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Evaluation of a Rapid Processing Protocol for Sexual Assault Samples using Rapid DNA Instrumentation: Final Report (DFSC-19-029)

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Table of Contents

Table of Tables

Table of Figures

Abstract

Rape and sexual assault are prevalent forms of crime worldwide. Due to the nature of these crimes, the evidence collected is often a mixture of DNA from two or more individuals, with the victim usually representing the majority of the recovered sample. The differential extraction process was developed to increase the likelihood of successfully obtaining a DNA profile from the assailant. However, this process is time-consuming to do manually or requires additional robotic equipment to automate. ANDE® has developed a protocol that supports the rapid processing of sexual assault samples. This new method utilizes a short pre-processing step that can be done using basic laboratory equipment to separate the alleged victim's DNA from the subject's DNA and enables the generation of the DNA profiles on the I-Chip, which was designed to process samples with low DNA content. Although this data is not currently eligible for upload to or searching against the national DNA index system (NDIS) it could be used to search against locally controlled databases, potentially enabling faster identification and apprehension of the perpetrator. This analysis method could be performed at the site of collection by a sexual assault nurse examiner (SANE) or could be utilized by DFSC DNA examiners in deployed locations to support rapidly testing and evaluating sexual assaults that occur on bases where an ANDE® instrument is co-located.

The Office of the Chief Scientist (OCS) has evaluated the success rate of this new protocol across a range of post-coital samples collected from 20 donor couples between 1 and 72 hours after intercourse. An additional set of mock post-coital samples, generated by placing semen dilutions onto buccal swabs were also run to further evaluate the success rate and the method's limits. A replicate swab collected at the same post-coital interval or generated with the same semen dilution was also processed through the traditional lab-based differential method to enable a comparison with current state-of-the-art testing at the Defense Forensic Science Center (DFSC). The ANDE® method successfully provided actionable DNA profiles for the majority of samples tested in this study and provided more single-source profiles than traditional typing. Traditional typing methods were generally more sensitive than the rapid method providing more data overall, but resulted in more mixed profiles than the rapid method.

1.0 Introduction

Rape and sexual assault are prevalent forms of violent crime within the U.S. Military, across the United States, and worldwide. In the 2019 survey year, the U.S. Department of Justice (DOJ) estimated there were 459,310 victims over the age of 12 who survived a completed or attempted rape or sexual assault within the U.S. population[1]. Although this represents a decrease from the previous survey year, which reported nearly 735,000 rapes and sexual assaults, it is higher than any of the previous three years[1]. Within the U.S. military, a population not included in the DOJ report, 6,236 victims reported sexual assaults in Fiscal Year 2019, an increase of 3% from the previous year[2]. Rates of reporting for rape and sexual assault are low, usually estimated at approximately 1 in 3, and reporting may often be delayed, indicating there could be hundreds of thousands of additional victims within the U.S. per year[1-3]. Backlogs of unprocessed rape kits and lengthy turnaround times for sample processing contribute to delayed justice for those victims that do report and complete the lengthy evidence collection process. In addition, before their identification, perpetrators remain free and may re-offend, creating new victims in the time it takes to process and investigate the initially reported incident.

In an effort to enable faster sample processing, ANDE® has developed a protocol that supports rapid processing of sexual assault samples[4]. The method includes a short pre-processing protocol that separates the sample collected as part of a sexual assault kit into two fractions, fraction 1 (F1 or epithelial) which usually represents the alleged victim's DNA profile, and fraction 2 (F2 or sperm) which usually represents the subject's DNA profile. Following separation, both the epithelial and sperm fractions can then be processed with the ANDE® Rapid DNA System (ANDE®, Waltham, MA) using the I-Chip, which is designed for samples with lower DNA quantity, in under 2 hours. This new analysis method could be performed at the site of collection by a sexual assault nurse examiner (SANE) or other trained hospital personnel, or could be employed by DNA examiners in deployed locations to support rapid testing of evidence for sexual assaults that occur on military bases where an ANDE® instrument is co-located. This technology has already been implemented in one state crime lab[5].

Although forensic (not reference) DNA profiles generated from rapid DNA instrumentation are not currently approved for upload into the Combined DNA Index System (CODIS), several agencies including the Federal Bureau of Investigation (FBI), the Scientific Working Group on DNA Analysis Methods (SWGDAM), and the European Network of Forensic Science Institutes (ENFSI) are evaluating the potential for future implementation of rapid DNA instrumentation for forensic sample processing[6]. Additionally, DNA profiles from crime scene samples processed on rapid DNA instruments are being used to search against locally controlled databases for jurisdictions that have established rapid DNA programs in advance of broad support[7, 8]. Rapid processing of these samples has been demonstrated to enable faster identification and apprehension of offenders.

To evaluate the utility and success rate of the ANDE® sexual assault processing protocol, post-coital and mock post-coital samples were processed. Post-coital samples were collected from 20 donor couples, over a range of post-coital intervals from 1 hour to 72 hours. Mock post-coital samples were generated by adding semen dilutions onto buccal swabs. Replicate samples for both sample types were also processed using traditional, differential processing methods. The results demonstrate that the ANDE® processing method can efficiently separate mixtures from

sexual assault swabs, more frequently resulting in a single-source profile than the replicate sample processed using traditional methods. However, traditional methods were more sensitive, resulting in a greater portion of samples providing usable data than the ANDE® processing.

2.0 Materials and Methods

2.1 Post-coital Sample Acquisition

Donor-collected post-coital vaginal swab samples were ordered from Lee Biosolutions, Inc. (Maryland Heights, MO). Each female donor was required to abstain from intercourse 72 hours prior to having unprotected intercourse with a non-vasectomized male. At the designated post-coital sample collection interval (1, 12, 24, 48, or 72 hours), the donor collected two vaginal swabs using a sterile rayon swab, labeling the order of collection. Ten donor couples provided samples at all five collection intervals, and an additional ten donors provided a set of swabs at 24 hours only. Due to a mistake in donor collection, 11 samples were provided at 1 and 12 hours post-coitus, and 12 samples were provided at 48 hours post-coitus. Only one collection interval was obtained from each intercourse event to prevent sample depletion that may occur if all swabs were collected from a single intercourse event. Swabs were dried before returning to the swab's plastic tube and were shipped and stored at room temperature before processing.

2.2 Mock Post-Coital Sample Generation

Ten female donors provided twenty buccal swabs each collected on cotton swabs. Swabs were dried and stored at room temperature until further use. Semen samples were ordered from five non-vasectomized donors (Lee Biosolutions, Inc., Maryland Heights, MO). Semen samples were frozen at the time of collection and throughout shipping. Samples were thawed, and diluted between 10 and 1600-fold in sterile water. A 100 µL aliquot of each dilution was pipetted into a round bottom tube and one dried buccal swab was placed head down into the tube to absorb the sample. Semen donor 1 was paired with buccal donors 1 and 6; semen donor 2 was paired with buccal donors 2 and 7; semen donor 3 was paired with buccal donors 3 and 8; semen donor 4 was paired with buccal donors 4 and 9, and; semen donor 5 was paired with buccal donors 5 and 10. Two swabs were made for each dilution. The 100-fold and 200-fold dilutions for each semen donor were also pipetted onto sterile cotton swabs. Half of each of these swabs were processed using DFSC's standard differential protocols (see 2.7 below) to estimate the quantity of DNA expected for each donor's dilution and assist in targeting the mock post-coital samples that would be most useful to evaluate on the ANDE.

2.3 Sample Truth Typing

Truth profiles were established for all ten buccal and five semen donors using both the GlobalFiler™ PCR Amplification Kit (Applied Biosystems™, Foster City, CA) and PowerPlex® Fusion 6C (Promega™, Madison, WI) short tandem repeat (STR) kits. Truth profiles for post-coital donors were established, by typing the F1 (epithelial) and F2 (sperm) fractions from at least one swab, targeting the sample that had the most favorable male to female ratio, where possible. For one donor couple who provided swabs at only 24 hours, the male's truth profile could not be established using traditional methods alone, but rapid methods provided a clear single-source male DNA profile. Additionally, no male DNA was detected from two of the donor couples who provided swabs at 24 hours only.

2.4 Rapid Buccal Swab Processing

Buccal swabs from all ten female donors utilized in the mock post-coital sample generation were also collected on the ANDE®-provided desiccant swabs. Swabs were sealed and stored at room temperature before processing. A total of eight ANDE® A-chips were processed across two ANDE® Rapid DNA Systems (ANDE®, Waltham, MA) using four swabs from each donor for a total of 40 samples.

