conditions for the pre-amplification were: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 60 sec; 68°C 10 min (final extension). Positive and negative controls were included with each amplification (positive controls consisted of a male DNA standard; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted.

Mini UHD Multiplex

The 25 μ l reaction mixture contained the following: 0.096 μ M to 1.2 μ M primers (see below), 250 μ M dNTPs, 1X PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.75 mM MgCl₂, and 2.0 units of AmpliTaq Gold DNA Polymerase (AB by Life Technologies). The primer concentrations were as follows: DYS481 – 0.16 μ M; DYS576 – 0.16 μ M; DYS598 – 0.096 μ M; DYS570 – 0.32 μ M; DYS556 – 0.104 μ M; DYS485 – 1.2 μ M; DYS508 – 0.24 μ M; DYS446 – 0.4 μ M; DYS607 – 0.24 μ M. All amplifications were performed using 9700 thermal cycler (AB by Life Technologies) and the cycling conditions consisted of the following: 95°C 11 min; 32 cycles: 96°C 30 sec, 59°C 1 min, 72°C 1.5 min; 72°C 45 min. Positive and negative controls were included with each amplification (positive controls consisted of a male DNA standard; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted.

PCR Product Detection - Capillary Electrophoresis

All amplified fragments were detected with the ABI Prism 3130 Genetic Analyzer capillary electrophoresis system (AB by Life Technologies). A 1.0 μ L aliquot of the amplified product was added to 9.7 μ L of Hi-Di™ formamide (AB by Life Technologies) and 0.3 μ L of GeneScan™ 500 LIZ® (G5 dye set) (AB by Life Technologies), GeneScan™ 500 ROX® size standard (F dye set) (AB by Life Technologies), or ILS-600 (Any4Dye dye set) (Promega). The electrophoretic conditions used were as follows: 16 sec injection time, 1.2 kV injection voltage, 15 kV run voltage, 60°C, 20 min run time, dye set G5 (Yfiler®, Identifiler®, Mini UHD

multiplex), F (Profiler Plus[®]), or Any4Dye (PowerPlex[®] Y). All samples were analyzed with GeneMapper[®] Software v4.0 (peak detection thresholds of 25 RFUs).

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III. RESULTS

A. Statement of Results

Y-STR Amplification and Analysis

Our previous work resulted in the ability to recover profiles of the semen donor in post-coital samples collected ≤ 5 days after intercourse. The initial goal of the current work was to re-evaluate our previously developed methodologies to determine if we could refine these methods in order to improve profile recovery from extended interval post-coital samples. Therefore, initial experiments were performed to evaluate profile recovery (using both Yfiler[®] and PowerPlex[®] Y multiplex systems) from extended interval post-coital swabs taken 2.5, 3, 3.5, 4, 5 and 6 days after intercourse. These samples were collected from a new donor couple that had not previously provided samples and, hopefully, would help confirm our ability to reproducibly obtain DNA profiles 4-5 days after intercourse. Additionally, these same sample extracts could be used in subsequent experiments to test alternative strategies (e.g. alternate polymerase, increased cycle number) for improving DNA profile recovery.

Each sample was extracted using a differential extraction and the sperm and non-sperm fractions were quantitated using both the Quantifiler[®] Y and Quantifiler[®] Human real time PCR quantitation kits. The amount of male DNA detected ranged from 0.01 to 0.53 ng/ μ l (Table 1). The results from quantitation using the Quantifiler[®] Human system indicated the presence of female DNA in the sperm fractions despite the use of a differential extraction, which is not ideal if these samples were to be analyzed with autosomal STRs. Therefore, the efficiency of the differential extraction for use with autosomal STR typing would need to be evaluated (see later autosomal STR section). Each sample was evaluated with both Yfiler[®] and PowerPlex[®] Y,

initially using standard reaction and amplification conditions. The results from this analysis are provided in Table 1. Full profiles were obtained for all sperm fractions using both multiplex systems at the various time intervals, with the exception of the Yfiler[®]-amplified 3.5 day sperm fraction where one allele drop-out was observed (Table 1). The Y-STR profiles for the 4-day sperm fraction are shown in Figure 1. Full profiles were obtained for the PowerPlex[®] Y amplified samples with adequate signal intensities from all detected alleles. However, like the Yfiler[®] results, allele drop out was observed for the 3.5 day PowerPlex[®] Y-amplified sample as well as low overall signal intensity for the rest of the alleles.

Effects of DNA Polymerase on DNA Profile Recovery. We used increasing amounts of Taq Gold (2-fold increase) for the Yfiler[®] amplification in order to determine if improved allelic signal intensity would be obtained. Full profiles were obtained again for all samples with a slight increase in signal intensity (Table 1). A full profile was also obtained for the 3.5-day sample (Table 1). We also evaluated the use of an alternative polymerase, Expand High Fidelity (HiFi) polymerase (Roche Applied Science, Indianapolis, IN), which is a mixture of *Taq* and *Tgo* polymerases that has been reported to be better than *Taq* polymerase for high sensitivity analysis. Indeed we have successfully employed this enzyme in other LTDNA projects. HiFi Amplifications were performed using standard and increased amounts of the High Fidelity polymerase and, surprising, a significantly lower number of alleles were obtained for both Yfiler[®] and PowerPlex[®] Y (Table 1). Therefore it was determined that this alternative polymerase was not compatible with the commercially available Y-STR kits and not suitable for further use. We also evaluated a number of other alternative polymerases, such as SuperTaq[™] Plus polymerase (Ambion/AB by Life Technologies), Platinum[®] *Taq* High Fidelity polymerase (Invitrogen), PicoMaxx High Fidelity (Agilent Technologies, Santa Clara, CA) and ExTaq Hot

Start (Clontech, Mountain View, CA) [29], to determine if an improvement in profile recovery in extended interval post-coital samples could be obtained. There were no significant differences observed with amplification using the alternative polymerases (data not shown).

Effects of PCR Additives on DNA Profile Recovery. The new-donor couple 5- and 6- day samples were extracted using differential extraction and amplified using standard conditions. No male DNA was detected in the sperm fraction so more of the extract volume (10%, i.e. 10 μ l) was used for amplification. The Yfiler[®] profiles from the 5- and 6-day sperm fractions are shown in Figure 2 (A and B, respectively). As can be seen, a full Yfiler[®] profile was obtained for the 5-day sample. Significantly, a potentially probative partial profile was obtained for the 6-day sample as well (12/17 alleles) (Figure 2B). This result was quite surprising since only a small portion (10%) of the sample extract was amplified. It was interesting to note that many of the higher molecular weight alleles were recovered. Alleles were not recovered for DYS19, DYS385, DYS439 and DYS 456. DYS 456 and DYS19 are both below 200bp. With the recovery of larger alleles at the DYS448 and DYS392 loci (\geq 300bp), it may indicate that the loss of alleles was due to amplification inefficiencies. This indicated that it might be possible to increase amplification efficiency using various enhancers, additives or purification steps in order to improve the degree of allele recovery in 6-7 post-coital samples.

A novel PCR additive, PCRBoost[®] (Biomatrica, San Diego, CA) is reported to enhance end-point PCR reactions five-fold or more by improving sensitivity and specificity during amplification. PCRBoost[®] can be added to most existing amplifications without modification to cycling programs or polymerases and is compatible with most types of *Taq* polymerases. The PCRBoost[®] reagent is used in the amplification reaction in place of water. Therefore, no additional purification steps or sample manipulation is required. For initial testing of the

PCRBoost[®] reagent, we amplified a low template sample (100 pg male DNA) using the Yfiler[®] multiplex kit. This was done to ensure that the reagent would be compatible with the multiplex prior to consuming any post-coital samples. Figure 3 shows a Yfiler[®] profile with (A) and without (B) the PCRBoost[®] reagent. As can be seen from the peak heights in the PCRBoost[®]-sample, there is an increase in allelic signal when the PCRBoost[®] reagent is used in place of water in the reaction mix. However, when PCRBoost[®] was used with post-coital samples, no significant improvement was observed (data not shown). This could be due to the limited amount of the reagent that could be used (only ~1-2 µl). Typically a maximum volume of sample extract is used in the amplification of post-coital samples and therefore does not leave sufficient room in the reaction volume for additional reagents to be added. As a result of these difficulties, no further experiments were conducted with PCRBoost[®].

Effects of Sample Extraction Strategy on DNA Profile Recovery. Since no significant improvement was observed in DNA profile recovery from commercial kits using increased polymerase, alternative polymerase or PCR additives, we next focused on the sample extracts themselves to determine if DNA of higher quantity or quality could be isolated for subsequent PCR analysis.

Up to this stage our experiments had all involved the use of a small portion of sample extract (typically 10%, or 10 µl from a 100 µl extract). Only trace amounts of male DNA are expected to be present in the extended interval post-coital samples and would therefore be ‘diluted out’ in the standard extract volume. Ideally if the entire sample extract could be added to the reaction, this would allow for amplification of all male DNA present in the sample and improve analytical sensitivity.

There are at least two possible approaches to obtaining a concentrated sample volume. The first would be to use an alternative extraction method that employs the use of small elution volumes (likely involving the use of silica column based methods). Two commercially available kits were evaluated: 1) Differex™ System (Promega) and 2) PrepFiler™ Forensic DNA extraction kit (AB by Life Technologies). The Differex™ system is specifically designed for differential extractions. It utilizes a “separation solution” that forms a barrier between the aqueous phase containing the epithelial DNA and the sperm pellet. It is reported to provide better separation of fractions during a differential extraction. The separated fractions are then extracted using the DNA IQ™ system (magnetic bead separation). The PrepFiler™ kit utilizes magnetic particles with an optimized surface chemistry in order to maximize sample recovery. The kit is designed to remove inhibitors and therefore improve the purity of the isolated DNA. The kit is reported to be suitable for use with both routine and challenging samples. We evaluated each kit with several post-coital samples. Limited partial or no profiles were recovered from 5-day samples (data not shown) suggesting that, without further optimization, the use of these non-organic extraction kits would not improve profile recovery with extended interval post-coital samples.

We considered two options to obtain a smaller elution volume using our standard organic extraction protocol: 1) continue with the full organic extraction and ethanol purification steps with elution into a smaller volume, or 2) replace the ethanol precipitation purification steps with alternative purification steps, such as the use of silica based columns, that permit the use of smaller elution volumes. In order to avoid having to include the use of additional steps or reagents such as silica columns, we first tried the full organic extraction but with elution into a smaller volume. We evaluated the use of 10 – 30 µl elution volumes for both differential and

non-differential extractions. However, even with an overnight incubation at 56°C, we were unable to successfully re-solubilize the pellet for the non-differential extractions or the non-sperm fractions from the differential extraction. It would not be ideal to increase incubation times or temperatures in order to improve re-solubilization due to an increased propensity for sample degradation. Therefore, it was likely that the use of modified purification steps would be needed. We therefore evaluated two silica column based purification methods, NucleoSpin® DNA Clean-up XS columns (Machery-Nagel, Bethlehem, PA) and MinElute columns (QIAGEN).

