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Development of Novel, Miniaturized Sperm Cell Isolation Techniques for Sexual Assault Samples

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DEVELOPMENT OF NOVEL, MINIATURIZED SPERM CELL ISOLATION TECHNIQUES FOR SEXUAL ASSAULT SAMPLES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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DEDICATION

First and foremost, I would like to thank my committee chair, Dr. Tracey Dawson Green. You have provided me with a lot of advice and opportunities throughout my academic career. In 2016, you made a decision against the norm to allow me, an undergraduate student at the time, to perform an independent study project within your lab – and I will forever be thankful for that opportunity. Not only did this allow me to build upon my wet lab skills, but it also made me realize that I was not satisfied with just a bachelor's degree in forensic science. Ever since that time, you have always supported me, challenged me, and prompted me to be better. Despite being an NC State Wolfpack fan, you're a great person who truly cares and fights for her students – so, thank you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α	Alpha
ΔR_n	Change in normalized reporter signal
ρ	Rho
µg	Microgram
µL	Microliter
µm	Micrometer
µTAS	Micro total analysis system
ν	Velocity
π	Pi
a	Area
ANOVA	Analysis of variance
BACS	Buoyancy activated cell sorting
bPeT	Black polyethylene terephthalate
BSA	Bovine serum albumin
C	Celsius
CAD	Computer-aided design
CD	Cluster of differentiation
CE	Capillary electrophoresis
cm	Centimeter
C_q	Quantification cycle (cycle threshold)
CV of LPH:TPH	Coefficient of variation for locus peak height-to-total peak height ratio
Cy5	Cyanine 5
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F	Fahrenheit
FACS	Fluorescence activated cell sorting
F_b	Buoyant force
F_d	Drag force
F_g	Gravity force
g	Gravity
HCl	Hydrochloric acid
HGH	Modified HTF Medium + Glutathione + Heparin
HSA	Heat sensitive adhesive
HTX	HEPES buffer + Triton X-100
ILS	Internal lane standard
IPC	Internal PCR control
IRB	Institutional Review Board
kDa	Kilodalton
KPICS	Kernechtrot-Picroindigocarmine stain

kV	Killivolt
LCM	Laser capture microdissection
M	Molar
M:F ratio	Male-to-female ratio
MACS	Magnetic activated cell sorting
MBG	Molecular biology grade
mg	Milligram
min	Minutes
mL	Milliliter
mm	Millimeter
mM	Millimolar
MP	Mustang Purple®
mW	Milliwatt
n	Sample size
NaOH	Sodium hydroxide
ng	Nanogram
NIJ	National Institute of Justice
nm	Nanometer
NP-40	Nonyl phenoxypolyethoxylethanol
NTC	No template control
p	Probability (p-value)
P-C-L	Print-cut-laminate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Pressure cycling technology
PeT	Polyethylene terephthalate
pH	Potential Hydrogen
PH	Peak height
PHR	Peak height ratio
PMMA	Polymethyl methacrylate
PMN	Polymorphonuclear
POP-4	Performance optimized polymer
proK	Proteinase K
PSA	Prostate-specific antigen
PSA	Pressure sensitive adhesive
QAS	Quality Assurance Standards
qPCR	Quantitative/real-time polymerase chain reaction
RB	Reagent blank
RFU	Relative fluorescence units
rpm	Revolutions per minute
SAECK	Sexual assault evidence collection kit
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
SF	Seminal fluid

SPAM-1/PH-20	Sperm Adhesion Molecule 1/Hyaluronidase protein 20
STR	Short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
TCEP	Tris(2-carboxyethyl)phosphine
TE	Tris-EDTA buffer
UVA	University of Virginia
VCU	Virginia Commonwealth University
VF	Vaginal fluid

