Document Title:	**Adaptation of the DNase I Procedure to the Biomek® NXP Robotic Platform for More Efficient and Automated Sexual Assault Sample Processing**
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Document Number:	**305803**
Date Received:	**January 2023**
Award Number:	**2019-NE-BX-0002**

Adaptation of the DNase I procedure to the Biomek® NX^{P} robotic platform for more efficient and automated sexual assault sample processing

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NIJ Award:
2019-NE-BX-0002

Project Period:
January 1, 2019 – December 31, 2022

Award Amount:
$250,636

Note: Application contributes to "Comparison of methodology or instrumentation new to the laboratory or field to instrumentation that is currently used in the laboratory"

Key Words: ANSI National Accreditation, forensic biology, sexual assault samples, differential extraction, DNase I, Biomek® NXP workstation, STR profiling

PROJECT SUMMARY

Major goals and objectives

The major goal of this project was to adapt, optimize, validate and integrate a DNase I differential extraction protocol (DNase DE) into the current sexual assault casework workflow on the Beckman Coulter Biomek® NX^{P} (NXP) automation workstations (Brea, CA) at the Virginia Department of Forensic Science (VADFS). The end goal is to expedite, without compromising quality, the processing of sexual assault samples. The objectives of this project are: 1. Combine and optimize published DNase DE protocols with the current VADFS protocols for seamless integration into automated DNA extraction using Promega's DNA IQ™ System (IQ) (Madison, WI) on the NXP; 2. Demonstrate that the semi-automated DNase DE protocol performs comparably to or better than the current VADFS manual differential extraction (DE) protocol in terms of sperm fraction (SF) DNA quantity and quality and STR profile quality; and 3. Validate optimized DNase DE protocol for sexual assault casework on the NXP with respect to reproducibility, sensitivity, effect of common contaminants and substrates, and lack of cross contamination.

Key outcomes-The optimized semi-automated DNase DE method:

- Produced Y-DNA yields and A/Y ratios comparable to the manual VADFS DE method with reduced manual steps involved (no sperm pellet washes)
- The two methods had comparable sensitivities for the SF.
- Demonstrated reproducibility comparable to the current manual VADFS DE method.
- Produced STR profiles comparable to the manual VADFS DE method, including being able to generate sole-source male profiles from SF samples.

- Was impacted by the presence of contaminants comparably to the VADFS DE method.
- Had a low cross-contamination frequency similar to the VADFS DE method.
- **Meets the criteria for replacing the current VDFS DE method for sexual assault casework.**

Research questions

Backlogs of sexual assault casework have long been, and continue to be a challenge throughout the US.[1-3] The foundational DE protocol to separate sperm from epithelial cells prior to DNA isolation developed by Gill et al.,[4] remains a standard method in the forensic science community, including at VADFS.[5] The DE method is based on lysing epithelial cells, and using centrifugation to pellet intact sperm cells to the bottom of centrifuge tubes. The supernatant containing lysed epithelial cells and their components is aspirated and transferred to another tube to create the "non-sperm fraction" (NSF). Sperm and other dense components pelleted at the bottom of the tube form the "sperm fraction" (SF). The SF is subsequently washed two or more times, without lysing sperm cells, to remove residual non-sperm DNA. Genomic DNA is isolated separately from the NSF and the SF, quantitated, normalized, and amplified to develop potential victim and offender STR profiles, optimally each being a single-source profile if there is only one victim and one semen contributor.

Challenges to manual and prior semi-automated DE protocols are that they require significant 'hands-on' time to prepare the samples due to incubation times and numerous sperm pellet wash steps, each wash step can result in some loss of sperm, and/or cross contamination, and retention of residual epithelial cell DNA, resulting in mixed or complex STR profiles, making it more challenging to identify contributors.[6]

Multiple methods have been developed to improve DE efficiency and the ability to develop single-source SF profiles from sexual assault samples through automation on multiple automation workstations and laser-, acoustic-, pressure-, pH-, and enzyme-based approaches (reviewed in Chong et al.).[6]

This project focuses on integrating three published manual DNase I-based protocols[7-9] with the current VADFS manual sexual assault DE protocol[5] to generate a semi-automated method to perform sperm pellet clean-up without the need for numerous pellet washes, thereby developing SF and NSF fractions in a more automated fashion. DNA purification will be performed following standard VADFS protocol using IQ on the NXP automated workstation, along with subsequent development of STR profiles on the same platform. A seamless transition will be in place linking the generation of SF and NSF fractions and DNA extraction on the automated platform. This protocol will be made available to other forensic laboratories using or considering using the DNase DE in an automated manner. The question addressed is how to optimally combine the published DNase DE protocols with the VADFS DE protocol to reduce manual steps and overall time and to increase throughput. Time savings and increased throughput are important, but DNA yields and quality must be maintained and potentially improved, as well as the quality of STR profiles developed from SFs. Critical considerations were cost, speed, ability to automate (increase throughput), ease of operation, sensitivity, reproducibility, cross contamination rates, quality of STR profiles, and potential disruptions to the current workflow for sexual assault samples.

VADFS's manual DE method requires a minimum of 3 SF washes for both the manual and automated DNA extraction, and **frequently** does not remove all epithelial cell DNA from the SF, especially when epithelial cells are in vast excess, resulting in complex profiles.[5,6]

Theoretically, and reportedly, DNase-based DE protocols can eliminate all or nearly all epithelial DNA, enhancing the development of single-source STR profiles from the SF fraction.[7-9]

In order to optimize efficiency, standard operating procedure at VADFS allows for multiple examiners to add samples and controls from different cases to a common 96-well deep well plate for automated DNA isolation with IQ, quantitation setup, sample normalization and STR setup.[5] Manual epithelial cell lysis is regularly performed the day prior to automated DNA purification, with samples added to a common 96-well plate the prior evening and left covered at room temperature overnight. Alternatively, examiners may perform epithelial lysis reactions overnight, and add those samples to the common 96-well plate in the morning. Together, these approaches permit automated DNA purification, quantitation, concentration normalization, and STR PCR reaction assembly, followed by manual loading for capillary electrophoresis of up to 80 samples plus controls in one day per automation workstation. One examiner or Forensic Laboratory Specialist (FLS) performs all these steps, freeing up other examiners for additional casework-related activities.

Summary of Research Project Design and Methods

The project proceeded in 3 phases: 1. Evaluation and optimization of a manual DNase DE protocol by combining epithelial cell lysis and DNase I digestions of mock sexual assault sample conditions from Garvin et al.,[7,8] and Wong and Mihalovich[9] DNase DE protocols with the VADFS DE protocol[5]; 2. Programming, evaluating and optimizing automated DNase I digestion conditions on the NXP); 3. Validating the optimized, semi-automated DNase DE protocol on the NXP.

Sample collection

Anonymized samples were collected and used under WCG IRB Protocol #20222596. Female donors S17, S27, S28 and S29 collected buccal, vaginal/cervical and/or rectal cell samples with cotton tipped swabs (Fisher, Puritan 806), which were air dried and stored at room temperature. Semen was collected from male donor S11 and frozen at -20^{o} C in 250 µL aliquots. As needed, aliquots were thawed, inverted repeatedly to mix prior to removing samples for use. After use, aliquots were immediately refrozen. Sperm cell counts were estimated with 1/20 dilutions in Type 1 water (DNase-free, 18 Ω) using a hemacytometer (Fisher Scientific, Hampton, NH) as described.[11] Fresh seminal fluid was diluted to 1:50, 1:500, 1:1,000, 1:5,000, 1:10,000 and 1:50,000 in Type I water and other than contamination studies, 50 µL was pipetted onto swabs containing female samples, and dried prior to use. The entire swab was transferred to 1.5 mL microcentrifuge tubes for epithelial cell lysis for all samples.

For tests of the effects of contamination, two approaches were taken. The first was for one donor to use Replens Vaginal Moisturizer (Replens) one to 7 seven days prior to collecting vaginal/cervical samples. In the second approach, 100 µL of 1:500 semen dilutions were pipetted directly onto common substrates in sexual assault cases (white panties (new, unwashed Hanes-Her-Way), white denim jeans (worn, washed), floral-patterned plastic-backed toiletries bag (used)), resulting in a US quarter-sized sample area, and dried prior to use. Commonly encountered contaminates (Canola Oil, Personal Lubricant (Silky), Astroglide female lubricant, Hair Spray (Smooth Sheen), 10-W30 Motor Oil, and Nivea Hand Cream), were applied to samples to saturate the side of the fabric to which diluted semen was applied, dried and stored in individual plastic bags until use. Samples were inspected with a 430-470 nm Crime-Lite (Foster+Freeman, Vale Park, Worcestershire, UK) to ensure fluorescence was spread approximately uniformly prior to cutting quarter-sized areas into nine equal radial pieces.

Evaluation of cross contamination during automated DNase I digestions was performed creating a checkerboard pattern of alternating reagent blank and sample wells in the first six columns of 96-well plates (24 reagent blanks), previously described in order to evaluate the susceptibility of the process to the introduction of contamination.[12]

Epithelial cell lysis

The working concentration compositions of epithelial cell lysis buffers and reaction volumes are shown in Table 1. During manual optimization, the DFS buffer was found to be incompatible with subsequent DNase I digestion, and subsequently used only in the standard VADFS DE method. Buffer E80+ incorporates the Tris, NaCl and EDTA concentrations from DFS, along with the higher ProK concentration and Tween-80 (present in the W80 buffer). E80+ contains NaCl, which W80 and GLB do not.