The NucleoSpin® DNA Clean-up XS kit is designed for the purification of DNA samples, particularly from phenol-chloroform based extractions. The kit claims to have a high level of sensitivity making it ideally suited for use with low template samples. The silica columns used in this kit are directed through a very small silica membrane. This minimizes the dead volume of the column and allows for efficient recovery of sample. The purification process is quick (~20 minutes) and easy to perform. The NucleoSpin® DNA Clean-up XS kit was initially evaluated using a 2- and 3-day post-coital sample. Using this kit, it was possible to utilize a 12 µl elution volume, from which 10 µl were used for amplification. Full male profiles were obtained for the sperm fractions of both the 2- and 3-day samples (data not shown). A 6-day post-coital sample then became available for examination. Surprisingly, a partial profile was recovered from the sperm fraction of this sample after extraction (Figure 4). These results supported the use of sample purification and concentration to improve profile recovery for samples collected at least 6 days after intercourse.

The use of MinElute columns permitted an elution volume of 10 µl and also could be semi-automated on the QIACube robot. We again evaluated a 6 day post-coital sample to

determine whether comparable results with the NucleoSpin[®] kit could be obtained. We were again able to obtain a partial profile from the sperm fraction of the 6-day sample (Figure 5). Since MinElute offered the advantage of a semi-automatable protocol, we decided to use this instead of the manual NucleoSpin[®] kit in subsequent experiments.

Selective Genomic Enrichment of Y-STR Loci

The above described experiments using sample purification and concentration resulted in the ability to obtain partial profiles from 6- and 7- day post-coital samples. However, we were unable to obtain full profiles from these samples. Since it was evident that male DNA was present in these samples, we wanted to develop additional strategies to permit the recovery of full profiles from these samples, and to possibly extend the post-coital interval even further. Other research in our laboratory resulted in the development of a chromosome-specific nested PCR-based pre-amplification multiplex system that specifically targets STR loci on the Y chromosome. With nested PCR, first round amplification is performed with a single primer set for each target. As with any standard PCR method, it is possible during this initial amplification to obtain unwanted amplification products (primer dimers, non-specific amplification due to mis-priming) which can reduce the efficiency of amplification of intended targets. However with a nested-PCR approach, the resulting first round amplification product is subjected to a secondary amplification with a second set of primers (nested primers, binding sites contained within the first round amplification products). If incorrect amplification products were obtained in the first round amplification, they would not be amplified a second time using the specific “nested” primers in second round amplifications. This improved specificity therefore can increase sensitivity as the targets of interest are amplified more efficiently (i.e. enhancement of the

desired targets, Y-STRs in this case). This improvement in specificity and sensitivity therefore may not be observed with amplifications that, for example, simply increase the number of amplification cycles of a single reaction using single primer sets. Additionally, increased cycle number reactions can result in depletion of critical PCR reagents thereby reducing the efficiency of the amplification in higher cycle numbers. This can result in little, if any, improvement in sensitivity.

Our nested PCR-based pre-amplification reaction utilizes Y-chromosome specific primers in the first round amplification located in the regions flanking commonly used Y-STR loci. The first round amplification primers in the developed Y-chromosome specific nested PCR reaction were designed to accommodate the various primer sets used by all commercially available Y-STR amplification kits. While the development and validation of this pre-amplification system will be presented in a separate publication and report, the results indicate that full Y-STR profiles can be obtained from as little as 5 – 10 pg of input male DNA. Therefore, it was reasonable to assume that the use of this Y-chromosome specific pre-amplification multiplex, with the additional extract purification and concentration step, would be ideal for the analysis of extended interval post-coital samples.

Since we had obtained partial success with ≥ 6 day samples, we wanted to determine if new enhanced profiling strategies (extract concentration/purification and the pre-amplification multiplex) would not only improve profile recovery from these samples but also increase the post-coital time interval even further beyond the 6-7 days. Four volunteer donor couples were used for this study to evaluate the reproducibility of the method and potential intra-donor variation. Post-coital cervico-vaginal swabs (x2) were taken by each of the four females at specified intervals after sexual intercourse (6 – 9 days). Each time point sample was collected

after a separate act of sexual intercourse. Donor couples were asked to abstain from sexual intercourse for an additional 2-3 days after previous sample collection in order to provide an overall 8-12 day period in between sexual intercourse in order to reduce the potential for residual semen to be present before starting the collection process for the next time interval. With the exception of couple 1, all donor couples collected a pre-coital swab prior to coitus for each sampling as a control to demonstrate the amount, if any, of residual semen from a previous sexual act. No male alleles were detected in the pre-coital swabs for couples 2 and 3 (6 and 7 day time points) (data not shown). A small number of alleles matching the profile of the male participant were detected in the pre-coital swabs from couple 4 for the 7-, 8- and 9-day samples (3, 2 and 1 allele respectively) after the enhanced profiling techniques described below (data not shown). The presence of these trace alleles did not preclude the use of these samples in subsequent studies. Donor couples were asked to abstain from sexual intercourse only and therefore trace amounts of male DNA may have been present from other forms of sexual contact. Regardless, the presence of only a minor number of alleles indicates that only trace amounts of male DNA were present and therefore would not significantly contribute to any profiles obtained from the sexual act at the specified time period.

A summary of the analysis schema used for all post-coital samples is provided in Figure 6. One of the two swabs collected at each time point was extracted using a non-differential organic extraction. The remaining swabs from each time point were stored for possible further analysis, with the exception of the 9 day samples for couples 1 and 4 in which both swabs were extracted for comparison (described below). The extracted DNA was re-solubilized in 75 μ l of TE⁻⁴. This large sample volume was not ideal for use with the post-coital samples as only a small aliquot could be used in subsequent amplifications due to reaction volume limits. Since

only a small amount of male DNA is expected to be present in these extended interval post-coital samples, ideally it would be best to add the entire extract so as not to exclude any of the male DNA present in the extract. However, we were not able to decrease the extract volume as lower amounts of TE⁻⁴ would result in failure of the DNA pellet to be completely re-solubilized due to the significant amount of female DNA present. We therefore employed the use of the MinElute PCR purification kit (QIAGEN) to purify and concentrate the sample extracts. In this study, samples were eluted into 12 µl of nuclease free water to use for quantitation (2 µl), nested PCR pre-amplification (5 µl), and a non-pre-amplification control in subsequent Y-STR analysis (5 µl). While both 5 µl aliquots were required for this study, non-pre-amplification controls will of course not be taken in the course of casework and therefore this second aliquot could be reserved for additional testing or re-analysis. Although all samples were quantitated using the Quantifiler[®] Y Human Male DNA quantification kit (AB by Life Technologies), no male DNA was detected in the 2 µl sample extract (i.e 17 % of the total). As shall be seen below, the absence of male DNA in such samples does not preclude the possibility of a subsequently successful Y-STR analysis. The 5 µl aliquot of purified and concentrated extract was used in a subsequent Y-chromosome specific nested PCR pre-amplification (25 µl reaction volume). The pre-amplification PCR products were then purified using the MinElute PCR purification kit (QIAGEN) with an elution volume of 25 µl (nuclease free water). A 0.5 µl aliquot of the purified pre-amplification product was used in a subsequent amplification using Yfiler[®] (AB by Life Technologies) (12.5 µl reaction volume). Five microliter aliquots of the non-pre-amplified extract were also amplified for comparison purposes.

The typing results of the post-coital samples subjected to a non-differential extraction followed by extract purification and concentration are summarized in Table 2. Representative

electropherograms from 8- and 9-day post-coital samples from one of the donor couples are shown in Figures 7 and 8, respectively. For each sample, two electropherograms are shown: A) without pre-amplification and b) with pre-amplification. For a majority of samples no alleles were detected without the use of the pre-amplification. However, it was interesting to note that a small number of alleles were present in a few non-pre-amplified samples (couple 1 – 6 day (1 allele), couple 2 – 7 day (4 alleles), and couple 4 – 8 day (4 alleles)) (Table 2, Figure 7A) thus indicating the partial success of the sample concentration/purification step alone in improving allele recovery.

As can be seen from Table 2, the use of the Y-chromosome specific nested PCR pre-amplification resulted in a significant increase in the number of alleles detected, with the recovery of $\geq 70\%$ of the male donor alleles (with the exception of couple 3) (Table 2). Remarkably, full or nearly full profiles were even obtained from samples collected 8 and 9 days after intercourse (couples 1 and 4) (Table 2, Figures 7 and 8). To our knowledge this level of typing success has never been previously demonstrated in any other forensic study. Our own previous work led to the routine recovery of male donor profiles from samples collected only up to 5 days, with only limited partial profiles obtained for 6 days and a single 7 day sample [27]. The use of the Y-chromosome specific nested PCR pre-amplification, therefore, significantly improved the time interval in which the profile of the semen donor can be obtained. With the strength of the profiles recovered from the 9-day samples for couples 1 and 4, it is possible that we have not yet reached the limits of detection. Additional samples beyond the 9-day period will be collected and evaluated in future studies.

While we obtained successful results for a majority of samples in this study, it is expected that not all real world samples will yield such good results. A variety of factors will influence the

amount of sperm and/or male epithelial cell remaining in the female victim such as (but not limited to): 1) activity level of the victim after assault; 2) victim showering, bathing, douching after assault; 3) occurrence of the assault during the victim's menstruation cycle; 4) sperm count of the perpetrator; 5) volume of semen ejaculated during the assault; and 6) number of times of ejaculation during the assault. These factors will all contribute to the amount of male DNA available for collection and analysis. The potential differential success in profile recovery from samples collected from different individuals within the same time interval is evident even in this study with only four donor couples, with significantly less alleles recovered for couple 3 (6 day – 24% of alleles; 7 day – 18% of alleles) (Table 2). Additionally, the samples in this study were self-collected and it is therefore possible that some donors experienced greater difficulty with sample collection thereby affecting the amount of biological material recovered on those samples.

For all of the samples evaluated in this study, only one of the two swabs collected at each time point was analyzed (Figure 6). In some cases, the donor couples indicated which swab in the set was collected first. It is possible that the first swab in the set of two would contain more biological material than the second due to the recovery of most, if not all, of the male cells during this initial swabbing. Since partial profiles were obtained for the 9-day samples from couples 1 and 4 (full or nearly full profiles obtained from 6 – 8 day samples), the second swabs from these sets were analyzed to determine if additional male donor alleles could be obtained. The results of the comparison of profile recovery from the two swabs collected at the 9-day time point are provided in Table 3. Couple 1 provided an indication of which swab was collected first and therefore the samples are labeled “1st” and “2nd”. This information was not available from couple 4 and the samples are therefore labeled “swab 1” and “swab 2”. For couple 1, a

significantly larger number of alleles (12/17 or ~71%) were recovered from the 1st swab (Table 3). Only five alleles were recovered on the 2nd swab collected at that time point. Four of these five alleles (DYS458, DYS19, DYS439, DYS437) were ones present in the 1st swab profile. However, the allele at DYS438 was only observed in the 2nd swab profile (Table 3). Therefore, if the profiles were used in combination, a total of 13 male donor alleles could be recovered. For couple 4, 12 (~71%) and 14 (~82%) alleles were recovered from swab 1 and 2, respectively (Table 3). While this is a significant number of alleles from each individual swab, if the two profiles were used in combination, a total of 15 of the 17 alleles would be present (~88% of the male profile). Therefore, it is possible that analysis of the second swab collected could further improve the success of male profile recovery in these extended interval post-coital samples. It is clear from this study that biological material was present even after the first swabbing thereby demonstrating the need for additional swabbing in order to increase the amount of male biological material recovered. In this experiment, both swabs were extracted and analyzed individually. Alternatively it might be possible that both swabs could be co-extracted together in an attempt to obtain the most alleles in a single profile rather than relying on the deduction of a consensus profile. Also, while the signal intensity for most of the alleles in these profiles was > 500 RFUs, it is possible that additional alleles may still have been present but were below the limit of detection of the amplification systems. Future studies will be conducted to determine if the extraction of both swabs in a single sample would indeed result in improved profiling success.