GLOSSARY

ΔR_n	The change in normalized reporter signal during real-time quantitative PCR. The change in the fluorescence signal from the reporter dye normalized to the passive dye signal per cycle.
Antibody	A protein (immunoglobulin) produced by the immune system in response to and targeting a specific antigen (foreign compound).
Antigen	A substance or compound foreign to the body that induces an immune response.
Buoyancy activated cell sorting (BACS)	The process of sorting or isolating target cells using a solid support that floats (or is buoyant).
Buoyant force	The upward force acting on a mass that is immersed in a fluid.
Centrifugal force	The apparent ("pseudo") outward force acting on a mass when it is rotated.
Centrifugal microfluidics	The behavior, control, and manipulation of fluids on a small scale using inertial pseudo forces such as centrifugal force.
Decondensation	The loosening or unwinding of tightly packaged DNA molecules.
Differential lysis	The process of using different reaction components to sequentially lyse different cell types that are present within a sample. For example, non-sperm cells may be broken open using certain reagents prior to centrifugation, removal of the supernatant, and subsequent lysis of sperm cells with different reagents.
Direct amplification	The process of adding a sample directly to a PCR amplification reaction without first subjecting it to DNA extraction, purification, or quantification.
Direct binding order	The process of conjugating antibodies to a solid support prior to introducing that complex to a sample during immunoprecipitation.

Eluant	A solution or fluid used to elute a substance from a substrate.
Eluate	A solution obtained by eluting a liquid from a substrate.
Immunoprecipitation	The isolation, separation, or affinity purification of a specific antigen using antibody/antibodies immobilized on a solid support.
Indirect binding order	The process of introducing free floating antibodies to a sample for antibody-target binding prior to adding a solid support during immunoprecipitation.
Internal PCR Control (IPC)	A form of positive control during real-time quantitative PCR that is present in all samples and serves as a gauge of proper amplification, reliability, STR amplification success, and presence of inhibitors.
Lysate	A solution that contains all products as the result of lysing or breaking open cells.
Magnetic activated cell sorting (MACS)	The process of sorting or isolating target cells using a solid support that is magnetic/paramagnetic.
Microfluidics	The behavior, control, and manipulation of fluids on a small scale within an enclosed environment.
No Template Control (NTC)	A form of negative control during real-time quantitative PCR that contains all components of the reaction but does not contain DNA.
Passive reference dye	A fluorescent dye present in all reactions during real-time quantitative PCR that emits the same signal throughout the procedure and can be used to normalize the fluorescent data in each reaction to account for variation from sample to sample.
qPCR	Amplification of a target sequence measured in real time using fluorescent dyes; quantitative PCR.
“Subnatant”	The liquid phase below a solid.
Terminal velocity/settling time	The maximum velocity an object can reach as it falls through a fluid.

ABSTRACT

DEVELOPMENT OF NOVEL, MINIATURIZED SPERM CELL ISOLATION TECHNIQUES FOR SEXUAL ASSAULT SAMPLES

By Brittany Celeste Hudson, Ph.D.

Virginia Commonwealth University, 2022

The ever-increasing sexual assault evidence collection kit backlog within the United States has prompted the search (and desperate need) for a more efficient, cost-effective, and rapid processing technique that can separate male and female contributions from evidentiary samples. Although backlogs have largely been tackled across the United States and many technological advancements have made the forensic DNA analysis workflow quicker, techniques for handling sexual assault samples remain time-consuming, tedious, and inefficient at separating cellular fractions from the victim and perpetrator. Thus, this research sought to identify a reproducible sperm isolation method using antibodies and/or alternative differential cell lysis techniques as a possible solution. Automation of such techniques within a centrifugal microfluidic platform was also explored.

Although dithiothreitol (DTT) has been the primary reagent employed for the lysis of spermatozoa, results of studies herein revealed that residual DTT can artificially increase DNA quantities of various qPCR targets *and* impact predicted male-to-female DNA ratios. Thus, this research identified Promega's Casework Direct kit, NP-40 cell lysis buffer, HGH (i.e., HTF media + glutathione + heparin), and alkaline (i.e., 1M NaOH) solution as promising direct-to-amplification lysis techniques which could generate sufficient DNA quantities and high quality STR profiles.

This research also developed a 35-minute sperm cell isolation assay using biotin-conjugated polyclonal PH-20 antibody and streptavidin-coated Microbubbles that could retain 58.0 ± 15% of seminal DNA within semen, as well as significantly *and* practically improve M:F ratios 2.76 ± 0.92-fold ($p = 0.041$) within STR profiles of processed mixture samples. Proof of concept studies demonstrated the feasibility of employing Microbubbles and buoyancy activated cell sorting (BACS) on a centrifugal microdevice platform, serving as the first assessment of BACS in such a format. Microbubbles were sufficiently mixed and aggregated, the position of Microbubbles within the microdevice was precisely controlled, and "subnatant" transfer was performed without significant loss of Microbubbles.