Buffer	Total Volume	Tris	NaCl	EDTA	Protein-ase K	Detergent
DFS*	505 ul	7.9 mM	79 mM	0.08 mM	0.2 ug/ul	1 % Sarkosyl
E80+	510 µL	7.9 mM	79 mM	0.08 mM	0.4 µg/µL	2 % Tween-80
W80**	510 µL	20 mM	0 mM	1 mM	0.4 µg/µL	2 % Tween-80
GLB***	510 µL	20 mM	0 mM	1 mM	0.4 µg/µL	2 % Triton X-100

Table 1. Volumes and working concentrations of epithelial cell lysis buffers tested.
Key: *=from VADFS standard procedures.[5] **=from Wong and Mihalovich.[9] ***=from Garvin et al.[7,8]

Epithelial cell lysis was performed in the presence of Proteinase K (ProK) (Sigma-Aldrich, St. Louis, MO, P2308), which degrades proteins.[13] ProK (>30 units/mg) was dissolved to 20 mg/mL in Type I water and 500 µL aliquots frozen in a frost-free freezer at @ -20^{o} C. Following thawing at room temperature, ProK was added to epithelial cell lysis buffer. Pooled solution was gently mixed/inverted and added to tubes containing swab heads or to empty tubes to serve as reagent blanks (RBs). Solutions containing ProK were not vortexed prior to

incubations due to the enyzyme's sensitivity to physical denaturation.[13] Pipet tips were used to submerge swabs in buffer. Based on VADFS standard procedures[5] and published DNase DE protocols,[7-9] tubes were transferred to a drybath incubator at 37° C or 56° C (Fisher Scientific, Hampton, NH, 11-718-4 and 11-7-8-2, respectively) for designated times. Following incubation, tubes were vortexed at top speed for 20 seconds (Fisher Scientific, Hampton, NH Genie-2 12-812), and centrifuged for 5-6 seconds (Eppendorph Biotech Co., Hamburg, Germany, 5425 with FA-24-2 rotor) to remove condensation from tube lids. Swabs were manually transferred to spin baskets and inserted into the original tubes and centrifuged. Following centrifugation, spin baskets were discarded, and all but ~50 µL of supernatant transferred to a clean, pre-labelled tube to serve as the NSF. Any visible pellets were left undisturbed when aspirating and transferring supernatants. Tubes with ~50 µL of supernatant plus pelleted materials, if any, served as unwashed SFs (unless using VADFS DE 'standard' protocol where three sperm cell washes would be performed). SF and NSF reagent blanks (RBs) were created in parallel for conditions tested.

DNase I Digestions

DNase I was purchased from Worthington (Lakewood, NJ) (LS006324) and dissolved in 10 mM Tris, 2 mM $CaCl_2$ in 50% glycerol at a concentration of 18 Units/µL. Based on the Wong and Mihalovich report,[9] and manufacturer recommendations for the use of DNase I,[14-16] the DNase I digestion buffer BE80 (1x = 10 mM Tris, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5% Tween-80) was used in all DNase DE tests. Because SFs were transferred with ~50 µL of epithelial cell lysis buffer to 96-well plates, ~one-fourth of the total DNase I digestion volume of 200 µL, residual components from epithelial cell lysis buffers were included in all automated DNase I digestions component concentration estimates. Manual tests found that DNase I

digestion volumes of 100 and 150 µL following epithelial cell lysis with Buffer E80+ resulted in elevated A-DNA/Y-DNA ratios relative to 200 µL digestions, potentially due to suppression of DNase I activity by NaCl carry-over into DNase I digestions (data not shown). The 200 µL reaction volume was used in subsequent experiments to reduce carryover of NaCl and ProK because NaCl concentrations > 30 mM reduce DNase I activity by 50% or more,[17] and ProK degrades DNase I.

Prior to addition to SFs (manual or automated), DNase I was manually combined with 2x DNase Buffer. DNase I was added immediately prior to use and gently mixed due to DNase I being physically denatured by vortexing and up to one-half of enzyme binding to container walls within 10 minutes.[14,16] Reactions were stopped by the addition of 1/10 volume (20 µL) 0.5 M EDTA (8.0), pipet mixing, and incubating at room temperature for at least 10 minutes to chelate Mg^{++} and Ca^{++}, which inactivates DNase I, prior to automated DNA isolation with DNA IQ™.[15]

Optimized semi-automated DNase DE protocol

Manual Steps

1. Add 510 µL epithelial cell lysis Buffer W80 to 1.5 mL microcentrentrifuge tubes containing swabs, or empty tubes for RBs.
2. Incubate tubes at 56° C for 60 minutes in drybath incubator.
3. Vortex tubes 20 seconds on highest setting.
4. Pulse spin tubes for 5-6 seconds to remove condensation from inside of lids.
5. Transfer swabs to spin baskets and insert baskets into original tubes.
6. Centrifuge tubes for 3 minutes at 4.61K RPM (2,000 x g). (centrifugation speed adopted from Wong and Mihalovich)[9]
7. Discard spin baskets containing dried swabs.

8. Carefully remove all but ~50 µL of supernatant without disturbing any visible pellet and transfer to clean 1.5 mL microcentrifuge tube to create NSFs. Set aside for parallel automated DNA purification with DNA IQ™.
9. Gently aspirate pellets in remaining 50 µL volumes (SF) with pipet, and transfer to 96-well deep well processing plate.
10. Add 50 µL Type 1 water to tubes, gently pipet 2-3 times, rinsing the walls near the bottom of the tube to release any sperm cells sticking to the sides of the tube and transfer to corresponding well in processing plate to maximize DNA recovery.

Preparation of automated platform

11. Add 0.5 M EDTA to one-half of quarter trough divided lengthwise (Beckman Coulter 372788) (estimate 20 µL/200 µL DNase DE reaction, a minimum of 3 mL).
12. Add DNase to 2x DNase I buffer BE80 in 15 mL Falcon tube, gently mix, and add into other half of one-half of quarter module divided by length as the final step after adding tips, plates, and buffer trough to the NXP deck. Include a minimum of 3 mL, including 1 mL extra volume beyond volume required.
13. Place tip boxes and other necessary labware on the robot deck (e.g., sample processing plate containing unwashed sperm cell pellets).

Automated steps

14. NXP transfers 100 µL of DNase I/DNase Buffer to each sample and mix using a 50 µL volume five times.
15. NXP moves 96-well plate to Watlow heater connected to a V&P Scientific (San Diego, CA) heat transfer unit (VP581A) set to 62.5° (in-well temperature 54-56 °C after 10 min.) for 25 minutes.

16. NXP moves 96-well plate to magnabot stand (Promega, Madison, WI). Plate expansion caused by heating prevents plate from seating properly at other deck positions until cooled.
17. NXP Transfers 20 µL 0.5 M EDTA (1/10 volume) from one-quarter trough to individual wells and mixes using a 50 µL volume five times.
18. NXP Transfers samples to corresponding wells in 96-well deep well sample plate containing NSFs and NSF RB, and any other non-DE samples slated for automated DNA purification with IQ.
19. Samples are now ready for DNA IQ™ System DNA purification using VADFS procedures as described.[5]

DNA Purification and Quantitation

Following manual or semi-automated DEs, automated DNA purifications were performed with an NXP using IQ and standard VADFS procedures, which elutes DNA in ~35 µL final volume.[5] DNA was quantitated with PowerQuant® (Promega) using a QuantStudio™ 5 Instrument (Applied Biosystems, Waltham, MA) following manufacturers' recommendations.[17,18] Reactions were assembled manually due to worldwide pipet tip shortages during the grant period. Samples were evaluated for Y-DNA concentration (Y-DNA), autosomal-DNA concentration (A-DNA), A-DNA to Y-DNA ratio (A/Y ratio), and the degradation index (DI). The A/Y ratio measures the relative amount of female to male DNA in a sample, with values of ~1 indicating the presence of only male DNA. Values > 1 indicate the presence of female DNA.[17] The DI measures the level of DNA degradation in samples (Promega). Samples with DIs > 2 were designated as progressively more degraded the higher the value as recommended by the manufacturer.[17]

STR Profiling

STR profiles were developed by PCR amplification of up to 0.5 ng of autosomal DNA using PowerPlex® Fusion (Promega) in half reaction volumes on a GeneAmp PCR System 9700 (Thermo Fisher, Waltham, MA) as recommended by the manufacturer and VADFS standard procedures.[19] The recommended DNA concentration is 0.1 ng/µL of A-DNA. Amplified products were size fractionated and visualized with a 3500*xl* Genetic Analyzer (Applied Biosystems) capillary electrophoresis instrument using 12 and 24 second injection times. Electropherograms were created and evaluated with GeneMapper® ID-X v1.4 software (Applied Biosystems), with limits of detection (LOD) set at 75 RFUs for all channels as recommended by the manufacturer and VADFS standard procedures.[20,21] STR profiles were evaluated for allelic and locus drop in/drop out, artifacts, and presence of male- and female-specific alleles, or any alleles in contamination studies. Donor allele assignment was performed manually by comparison to a staff index. The frequency of gender-specific alleles in a profile was determined by totaling the number of gender-specific alleles observed and dividing by the number of gender-specific alleles possible in a profile, excluding the X, Y, and DYS391 loci. Allele gender specific alleles that were the same were considered present as long as other alleles from that donor were also present.