As shown above, an increased number of alleles can potentially be recovered by utilizing the additional swab available at each time point. Another way of increasing the recoverability of alleles may be to use more of the pre-amplified sample in the subsequent Y-STR amplification.

As described previously, only a 0.5 μ l aliquot of the 25 μ l (2%) purified pre-amplification product is used in the subsequent Y-STR amplification (Yfiler[®], 12.5 μ l reaction volume). We therefore compared the use of a 5-fold increase in the volume of pre-amplification product used in the Y-STR amplification (increase from 0.5 μ l to 2.5 μ l) again using the 9-day samples from couples 1 and 4 (both swabs). There were no instances in which the use of the increased volume of pre-amplification product resulted in the recovery of additional alleles (data not shown). All Yfiler[®] amplifications up to this point were performed using the recommended 30 amplification cycles. We repeated the amplification of 0.5 and 2.5 μ l aliquots using increased cycle numbers (32 and 36 cycles) since increased cycle numbers are often used in the analysis of low template DNA (LTDNA) samples. For couple 1, one additional allele (77 RFUs) was observed using 0.5 μ l of pre-amplification product and 32 amplification cycles (data not shown). While this additional allele matched the male donor reference profile, it was not observed with 36 amplification cycles or using 2.5 μ l of pre-amplification product (data not shown). For couple 4, one additional allele was observed using 2.5 μ l of pre-amplification product and 32 and 36 amplification cycles (269 and 88 RFUs, respectively) (data not shown). While an additional allele was recovered for each sample using the increased sample input and cycle number, a significant increase in the number of alleles recovered was not observed. Since the signal intensities of most recovered alleles in the Y-STR profile after pre-amplification are large, the use of the increased sample and cycle number also led to saturation of these alleles (data not shown). The lack of a significant increase in alleles and the increased interpretation difficulty due to saturation effects, therefore, did not warrant increasing the amount of pre-amplification product added to the Y-STR amplification reaction (Figure 6).

In summary, the results of this study demonstrate that the novel enhanced profiling strategies developed resulted in the ability to significantly extend the time frame in which profiles of the male donor in extended interval post-coital sample can be recovered. Due the success of the 9 day samples, it is also possible that we have not yet reached the detection limit and therefore future work will be performed to evaluate ≥ 9 day samples.

Mini Y-STRs

We evaluated the use of mini Y-STRs for the analysis of post-coital samples. It is possible that the DNA in male cells present in extended interval post-coital samples is somewhat degraded and therefore analysis of these samples could be improved with the use of mini Y-STRs. It is also possible that when the sperm cells' integrity is compromised and structurally fragile, the protamine-protected DNA is not. However we know of no formal studies that have been reported that correlate sperm damage with DNA profile recoverability. Interestingly in our previous experiments with Yfiler, alleles >300 bp in size were recovered from 6-day old sperm, indicating that despite the probability of sperm damage, at least some of the male DNA is not degraded.

Two different mini-YSTR multiplex systems were developed. The first was the mini-UHD multiplex (Figure 9) which is based on the Ultra Highly Discriminating (UHD) multiplex previously developed and validated in our laboratory [22]. Attempts were made to include only loci contained within the original UHD multiplex. However due to difficulties in designing primers for amplification of reduced size amplicons for several loci, only 6 original UHD loci are contained within the multiplex. The remaining three loci were selected from other in-house Y-STR multiplexes based on their discriminating ability and the ability to design primers for

reduced size amplicons that were compatible with the other loci in the multiplex. The second mini-YSTR multiplex system developed was the mini-Yfiler multiplex (data not shown). All loci contained in this multiplex are also found in the Yfiler[®] amplification system; however the size of the amplification products for most loci are smaller. Since we were successful with our use of the standard Yfiler[®] kit, we chose to focus on the mini UHD multiplex system since it could offer additional discrimination if used in conjunction with the Yfiler[®] loci. The miniUHD multiplex system was evaluated in terms of discrimination potential, sensitivity, and specificity.

In-house population data for the miniUHD multiplex loci from ~1200 samples comprising various population groups including Caucasian, African American, Hispanic, Asian and Native American was available. For the mini-UHD system 762 unique haplotypes in 1209 samples were observed, with an overall multiplex discriminatory capacity of 73.7%. The most frequent haplotype was observed 26 times within the sample set. The sensitivity of both multiplexes was examined by amplification of a range of input DNA amounts from 25 pg to 5 ng. The sensitivity of the multiplex system was determined to be 50 pg (data not shown), with partial profiles recovered using 25 pg. The specificity of the miniUHD multiplex was also evaluated by amplification of various amounts of input female DNA ranging from 100ng to 1µg. No significant amplification products were observed when as much as 1µg of female DNA was amplified (data not shown).

Since the sensitivity of the miniUHD multiplex was determined to be ~50 pg, it was not likely that this would be sufficient for the analysis of the extended interval post-coital samples. Akin to the situation with the standard Y-STR loci, we reasoned that a miniUHD pre-amplification multiplex could be developed to improve sensitivity. Since mini-primer sets were designed for some of the loci in the miniUHD multiplex, the original reported primers for these

loci could be used as the first round amplification primers without the need for additional primer design. Nevertheless, we found that first round amplification primers still needed to be designed for several loci. This process proved to be very challenging and we encountered some difficulty with a few of the loci due to homology with the X chromosome. However, after exhaustive primer design work, recently we were able to develop suitable potential outer PCR primer sets for each of the nine loci in the mini-plex. Thus far, we have not been able to develop a highly robust and efficient pre-amplification multiplex. Future work will include further optimization of the miniUHD pre-amplification multiplex and its possible use with extended interval post-coital samples.

Autosomal STRs

We tested 2.5 – 4 day post-coital samples from a single donor using autosomal STR analysis (Profiler[®] Plus). A male-female mixture was obtained for the sperm fractions of all the time points (4 day profile is shown in Figure 10). We utilize only one wash step during a differential extraction of extended interval post-coital samples so as not to cause additional sample loss during the physical manipulations of the extraction. Since admixed profiles were obtained in all sperm fractions, the single wash step is inefficient in removing residual non-sperm (i.e. mainly female) DNA. Despite the challenges of mixture profile interpretation, the recovery of the minor male profile in a sample collected 4 days after intercourse was promising. However, this was only one 4-day sample. We next needed to determine if these results were reproducible and therefore two additional 4-day samples were analyzed from two additional donors. No male profile was obtained for these samples (data not shown). It is possible that the

limit of detection for autosomal STR profiles may be 3-4 days post-coitus, with variable recovery success at 4 days.

An improvement in male autosomal profile recovery from extended interval post-coital samples would require successful elimination or removal of the overwhelming amount of female DNA present. Our previous work with low copy number samples and laser capture micro-dissection permits the recovery of STR profiles from a small number of cells (unpublished data). When an extended interval post-coital sample is hydrated and placed onto a proprietary slide for microscopical examination, the significant amount of female vaginal epithelial cell that are present make it difficult to find and isolate the few sperm that may be present. The entire swab is used to make the slide in order to recover all sperm. In a differential extraction an overnight incubation is performed in order to lyse the non-sperm cells and leave the sperm cells intact. We hypothesized that perhaps this same overnight incubation at a lower temperature (37°C) would allow for the removal of the female vaginal cells and the intact sperm cells could then be placed onto a slide for collection using laser capture micro-dissection. This process was originally evaluated with an artificial semen-vaginal mixture. When this sample was examined, intact sperm cells could be identified. However, there was still a considerable amount of cell debris remaining from the non-sperm cellular material (data not shown). This process was repeated with *bona fide* post-coital slides (2.5 – 4 days after intercourse). However, very few sperm cells could be identified on these slides. It is possible that the few sperm that would be present in extended interval samples were too fragile and had prematurely lysed during the overnight incubation or were hidden by the significant amount of cell debris still remaining in the sample. Ultimately, the aforementioned strategy did not appear to be a viable approach for improving the ability to recover male autosomal STR profiles from the post-coital samples.

Recently, a new DNA extraction kit became available (Erase Sperm Isolation kit, Paternity Testing Corporation, Columbia, MO) that is reported to provide more efficient removal of epithelial cell DNA from samples containing mixtures of sperm and epithelial cells, such as in sexual assault evidence. Samples can be processed by hand in less than 2 hours, but it is also possible to automate kit extraction. Since effective strategies for the removal of non-sperm DNA are going to be required to improve autosomal STR profile recovery, we evaluated this kit to determine if more efficient fraction separation could be achieved.

We first evaluated the kit's ability to detect male profiles containing a small amount of semen present in vaginal-semen admixed samples. We created a series of vaginal swabs (1/2 swab) and added decreasing volumes of liquid semen. The semen volumes used included: 50 μ l, 25 μ l, 15 μ l, 10 μ l, 5 μ l, 1 μ l, 0.5 μ l and 0.25 μ l. Each sample was extracted using the Erase Sperm kit (differential extraction). The sperm and non-sperm fractions were typed using both Y-STR (Yfiler[®]) and autosomal STR analysis (Identifiler[®]). Full male Y-STR profiles were obtained for the sperm fractions of all samples (data not shown). However, full and partial profiles were also obtained in the non-sperm fractions for all samples: 50 μ l semen (full male profile in the non-sperm fraction); 25 μ l semen (10/17 alleles in the non-sperm fraction); 15 μ l semen (15/17 alleles in the non-sperm fraction); 10 μ l semen (full male profile in the non-sperm fraction); 5 μ l semen (full male profile in the non-sperm fraction); 1 μ l semen (15/17 alleles in the non-sperm fraction); 0.5 μ l semen (full male profile in the non-sperm fraction); 0.25 μ l semen (full male profile in the non-sperm fraction) (data not shown). The male profiles obtained in these samples were compared to a reference profile obtained from a male donor buccal swab. All observed alleles matched the reference profile. The results of the Y-STR analysis indicate that there is pre-mature lysis of the male cells into the non-sperm fractions. While this was one

sample set, if pre-mature lysis is observed with standard samples it is unlikely that this kit will be suitable for use with extended interval post-coital samples in which the sperm cells would be likely found in a more fragile state. With a high probability of premature lysis of these cells into the non-sperm fraction, an improvement in the ability to obtain autosomal STR profiles of the male donor is not likely.