2.5 Rapid Sexual Assault Sample Processing

Post-coital and mock post-coital samples were prepared for processing following the ANDE® Sexual Assault Kit (SAK) Pre-Processing Protocol with associated reagents and consumables available from ANDE®. Briefly, the entire swab head was cut and placed into a tube containing a lyophilized reagent cake. A volume of 300 µL of Reagent 1 was added, the tube was vortexed to re-suspend the reagent cake and hydrate the swab. The swab was then transferred to a spin basket and spun to recover the liquid absorbed by the swab and to pellet sperm cells. The swab head was removed and stored at -20 ºC for potential future processing. The liquid was removed without disturbing the cell pellet and placed in a new, clean tube, and was reserved for processing the F1 (female/non-sperm) fraction, using 5 µL placed onto an ANDE® swab. The cell pellet was washed by adding Reagent 2, vortexing to mix, and spinning to re-pellet the sperm cells. The supernatant was removed as before and discarded. Reagent 3 was then added and mixed with the remaining sperm pellet by pipetting, spun briefly, and incubated for 10 minutes at 37 ºC. Reagent 4 was added, mixed by pipetting, spun briefly, and incubated for 10 minutes at 56 ºC. Reagent 5 was added and mixed into the sample by pipetting. The sample was then absorbed onto an ANDE® swab and processed as the F2 (male/sperm) fraction. The processing protocol takes approximately 50 minutes when processing four samples simultaneously from start to finish which includes 27 minutes of timed incubation or centrifugation steps. The F1 (if used) and F2 sample swabs were then scanned during the ANDE® chip loading process and the I-Chip was loaded into the instrument.

2.6 ANDE Data Analysis

The onboard ANDE® software was set to require at least eight CODIS loci for the I-Chip and 20 autosomal loci for the A-Chip. The low DNA setting, a conservative approach to allele calling when enabled, was enabled for the A-Chip and disabled for the I-Chip. After the run was complete, data was exported, decrypted with the ANDE® FAIRS software and electropherogram data was compared to truth profiles previously generated.

2.7 Traditional Sexual Assault Sample Processing

Post-coital and mock post-coital swabs were prepped for processing using DFSC's validated differential processing protocols. Briefly, half of each swab sample was used for processing, initial lysing agents were added and incubated for approximately 1 hour. The swab cutting was removed using a spin basket and the remaining sample was processed using a QIAcube® (Qiagen®, Hilden, Germany). Following the completion of the first part of the automated differential protocol, the F1 fraction was removed and the DNA was purified following the Large Volume protocol on the EZ1® Advanced XL, (Qiagen®, Hilden, Germany), eluting the DNA in 50 µL of Tris-HCl EDTA (TE) buffer. After the second part of the automated differential protocol, the F2 fraction was removed, the sperm pellet was re-suspended and a sperm slide was prepared. The remaining

sample was lysed using G2 buffer (Qiagen®, Hilden Germany) and Dithiothreitol (DTT), and the DNA was purified using the Trace protocol on the EZ1® Advanced XL, (Qiagen®, Hilden, Germany), eluting the DNA in 50 µL of TE buffer. All F2 fractions were then dried using an Eppendorf® Vacufuge® plus, (Eppendorf®, Hamburg, Germany) and were re-suspended in 14 µL of sterile water. Samples were then quantified using Quantifiler™ Trio Quantification Kit (Applied Biosystems™, Foster City, CA) and amplified with the GlobalFiler™ PCR Amplification Kit (Applied Biosystems™, Foster City, CA).

2.8 STRmix™ Analysis of Traditional Samples

Mixture analysis was performed using the continuous probabilistic software STRmix™ (ESR, Auckland, New Zealand) using DFSC protocols and casework input parameter settings. Input profiles were exported in table format from the DNA analysis software ArmedXpert™ (NicheVision Forensics, LLC, Akron, OH). The number of contributors was determined by counting the number of alleles detected at each locus and using truth profile information when possible. Single-source profiles were modeled as one contributor with no conditioning and included profiles with only one or two extra alleles in stutter positions. Two contributor profiles were modeled by conditioning the female donor under both Hp and Hd when possible and the contributor proportions taken from the STRmix™ Advanced Summary Report. Likelihood ratios (LRs) were calculated by conditioning the female and male donor truth profiles under Hp and the female donor truth profile alone under Hd. DFSC protocol states that the LR will be capped at 1.0 quintillion for casework DNA profiles. CODIS eligibility was determined by running an in-house software on the male contributor profile from the STRmix™ output. DFSC protocol states that CODIS eligible genotype combinations must be weighted greater or equal to 99% and mixtures satisfy CODIS upload criteria.

3.0 Results and Discussion

3.1 ANDE® Results Overview

The ANDE® instrument and expert system software provide automatic results based on instrument-defined success metrics (e.g. internal lane standard sizing, on-chip ladder success) as well as user-defined settings (e.g. number of passing autosomal or CODIS loci required). Samples meeting or exceeding the set thresholds and which are not detected as mixtures will provide allele calls in an allele table and XML document which can be used for searching and matching. All samples will provide an electropherogram (EPG) image as a PNG document. These EPGs indicate confident allele peaks that will be included in the allele table and XML in grey boxes. Allele peaks that will not be included in the allele table or XML document and warrant further review are provided in red. These alleles may be marked in red for many different reasons including not meeting peak height or morphology thresholds, peak height imbalance within the locus, observing more than 2 alleles at a locus, detection of a mixture across the profile, and/or issues detected with the on-chip or on-board ladder. Figure 1 below provides examples of a fully confident and correct profile as well as a profile that failed one or more thresholds for the same donor and ultimately resulted in a fully correct profile called in red alleles.

Figure 1: Example ANDE® electropherograms with a fully correct allele calls that would be (A) and would not be (B) automatically exported for searching and matching. Full EPGs for both samples can be seen in Supplementary Figures 1 and 2.

While Figure 1 illustrates results for full profiles obtained in this study, several samples provided partial profile results. Figure 2 below illustrates an example of a profile that provided partial profile information in grey alleles which are exported into allele tables and XML files, with additional profile information in red allele calls. This profile, generated from an F1 (epithelial) fraction, was the profile with the least information that met the minimum ANDE® expert system thresholds generated in this study. All alleles, including red allele calls, correspond to the donor's truth profile, however, several alleles include only one of two expected alleles.

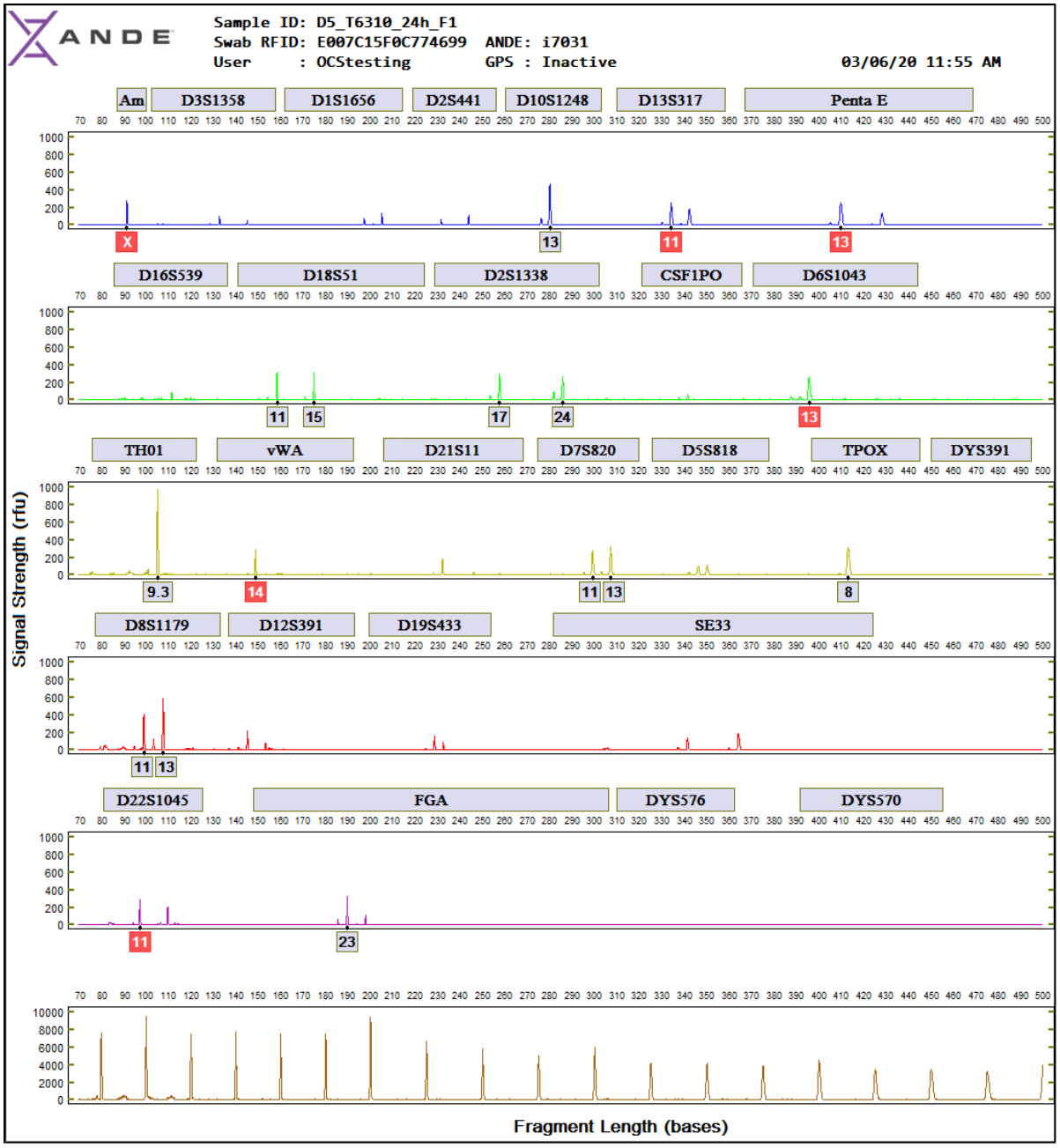

Figure 2: Example partial ANDE® profile. All alleles observed are correct, the grey alleles were automatically exported into an allele table and XML. The allele calls at D13S317, PentaE, vWA, D22S1045, and FGA include one of two expected alleles based on the donor's truth profile.