Finally, a modified differential cell lysis method using a combined *prep*GEM™ and alkaline approach was developed. With this assay, STR profiles of processed mixture samples exhibited 3.01 ± 2.3-fold improvement in M:F ratios and recovered 5.90 ± 7.8 unshared male contributor alleles in sperm fractions that were otherwise undetected in unseparated controls. Overall, the data indicated that using *prep*GEM™ enzyme and alkaline solution for lysis of non-sperm and sperm cells, respectively, could enrich for male DNA within sperm fractions and required only 25 minutes of overall processing time. Transition of this assay onto a centrifugal microdevice with a cell trap module served as proof of concept for accomplishing cell trapping and fractional separation in an automated format – which could be formatted for numerous biomedical and forensic applications.

KEYWORDS: forensic genetics, forensic DNA analysis, sexual assault, sperm, direct-to-PCR, alkaline lysis, PH-20 antibody, BACS, Microbubbles, *prep*GEM™, centrifugal microdevice, microfluidics

CHAPTER ONE:

INTRODUCTION

STATEMENT OF PROBLEM

The true extent of sexual assault cases in the United States has been notoriously difficult to both calculate and comprehend. In 2015, the National Institute of Justice (NIJ) estimated a backlog of approximately 350,000 forensic cases nationwide, with sexual assaults comprising more than 50% [1]. While the NIJ defines a backlogged case as one that has not been tested within 30 days of its receipt by the laboratory, other sources note that the term can also refer to cases residing with law enforcement that have yet to be submitted to the lab, either due to differences in policies regarding when and where sexual assault evidence collection kits (SAECKs) are directed or limited resources and police discretion [2,3]. Although these inconsistencies make it hard to grasp the true extent of backlogged sexual assault cases in the United States, the recognition that untested SAECKs are a major problem is undeniable. Early studies in 2004 and 2010 conducted nationally representative surveys and discovered that there were almost 200,000 unsolved rape cases which had never been submitted to a lab for testing [4,5]. Even further, the 2018 Criminal Victimization bulletin compiled by the Bureau of Justice Statistics noted the rate of rape or sexual assault experienced an almost two-fold increase from 2015, highlighting the current underestimation of such cases and their associated backlogs [6]. Additionally, despite a decline in the rate of rape or sexual assault each year since 2018 according to the most recent 2021 Criminal Victimization bulletin, these occurrences still comprise approximately 22% of victimizations reported by police [7]. Ultimately, the volume of sexual assault backlog cases — whether due to increased crime rates, lack of resources, or other proposed factors — undoubtedly poses a significant social justice issue, as the inability to punctually process cases and convict criminals not only risks the occurrence of more sexual assaults, but also prevents victims from obtaining justice and closure. In fact, one study projected that although testing all backlogged SAECKs would increase labor and cost $1,600 per

kit, it would potentially lead to an estimated savings of $130,000 for the testing laboratories and the criminal justice system per sexual assault (as well as avert 26 additional sexual assaults) in Detroit, Michigan alone [8]. Extending this practice nationally could provide tremendous benefits.

From a scientific perspective, the SAECK backlog poses additional issues for forensic laboratories. While the total number of violent crimes has decreased each year since 2018 [7], the ability of forensic scientists to keep pace with both previous and current submissions – as well as process evidence – in a timely manner is still extremely challenging. This inability to keep pace, despite the reduction in violent crimes, stems from many reasons – including an increased submission of samples relating to touch evidence *and* the fact that evidence from misdemeanor crimes must also still be processed. Further, although there have been tremendous and moderately successful efforts to tackle SAECK backlogs across the country in recent years, new legislation requiring the submission and testing of all collected SAECKs in many states is likely generating new backlogs [9]. There are several reasons for the tendency of SAECK backlogs to form. First and foremost, sexual assault samples are often comprised of cells from both the perpetrator and the victim; therefore, it is critical to separate cells from each contributor as much as possible before DNA extraction in order to reduce or avoid subsequent mixture DNA profiles. Probabilistic genotyping software, such as TrueAllele® and STRmix™, has been introduced to more efficiently handle interpretation and deconvolution of mixed forensic DNA profiles during the final step of the forensic DNA workflow (i.e., data analysis). This approach utilizes statistical modeling to make sense of complex genotyping results, predicting the most likely explanations for observed DNA patterns (i.e., genotypes) in a more consistent manner, absent human error and bias [10]. However, despite some success in limiting subjective decisions and providing more accurate mixture