Based on the Y-STR analysis results, we were able to determine that pre-mature lysis of some of the male cells was occurring. However, a reported benefit of the Erase Sperm kit is the removal of any residual non-sperm fraction from the sperm fraction. We therefore decided to perform autosomal STR analysis on these same samples in order to determine if a more efficient separation of the sperm and non-sperm fractions was in fact achieved. For the non-sperm fractions, a mixture was observed for the 50 μ l sample (data not shown). All other non-sperm fractions contained only the profile of the female donor (data not shown). From the Y-STR results it was expected that mixtures would be obtained for all non-sperm fractions since male DNA was detected in each of the non-sperm fractions. Since mixture samples were not observed for the non-sperm fractions using Identifiler[®], it is possible that the female profile was masking the small amount of male DNA present in the sample. The Identifiler[®] amplifications were performed using 28 cycles and therefore the male DNA may be present in low quantities and thus fall below the analytical sensitivity of the amplification system. For the sperm fractions, mixture profiles were obtained for several of the samples indicating the potential ineffectiveness of sperm and non-sperm fraction separation (data not shown).

While the results of the initial evaluation of the Erase Sperm kit were not particularly promising, we evaluated its performance with real cervico-vaginal post-coital samples. We extracted a 4- and 5-day post-coital sample using the Erase Sperm kit. We initially tested the

extracts with Yfiler[®] in order to determine if male DNA would be detected. No male profiles were recovered from the 4- or 5- day samples (data not shown). We next determined if we could still recover male Y-STR profiles from these samples using our developed pre-amplification multiplex. We concentrated the extracts and then performed the pre-amplification. Following pre-amplification, we were able to obtain a partial profile from the 4-day sample (11/17 alleles) but were still unable to recover any male alleles from the 5-day sample (data not shown). This result was disappointing since, even with standard extractions and amplifications, we have been able to routinely obtain profiles from samples collected up to 5 days after intercourse.

While perhaps suited for use with routine rape kit and other sexual assault samples in which sufficient quantities of male DNA is present, the results from the sensitivity and post-coital studies provide an initial indication that the Sperm Erase extraction kit may not be suitable for use with extended interval post-coital samples. The principle of the extraction kit is simple: removal of non-sperm fraction DNA using DNase or other degradative enzyme, thus isolating the sperm fraction. However, in practice this is not so easily accomplished. The sperm cells in extended interval post-coital samples are likely to be structurally fragile and therefore may be prematurely lysed during initial extraction steps and therefore destroyed by subsequent DNase treatment. Therefore, the ability to improve autosomal STR profile recovery in extended interval samples awaits the development of novel strategies for the efficient separation of an exceedingly small number of sperm cells from very large numbers of non-sperm cells.

B. TABLES

Table 1. Y-STR Profile Recovery From Differentially Extracted Post-Coital Cervico-Vaginal Samples (2.5 – 4 days after intercourse) from One Donor Couple

The number of alleles recovered from each sample is shown. The (low) designation indicates low signal intensity of the recovered alleles.

		Standard Cylce # (Yfiler - 30; PPlxY - 32; Profiler Plus - 28)								
		Real-Time Quant (ng/ul)		Yfiler (out of 17)				PowerPlex Y (out of 12)		
Interval	Fraction	Human	Y	4U Taq Gold	8U Taq Gold	4U HiFi	8U HiFi	2.75U Taq Gold	2.8U HiFi	8U HiFi
2.5 days	Non-sperm	326.8	0.04	0	NT	0	NT	0	0	0
	Sperm	0.46	0.13	17	17	13 (low)	4 (low)	12	4	7
3 days	Non-sperm	611.3	0.02	2	NT	0	NT	1	0	0
	Sperm	1.24	0.53	17	17	16 (low)	6 (low)	12	0	10
3.5 days	Non-sperm	260.2	undet	0	NT	0	NT	0	0	0
	Sperm	0.29	0.01	16 (low)	17	8 (low)	0	12	2	7
4 days	Non-sperm	407.5	0.01	0	NT	0	NT	0	0	0
	Sperm	0.37	0.07	17	17	12 (low)	5 (low)	12	4 (8 low RFU)	8
RB1		undet	undet	0	0	0	0	0	0	0
RB2		undet	undet	0	0	0	0	0	0	0
+ cntrl		NA	NA	17	17	6 (low)	2 (low)	12	12	12
AB		NA	NA	0	0	0	0	0	0	0

NT = not tested

Table 2. Profile Recovery from Extended Interval Cervico-Vaginal Samples (6-9 days after intercourse)

The number of alleles recovered from one of the two swabs collected per time interval (6, 7, 8 and 9 days). The shading indicates the average RFU value of all alleles within the profile (white – not detected; light grey < 500 RFUs; dark grey > 1000 RFUs).

Post-coital Interval (Days)	Pre-Amplification?	Allele recovery (out of 17 possible)			
		Couple 1	Couple 2	Couple 3	Couple 4
6	Yes	17	10	4	17
	No	1	0	0	0
7	Yes	17	16	3	17
	No	0	4	0	0
8	Yes	16	NT	NT	17
	No	0	NT	NT	4
9	Yes	12	NT	NT	14
	No	0	NT	NT	0

NT = not tested

Table 3. Comparison of Profile Recovery in Multiple Cervico-Vaginal Samplings 9 days after intercourse

A “+” or “-“ designation represents the presence or absence of an allele at a given locus, respectively. Two designations are provided for DYS385 since it is a bi-local locus and each donor is heterozygous. Couple 1 indicated which swab was collected 1st in the set of two swabs collected 9 days after intercourse. Couple 4 did not make this designation and therefore the samples are labeled swab 1 and 2 (not 1st and 2nd). The shading indicates the RFU value (white – not detected; light grey < 500 RFUs; medium grey 501-999 RFUs; dark grey > 1000 RFUs).

Locus	Couple 1		Couple 4	
	1 st Swab	2 nd swab	Swab 1	Swab 2
DYS456	+	-	+	+
DYS389I	+	-	+	+
DYS390	-	-	+	+
DYS389II	+	-	+	+
DYS458	+	+	-	-
DYS19	+	+	+	+
DYS385	++	--	-+	-+
DYS393	+	-	+	+
DYS391	+	-	-	+
DYS439	+	+	+	+
DYS635	+	-	+	+
DYS392	-	-	+	-
Y-GATA-H4	-	-	+	+
DYS437	+	+	+	+
DYS438	-	+	-	+
DYS448	-	-	-	+
Total Alleles	12	5	12	14

C. FIGURES

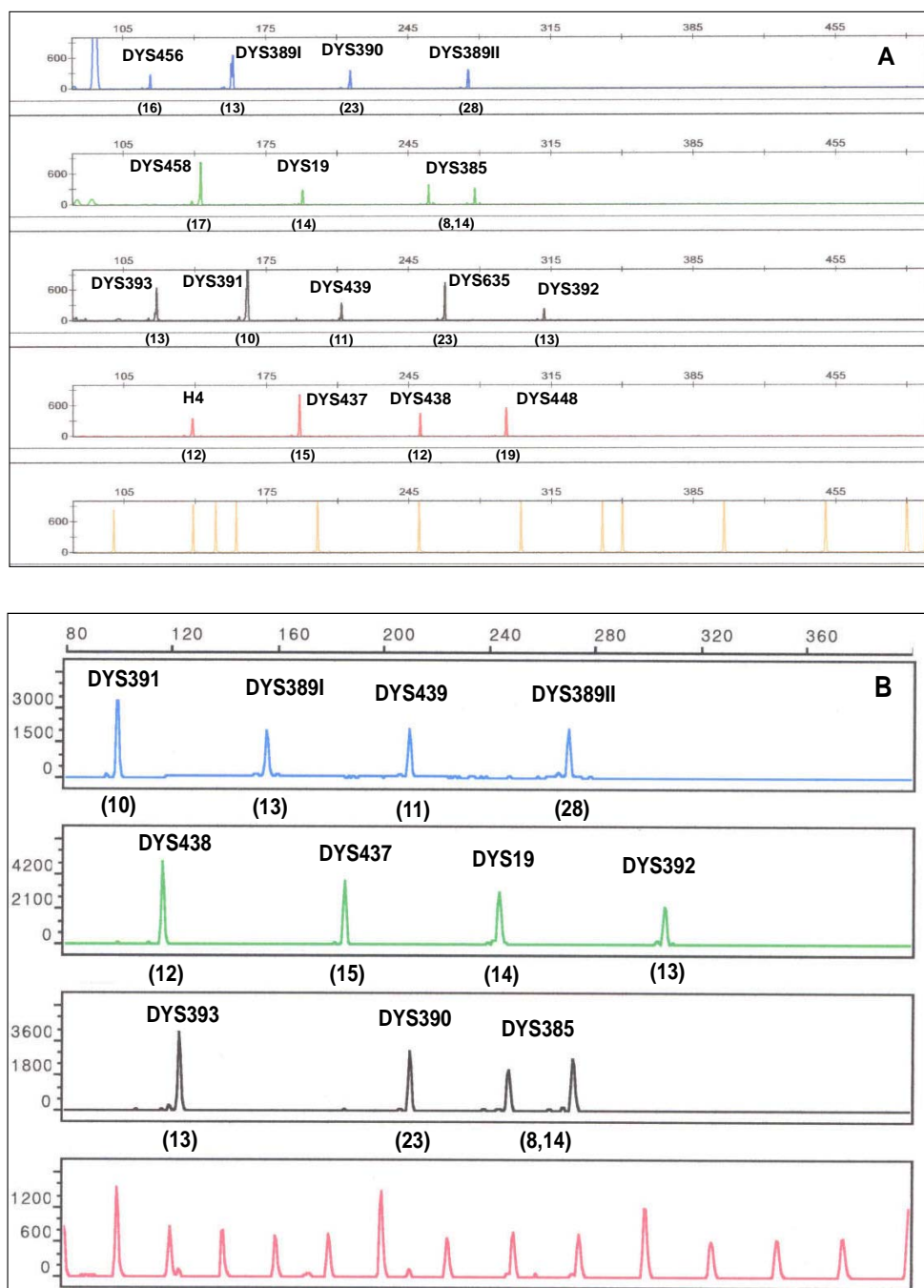


Figure 1. Yfiler[®] (A) and PowerPlex[®] Y (B) Profile From a 4 day Sample Sperm Fraction (Standard Conditions)