Mixtures were infrequently obtained with ANDE® runs and primarily occurred with low DNA profiles. The ANDE® expert system will flag a profile as a mixture if it detects more than two alleles at more than two loci. If more than two alleles are detected at a single locus, only that locus will be flagged in red. Figure 3 below provides an example of a mixture profile obtained from the F2 fraction of a post-coital swab collected at 72 hours. The profile includes a partial profile from the male donor with four (4) alleles that can only be attributed to the female donor. Profiles like

these do contain valuable information but require careful review by a technical expert to make use of the data available.

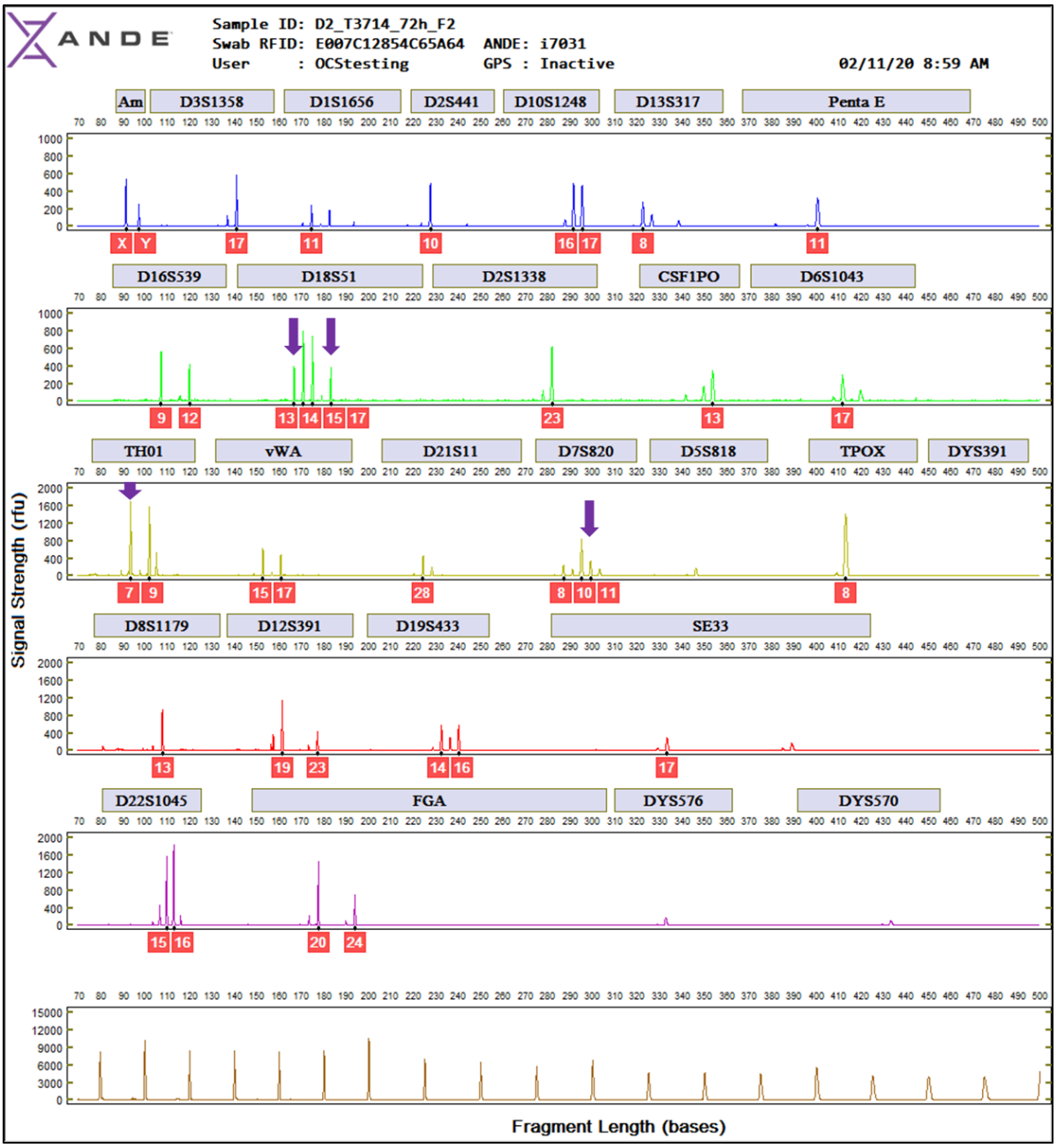

Figure 3: Example mixture profile obtained from an F2 fraction of a post-coital sample collected 72 hours after intercourse. The male donor is the major, but four (4) alleles corresponding only to the female donor are observed and indicated by purple arrows pointing to the allele peak but not necessarily the allele call box.

The results for all sexual assault samples processed are displayed in Table 1 below. Overall, 68.1% of the 144 samples processed met the instrument metrics for success and resulted in at least a partial profile. One sample provided confident allele calls at exactly eight loci, the threshold for passing, but included drop-out of one allele in a heterozygous pair at two loci. Full profiles were

obtained for 38.9% of the samples with confident allele calls. When considering the profiles that provided red alleles (the profiles that would require manual review) the proportion of full profiles increased to 54.9% and the samples providing fully correct calls for at least eight loci rose to 82.6%. Four of the 144 samples were lane failures (as determined by poor internal lane standard (ILS) sizing) in which no usable data was collected or could be recovered via manual review.

Samples were determined to be a mixture when alleles from both donors were observed at a minimum of least two loci. All mixture samples were partial profiles, providing allele data for 8 to 23 of the 27 possible loci. All samples that contained carryover of the paired donor's profile were F1 profiles detected in the F2 sample. These profiles ranged from allele data at 3 to 12 loci and had at most one allele that could be attributed only to the F2 donor's profile.

Drop-out of one of two alleles in a heterozygous pair was observed infrequently among post-coital samples (two F1 samples) when evaluating the confident data (grey allele calls) but was observed more frequently when evaluating the red allele calls and mock post-coital samples. When considering confident data, only one profile included a drop-in allele that did not belong to either of the donors in the sample. When considering red allele calls, drop-in was found in 11 profiles that were not mixtures with several alleles from both donors or carryover of only the paired donor's profile. Eight of those 11 included drop-in at only a single locus. Within these profiles, half of the 14 total drop-in alleles could be attributed to the paired donor and the other half were either artifacts or true drop-in. Due to the increased potential for observation of drop-in or drop-out when utilizing red allele calls, moderate match stringencies may be more appropriate to apply to this data.

Table 1: Overall results for all sexual assault samples processed using the ANDE®. Samples were evaluated based on the number of loci that provided only the expected donor's fully correct alleles, irrespective of drop-in or drop-out at other loci.

All ANDE® Samples (n=144)	Grey Allele Calls		Red Allele Calls	
Full PP27 Profile	56	38.9%	79	54.9%
17-26 Full Loci	36	25.0%	29	20.1%
8-16 Full Loci	5	3.5%	9	6.3%
1-7 Full Loci	1	0.7%	5	3.5%
No Confident/Correct Loci	42	29.2%	9	6.3%
Mixture	0	0.0%	4	2.8%
Paired Donor Partial Profile	0	0.0%	5	3.5%
Failed Lanes	4	2.8%	4	2.8%

3.2 ANDE® Sexual Assault Results by Sample Type

The focus of this study was to evaluate the success rate for F2 sample processing. This evaluation included a set of post-coital samples self-collected at 1, 12, 24, 48, and 72 hours after a single coital event, purchased from 10 donor couples. An additional 10 donor couples provided 24-hour post-coital swabs only. For complete details on post-coital sample collection, see section 2.1. A set of mock post-coital samples were generated using female buccal swabs and semen dilutions. After processing one set via traditional methods, samples that provided quant data in the most informative range for the ANDE® processing method, that is samples that had DNA quantities

consistent with post-coital samples on the border of success and failure (see Figure 4 and 5), were run using the rapid typing protocol. For additional details on mock post-coital sample generation, see section 2.2. Results for each set of samples are shown in Table 2 below.

Table 2: All ANDE® F2 sample results.