Expected applicability of the research

The development of a more hands-free, fully integrated and seamless differential extraction method, the DNase DE method, will relieve examiners from performing the three or more required sperm pellet washes after non-sperm cell lysis, prior to loading the sample onto the robot. Given that each wash step takes ~five minutes with centrifugation and sample handling, the entire process takes progressively more time with each additional sample meant for

differential extraction. The DNase DE method is automated so that examiners will add the unwashed sperm pellets to the designated plate and the automated platform will perform the DNase I sperm pellet clean-up in an automated manner, all hands-free on the robot deck. Once completed, sperm pellets will be transferred by the robot to the sample plate containing the non-sperm fractions and any other forensic sample, and the DNA will be purified from all samples using the existing DNA IQ™ System by the NXP platform.

The expected applicability of this seamless, more automated DE method will allow examiner's time for other casework activities while not compromising quality or quantity of sperm cell DNA. Moreover, other laboratories utilize this same automated platform or a very similar one and the method will be provided to any interested forensic laboratory.

Participants and other collaborating organizations

The primary scientist on this project was Dr. William Eggleston, as a post-doctoral research fellow. He designed and performed nearly all experiments in consultation with Dr. Susan Greenspoon. Both Dr. Greenspoon and Ms. Cathryn Shannon provided assistance to Dr. Eggleston as needed. Dr. Andrew Timmes participated briefly at the beginning of the research period, assessing the DNase I enzyme's removal of non-sperm DNA as well as different buffers. There were no outside collaborating organizations.

Changes in approach from the original design and the reason for change

No substantive changes in the original design of the project occurred. Alterations to the non-sperm cell lysis buffer (as described in the results section) were necessary for optimal sperm cell DNA recovery, as described.

Outcomes

Activities

Epithelial cell lysis and DNase I digestions were initially performed manually to test and optimize multiple conditions based on the three published methods and the VADFS standard DE protocols.[5,7-9] Only a subset of conditions were retested following automation of the DNase I step on the NXP. Epithelial cell lysis and automated DNase I digestion conditions tested included buffer composition, enzyme concentration, and temperature and duration of incubation.

Analyses focused on the quantity of Y-DNA recovered as a primary measure of the ability to recover sperm DNA, on A/Y ratios as a measure of the ability to enhance for sperm versus epithelial cell DNA, and proportion of male- and female-specific alleles in STR profiles as a measure of the ability to generate sole-source genotypes. Other than sensitivity and contamination studies, 50 µL, of 1:1,000 dilutions of seminal fluid were used (1/20 dilutions) per sample. Based on an average of ~124,000 sperm/µL among initial aliquots measured with the hemacytometer, ~6,200 sperm were applied to each swab head. Given DNA extraction methods are estimated at only ~20% efficiency and these are haploid cells, then only approximately 3.7 ng of DNA/swab would be an expected yield. That works out to a final concentration under the conditions reported here of ~106 pg/µL, which coincides with the target male yield of 0.1 ng/µL.[22] This dilution was chosen to produce moderately challenging conditions in which increases and decreases in Y-DNA recovery, A/Y ratio and percent of male- and female- specific alleles could be detected to determine the effect of modifying conditions, and in comparisons of the manual VADFS DE method to the semi-automated DNase DE method (S. Greenspoon, unpublished observations).

An important complication must be addressed. The results from multiple experiments were excluded from consideration due to robotic failures during automated DNA IQ™ DNA purification during a period of testing. The failures were eventually attributed to a specific lot of

P1000 tips used on the NXP. Once the defective tips were discovered, that lot was not used further. Additionally, during a regularly scheduled preventive maintenance check performed immediately after the end of the experimental period, two of eight NXP's liquid handling pumps failed QC/QA tests. It was uncertain how long prior to the QC/QA test the problems had developed and how consistent they were. A review of the data found no consistent large differences between samples processed with the two pumps versus the other six pumps, but small differences and increased variation cannot be ruled out.

Results and findings

Optimizing epithelial cell lysis

Figure 1 shows the effects of varying epithelial lysis buffer, incubation temperature and/or incubation time on mock sexual assault vaginal/cervical tissue samples. Samples from three female donors (S17, S27 and S28) were combined with 50 µL of a 1:1,000 seminal fluid dilution from male donor S11 and the Y-DNA yield and A/Y ratios were measured for DNA purified from SFs. Throughout the summary, color coding in figures is: S17-blue, S27-orange, S28-grey, S29-unfilled. Panel A shows 3- to 5-fold differences in average Y-DNA yields among treatments for the three donors tested. Epithelial cell lysis in Buffer W80 yielded the highest average Y-DNA concentration for donors S17 and S28, and the VADFS DE the highest average yield for donor S28. The VADFS DE method yielded significantly more Y-DNA than two of the seven DNase DE treatments tested with donor S17 and all three of the treatments tested with donor S28. All other conditions were statistically the same. Taken together, the data are consistent with no epithelial cell lysis condition consistently yielding significant more or less Y-DNA than the VADFS DE method.

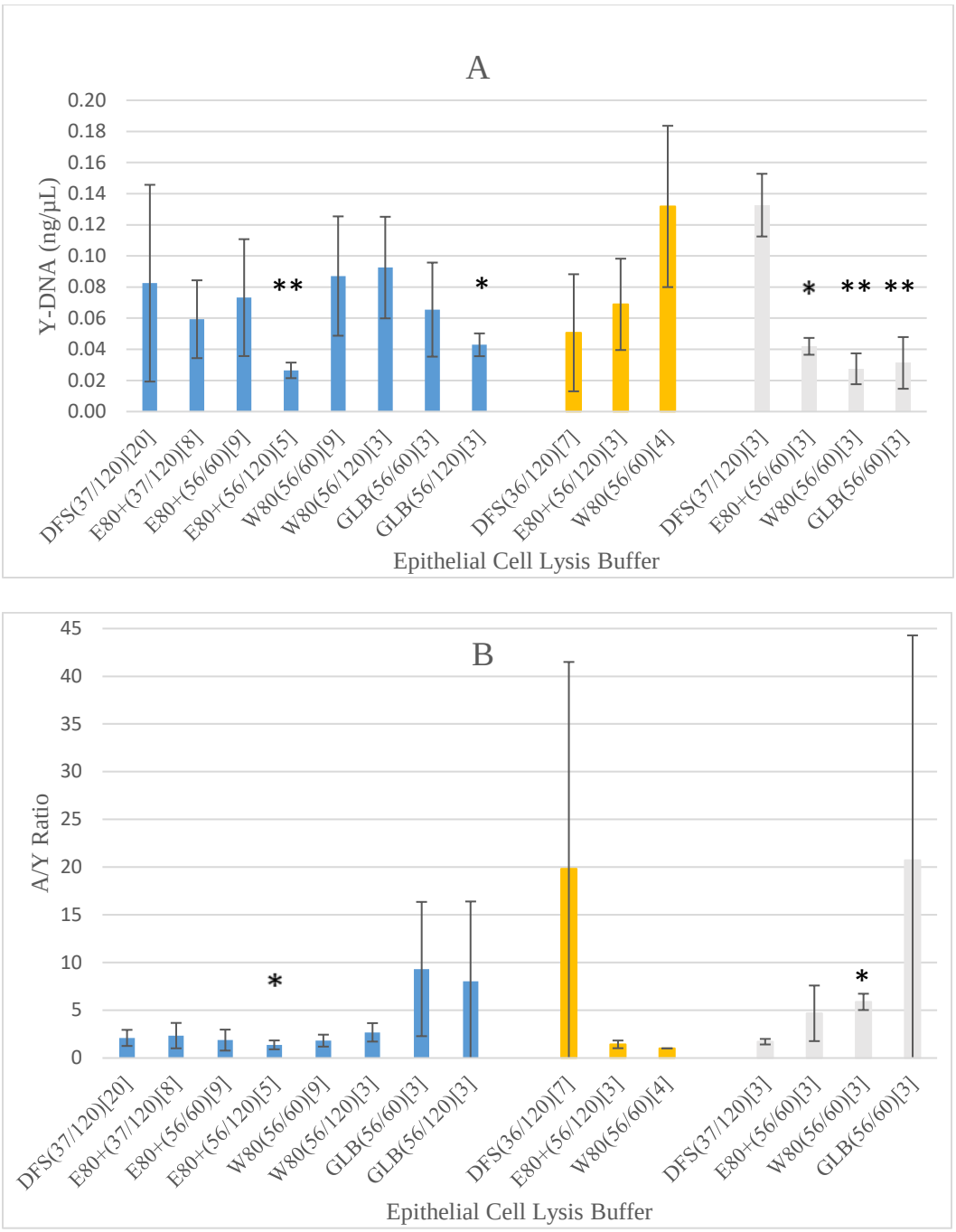

Figure 1. Effect of varying epithelial cell lysis of mock sexual assault vaginal/cervical tissue samples with 50 µL 1:1,000 diluted S11 seminal fluid on average Y-DNA yield and A/Y ratio. Here and in subsequent Figures, DFS indicates samples manually processed with the VADFS DE method. For other treatments, following manual cell lysis, automated DNase DE was performed (5 U DNase I in 200 µL, at a heater temperature of 62.5° C for 25 minutes). Panel A shows Y-DNA yields. Panel B shows A/Y ratios. Key: Values in () denote the temperature (°C)/ length of epithelial cell lysis in minutes, and values in [] denote number of samples tested. Blue columns indicate donor S17, orange columns donor S27, grey columns

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donor S28. * and ** indicate significant difference ($p < 0.05$ and $p < 0.01$, respectively) in two-tailed t-tests versus the manual VADFS DE method for the same vaginal/cervical tissue donor.