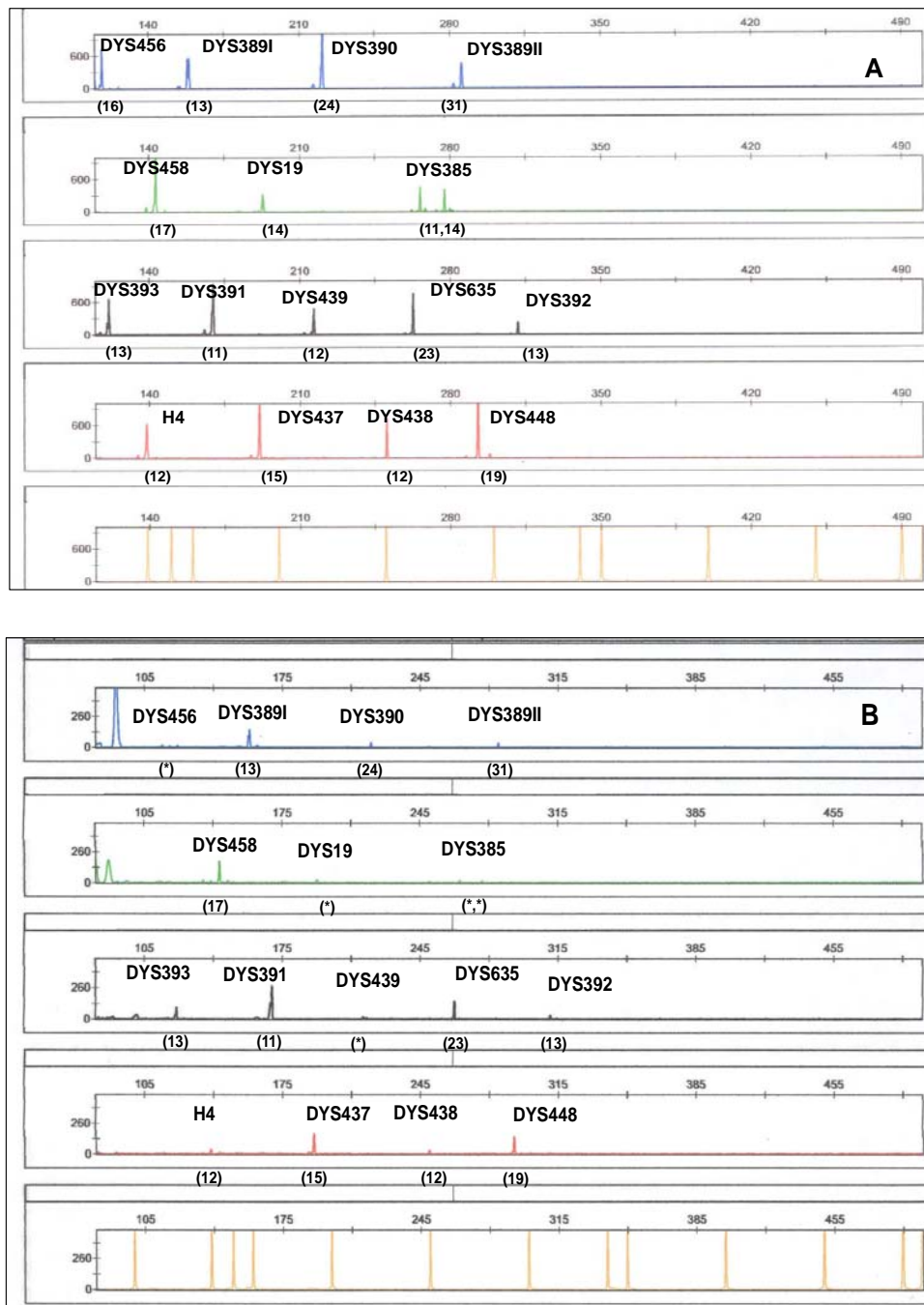


Figure 2. Sperm Fraction Yfiler® Profiles From a 5 (A) and 6 day (B) Post-coital Sample (Standard Conditions)

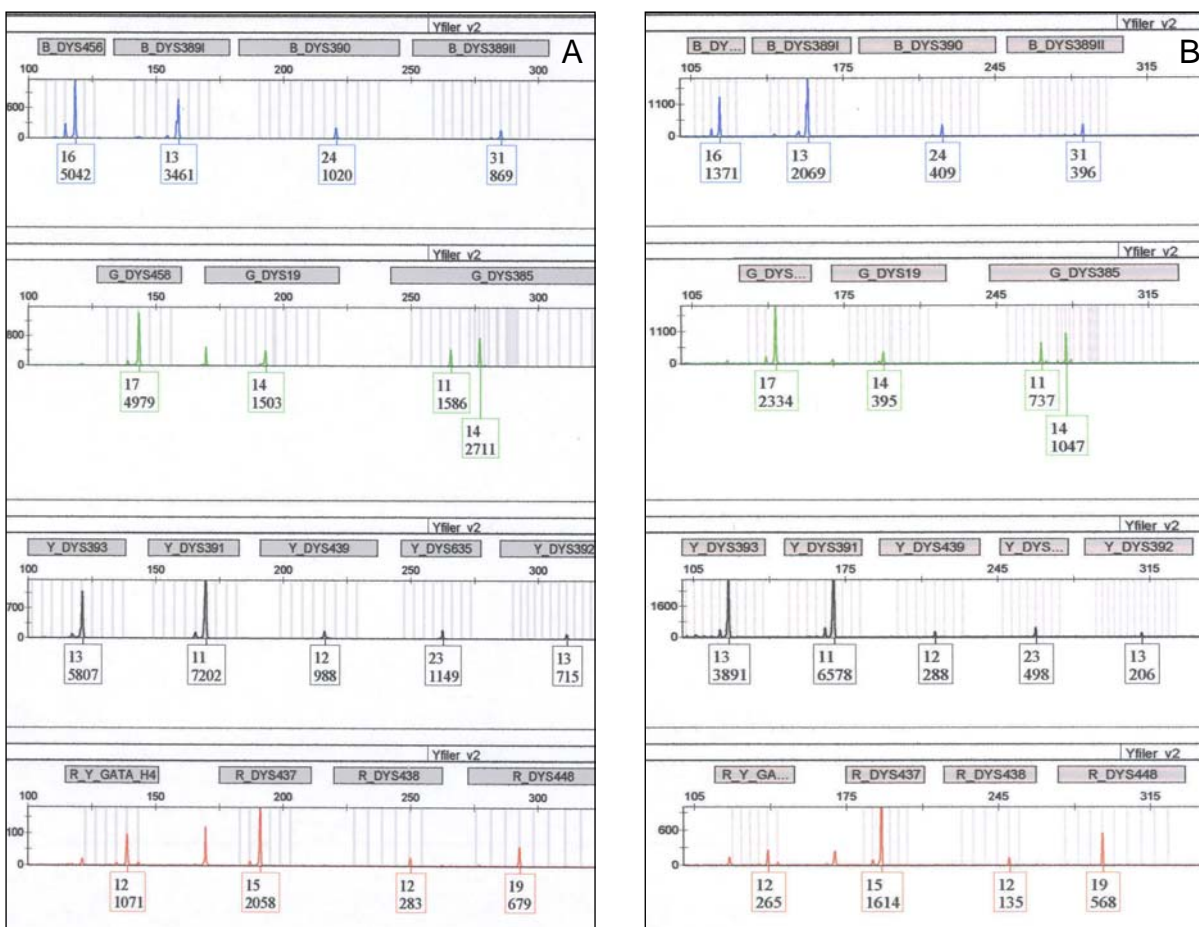


Figure 3. Comparison of Yfiler[®] profiles with (A) and without (B) PCRBoost[®] enhancer

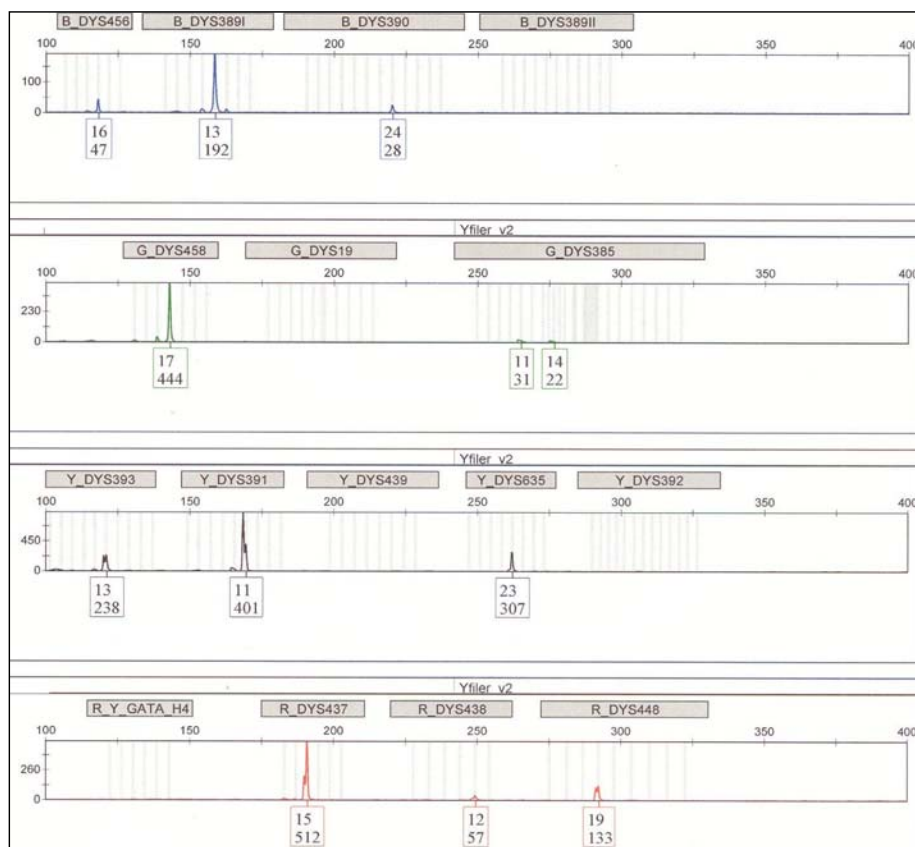


Figure 4. Yfiler[®] profile obtained from the sperm fraction of a 6 day post-coital sample extracted with an organic extraction and purified with NucleoSpin[®] DNA Clean-up XS kit

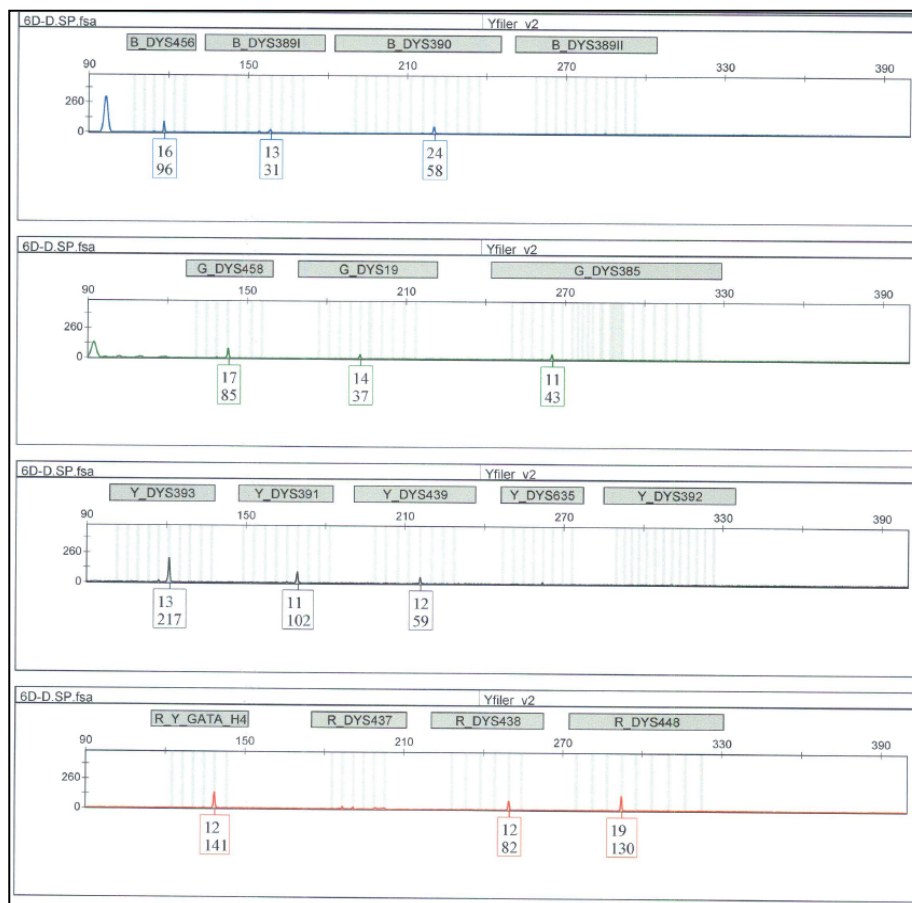


Figure 5. Yfiler[®] profile obtained from the sperm fraction of a 6 day post-coital sample extracted with an organic extraction and purified with MinElute