ANDE® F2 Samples	Post-Coital (n=64)		Mock Post-Coital (n=39)	
	Grey Alleles	Red Alleles	Grey Alleles	Red Alleles
Full PP27 Profile	32.8%	53.1%	30.8%	38.5%
17-26 Full Loci	21.9%	15.6%	35.9%	30.8%
8-16 Full Loci	0.0%	3.1%	10.3%	15.4%
1-7 Full Loci	0.0%	4.7%	2.6%	5.1%
No Confident/Correct Loci	42.2%	6.3%	20.5%	10.3%
Mixture	0.0%	6.3%	0.0%	0.0%
Paired Donor Partial Profile	0.0%	7.8%	0.0%	0.0%
Failed Lanes	3.1%	3.1%	0.0%	0.0%

When considering only the grey alleles, both post-coital and mock post-coital samples generated full profiles corresponding to the expected donor at similar rates, 32.8% and 30.8% respectively. The post-coital samples had a significant portion of samples, 42.2%, that did not provide confident data, more than double the rate of samples from mock post-coital samples. If red alleles are considered, the success rate of obtaining full profiles for post-coital samples increases by more than 20% and 71.5% of samples provided at least eight fully correct loci corresponding to the expected donor. The increase in observation of full profiles for mock post-coital samples is less dramatic, but this result is expected as these samples were designed to further evaluate the parameters for success.

Mixtures and carryover of the F1 profile were obtained for four and five of the 64 post-coital samples, respectively, but were not observed for mock post-coital samples. Samples that did not provide any allele calls and which were not determined to be lane failures occurred infrequently. Except for two post-coital samples (collected at 1 and 12 hours post-coitus), results primarily corresponded to samples with low male DNA quantities. Because the entire F2 fraction generated through the rapid differential protocol is consumed in the ANDE® run, re-processing the sample is not possible without using an additional swab.

While the primary aim of many sexual assault investigations is to identify the assailant, who is usually represented in the F2 fraction, the profile from the F1 fraction, usually representing the victim, is also frequently generated for traditional processing methods. A total of 41 F1 fractions were run on the ANDE®, 39 from post-coital samples and five from mock post-coital samples. Results for all F1 fractions are provided in Table 3 below.

Table 3: All ANDE® F1 sample results.

ANDE® F1 Samples (n=41)	Grey Alleles	Red Alleles
Full PP27 Profile	56.1%	73.2%
17-26 Full Loci	19.5%	17.1%
8-16 Full Loci	2.4%	2.4%
1-7 Full Loci	0.0%	0.0%
No Confident/Correct Loci	17.1%	2.4%
Mixture	0.0%	0.0%
Paired Donor Partial Profile	0.0%	0.0%
Failed Lanes	4.9%	4.9%

The large majority of F1 fractions provided full or partial profiles with at least eight loci corresponding to the expected donor and automatically provided files for searching and matching. Nine of the F1 samples did not provide immediately actionable results, two of which were lane failures.

After reviewing the red allele calls for these profiles, the success rate of samples providing full and correct profiles rose to 73.2%. A further 19.5% provided at least 8 loci matching the expected donor. Only one profile that was not a lane failure did not provide any correct loci, and in fact, did not provide any allele calls at all. The majority of the F1 fractions that did not provide full profiles, and were not lane failures, had low signal. If obtaining the profile from this fraction was essential, an increased quantity of the remaining fraction could be run on a subsequent chip to obtain a profile, however, this was not done during this study.

Processing the F1 fraction may become important for samples run on the ANDE® if utilizing red allele call data, particularly if only a partial profile is obtained. During this study, nine partial profiles were obtained from F2 samples that corresponded to mixtures or F1 carryover. Analysis of the F1 profile is useful in assessing whether partial profiles contain probative data and may be essential when both the victim and assailant are male.

3.3 Comparison of Rapid and Traditional Typing Results

Differential extraction and traditional typing methods are well-established and because the success of these methods was not the focus of this study, results for laboratory processing will only be compared to rapid results rather than described separately. Half of a second swab collected at the same post-coital interval or prepared at the same semen dilution using the same buccal swab donor were processed using traditional laboratory methods. For additional details on processing methods, see section 2.7. All fractions were quantified, and all F2 samples and a sub-set of F1 fractions were amplified using GlobalFiler™ chemistry.

Mixtures are more frequently generated in both the F1 and F2 fractions when samples are processed via traditional methods. However, these mixtures often provide data that can be deconvoluted with the assistance of software designed for this purpose. Mixtures generated through traditional sample processing methods were run through STRmix™, conditioning the run based on the female profile, often resulting in likelihood ratios greater than 1 quintillion. Because

mixture deconvolution of samples generated through rapid instrumentation is not currently possible and due to the different limitations on the use of data resulting from rapid or traditional processing, results are analyzed differently as shown in Table 4 below.

Table 4: Comparison of sexual assault samples processed using rapid and traditional methods. Results indicate the number of samples within each category, separated based on the ability to utilize the data generated. Numbers in parentheses within the dark green column for traditional processing indicate the number of the total samples in that category which were single-source. *Samples processed from two donors at 24 hours did not provide any male DNA in the F2 fraction from either rapid or traditional methods, likely indicating a collection issue. Note: F1 samples would not be uploaded into a database, the data below is to assist with comparing profile quality and completeness.

	ANDE®					Traditional			
	Single-Source Full/ Partial	Single-Source Full/ Partial Review Req.	Mixture	Insufficient Data/Paired Donor Profile	Failed Lane	National (# Single-Source)	National Mix	State	No upload
Post-coital F1 Samples	27	6	0	1	2	32 (23)	0	0	0
F2-1 hour	7	1	0	1	2	11 (8)	0	0	0
F2-12 hours	10	0	0	1	0	11 (2)	0	0	0
F2-24 hours	9	8	0	3*	0	16 (1)	2	0	2*
F2-48 hours	7	2	1	2	0	6 (0)	5	1	0
F2-72 hours	2	1	3	4	0	3 (0)	2	1	4
Mock F1	5	0	0	0	0	5 (1)	0	0	0
Mock F2	31	3	0	5	0	39 (17)	0	0	0

A total of 98 of the 144 samples (68.1%) processed using the ANDE® protocol provided data that could be immediately utilized for comparison without further review. If a review was included, up to 119 samples (82.6%) provided at least partial data consistent with the expected donor's profile for at least 8 loci, with no more than 1 allele corresponding to the paired donor's profile. The remaining 25 samples (17.4%) provided either a mixture, data at fewer than 8 loci, profile information that corresponded to the paired donor (F1 observed in the F2 fraction), or were lane failures.

Samples processed using traditional methods were analyzed based on the eligibility for upload to national (NDIS) or state (state DNA index system, SDIS) databases. A total of 123 of these 140 samples (87.9%) provided data that could be uploaded as a single-source profile to the national database. Of those 123 samples, only 52 provided single-source profiles after traditional differential extraction. A further 9 samples (6.4%) could be uploaded to NDIS as a mixture, and two (1.4%) could be uploaded to SDIS. Only six samples (4.3%) did not provide results that could be uploaded to any database.

Comparison of the results between rapid and traditional processing indicates that the ANDE® method is more successful at generating single-source profiles. At extended intervals (48 or 72 hours) only the rapid protocol provided any single-source profiles from F2 fractions. Seven of those profiles, five 48-hour and two 72-hour, provided full profiles either automatically or after review of red allele calls.

Although the rapid method was more successful at generating single-source profile data, the traditional method was more sensitive and provided more actionable data overall. To further evaluate the sensitivity of the rapid method the quant data obtained via truth typing was utilized to estimate the DNA quantity-based sensitivity of the rapid method. It cannot be presumed that the rapid and traditional methods provide the same recovery of DNA in the F2 fraction. Therefore, the total quantity of male DNA obtained in the F1 and F2 fractions after traditional processing of a half swab was combined and doubled to estimate the total male DNA available on the sample swab. Figures 4 and 5 provide a comparison of success rates for post-coital samples. In this comparison, any sample that provided a profile with actionable data (including red allele calls which would require manual review) was characterized as successful. Mixtures, samples that provided the paired donor's profile, or very low-level results were not considered successful.