interpretations, this black box technique suffers from complex principles that are rather difficult for most to understand or convey to a jury. Further, although several probabilistic genotyping software programs have been validated within forensic labs across the country, substantial purchasing and usage costs have driven most research efforts towards seeking ways to improve the front-end isolation of specific target cells to minimize the occurrence of mixture DNA profiles in the first place [11–13].

Differential lysis has been the most widely accepted, traditional technique for separating the predominant cell types found in sexual assault samples, but this method often inefficiently isolates sperm and non-sperm cells, requires long incubations, relies heavily on manual pipetting, and is limited to mixtures that contain sperm cells. The federal government has attempted to address and overcome this issue through financial assistance to scientists to fund research and development of new techniques capable of handling sexual assault cases in a more cost effective and timely manner. Some advancements that have been reported in the recent literature include the use of: alternative lysis reagents and conditions to reduce incubation times [14,15], DNase digestion [16–19] or additional wash steps to minimize female DNA carryover [20,21], and alternate techniques such as those involving lasers that use physical properties to capture target cells [22,23]. Unfortunately, many of these procedures still result in mixtures and/or lead to incomplete male DNA profiles. Further, these methods generally lack complete automation, requiring significant hands-on processing. These limitations have led to few of these procedures being implemented in forensic labs and have prompted a surge in research involving affinity methods (e.g., antibodies and aptamers) in an effort to more selectively and efficiently isolate specific cells from sexual assault samples. The transition of many of these cell isolation techniques (along with cell lysis and

DNA purification techniques) to a microfluidic environment has also been heavily explored for the reduction of sample and reagent consumption, contamination risk, and processing time; however, more research is needed to develop a cheap, disposable, and automatable microdevice that can be easily implemented within the current DNA workflow. Regardless of the approach, there is little doubt that a new method which simultaneously increases the probability of successfully generating perpetrator DNA profiles from sexual assault samples *and* reduces time and cost requirements is sorely needed in the forensic DNA community.

COMMON CELLULAR COMPONENTS OF SEXUAL ASSAULT SAMPLES

It is well known that cells are differentiated early during development based upon their ultimate target tissue and function. While all cells may share some common characteristics, such as the presence of organelles or DNA, those residing in one tissue or body fluid will ultimately have biomarkers (e.g., proteins, carbohydrates) that are unique compared to cells from a different tissue or body fluid. Thus, several research efforts have focused on identifying and exploiting these unique characteristics to target and isolate cells of interest (e.g., spermatozoa). Before applying this concept to sexual assault samples, one must acknowledge all possible cell types within each commonly encountered body fluid and the unique properties that accompany them.

Semen

Semen consists of a mixture of spermatozoa, non-spermatozoa cells, and various fluids. Although non-sperm cells are present in normal semen, spermatozoa (the male gametes) are the predominant cell type. According to the literature, the number of spermatozoa can vary from 20 – 250 million cells per milliliter of normal ejaculate [24–28], with the average being 61 million per milliliter

[29,30]. The average ejaculate dispenses 2 – 7 mL of semen [25]; thus, it is unsurprising that sperm cells are commonly encountered within sexual assault samples. The unusual morphology and highly specialized roles and functions of spermatozoa result in many unique characteristics, some of which have already been exploited by forensic scientists. Ranging from 4 – 5.5 μm in length and 2.5 – 3.5 μm in width, sperm cell heads contain a highly condensed nucleus and are surrounded by a protective acrosomal cap comprised of hydrolytic enzymes [31]. The sperm cell body also includes a flagellum/tail and a midpiece consisting of mitochondria surrounded by a sheath, making the total length of morphologically normal spermatozoa approximately 40 – 50 μm [31].