Figure 1B shows that average A/Y ratios for the samples varied up to 19-fold for a single donor, and that 4 out of 15 DNase DE treatments also had very high standard deviations. For two out three donors, the VADFS DE method produced the lowest A/Y ratios (lowest amount of contaminating female DNA), and for the third donor (S27), the highest (highest amount of female DNA). Only two significant differences were found between the VADFS DE method and the 12 epithelial cell lysis methods used with the DNase DE treatments, one lower (S17) and one higher (S28). As with Y-DNA yields, no epithelial cell lysis method consistently produced significantly higher or lower A/Y ratios than the VADFS DE method, or other buffer treatments. However, for the two donors tested, buffer GLB produced the highest A/Y ratios. It also is noted that the A/Y ratio for donor S27 varied using the VADFS DE method. In some trials it was slightly higher than for donor S17 and other trials, considerably higher over the course of the experimental period.

Together, the results in Figure 1 are consistent with there being no significant difference between the current manual VADFS DE and the variations of the epithelial cell lysis buffer used in conjunction with the semi-automated DNase DE method tested in terms of both Y-DNA yield and A/Y ratios, a necessary element in implementing the latter.

Figure 2 shows the effects of varying epithelial lysis buffer, incubation temperature and/or incubation time of mock sexual assault buccal tissue samples. Samples from all female donors (S17, S27, S28, and S29) were combined with 50 µL of a 1:1,000 seminal fluid dilution from male donor S11. Y-DNA yield and A/Y ratios were evaluated in DNA purified from SFs. As found in prior manual DNase DE tests, buccal samples generally produced higher average Y-

DNA yields, lower average A/Y ratios and had lower variation than vaginal/cervical samples from the same donors (Figures 1 and 2).

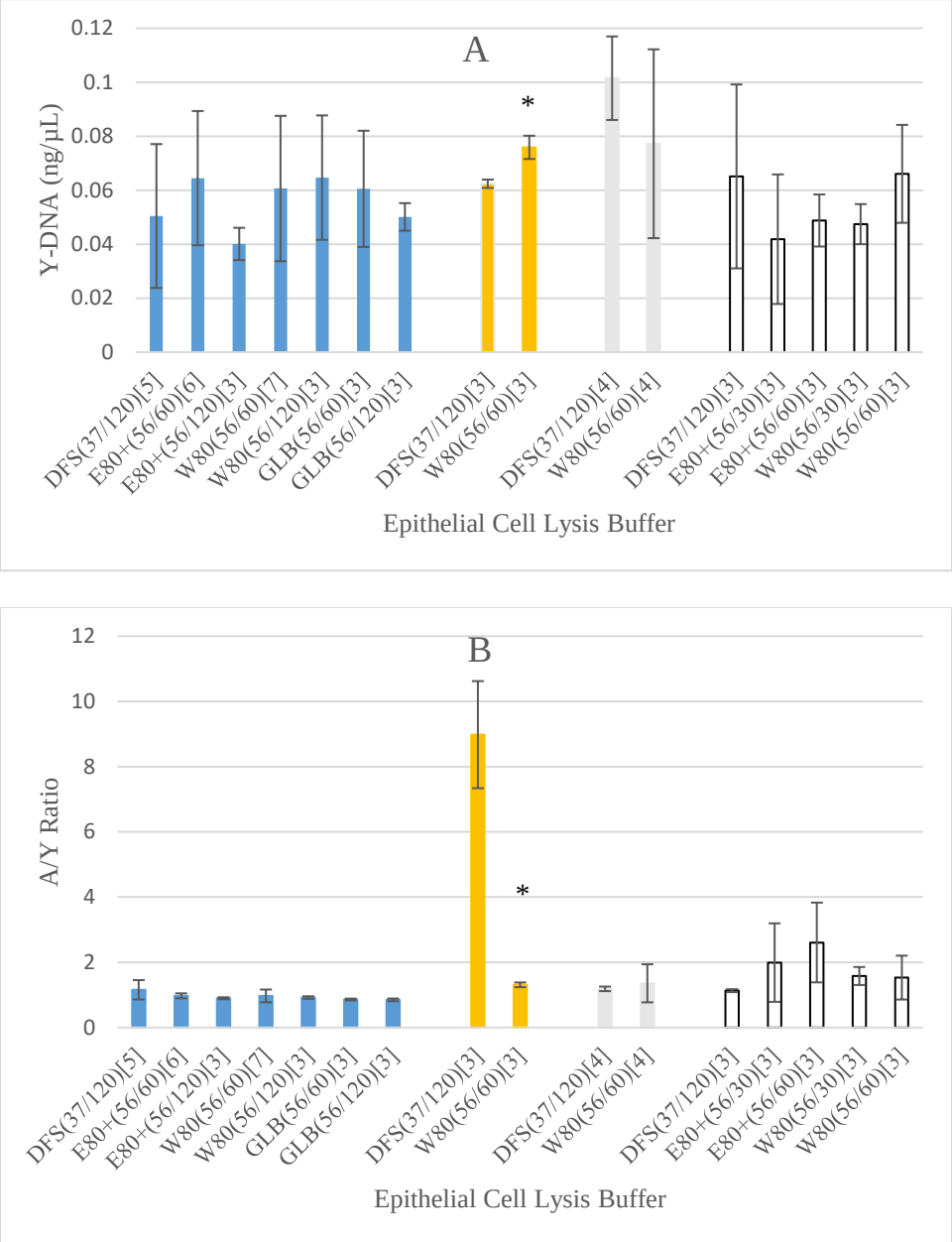


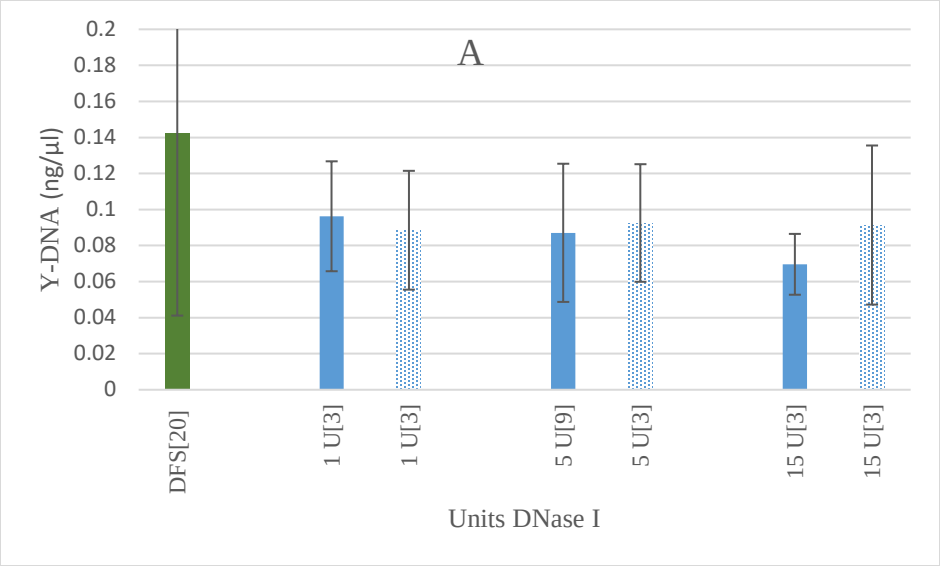
Figure 2. Effect of varying epithelial cell lysis of mock sexual assault buccal tissue samples with 50 µL 1:1,000 diluted donor S11 seminal fluid on average Y-DNA yield and A/Y ratio for DNA purified from SF samples. VADFS (DFS) and automated DNase DE conditions as in Figure 1. Panel A shows Y-DNA yields. Panel B shows A/Y ratios. Key: (), [], column colors, * and ** as in Figure 1. Unfilled columns indicate samples from donor S29.

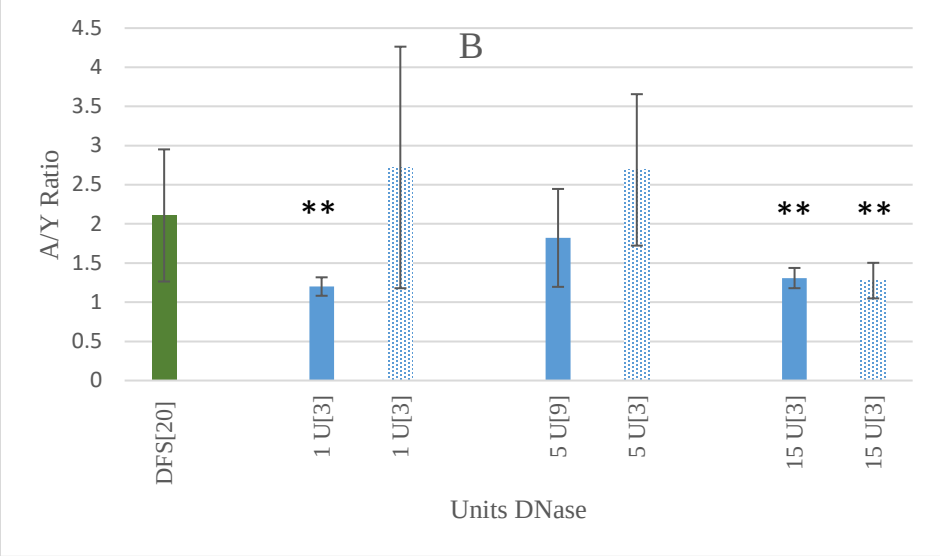
Other than for donor S27, no significant difference was found between the VADFS DE method and variations of the epithelial cell lysis buffer used in conjunction with the semi-automated DNase DE variations tested in terms of Y-DNA yields and A/Y ratios. As found for vaginal/cervical samples, no treatment or method consistently produced the highest or lowest average values for Y-DNA yield or A/Y ratio.