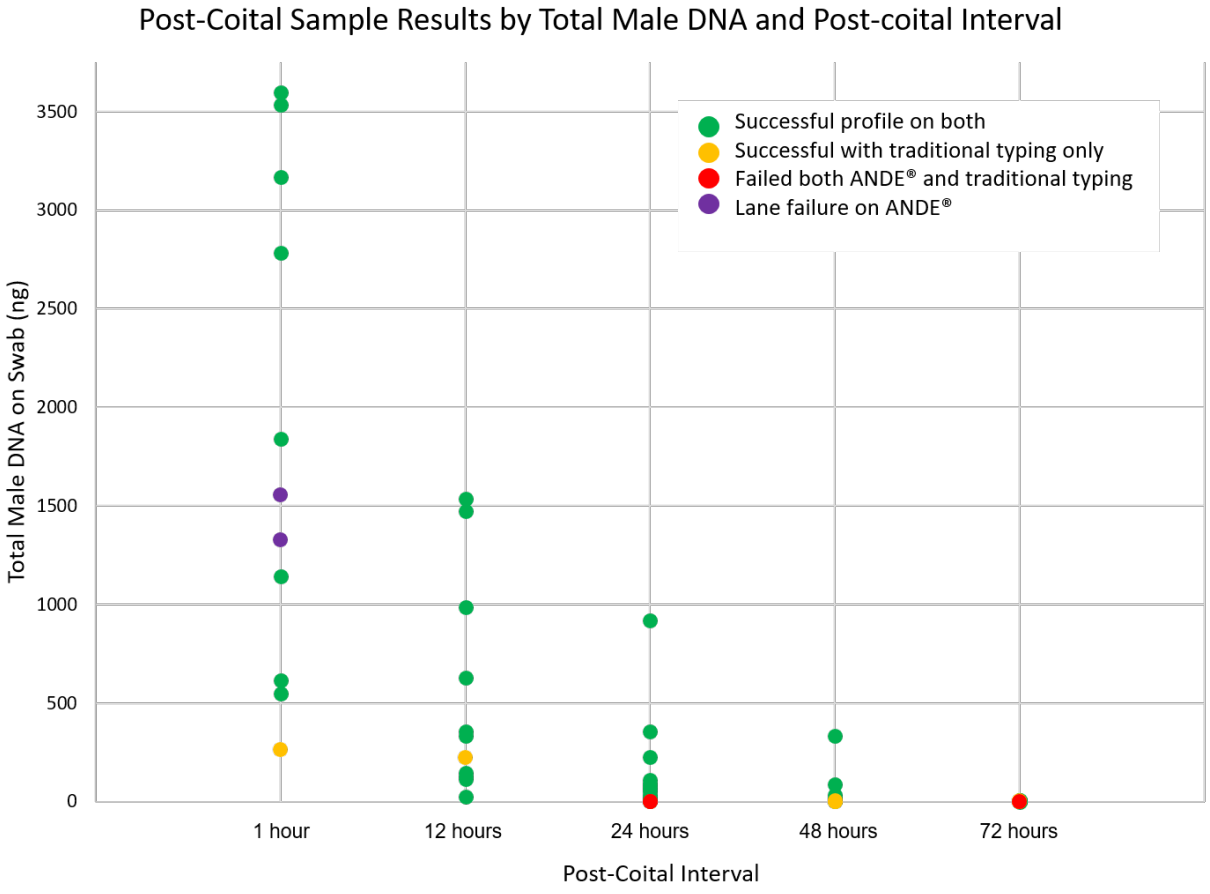

Figure 4: Post-coital F2 results plotted based on estimated total male DNA on the swab. Sample success is color-coded based on the key in the top right.

To further visualize the expected sensitivity limits of rapid typing, the Y-axis, representing total male DNA on the swab, was scaled to a maximum of 10 ng, Figure 5. Although there were two post-coital samples (collected at 1 and 12 hours after intercourse) that had DNA quantities over 200 ng which were successful with traditional typing but not rapid and did not correspond to a lane failure, the borderline for success with rapid processing of post-coital samples appears to be near

4 ng of total male DNA recovered from the swab via traditional methods. This does not, however, indicate that the limit of sensitivity for the ANDE® I-Chip is approximately 4 ng. The lowest post-coital sample which provided a full F2 profile corresponded to an estimate of 5.7 ng of male DNA recovered from the swab via traditional extraction methods.

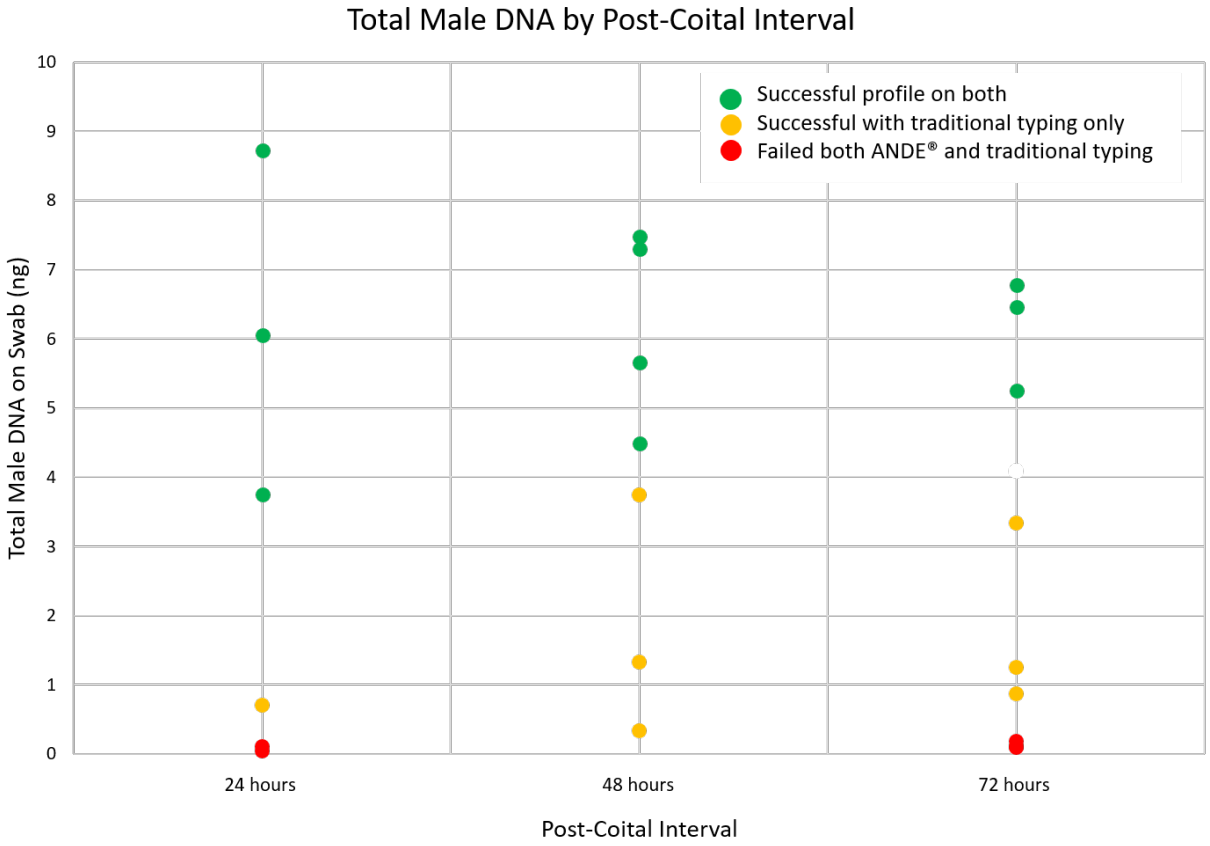

Figure 5: Post-coital F2 results plotted based on estimated total male DNA on the swab, scaling to 10 ng of total male DNA. Sample success is color-coded based on the key in the top right.

Results for mock post-coital samples in comparison with the male DNA quantity recovered through traditional processing methods are provided in Supplementary Tables 3 and 4. In these samples, there was not as clear of a delineation on likelihood of success based on the total male DNA recovered via traditional typing. Three samples provided actionable data at even lower ranges than post-coital samples, corresponding to an estimate of between 1.28 and 1.50 ng of total male DNA recovered. Conversely, one sample with an estimated total male DNA quantity of 23.6 ng failed to provide usable data with rapid processing. In general, the mock post-coital samples provided better separation in the F1 and F2 fractions than the actual post-coital samples and all mock samples provided actionable data via traditional typing.

3.4 Evaluation of Swab Order

At the time of collection, each donor labeled which swab was collected first and second. Because there may be a chance that the semen and/or sperm cells available for collection could be partially

depleted by collecting the first swab, an effort was made to eliminate this potential impact on processing methods. For post-coital swabs, the first swab from all odd-numbered donors and the second swab from all even-numbered donors was run via traditional processing and vice versa for the rapid protocol. Due to a collection issue with an even-numbered donor, there are four more samples for which swab 1 was processed on the ANDE® than for swab 2. Table 5 provides the success metrics for swabs processed with the ANDE®.

Table 5: ANDE® results by swab order.

	Swab 1 (n=34)		Swab 2 (n=30)	
	Grey Alleles	Red Alleles	Grey Alleles	Red Alleles
Full PP27 Profile	32.4%	55.9%	33.3%	50.0%
17-26 Full Loci	26.5%	14.7%	16.7%	16.7%
8-16 Full Loci	0.0%	5.9%	0.0%	0.0%
1-7 Full Loci	0.0%	2.9%	0.0%	6.7%
No Confident/Correct Loci	38.2%	0.0%	46.7%	13.3%
Mixture	0.0%	8.8%	0.0%	3.3%
Paired Donor Partial Profile	0.0%	8.8%	0.0%	6.7%
Failed Lanes	2.9%	2.9%	3.3%	3.3%

Initially, these results appear to indicate a higher rate of success with the rapid protocol for the set of samples in which the first swab was processed. The first swab provided 8.9% more data with greater than 8 fully correct loci when considering the confident data (grey alleles) and 9.8% more data if red allele calls are considered. However, upon further review of the results, two samples in the second swab set did not provide any allelic data despite the paired swab providing more than 200 ng of total DNA with traditional processing (yellow data points at 1 and 12 hours in Figure 4), well above the expected sensitivity limit for rapid processing. These swabs may likely have represented an alternate failure method for the ANDE® processing that did not also correspond to poor ILS sizing, for example, a blockage in the fluidics prior to injection of the PCR product into the on-chip capillaries. Post-processing of the reserved swabs indicated sufficient DNA remaining on the swabs, see section 3.5. These two failures reduced the success rate for the second swab set by 6.7%. If these samples had been successful, the difference in success rates between the first and second swab sets would have been negligible.