Spermatozoa are produced during spermatogenesis, a process which takes place in the seminiferous tubules within the testis and consists of several sub-processes. The sequence of events for mature sperm cell production are as follows: 1) Spermatogonia, which are stem cells, divide mitotically within the seminiferous tubules to produce additional spermatogonia and primary spermatocytes; 2) Meiosis I produces haploid secondary spermatocytes, which become haploid spermatids after meiosis II; 3) Mature spermatozoa are developed from spermatids during a process called spermiogenesis, which condenses the chromatin and creates the sperm head, midpiece, and tail; 4) Spermatozoa are released into the lumen of the seminiferous tubules during spermiation, where they will further mature and travel through the epididymis for almost two weeks prior to their discharge via ejaculation [30,31]. Each spermatogenesis cycle generates millions of sperm cells alongside other cell types that provide support for the maturing spermatozoa. This development process prepares mature spermatozoa for ejaculation into the female genital tract and transport to the oocyte for their ultimate role in fertilization.

In order to efficiently deliver the male genome during fertilization, spermatozoa take on a small, highly condensed head and a motile tail powered by mitochondria within the midpiece. Chromatin re-packaging and condensation within the sperm nucleus serves two major roles: development of a hydrodynamic head shape for enhanced motility and protection of paternal DNA from harmful components within the female genital tract [14,32,33]. Condensation is accomplished by the replacement of somatic histones with protamines during spermiogenesis. Protamines are arginine- and cysteine-rich, positively charged proteins unique to spermiogenic cells and spermatozoa that tightly bind to DNA ten times more efficiently than histones [30,32–43], resulting in the reduction of the sperm head to anywhere from 1/7 – 1/20 that of a somatic cell nucleus [32,37]. The ability of protamines to more tightly package DNA stems from their arginine and cysteine residues, which give them a highly positive charge that is strongly attracted to negatively charged DNA and promote the formation of stable disulfide bonds that interlock neighboring protamines to prevent their dissociation, respectively [14,31,32,34,35,37,38,41,42,44]. The tight packaging of protamines around sperm DNA is what necessitates the use of a strong reducing agent during DNA extraction of spermatozoa, as one of the natural purposes of such a compact and stable structure is to remain intact and inactive until the sperm enters the egg [14,37,41]. Humans have two major types of protamines, Protamine 1 and Protamine 2, which normally occur in a 1:1 ratio and have not been observed in any other cells or tissues to date [30,32–37].

Replacement of histones by protamines is neither immediate nor comprehensive. In fact, most somatic histones are first replaced by transition proteins which are subsequently exchanged with protamines during spermatid elongation, but approximately 10-15% of the sperm genome remains packaged by histones throughout the life cycle of spermatozoa [14,30,32–35,37,41,44–46]. Studies

have shown that these remaining histones are mainly sperm-specific variants of H2B that bind to telomeres and regions of the DNA involved in sperm cell maturation, function, capacitation, and fertilization (i.e., regions of developmental importance) [32,35,37,44–46]. Preservation of histones enables certain loci to remain less condensed and thus more easily expressed during the life cycle of spermatozoa and early fertilization of the oocyte. Ultimately, the presence of sperm-specific protamines, as well as sperm-specific and testis-specific histones, means these components are unique and could potentially be targeted for sperm cell and/or sperm DNA isolation.

As previously mentioned, non-sperm cells are also involved in the production of mature spermatozoa. After development within the seminiferous tubules, spermatozoa travel from the epididymis through the vas deferens to the urethra, picking up cells and other components from these sources along the way. The seminal vesicles, prostate gland, Cowper and Littre glands, ampulla, and epididymis also secrete fluids (collectively referred to as semen) that provide sustenance and a transport medium for developing sperm cells [30,31]. A few studies have quantified and characterized the non-sperm cellular components of semen. These identified characteristics could potentially serve as unique targets for circumstances where sperm cells are limited or even absent from an evidence sample, rendering the isolation of the non-sperm cell fraction necessary for identification of the male contributor.