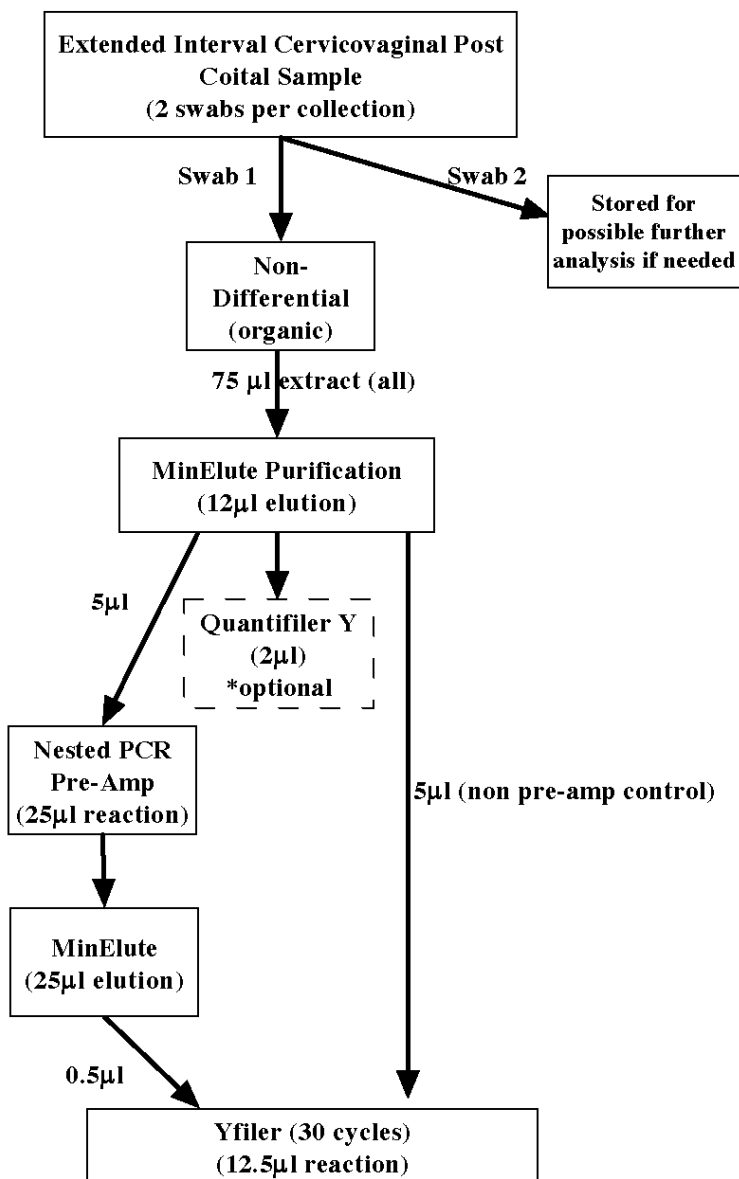


Figure 6. Experimental schema for enhanced profiling of the semen donor in extended interval post-coital samples

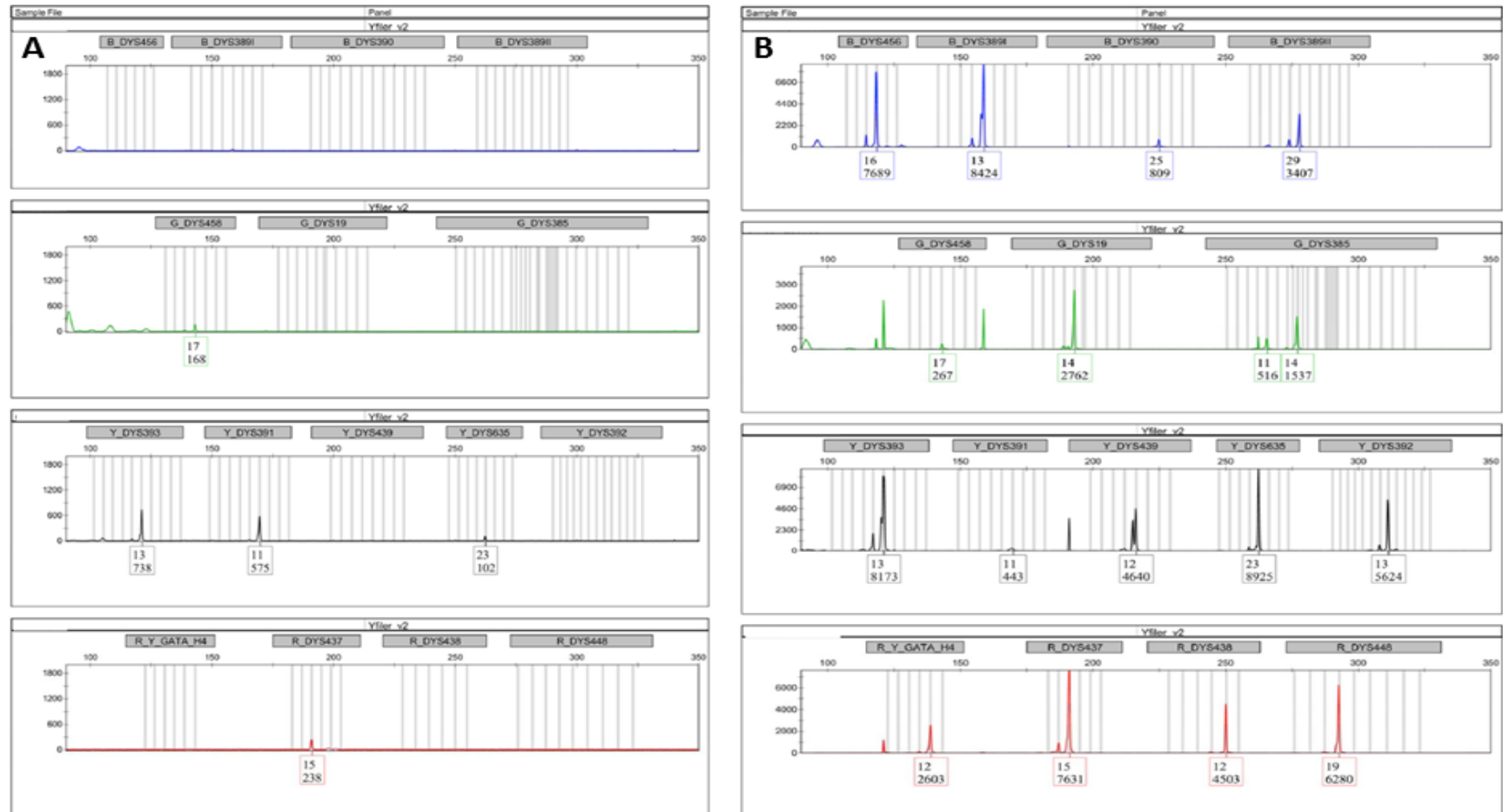


Figure 7. Improved Profile Recovery using Selective Enhancement of Semen Donor – 8 days after intercourse. Y-STR profiles of the semen donor in a 8-day post-coital sample without (A) and with (B) pre-amplification. Allele designations are indicated below each locus.

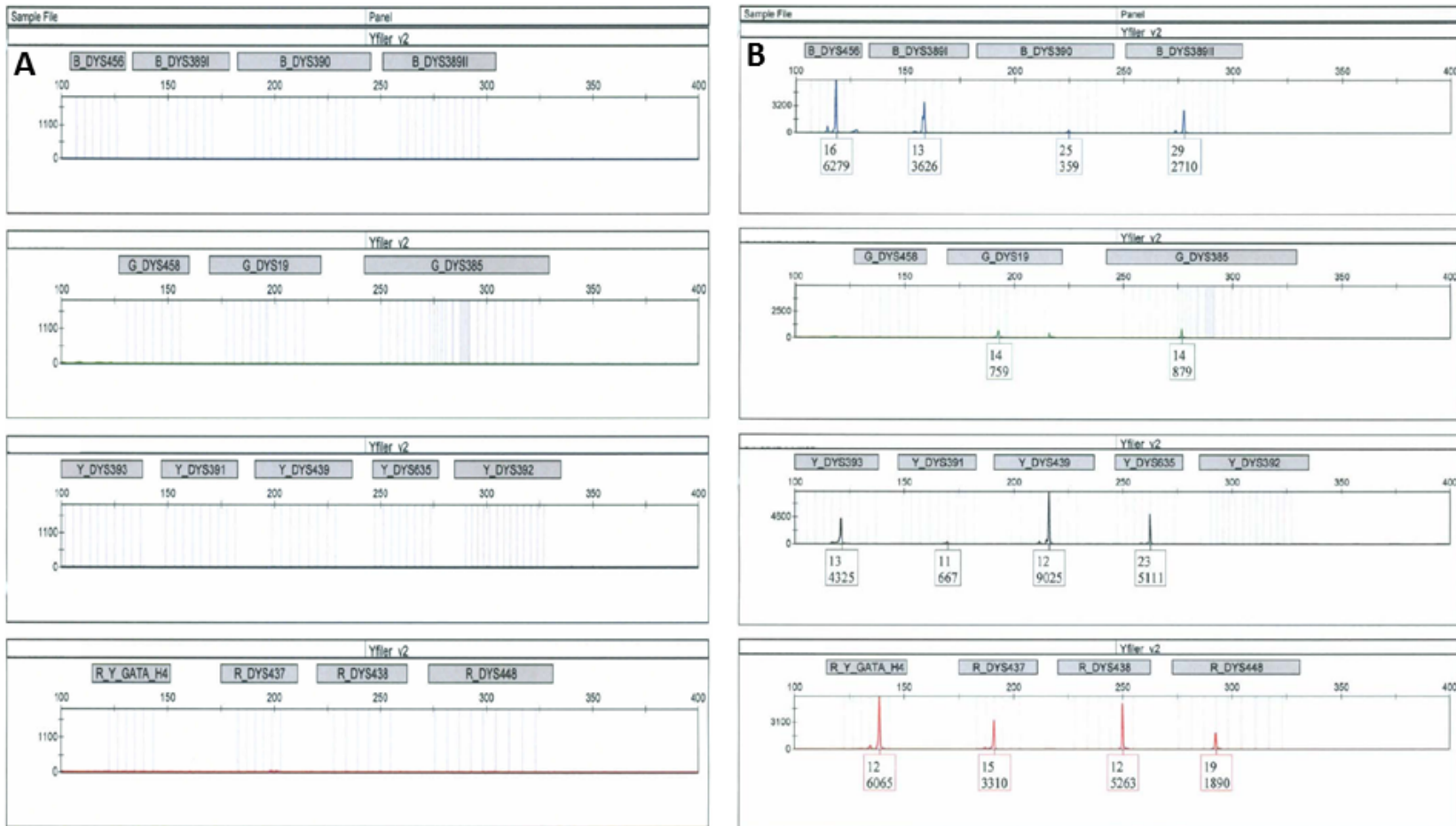
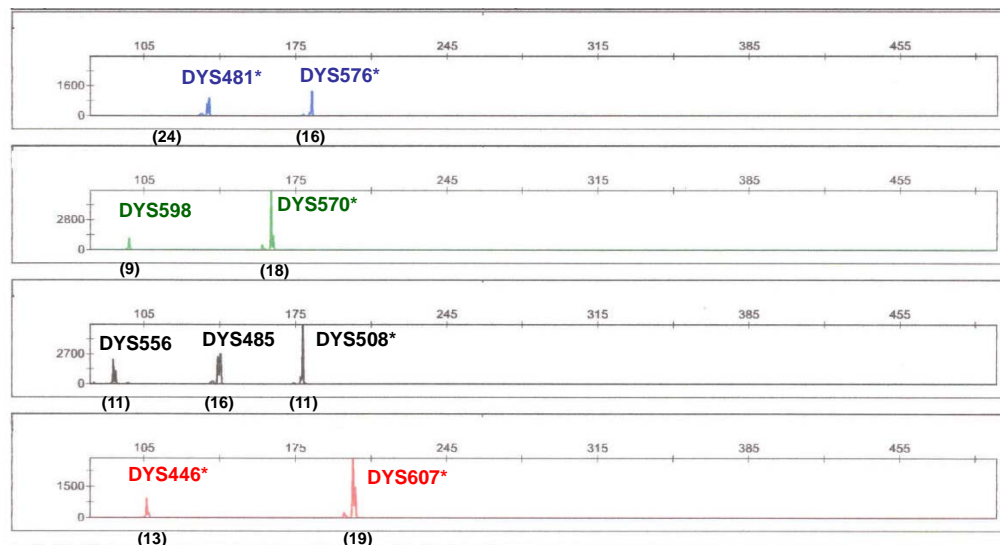