An evaluation of the DNA quantity recovered from traditional analysis methods further investigates the potential differences based on processing the first or second swab collected. Figure 6 below shows the estimated total male DNA recovered from each swab, color-coded by swab order. The data indicates that, although the set of swabs collected first often had the highest quantity of DNA recovered in each post-coital interval set, there was not a trend of swabs collected first providing the greatest quantities of DNA. The differences observed may correlate more strongly to differences between the sperm count and ejaculate volume for the male in the post-coital couple or efficiency of swabbing, rather than to the order of swab processing. The highest quantity of DNA recovered for each post-coital interval was primarily from two of the 10 donor

pairs that donated the full set of swabs and both of those donors had the first swab processed via traditional methods.

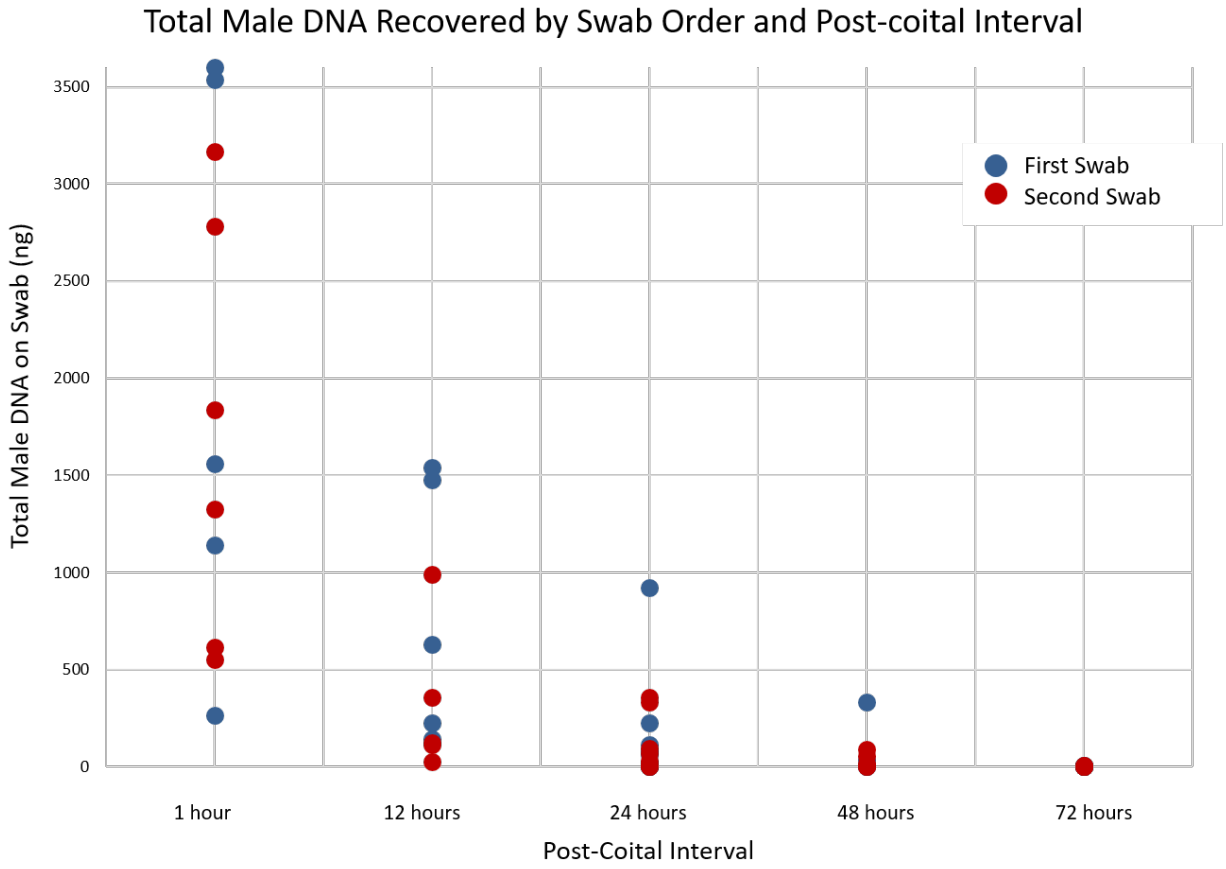

Figure 6: Quantity of DNA recovered based on swab order and post-coital interval.

Although sperm cells and DNA from the perpetrator may be depleted when additional swabs are taken, the data collected in this study indicate that reserving the first swab collected for traditional laboratory-based processing should not impact the expected success of rapid processing on the ANDE® instrument.

3.5 Traditional Processing of Reserved ANDE® Swabs

All swabs processed using the rapid method were recovered and reserved at -20 ºC for potential re-processing. Five of these reserved swabs were re-processed via traditional sexual assault processing methods to assess whether preservation of the swab may provide a backup method for obtaining the profile of the subject. Of the five samples, one was successful with original rapid processing and four did not provide any actionable data from the F2 profiles obtained from the ANDE®. Two of those four corresponded to samples that had an estimate of more than 200 ng of male DNA available on the swab collected at the same interval and processed via traditional methods, and two were swabs in which the F2 fraction was a lane failure as indicated by a poor ILS sizing. The entire reserved swab head was processed to maximize the potential of recovering

the remaining DNA and obtaining a DNA profile. The quantity of male DNA recovered from the F2 fraction of each of these re-processed swabs is displayed in Table 6 as compared to the quantity of DNA recovered by processing the original half swab.

Table 6: Quantity of male DNA obtained after re-processing swabs reserved from rapid analysis.

	Total Male DNA in F2 Fraction (ng)	
	Original ½ Swab	Re-process Full Swab
Donor 3 – 1h	1475.1	57.8
Donor 5 – 1h	111.8	53.4
Donor 5 – 12h	71.6	1.9
Donor 7 – 1h	697.8	41.9
Donor 10 – 1h	590.6	4.8

For all of the five samples re-processed using traditional methods, sufficient male DNA was recovered to generate a full GlobalFiler™ profile. One sample generated a single-source male DNA profile from the F2 fraction, and the remaining four were mixtures in which the male profile represented at least 90% of the signal. In addition, sperm cells were visualized on the sperm slide generated during the differential process for all re-processed samples. Although the quantity of male DNA available on the swab before rapid processing cannot be known, the reduction in DNA after rapid processing, based on assessing the DNA quantity obtained from the swab cutting collected at the same interval, appears to be significant. For samples that may have otherwise had limiting DNA quantities to begin with, the re-processing method may not provide successful profile generation, but it is clear that the rapid method does not deplete all of the available sample.

3.6 Rapid Buccal Swab Processing Results

A set of 40 buccal swabs were run on the ANDE® A-Chip designed for reference sample processing which accommodates 5 swabs per run. These samples were processed on both instruments utilized for this evaluation at the beginning, middle, and end of the evaluation in an attempt to ensure the instrument was working properly. Results from the buccal swab samples are displayed in Table 7 below.

Table 7: Rapid buccal swab processing results.

All Buccal Samples (n=40)	Grey Alleles	Red Alleles
Full PP27 Profile	65.0%	65.0%
17-26 Full Loci	2.5%	15.0%
8-16 Full Loci	0.0%	12.5%
1-7 Full Loci	0.0%	0.0%
No Confident/Correct Loci	27.5%	2.5%
Failed Lanes	5.0%	5.0%

A total of 26 of the buccal swabs (65.0%) provided full profiles and this number did not improve when considering red alleles. Eleven buccal swabs (27.5%) did not provide confident data, but ten of those improved to provide at least 8 full and correctly reported loci when red alleles were

considered. Two of the 40 buccal swabs processed were lane failures. Upon further review of the buccal swab results, one of the ten buccal swab donors failed to provide a confident profile on any of the four samples provided. Three of the four samples generated partial profiles when red alleles were evaluated, but one sample did not provide any allele calls. These results suggest that the buccal swab success rate was reduced due to poor sample collection rather than due to the performance of the ANDE® A-Chip. All other donors provided two or more swabs which generated at least partial, confident profiles.

Obtaining a reference profile for the victim may be important to the case, particularly when carryover of the F1 DNA into the F2 may be a concern. If the laboratory wishing to process sexual assault samples has sufficient demand for this testing, it may be reasonable to process the victim's reference profile using a buccal swab processed on the ANDE® A-Chip, and utilize I-Chips only for F2 sample processing. Processing reference profiles from buccal swabs would increase the chance of obtaining a full, single-source profile for the victim and may increase case throughput, particularly if two ANDE® instruments were available.

Conclusion

The data gathered through this study indicates that the successful rapid processing of sexual assault samples via the ANDE® is possible. The majority of samples run, evaluated overall or in any sample breakdown, provided results that met or exceeded the set sample success thresholds and could be utilized without further review. The rapid analysis method also more frequently provided single-source sample results than traditional processing, including two single-source profiles generated from samples collected at 72 hours post-coitus. Additionally, a subset of swabs reserved after rapid processing provided sufficient recoverable DNA for traditional processing methods, indicating that the rapid method does not utilize all of the available sample, although a significant depletion of DNA was observed.