Non-sperm cells normally occur at a concentration that is less than 15% that of sperm cells, or approximately 4 – 5 million cells per milliliter of semen [24,26,28,30,47]. These cells consist primarily of immature germ cells (e.g., primary/secondary spermatocytes and spermatids), with minor contributions from leukocytes and epithelial cells from the male genital tract (e.g., seminal

vesicles, prostate, urethra) [24,28,30,48]. When correcting for the fact that spermatocytes and spermatids are developing sperm cells, and that the majority of non-sperm cells are actually anucleate bodies, true nucleated non-sperm cells only constitute approximately 12% of total cells within human semen. Leukocytes make up approximately 13% of those non-sperm cells, with polymorphonuclear (PMN) leukocytes (e.g., neutrophils, eosinophils, and basophils) as the most frequently occurring type [24]. Sertoli cells (from the germinal epithelium), which surround developing spermatozoa and metabolically support them throughout spermatogenesis by providing an environment of hormones [30,31,48], can also be found within ejaculated semen.

Epithelial, sustentacular/support (e.g., Sertoli), and white blood cells are much easier to lyse than spermatozoa and therefore they naturally carryover into the non-sperm fraction of a sample when female epithelial cells are preferentially lysed using traditional differential lysis techniques [30,49]. While the quantity of non-sperm cells in semen is miniscule compared to that of sperm cells, they become increasingly important when encountering samples from vasectomized, infertile, and otherwise oligospermic males. Thus, new methods are needed to be able to efficiently target and isolate non-sperm cells in these situations, especially given that a traditional differential cell lysis would be futile.

Vaginal Fluid

Vaginal fluid, or vaginal discharge, is a general term for fluid that comes from the female vaginal tract. The vagina is an elastic reproductive organ that extends from the vulva (external genitalia) to the cervix of the uterus [50,51], serving as a support for both the bladder and the rectum [50]. Naturally, the environment and cellular makeup of the vagina are dependent upon many factors,

including age and menstrual cycle phase. While the quantity of normal vaginal discharge can vary greatly, it usually appears white and nonhomogeneous; it contains desquamated epithelial cells from the cervix and vagina, material from several glands (e.g., sweat, sebaceous, and Bartholin's), secretions from the cervix, PMN leukocytes, bacteria, mucus, water, as well as contaminating rectal, urethral, and vulval cells [52–54].

This discharge is secreted through the vaginal wall and contains many non-cellular components from cervical and vestibular glands, among other sources [55–57]. Columnar cells within the Bartholin glands, which are homologous to the male Cowper glands, secrete a clear, whitish mucus that helps to lubricate the vagina [50]. Other non-cellular components, such as water and electrolytes, help to maintain an acidic environment (pH ~4.5) within the vagina that inhibits the growth of certain pathogens such as *E. coli* [55]. Lactic acid is also a major component of vaginal fluid which, along with certain proteins (e.g., calprotectin and lysozymes) within the vaginal tract, contains antimicrobial activities and helps provide the vagina with resistance to exogenous microbes [55]. This lactic acid is produced by lactobacilli, whose growth and proliferation within the vagina are promoted by glycogen; vaginal epithelial cells contain high levels of glycogen, the byproducts of which serve as a food source to lactobacilli and therefore precursors to lactic acid [58].

Endogenous microbiota, such as various lactobacilli, are predominant within the vagina and help to protect it from harmful pathogens by producing the aforementioned lactic acid [59–63] and by forming a bacterial film on the vaginal epithelium [64]; however, bacteria aren't the only sources of cellular material within vaginal fluid. Stratified, squamous epithelial cells line the vagina and

contain many types of cytokeratin (e.g., cytokeratins 4-6 and 13-16)[65], some of which have been successfully targeted for the isolation of vaginal epithelial cells [51,54,55,66,67]. Many squamous epithelial cells are encountered in vaginal samples, likely because they continuously slough off to help remove any attached, exogenous bacteria [55]. Epithelial cells from other components of the genitourinary tract are also prevalent, stemming from migration of fluids from the cervix into the lower genital tract. Leukocytes and immunoglobulins are commonly present within vaginal secretions and the cervix, serving as the primary defense mechanisms for exogenous microbes and potential pathogens [68–71]. In addition, these immune cells and concomitant reactive oxygen species (ROS) may lead to the inadvertent detection and degradation of some spermatozoa (and corresponding DNA) within the female genital tract [68,72]. Studies have even shown that certain vaginal products, sexual intercourse, and infections can affect the level of lymphocytes [69,73–75], which is unsurprising since a major role of the human vagina is to defend the genitourinary tract against infections and pathogens [55–57]. Additionally, immunoglobulin A and G may inhibit the attachment of microbes to epithelial cells [55], while the retainment of lymphocytes within the epithelium is likely due to CD103, which is the ligand for E-cadherin that is expressed by mucosal epithelial cells [69,76]. Langerhans cells and melanocytes have also been reported in trace amounts within vaginal fluid [77].