Based on the data in Figures 1 and 2, in order to address critical questions, epithelial cell lysis buffer W80 (56º C incubation for 60 minutes) was selected for further testing. Buffer GLB had been found to regularly produce higher A/Y ratios in both manual and semi-automated DNase DE tests, and although not significantly different, buffer W80 produced slightly better results than buffer E80+ (higher Y-DNA yields and lower A/Y ratios) in tests.

Optimizing DNase I Amount

Figure 3 shows the results of varying the amount of DNase I on the semi-automated DNase DE, with other conditions held constant other than duration of epithelial cell lysis. Average Y-DNA yields varied about 2-fold (Figure 3A), with the VADFS DE method having the highest average yield with a large standard deviation, but did not differ significantly from the six semi-automated DNase DE treatments tested. Y-DNA yield was very comparable between the six DNase DE treatments. Average A/Y ratios (Figure 3B) showed a similar level of variation between treatments, but ratios for three of the six semi-automated DNase DE treatments were significantly lower than the VADFS DE method.





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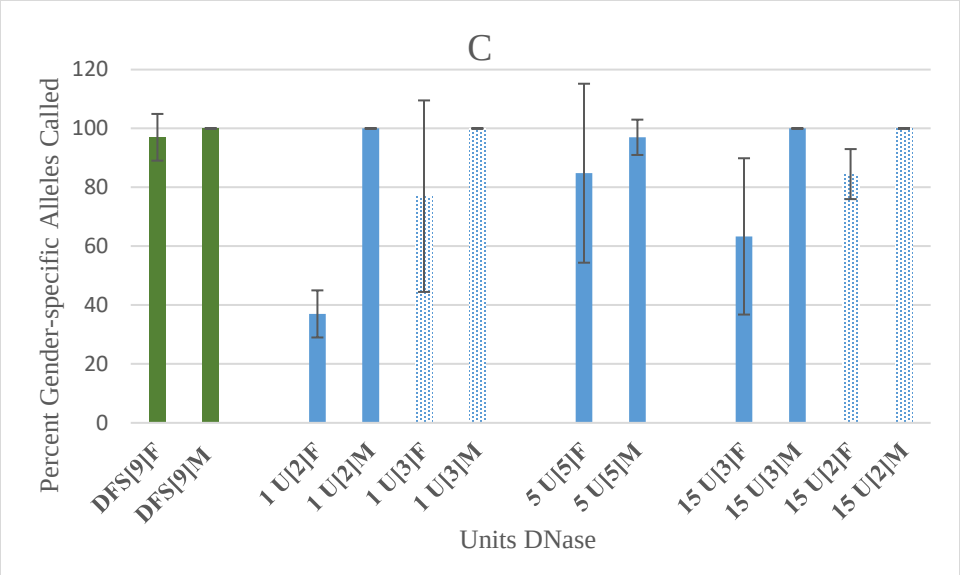

Figure 3. Effect of varying DNase I on average Y-DNA yield, A/Y ratio and gender-specific alleles in STR profiles generated from mock sexual assault vaginal/cervical SF samples. Donor S17 was used with 50 µL of 1:1,000 diluted S11 seminal fluid for DNA purified from SF samples. Panel A shows Y-DNA yields, B shows A/Y ratios, C shows percent of female(F)- and male(M)-specific alleles called during STR profiling. DFS defined as in Figures 1 and 2. Blue solid columns represent epithelial cell lysis Buffer W80 at 56° C for 60 minutes, and shaded blue columns for 120 minutes, followed by automated DNase DEs in 200 µL at a heater temperature of 62.5° C for 25 minutes with the indicated amounts of DNase I. Key: [], column colors (green indicates VADFS DE method), ** are defined as in Figure 1.

A critical aspect of the quality of a differential extraction is the proportion of alleles from the epithelial cell donor and sperm donor present in STR profiles of SFs. Figures 3C shows the average proportion of female- and male-specific alleles "called" (labeled by the GeneMapper ID-X software) in STR profiles, respectively. All five semi-automated DNase DE treatments produced a lower average proportion of female-specific alleles than the VADFS DE method. The VADFS DE method and four out of five DNase DE treatments produced full male-specific profiles. For the majority of conditions tested, the female alleles were lower in RFUs than the male alleles and for all tested, male profiles were enhanced by the differential extraction procedures. No significant difference was found between the VADFS DE method and the five DNase DE treatments tested, indicating that the latter produced comparable results to the former.

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The only significant difference found was in Figure 3C between 1 unit of DNase I (60 minute cell lysis) and 5 units of DNase I (60 minute cell lysis), where the 5 Units of enzyme results in a greater average percentage of female alleles detected ($p < 0.05$). Overall, the results in Figure 3 are consistent 1-15 Units of DNase I in the semi-automated DNase DE method producing results comparable to the VADFS DE method.

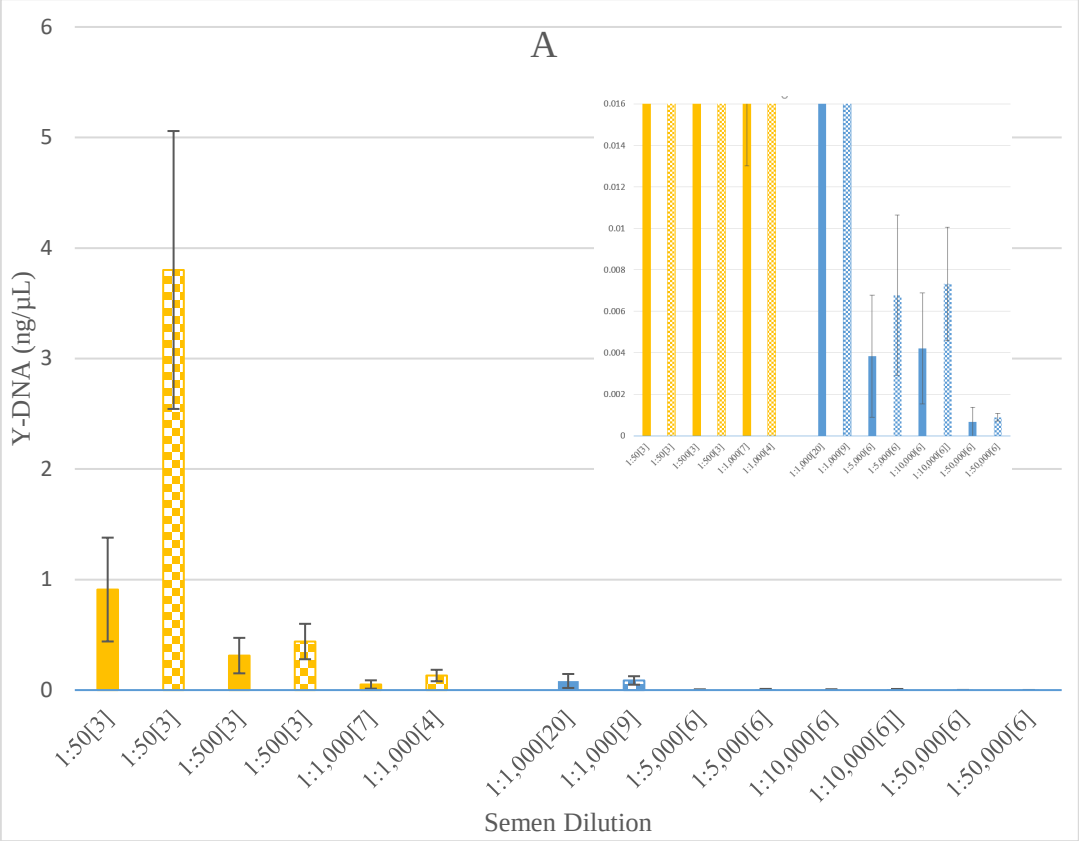
Sensitivity

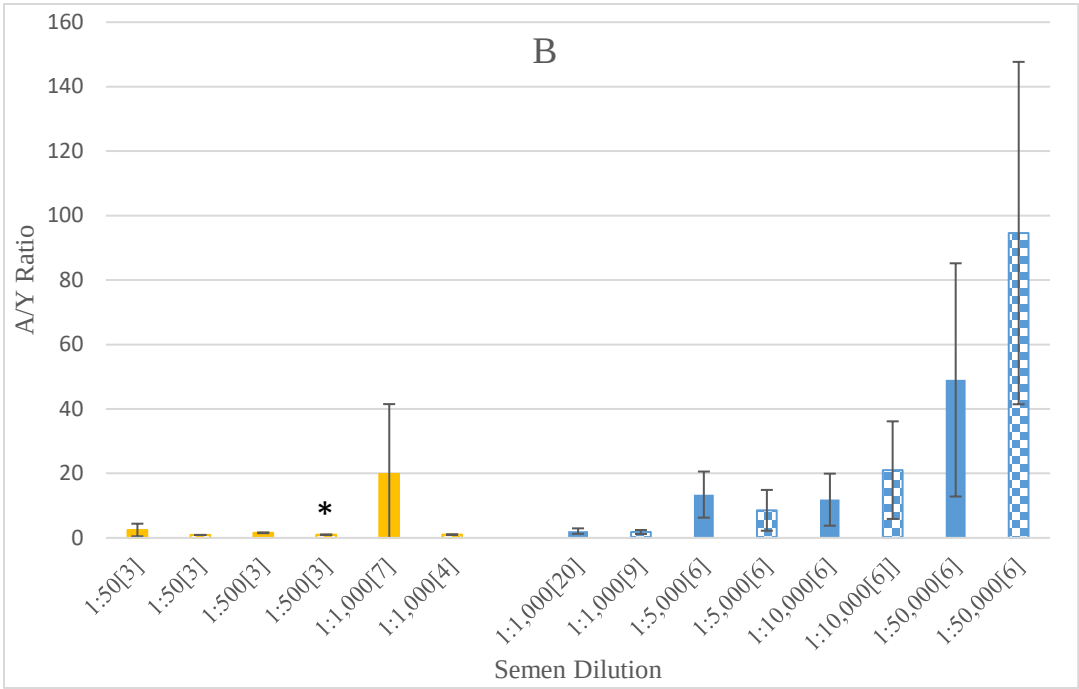
Figure 4 shows the results of comparing the VADFS DE method versus the semi-automated DNase DE method over a range of seminal fluid dilutions to test the relative sensitivity of the two methods with vaginal/cervical (VC) mock sexual assault samples for donors S17 and S27. In each case, 50 µL of diluted seminal fluid was applied to cotton swabs with female VC samples, at the dilutions 1:50, 1:500. 1:1,000, 1:10,000 and 1:50,000, which represented ~124,000, 12,400, 6,200, 620 and 124 sperm per swab, respectively.