Although the rapid method demonstrated success, it was not as sensitive as traditional DNA processing methods and did not provide actionable data for as many samples as the conventional protocols. Therefore, negative results with a rapid processing method should not be used to determine which samples are likely to yield actionable results for traditional processing methods. In addition, rapid processing does not currently replace the need for traditional processing. Forensic samples processed via rapid instrumentation are not currently approved for upload into NDIS and may face significant challenges within the courtroom when used as part of generating an investigative lead.

This study evaluated a set of post-coital and mock post-coital samples which were known to contain only two donors. The success rates of these samples as compared with the total quantity of male DNA observed on the swab collected at the same post-coital interval may provide a method for estimation of the success rates for sexual assault samples processed in a forensic laboratory, assuming similar DNA yields from the laboratory's differential method. However, additional challenges may be encountered for actual forensic samples, particularly for cases when sperm from more than one male may be present, either due to recent intercourse with a consent partner or for cases in which more than one assailant was involved. Similarly, this method is not expected to be successful for assaults in which sperm is not present.

Future study of the rapid sexual assault method could include testing of samples collected at intervals greater than 72 hours post-coitus, an evaluation of the success rates for samples recovered from bedding or clothing, as well as an assessment of the potential to recover the victim's DNA from the remaining F1 fraction. Additionally, samples collected years to decades previously may be interesting to study in order to evaluate whether this method may be suitable for more rapidly processing backlogged samples for which the statute of limitations has expired, but from which valuable information can be obtained and may result in connecting serial cases.

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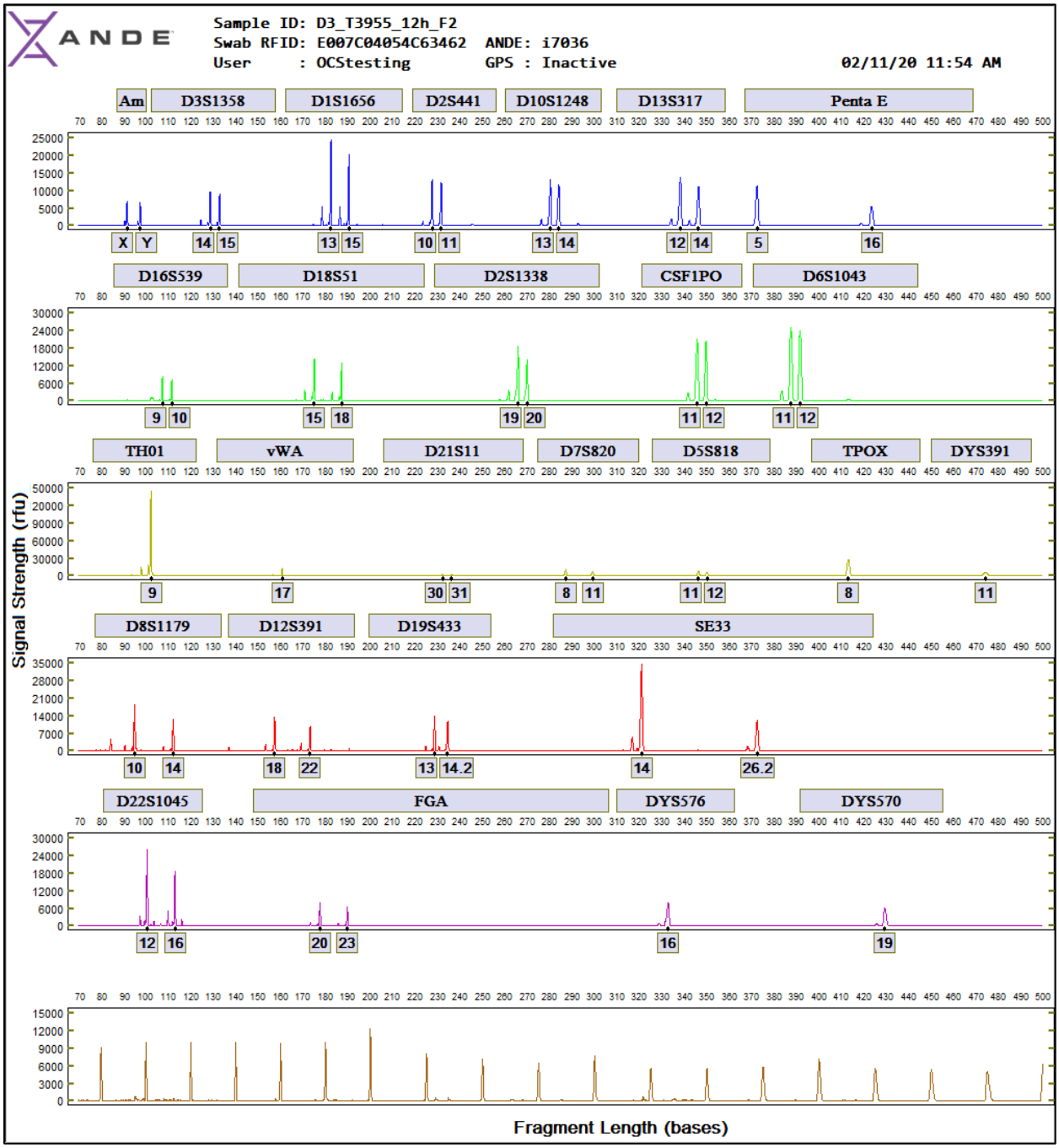
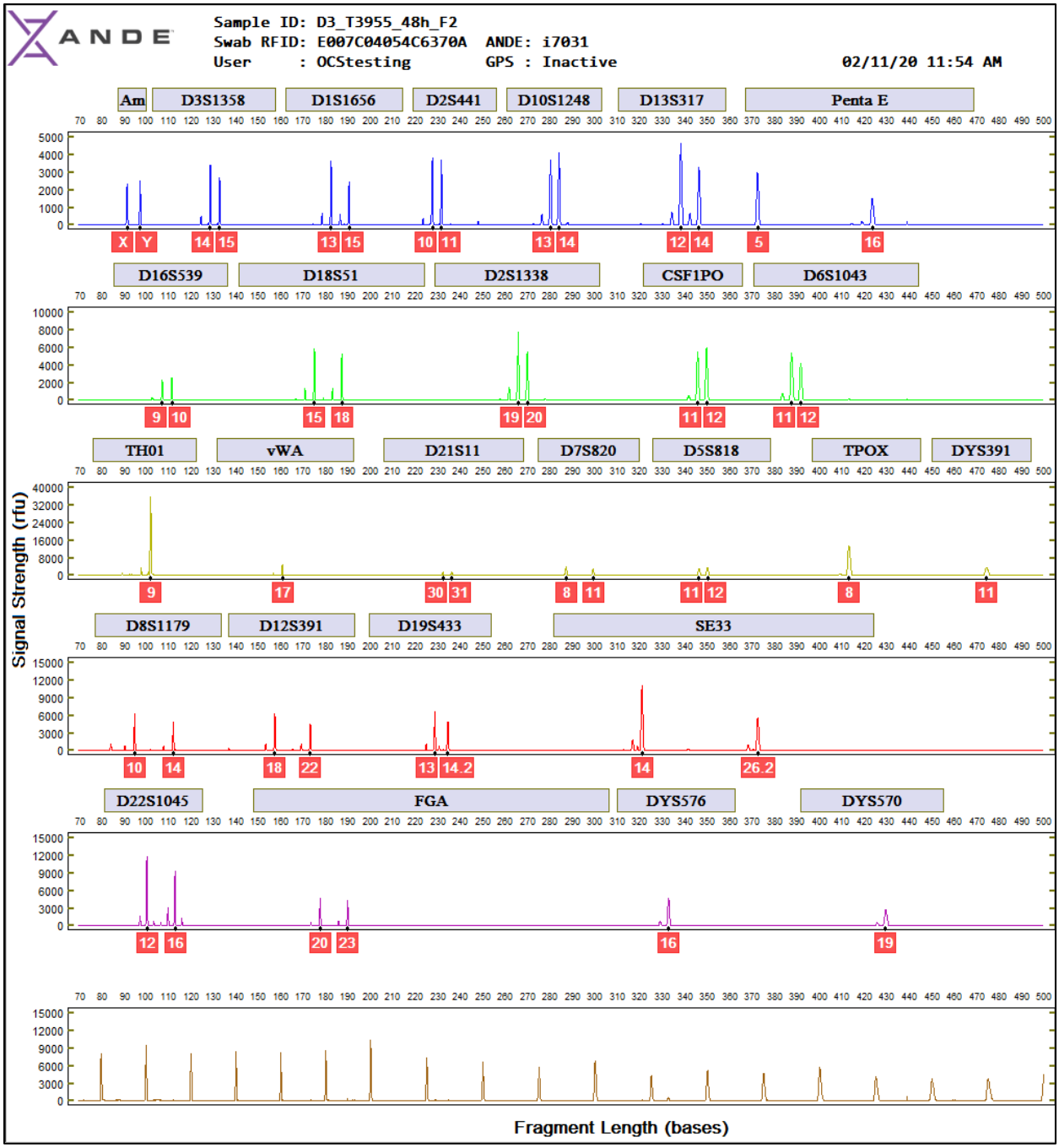
References

1. Morgan, R.E. and J.L. Truman, *Criminal Victimization, 2019*. 2020, U.S. Department of Justice.
2. *Department of Defense Annual Report on Sexual Assault in the Military Fiscal Year 2019*. 2020, Department of Defense.
3. Rennison, C.M., *Rape and Sexual Assault: Reporting to Police and Medical Attention, 1992-2000*. 2002.
4. *Reduce sexual assault with rapid DNA*. [cited 2021 2/17/2021]; Available from: https://www.ande.com/sexual-assault/.
5. *Kentucky first in nation to adopt rapid DNA for sexual assault investigations*. [cited 2021 February 19, 2021]; Available from: https://kentuckystatepolice.org/hq-4-10-19/.
6. Hares, D.R., et al., *Rapid DNA for crime scene use: Enhancements and data needed to consider use on forensic evidence for State and National DNA Databasing - An agreed position statement by ENFSI, SWGDAM and the Rapid DNA Crime Scene Technology Advancement Task Group.* Forensic Sci Int Genet, 2020. **48**: p. 102349.
7. Harran, F., *Community Policing and Forensics: Using a Local DNA Database to Reduce Crime.* Police Chief, 2014. **81**(10): p. 48-54.
8. Hynds, J. *The Need for Speed: Orange County District Attorney's Rapid DNA Program*. in *Green Mountain DNA Conference*. 2019. Burlington, Vermont.