Figure 8. Improved Profile Recovery using Selective Enhancement of Semen Donor – 9 days after intercourse. Y-STR profiles of the semen donor in a 9-day post-coital sample without (A) and with (B) pre-amplification. Allele designations are indicated below each locus.



* denotes a locus present in the original UHD multiplex

Figure 9. Mini-UHD Multiplex System.

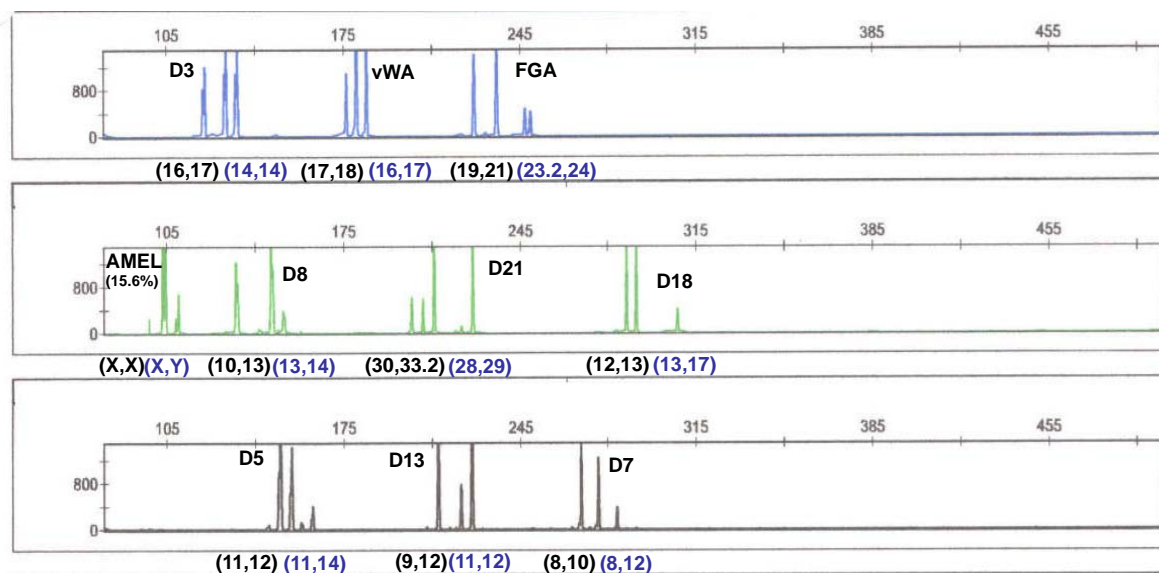


Figure 10. Admixed Profiler Plus profile obtained from the sperm fraction of a 4 day post-coital sample (male alleles are shown in blue)

IV. CONCLUSIONS

A. Discussion of findings

For a variety of reasons, some victims of sexual assault may not provide samples in the first few days after the assault occurs. This could be for instances of victim incapacitation (unconsciousness, intoxication by alcohol or drug), fear to come forward, or, in the case of assault of young children, the inability to recognize and report the assault. For many jurisdictions, due to a perceived inability obtain useful genetic information, samples may not be collected from victims who report sexual assault more than 72 – 96 hours after occurrence. While this time perception may in fact be valid using autosomal STR analysis, advanced DNA profiling methodologies and strategies have been developed that may provide the ability to obtain useful genetic information from the semen donor far beyond this time frame. Therefore, failure to collect samples after only 3 or 4 days may result in the loss of probative evidence that is crucial to the investigation and prosecution of these crimes. It is the responsibility of the forensic community to develop reliable and robust methods to obtain genetic profiles of the male donor in such cases. Based on advances made in our previous work, we were able to demonstrate the routine recovery of Y-STR profiles of the male contributor in cervico-vaginal samples collected 5 days after intercourse, with variable success with samples collected 6- 7 days after intercourse [27]. In the current work, we attempted to extend the time frame in which male profiles could be recovered after intercourse even further. Using a combination of DNA extract purification and concentration, as well as a novel Y-chromosome specific nested PCR pre-amplification, we demonstrate the ability to obtain probative Y-STR profiles from samples collected up to 9 days after intercourse. This is a significant increase in the time frame in which

male DNA profiles can be obtained and represents true advancement in the analysis of trace DNA samples.

Despite the successful analysis of these extended interval post-coital samples, there may be reluctance by forensic personnel (sexual assault nurses and/or operational forensic casework laboratories) to collect samples up to 9 days after intercourse due to a perception that this will significantly increase an already backlogged amount of DNA evidence to be processed. Additionally, there may also be concerns regarding the storage of additional sexual assault kits with already limited physical space for evidence storage. However, it is expected that the number of cases in which this extended length of time (≥ 6 days) between assault and sample collection would be relatively small thereby not significantly increasing the amount of work for sexual assault nurse and crime laboratories or the amount of storage space needed. For example, Morgan et. al reports that in 30 months of sexual assault evidence analyzed by the Canton-Stark Country Crime laboratory, only 4 of 83 (~5%) of cases involved samples in which there were more than 73 hours between assault and arrival at the medical center [30]. This is in broad agreement with an estimate of the number of sexual assaults from Orange County, FL that are reported > 72 hours after the incident (~4%) (Judy Bednar, Orange County Sexual Assault Treatment Center (SATC), personal communication). Regardless of the number of cases that would include samples collected from extended post-coital intervals, it is essential that the forensic community utilize novel DNA profiling methodologies in order to provide all victims will the reassurance that our best effort is made to obtain crucial DNA evidence in order to be used in criminal investigations and prosecution of potential perpetrators. Therefore, while we are not suggesting that the methods developed here will be necessary for every case, we are hopeful

that these methodologies will assist in the small number of challenging cases in which it was previously thought that successful DNA profiling was highly unlikely.

In addition to concerns regarding an increased volume of sexual assault evidence that will need to be processed (addressed in the preceding paragraph), there is likely to be initial concerns regarding an increased risk of contamination due to an increase in cycle number and need for sample manipulation during purification and secondary amplification steps. While there is always a potential risk for additional contamination when a larger number of amplifications cycles are used, significant contamination issues (i.e. contamination that was seen to be greater than the normal sporadic limited contamination events seen by all DNA laboratories) were not observed throughout the course of this study. As mentioned previously, extraction blanks were subjected to the same analysis as the post coital samples. This included extract concentration, pre-amplification, purification and subsequent Y-STR amplification and detection. Contamination was not observed in any of the extraction blanks and drop-in alleles (not originating from the sample donor) were rarely observed. Therefore, it should be possible, with proper controls and procedures to employ these methods without significant contamination issues. Once the pre-amplification reaction has been performed, further processing of the samples (purification and Y-STR amplification) would have to occur in a post-amplification room. It is possible that small separate bench-top PCR workstations could be designated for purification set-up as well as secondary amplifications in order to isolate these reactions from other areas of the post-amplification environment and minimize contamination. Other standard practices of minimizing the time and frequency of tubes containing amplified product being open, sterilization of pipets and work spaces, and use of sterile consumables and reagents should also reduce the risk of potential contamination.

While we were able to successfully improve the recovery of male Y-STR profiles, the ability to improve the recovery of male autosomal STR profiles proved to be more challenging and awaits the development of novel strategies to more efficiently isolate male DNA from the overwhelming amount of female DNA present.

B. Implications for policy and practice

Currently rape kits are often only taken from the victim up to 72 - 96 hours after the sexual assault. In this work we have successfully demonstrated the ability to obtain full and probative partial profiles from cervico-vaginal samples collected up to 9 days after intercourse. These results indicate that failure to collect samples after only 3 or 4 days may result in the loss of probative evidence that could be crucial to the investigation and prosecution of these crimes. We are hopeful that the results of our current work will change the time frame in which rape kit evidence is collected in many jurisdictions throughout the U.S. in order to permit an increase in the success rate of convictions for sexual assaults.

C. Implications for further research

We have demonstrated the ability to obtain full and probative partial DNA profiles from cervico-vaginal samples recovered 6 – 9 days after intercourse using enhanced profiling strategies. With the success of the 9-day profiles, it might even be possible to obtain profiles from samples collected beyond 9 days. Future work will therefore include an evaluation of > 9 day samples. Additionally, only four donor couples were utilized in this study. It will therefore be necessary to expand our analysis to include a much larger sample set. This would permit a determination of the variation in profile recovery between donors. We are currently involved in

a collaborative study with the University of Tennessee that involves the collection of 4, 7 and 9 day samples from ~100 (or more) donor couples. These samples will be collected by trained sexual assault nurses and will therefore eliminate potential deficiencies with self-collection. Additionally, variables including (but not limited to) the date of last menstrual period, birth control method, Gravid, Para, and medication will be evaluated in an attempt to correlate various biological factors with successful profile recovery.