As anticipated, higher dilutions overall resulted in lower average Y-DNA yields and higher average A/Y ratios (Figure 4A (inset) and 4B), although two tests for donor S27 did not fit with the overall trends (as found above). No significant differences were found between the VADFS DE and semi-automated DNase DE methods for the same dilution from the same female donor for Y-DNA yields and only one significant difference was found for A/Y ratios (S27, 1:500 dilution).

Figure 4C shows that for seminal fluid dilutions up to 1:1,000 for donor S27, the VADFS DE method (standard protocol) produced > 3-fold higher average proportions of female-specific alleles, in two cases, significantly higher (at dilutions 1:500 and 1:1,000) than the semi-automated DNase DE method. Also shown is that for seminal fluid dilutions of 1:1,000 and higher, both methods produced full or nearly full female-specific STR profiles for donor S17.

Semen dilutions up to 1:1,000 produced full or nearly full male-specific STR profiles with both methods and for both donors.





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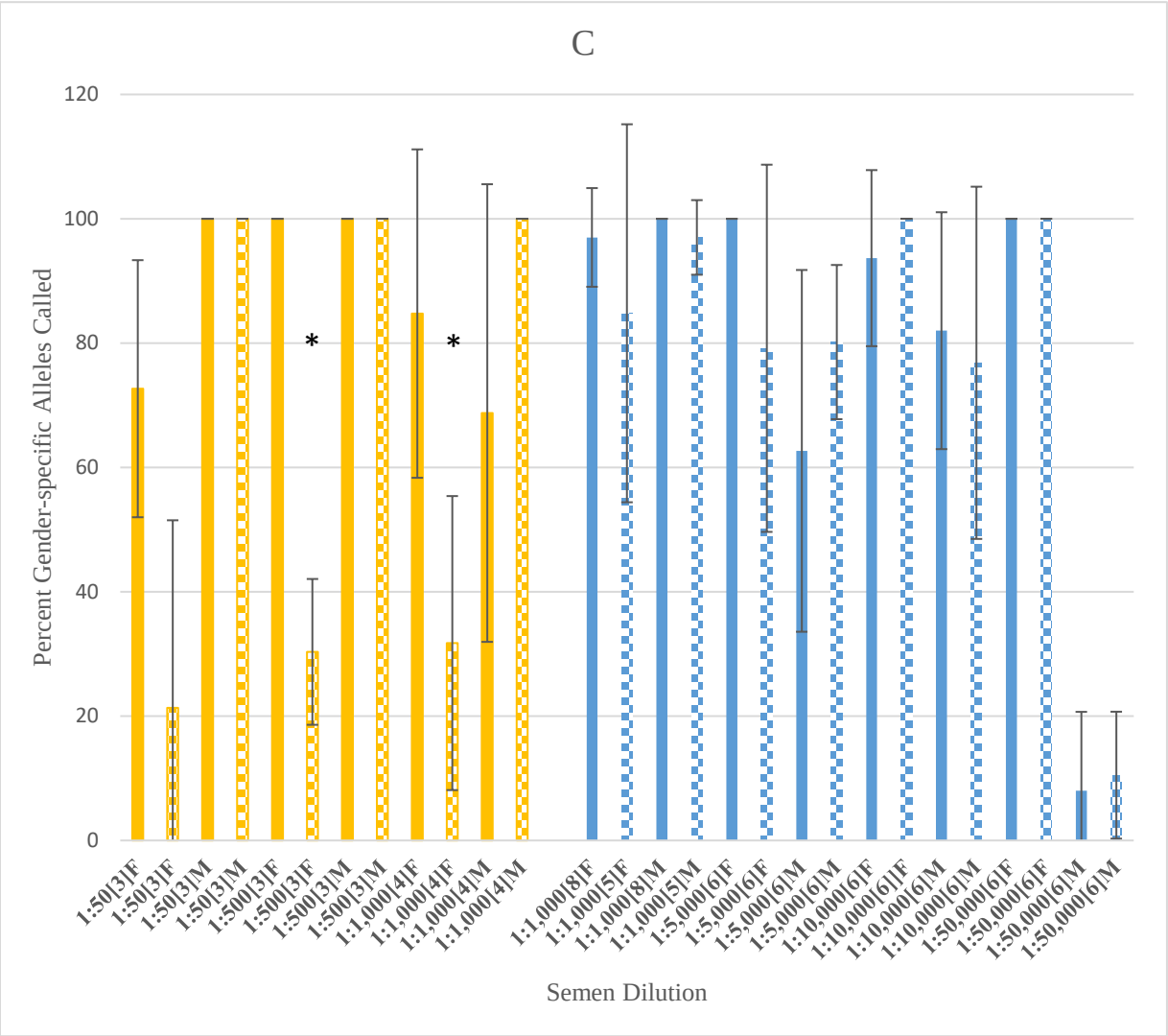

Figure 4. Effect of varying dilutions of S11 seminal fluid applied to mock sexual assault vaginal/cervical samples on average Y-DNA yield, A/Y ratio and gender-specific alleles. STR profiles were generated for donors S17 (blue) and S27 (orange) for DNA purified from SF samples. Panel A shows Y-DNA yields (insert shows results on enlarged scale), B shows A/Y ratios, C shows percent of female(F)- and male(M)-specific alleles called during STR profiling. Filled columns represent samples processed with the VADFS DE method and hatched columns samples processed with the semi-automated DNase DE method (epithelial cell lysis in Buffer W80 at 56º C for 60 minutes followed by automated DNase DEs with 5 Units DNase I in 200 µL at a heater temperature of 62.5º C for 25 minutes). Key: [] and * defined as in Figure 1.

The proportion of male-specific alleles called decreased with increasing semen dilutions, markedly between 1:10,000 (~620 sperm) and 1:50:000 (~125 sperm) for donor S27, but no significant difference was found between the methods at the same dilution, indicating that the two methods have comparable sensitivities.

Contamination Studies

Contaminants are a common component of sexual assault samples. Figure 5 shows the average Y-DNA yield and A/Y ratio for vaginal/cervical samples collected by donor S17 following use of Replens Vaginal Moisturizer (Replens). Figure 5A shows that the three DNase DE methods yielded ~3-6.5X more Y-DNA than the VADFS DE method, and epithelial cell lysis with W80 and GLB significantly more than the VADFS DE procedure. Figure 5B shows that the DNase DE methods E80+ and W80 both produced lower average A/Y ratios than the VADFS DE method, and the GLB method slightly higher, but that none of the differences were significant. Additionally, the presence of Replens resulted in sporadic clumping of DNA IQ™ resin during DNA purification which resulted in a lack of the resin at the elution step. Both the VADFS DE method and the epithelial lysis buffer E80+ in the semi-automated DNase DE method resulted in resin clumping and recovery of no measurable DNA in one and two samples, respectively. One possible explanation is the incorporation of NaCl in the DFS and E80+ buffers. The results also support the prior decision to focus optimization efforts on epithelial cell lysis buffer W80 during development of the semi-automated DNase DE method.