Many of the aforementioned cellular components of vaginal fluid have been targeted using antibodies and filtration. In 1999, Schoell et al. targeted vaginal cells based on a combination of human leukocyte antigen/major histocompatibility (HLA/MHC) class I, CD45, and cytokeratin expression [78]. Using antibodies for these targets alongside flow cytometry, this study was able to separate 10:1 vaginal-to-spermatocyte cell mixtures with 92% sensitivity [78]. Although

promising, these targets have yet to be implemented within sexual assault samples, likely due to the additional prevalence of white blood cells (and thus CD45 expression) within semen and buccal samples as immune responses to potential pathogens and infections. Further, studies have demonstrated that epithelial cells and (PMN) leukocytes occur in similar numbers within buccal samples, complicating the use of these targets with certain sexual assaults [79]. Isolation of vaginal mucosa over other epithelial cells (e.g., buccal) has also been attempted; however, similarities in both the roles and functions of these epithelial cells have made it difficult to identify unique markers for each. For example, antibodies against cytokeratins 4 and 15 have been utilized for the separation of mucosal epithelial cells because of their abundance on the surface of internal squamous stratified epithelia [66,80]; however, buccal and vaginal epithelial cells are of the squamous, stratified type and thus comparably bind to anti-cytokeratin antibodies [80]. Unfortunately, despite the promise of targeting vaginal mucosal cells, several studies have demonstrated the similarities of cytokeratin 4 expression for both reproductive and non-reproductive skin mucosa [81,82]. While targeting all mucosal epithelial cells within a sample may help with the indirect isolation of spermatozoa, the available literature still lacks evidence of unique biomarker(s) for vaginal mucosal cells despite numerous investigations into their protein expression.

While potentially not as unique as spermatozoa, further characterization of the cells present within vaginal fluid could prove them just as useful for forensic identification purposes. By targeting leukocytes and mucosal epithelial cells, one may be able to indirectly isolate spermatozoa. Ideally, however, an antigen unique to vaginal mucosal epithelial cells should be identified so that the female fraction of a sexual assault sample could be more efficiently targeted and pulled away from

the male fraction. Investigation into these vaginal cells could be especially critical when faced with sexual assault samples possessing limited or no sperm cells, as they could be targeted and isolated to indirectly enrich the detection of male cells and their associated DNA.

CURRENT PRACTICES & AVAILABLE METHODOLOGY

DNA purification is a critical step in the forensic DNA workflow. This process typically begins with breaking open or lysing cells found on evidence samples to access the DNA, followed by isolation of that DNA from other contaminants and components located both within cells and on substrates from which the sample was collected [83]. DNA extraction has the most impact on developing a DNA profile from evidence, as it is one of the first steps in the analytical process and determines both the quantity and quality of DNA obtained from a sample [67]. Sample type usually governs which DNA extraction method is utilized, and the expectation of DNA quality can ultimately be dependent upon the extraction method chosen. Regardless, an effective DNA extraction method should be able to obtain DNA from a variety of biological sample types, reduce the loss of DNA, attain DNA from small amounts of biological material, and isolate high concentrations of DNA [67].

Because sexual assault evidence is usually collected from an orifice of the victim, it may contain a mixture of cells from both the victim and perpetrator. A majority of sexual assaults are committed by males on female victims, so the cell types within sexual assault samples usually include spermatozoa from the semen of males and mucosal epithelial cells from the female vagina [84]. While spermatozoa and vaginal epithelial cells are most commonly encountered within these samples, other possible and less prevalent cell types do exist. In any case, a routine DNA extraction

method would merely lyse all existing cells and lead to a DNA profile containing a mixture of alleles from all contributors. Thus, with sexual assault samples it is critical to separate or isolate the male and female cells as much as possible prior to cell lysis and DNA purification, preventing the requirement for a mixture DNA profile interpretation at the end of the workflow.