V. REFERENCES

- [1] A. Hall, J. Ballantyne, Novel Y-STR typing strategies reveal the genetic profile of the semen donor in extended interval post-coital cervico-vaginal samples, *Forensic Sci.Int.* 136 (2003) 58-72.
- [2] A. Davies, E. Wilson, The persistence of seminal constituents in the human vagina, *Forensic Sci.* 3 (1974) 45-55.
- [3] V. Eungprabhanth, Finding of the spermatozoa in the vagina related to elapsed time of coitus, *Z.Rechtsmed.* 74 (1974) 301-304.
- [4] A.I. Morrison, Persistence of spermatozoa in the vagina and cervix, *Br.J.Vener.Dis.* 48 (1972) 141-143.
- [5] J.C. Rupp, Sperm survival and prostatic acid phosphatase activity in victims of sexual assault, *J.Forensic Sci.* 14 (1969) 177-183.
- [6] G.M. Willott, J.E. Allard, Spermatozoa--their persistence after sexual intercourse, *Forensic Sci.Int.* 19 (1982) 135-154.
- [7] W.H. Perloff, E. Steinberger, In vivo survival of spermatozoa in cervical mucus, *Am.J.Obstet.Gynecol.* 88 (1964) 439-442.
- [8] N. Sharpe, The significance of spermatozoa in victims of sexual offences, *Can.Med.Assoc.J.* 89 (1963) 513-514.
- [9] E.M. Silverman, A.G. Silverman, Persistence of spermatozoa in the lower genital tracts of women, *JAMA.* 240 (1978) 1875-1877.
- [10] E.M. Silverman, A.G. Silverman, Presence of spermatozoa in cervico-vaginal smears from young and old women, *Exp.Aging Res.* 5 (1979) 155-159.
- [11] P.J. Collins, L.K. Hennessy, C.S. Leibelt, R.K. Roby, D.J. Reeder, P.A. Foxall, Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifier PCR Amplification Kit, *J.Forensic Sci.* 49 (2004) 1265-1277.
- [12] W.E. Frank, B.E. Llewellyn, P.A. Fish, A.K. Riech, T.L. Marcacci, D.W. Gandor, D. Parker, R.R. Carter, S.M. Thibault, Validation of the AmpFISTR Profiler Plus PCR amplification kit for use in forensic casework, *J.Forensic Sci.* 46 (2001) 642-646.
- [13] C.L. Holt, M. Buoncristiani, J.M. Wallin, T. Nguyen, K.D. Lazaruk, P.S. Walsh, TWGDAM validation of AmpFISTR PCR amplification kits for forensic DNA casework, *J.Forensic Sci.* 47 (2002) 66-96.
- [14] B.E. Krenke, A. Tereba, S.J. Anderson, E. Buel, S. Culhane, C.J. Finis, C.S. Tomsey, J.M. Zachetti, A. Masibay, D.R. Rabbach, E.A. Amriott, C.J. Sprecher, Validation of a 16-locus fluorescent multiplex system, *J.Forensic Sci.* 47 (2002) 773-785.
- [15] B.E. Krenke, L. Viculis, M.L. Richard, M. Prinz, S.C. Milne, C. Ladd, A.M. Gross, T. Gornall, J.R. Frappier, A.J. Eisenberg, C. Barna, X.G. Aranda, M.S. Adamowicz, B. Budowle, Validation of a male-specific, 12-locus fluorescent short tandem repeat (STR) multiplex, *Forensic Sci.Int.* 148 (2005) 1-14.
- [16] M.J. LaFountain, M.B. Schwartz, P.A. Svete, M.A. Walkinshaw, E. Buel, TWGDAM validation of the AmpFISTR Profiler Plus and AmpFISTR COfiler STR multiplex systems using capillary electrophoresis, *J.Forensic Sci.* 46 (2001) 1191-1198.

- [17] D.L. Daniels, A.M. Hall, J. Ballantyne, SWGDAM developmental validation of a 19-locus Y-STR system for forensic casework, *J.Forensic Sci.* 49 (2004) 668-683.
- [18] A. Hall, J. Ballantyne, The development of an 18-locus Y-STR system for forensic casework, *Anal.Bioanal.Chem.* 376 (2003) 1234-1246.
- [19] E.K. Hanson, J. Ballantyne, A highly discriminating 21 locus Y-STR "megaplex" system designed to augment the minimal haplotype loci for forensic casework, *J.Forensic Sci.* 49 (2004) 40-51.
- [20] E.K. Hanson, P.N. Berdos, J. Ballantyne, Testing and evaluation of 43 "noncore" Y chromosome markers for forensic casework applications, *J.Forensic Sci.* 51 (2006) 1298-1314.
- [21] D. Corach, R.L. Filgueira, M. Marino, G. Penacino, A. Sala, Routine Y-STR typing in forensic casework, *Forensic Sci.Int.* 118 (2001) 131-135.
- [22] E.K. Hanson, J. Ballantyne, An ultra-high discrimination Y chromosome short tandem repeat multiplex DNA typing system, *PLoS.One.* 2 (2007) e688.
- [23] M. Kayser, A. Caglia, D. Corach, N. Fretwell, C. Gehrig, G. Graziosi, F. Heidorn, S. Herrmann, B. Herzog, M. Hidding, K. Honda, M. Jobling, M. Krawczak, K. Leim, S. Meuser, E. Meyer, W. Oesterreich, A. Pandya, W. Parson, G. Penacino, A. Perez-Lezaun, A. Piccinini, M. Prinz, C. Schmitt, L. Roewer, ., Evaluation of Y-chromosomal STRs: a multicenter study, *Int.J.Legal Med.* 110 (1997) 125-129.
- [24] J.J. Mulero, C.W. Chang, L.M. Calandro, R.L. Green, Y. Li, C.L. Johnson, L.K. Hennessy, Development and validation of the AmpFISTR Yfiler PCR amplification kit: a male specific, single amplification 17 Y-STR multiplex system, *J.Forensic Sci.* 51 (2006) 64-75.
- [25] M. Prinz, M. Sansone, Y chromosome-specific short tandem repeats in forensic casework, *Croat.Med.J.* 42 (2001) 288-291.
- [26] K.A. Mayntz-Press, J. Ballantyne, Performance characteristics of commercial Y-STR multiplex systems, *J.Forensic Sci.* 52 (2007) 1025-1034.
- [27] K.A. Mayntz-Press, L.M. Sims, A. Hall, J. Ballantyne, Y-STR profiling in extended interval (> or = 3 days) postcoital cervico-vaginal samples, *J.Forensic Sci.* 53 (2008) 342-348.
- [28] P.J. Smith, J. Ballantyne, Simplified low-copy-number DNA analysis by post-PCR purification, *J.Forensic Sci.* 52 (2007) 820-829.
- [29] J. Hedman, A. Nordgaard, C. Dufva, B. Rasmusson, R. Ansell, P. Radstrom, Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis, *Anal.Biochem.* 405 (2010) 192-200.
- [30] J.A. Morgan, Comparison of cervical os versus vaginal evidentiary findings during sexual assault exam, *J.Emerg.Nurs.* 34 (2008) 102-105.

VI. DISSEMINATION OF RESEARCH FINDINGS

A. Publications

1. Extending the Time to Collect DNA in Sexual Assault Cases. Taylor, T. The NIJ Journal, Issue No. 267, p. 22–27. Jack Ballantyne contributed to this publication through an interview with the author, Terry Taylor. (2008)
2. “Analysis of Late Reported (>6 days) Sexual Assaults by Y-chromosome Specific DNA Enhancement”. Hanson, E and Ballantyne, J.
*The manuscript has been prepared and is currently being edited before submission to Medicine, Science and the Law. Expected submission date: February 2012.
3. “Y-Chromosome Specific Nested PCR Pre-Amplification Method for Improved Detection of Male DNA.” Hanson, E., DiPasquale, F., Strauss, S., Engel, H., and Ballantyne, J.
*This manuscript in preparation and is expected to be submitted for publication to Forensic Science International Genetics in April 2012.

B. Presentations

1. DNA Profiling of the Semen Donor in Extended Interval (> 72 hr) Post-coital Cervico-vaginal Samples. Ballantyne, J. and Hanson, E. NIJ Applied Technologies Conference, Point Clear, AL. (2008)
2. Biology of the Y Chromosome and Y-STRs. New Technologies and Applications in Forensic Biology Workshop, New Jersey State Police, Hamilton, NJ. (2008)
3. DNA Profiling of the Semen Donor in Extended Interval (> 72 h) Post-coital Cervico-vaginal Samples. The NIJ Conference, Crystal City, VA. (2008)
4. Why the Y?: Y Chromosome Biomarker Applications in Sexual Assault Investigations. New Zealand Forensic Science Society, Auckland, New Zealand. (2008)
5. Y Chromosome Applications in Forensic Casework. Arizona Department of Public Safety, Phoenix, AZ. (2008)
6. Forensic Biology Research at the National Center for Forensic Science. The 20th EDNAP (European DNA Profiling Group) Meeting, Zurich, Switzerland. (2008)
7. Why the Y? Y Chromosome Biomarker Applications in Sexual Assault Investigations. Forensic DNA Initiatives 2009 Symposium – Sexual Assault Cases, Northeast Regional Forensic Institute & New York State Police Forensic Investigation Center. (2009)
8. Post-coital Interval for DNA Testing. The Fifth National SART (Sexual Assault Response Team) Training Conference, Seattle, WA. (2009)
9. Sexual Assault: Obtaining DNA From Evidence Collected up to a Week Later, The NIJ Conference, Washington, DC. (2009)
10. Recent Research into Extending the Post-coital Time Interval for DNA Profile Recovery.” Ballantyne, J. International Association of Forensic Nurses, Sexual Assault

- Forensic Examiner Technical Assistance (SAFETA) webinar on Timing Considerations for Sexual Assault Examination. (2009)
11. Extending the Post-coital Time Interval for DNA Profile Recovery. Orange County SART (Sexual Assault Response Team), Orlando, FL. (2010)
 12. From the Bed to the Bench: Defining the Vaginal and Cervical Environmental for Post-Coital DNA recovery. Speck, P., Faugna, D. and Ballantyne, J. AAFS Annual Meeting, Chicago, IL. (2011)
 13. Big Impacts from the Smallest Chromosome: The Effects of Y-STR Research on Practice and Policy. Ballantyne, J. The NIJ Conference, Arlington, VA. (2011)
 14. Enhanced DNA Profiling of the Semen Donor Can Assist the Investigation of Late Reported (> 5 days) Sexual Assault. Hanson, E. and Ballantyne, J. American Society of Crime Lab Directions (ASCLD) Annual Symposium, Denver, CO. (2011)
 15. Y-chromosome Specific Nested PCR Pre-amplification Method for Improved Detection of Male DNA. Hanson, E., Solivan, M, Strauss, S., DiPasquale, F., Engel, H., and Ballantyne, J. 22nd Annual Symposium on Human Identification, Natioal Harbor, MD. (2011)
 16. Y-chromosome biology and the National Y-STR database. Ballantyne, J. Y-Chromosome Short Tandem Repeat (Y-STR) Analysis and Typing, California Criminalistics Institute, Richmond CA (webinar). (2011)
 17. Enhanced DNA Profiling for Detection of the Male Donor in Trace DNA Samples. Hanson, E. and Ballantyne, J. European National Forensic Science Institutes (ENFSI) DNA Working Group Meeting, Athens, Greece (2011).