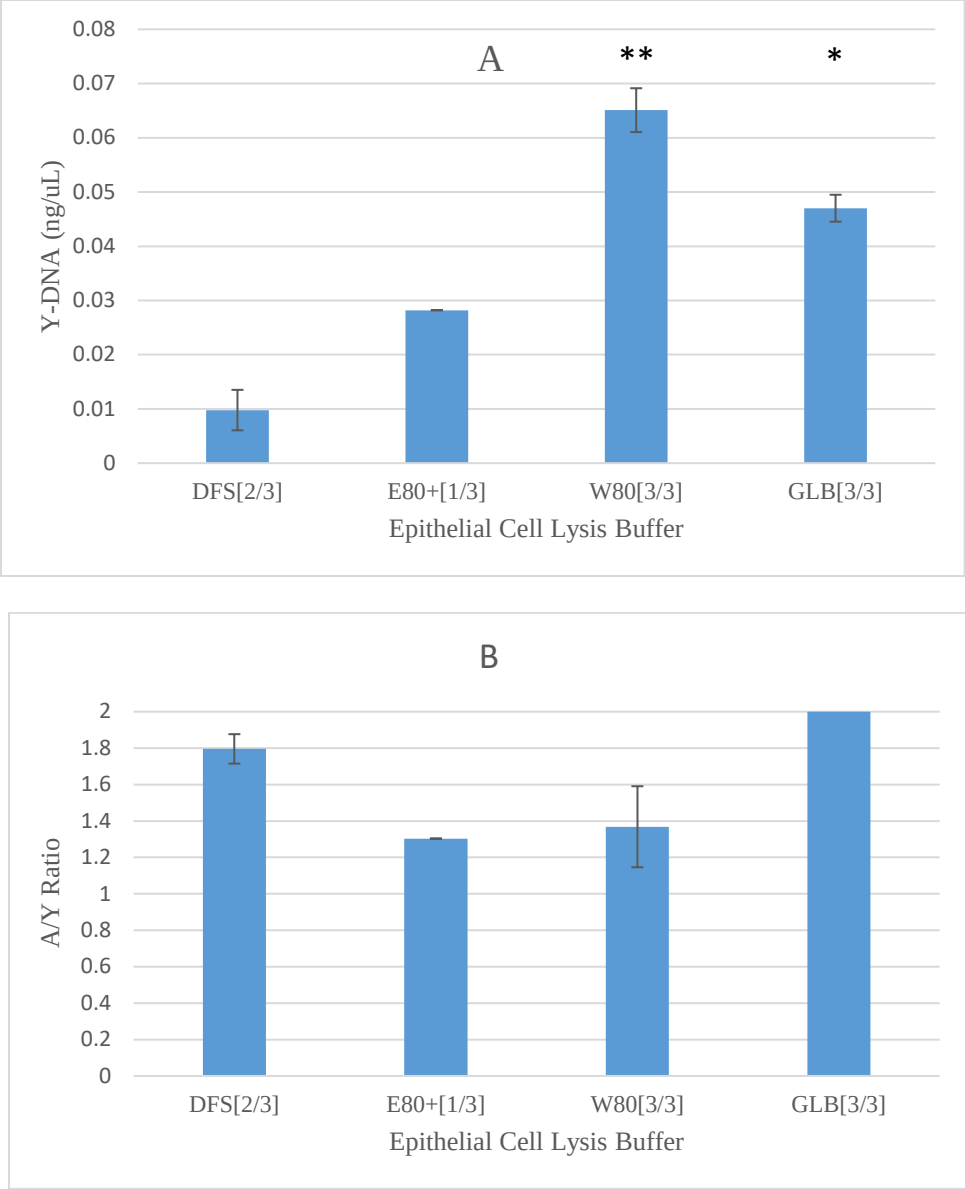

Figure 5. Effect of Replens use on average Y-DNA yield and A/Y ratio for vaginal/cervical samples from donor S17 with 50 µL 1:1,000 diluted donor S11 seminal fluid for DNA purified from SF samples. Panel A shows Y-DNA yields, B the A/Y ratios. VADFS DE samples labeled as in prior Figures. Semi-automated DNase DE method in epithelial cell lysis Buffers indicated at 56° C for 60 minutes followed by automated DNase DEs with 5 Units DNase I in 200 µL at a heater temperature of 62.5° C for 25 minutes). Key: [#/#] shows number of samples with measurable DNA/total number of samples tested, and * and ** as described in Figure 1. In B, the GLB A/Y ratio is truncated, the average ratio is 77.2, S.D. = +/- 68.9 (not shown).

Figure 6 shows the effects of six commonly encountered contaminants on three substrates for S11 seminal fluid diluted to 1:500 and applied to the substrates (16 total tests, including

uncontaminated substrates), and divided into nine ~equal pieces (~2,756 sperm in 1/9th cutting). The first observation is the high variation in results for both Y-DNA recovery and proportion of alleles called as indicated by the high standard deviations in a large proportion of tests, particularly for samples processed with the VADFS DE method. No STR results were obtained for the unwashed white panties with no contaminant applied and processed using the VDFS DE method. Figure 6A shows that while the VADFS DE method produced higher average Y-DNA yields than the semi-autonomous DNase DE method for paired samples in 88% of tests, no statistically significant differences were found between the two methods among the 16 tests performed. Unexpectedly, 50% (13/26) of contaminated samples had higher DNA yields than the corresponding uncontaminated sample.

Figure 6B shows that 50% produced profiles with 80-100% of male alleles called and 64% of contaminated samples produced STR profiles with more than 50% of male alleles. In all but one comparison (motor oil on toiletry bag), no significant difference was found between the percent of alleles called following processing with the VADFS DE method and the semi-automated DNase DE method. Together with Figure 6A, these results are consistent with the semi-automated DNase DE method producing results comparable to the manual VADFS DE method.

Together, the results in Figures 5 and 6 show that with buffer W80, the semi-automated DNase DE method performed as well, and some instances significantly better, than the VADFS DE method with contaminants commonly encountered in sexual assault casework samples.

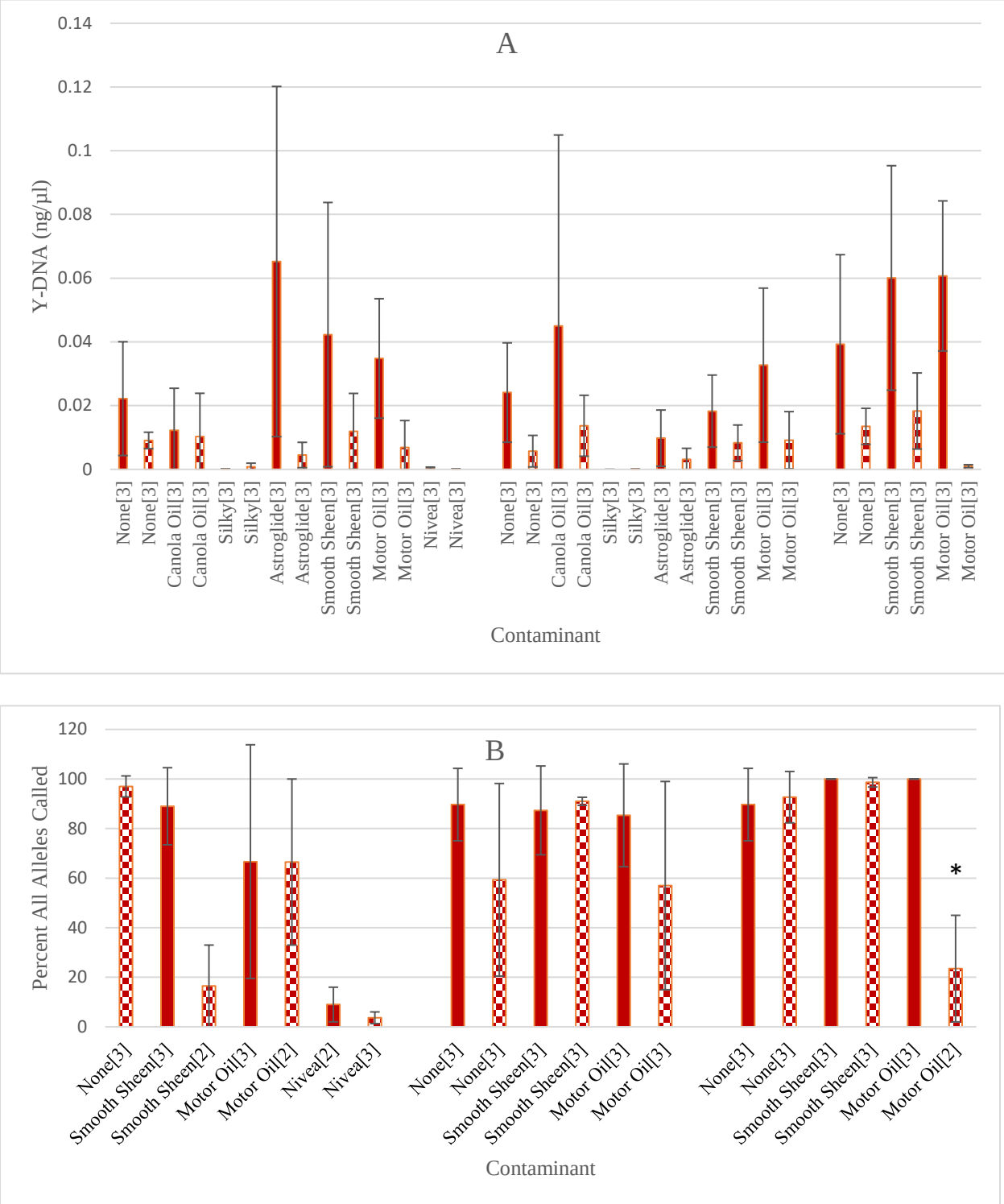

Figure 5. Effect of contaminants on Y-DNA yield and proportion of alleles called for 1:500 dilutions of S11 seminal fluid. Panel A shows Y-DNA yields, B shows the percent of male alleles called. Tests are grouped such that to the left are tests performed using unwashed white

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panties, in the center using washed white denim, and to the right using a toiletry bag. Filled columns represent samples processed with the VADFS DE method and hatched columns samples processed with the semi-automated DNase DE method (epithelial cell lysis in Buffer W80 at 56° C for 60 minutes followed by automated DNase DEs with 5 Units DNase I in 200 µL at a heater temperature of 62.5° C for 25 minutes). Key: [], * and ** as described in Figure 1. No STR profile results were available for uncontaminated unwashed panties.