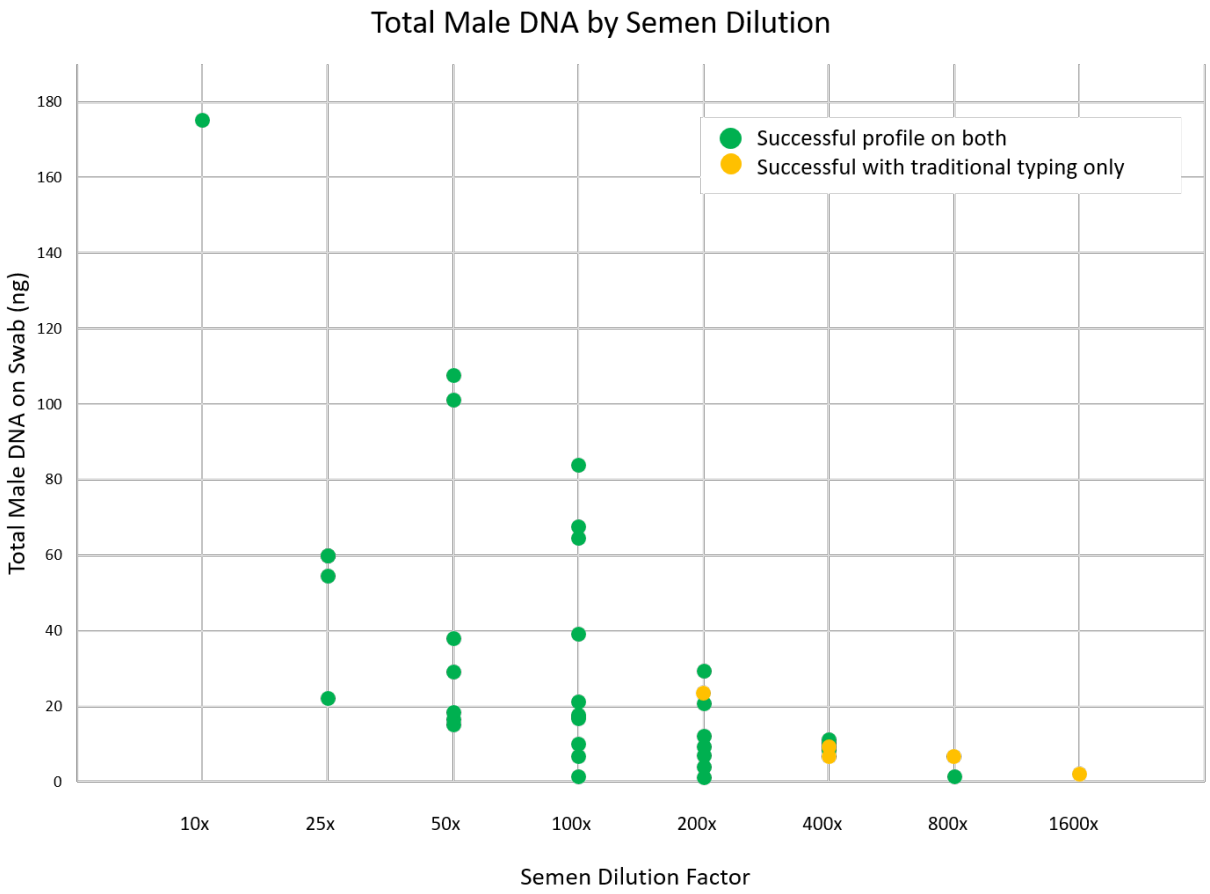
Supplementary Information



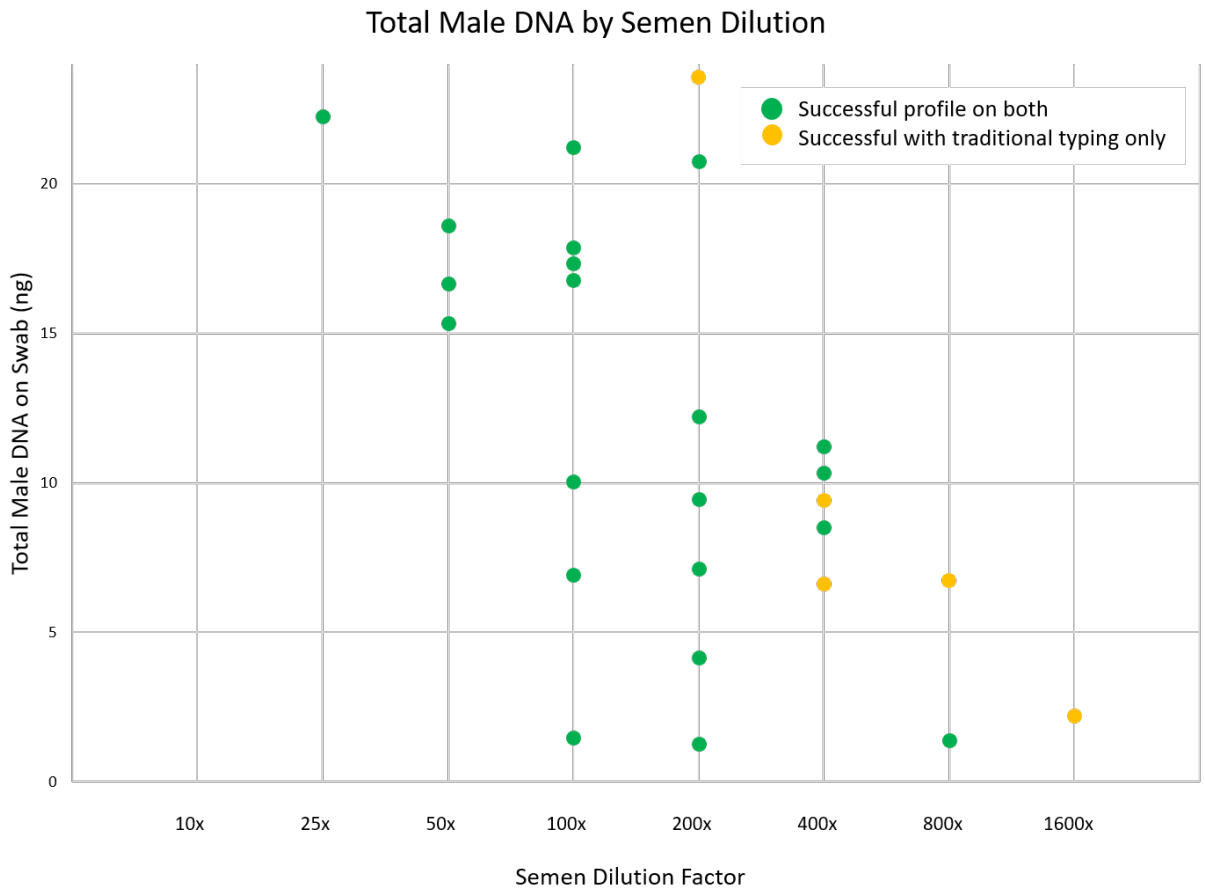
Supplementary Figure 1: Example ANDE® electropherogram providing a fully correct and confident profile. Note: This profile was generated from an F2 (sperm) fraction of a post-coital swab collected 12 hours after intercourse.



Supplementary Figure 2: Example ANDE® electropherogram with all allele calls in red. Upon review, the alleles represent a full and correct profile for the donor. This profile was generated from a run in which the on-chip ladder failed, which is likely the reason the profile was called in red and would not provide files for automatic searching and matching. Note: This profile was generated from an F2 (sperm) fraction of a post-coital swab collected 48 hours after intercourse. The donor is the same as observed in Supplementary Figure 1.



Supplementary Figure 3: Mock post-coital F2 results plotted based on estimated total male DNA on the swab. Sample success is color-coded based on the key in the top right. There were no mock post-coital samples that failed to provide usable data with traditional methods.



Supplementary Figure 4: Mock post-coital F2 results plotted based on estimated total male DNA on the swab, scaling to 25 ng of total male DNA. Sample success is color-coded based on the key in the top right.