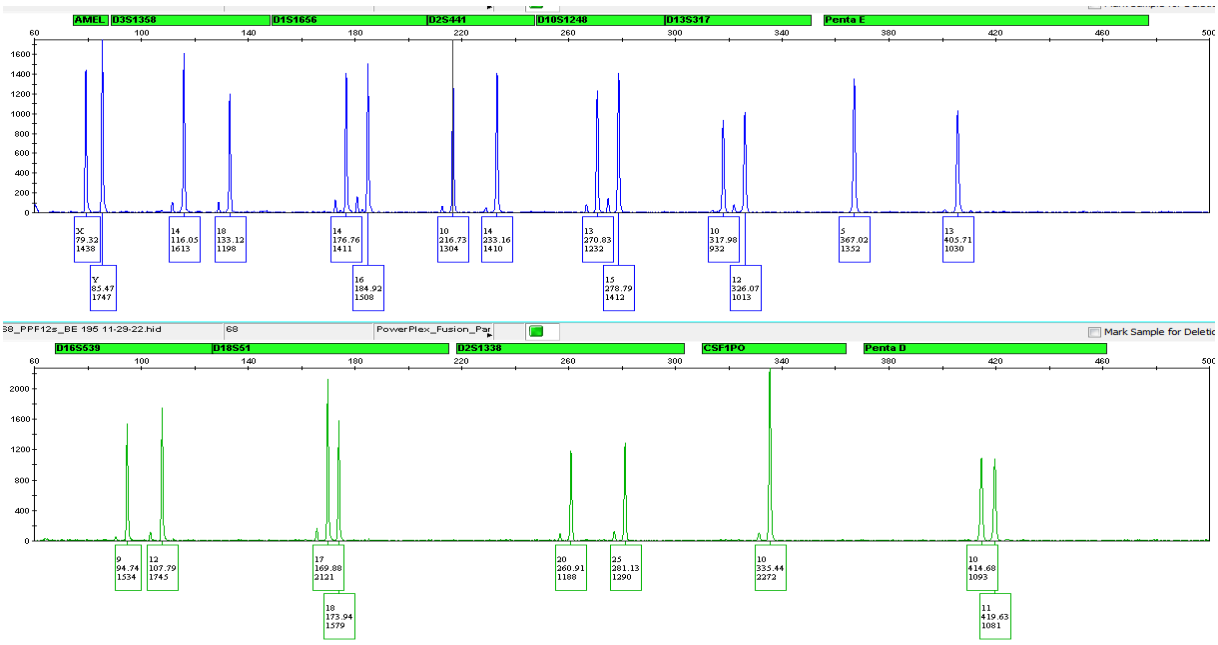
Cross Contamination Studies during automated DNase I digestions

All samples from the checkerboard tests were quantified using the PowerQuant® System and it confirmed that all sample wells contained DNA (data not shown). All blank samples were typed for PowerPlex® Fusion and two showed a single peak below 100 RFU. Upon re-amplification, neither peak was reproducible. The overall rate of cross contamination of 0/116 or 0%, which is similar to the 0.4% drop in/partial profile frequency measured for the Biomek NX^P (unpublished VADFS Validation Study).

STR Profiles

Figure 7 contains partial STR profiles of SFs for S17 rectal samples combined with 50 µL of a 1:1,000 dilution of S11 seminal fluid. The top two panels (VADFS DE) and bottom two panels (semi-automated DNase DE method), along with the two remaining panels in each profile (not shown) contain complete male profiles, with no female-specific alleles called by GeneMapper® ID-X. These results demonstrate that both the VADFS DE and semi-automated DNase DE methods can generate sole-source male profiles from mock sexual assault samples even at a 1:1,000 dilution.

A



B

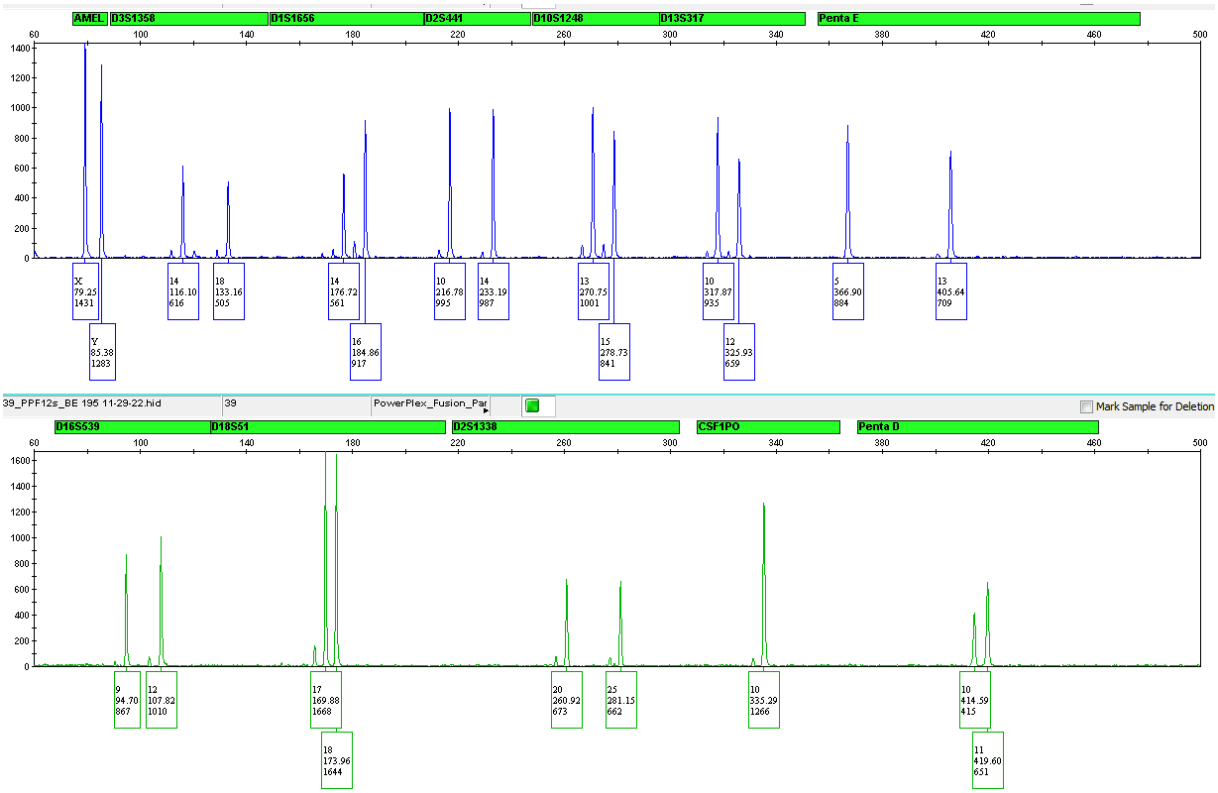


Figure 7. STR profiles generated by GeneMapper® ID-X for S17 mock sexual assault rectal samples with 50 µL 1:1,000 diluted S11 seminal fluid. The blue and green channels are

shown for samples processed with the VADFS DE method (A) and with the optimized semi-automated DNase DE method (B).

Limitations

The primary source of limitations with the DNase DE method are with the DNA IQ™ System extraction itself. The contaminants study provided some examples where DNA yields were reduced in the presence of saturated, oily compounds and this applied to the VADFS semi-automated DE method as well as the DNase DE method. Moreover, the DNA IQ™ resin has a limited capacity, so that DNA yields will taper off when large quantities of DNA are available (23). However, the Greenspoon, et al. 2004 report demonstrated that low template levels of sperm DNA were effectively captured by the DNA IQ™ system at an equal or superior efficiency to the standard, organic differential extraction method.

Applicability to Criminal Justice

This project has the potential to significantly impact DFS, and ultimately the broader forensic community, by streamlining the processing of sexual assault samples, removing manual wash steps, making the differential extraction procedure more automated, and simplifying sperm fraction DNA profile analysis by the enhanced removal of female (non-sperm fraction) alleles. Upon completion of the project, a fully validated and automated modified differential extraction process using DNase I will be developed. The incorporation of the modified differential extraction procedure into the current automated platform in a seamless fashion will avoid requiring a reconfiguration of laboratory procedure in order to accommodate the new approach and will require little change from DNA examiners other than to cut out the sperm pellet washes. It will simply result in the use of an automated sub-program to perform the DNase I digestion on sperm cell pellets prior to initiation of DNA purification for all of the casework samples on the

robot run. This is particularly important since at VADFS, all types of forensic casework samples, except for bone, can be processed together using the automated platform.

Artifacts

List of all products:

1. No scholarly products are available at this time.

2. A fully functioning, automated DNase DE automated method is available for dissemination to any interested laboratories, however, the automated method itself is designed for the NXP automated platform.

Data sets generated

While the data are reported herein, the DNA samples themselves and the electropherogram profiles will not be available to disseminate for other laboratories use due to privacy policies.

List of all dissemination activities:

1. Eggleston WB, Greenspoon SA, Shannon CA. Adaptation, Optimization and Validation of a semi-automated DNase I-based Differential Extraction Procedure on the Beckman Coulter Biomek NX^P Automation Workstation. To be presented at 2023 AAFS NIJ Awardee Symposium. February 14, 2023.

Upon completion of the project, the automated protocol will be made available to any laboratory that requests it, and there are several forensic laboratories in the United States that also employ the same automation platform. Moreover, DFS publishes all its departmental procedure manuals online and therefore, the procedure will be available to any interested parties including crime laboratories, members of the criminal justice system and customers. Any resulting publications will be submitted to peer-reviewed journals, such as the Journal of Forensic Sciences (JFS), Forensic Science International: Genetics, or the International Journal of Legal Medicine. The work performed for this project will be disseminated in the form of

presentations at scientific conferences such as the International Symposium on Human Identification, American Academy of Forensic Sciences or the Mid-Atlantic Association of Forensic Scientists**.**

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