Traditional Differential Cell Lysis

While forensic labs vary in methods of screening and selecting evidence items to be tested, the majority perform a differential lysis technique for sexual assault samples containing spermatozoa. The traditional differential cell lysis technique was first described by Gill et al. in 1985, where it was demonstrated that DNA from epithelial cells could be removed prior to lysing and releasing DNA from sperm cells [85]; it is still the most commonly used technique in labs today. Briefly, this method involves the use of proteinase K (proK) and sodium dodecyl sulfate (SDS) to initially break open epithelial cells, followed by centrifugation and removal of the supernatant containing DNA alongside cellular components from the lysed cells while leaving behind intact spermatozoa. After several washes, the remaining sperm cells are lysed using proK, SDS, and a reducing agent (often dithiothreitol [DTT]) that will break disulfide bonds present within the head of sperm cells and release the DNA. This procedure splits an evidence sample into two fractions – non-sperm and sperm – that can be processed in tandem for the remainder of the DNA workflow.

Ideally, this technique would culminate in a sperm fraction containing only DNA from the male perpetrator, as well as a non-sperm fraction containing DNA from the lysed female vaginal epithelial cells. Unfortunately, traditional differential lysis often fails to fully separate male and female contributions. Inefficient separation of sperm and non-sperm cells can stem from the

presence of old or degraded sperm cells that are susceptible to premature lysis, excess female epithelial cells that fail to completely lyse and remain within the sperm fraction, loss of sperm due to repeated wash steps, poor manual pipetting technique, or many other factors [27]. Further, other non-sperm cells exist in human semen, and these male cells will remain in the non-sperm fraction in minute amounts, often resulting in non-sperm mixture profiles despite attempted separation. On top of these limitations, differential extraction is a laborious process (taking anywhere from hours to an entire day to complete) that is difficult to automate; it requires multiple centrifugation steps (which few automated platforms can execute), includes two lysis incubation times at controlled temperatures, and involves manual pipetting and maneuvering of the sample (which can be slow, inefficient, and prone to increased risk of contamination) [20,21,85,86]. Not only do these shortcomings yield inefficient results, wasting both time and money, but they also often lead to mixture DNA profiles at the end of the DNA workflow.

Mixed DNA profiles pose significant problems in forensic DNA analysis because they add uncertainty to the assignment of alleles, impact statistical analysis, complicate court testimony/explanations, and add considerable time to the workflow. In fact, some studies report that mixture DNA profile interpretation during data analysis serves as the most complex process of the entire workflow, requiring a significant and excessive investment of both time and energy [27]. Even further, the time and effort given to mixture interpretation is often all for naught, as deconvoluting mixed DNA profiles is completed with neither 100% certainty nor consistency and ends with higher rates of inconclusive reporting [87,88].

Modified Differential Cell Lysis Methods

The many disadvantages and limitations of traditional differential extraction have prompted research aimed at either improving the current state of the art method or developing a new, more efficient technique. By using alternate chemicals and additives that simply enhance the separation efficiency of traditional differential lysis, labs could implement it with relatively little supplementary training for analysts. In addition, the adoption of a technique similar to one already enjoying widespread use throughout the field is much more likely to occur than implementation of a completely novel technique, especially if proven equally or more efficient.

In a 2015 publication, Cotton and Fisher generated a summary table of several modifications that have been explored thus far for differential extraction, which have mainly focused on reducing the carryover of cells between non-sperm and sperm fractions (Table 1) [30]. Some of the earliest modifications involved simple lysis condition adjustments, such as milder reagents and increased temperature, to avoid the unintended loss of sperm DNA and promote more efficient lysis of epithelial cells [20,89]. A second mild lysis step prior to sperm cell lysis has even reportedly improved male:female DNA ratios in sperm fractions by as much as 6-fold [21], as well as resulted in 5.5-fold reduction in non-sperm DNA carryover without reducing sperm DNA recovery [90].

Additional studies have focused on the replacement of DTT as the reducing agent for spermatozoa lysis, as it has limited stability, exhibits optimal functionality at a narrow pH range, possesses a strong and unpleasant odor, and often requires long incubations ranging from two hours to overnight (Table 1) [14,91]. Studies within our lab have also demonstrated that DTT can interfere with accurate DNA quantification when using certain kits due to its interaction with some

fluorescent dyes, rendering this compound unusable for direct amplification techniques [92]. In 2015, a five-minute mechanical homogenization step in the presence of guanidine-based lysis buffer and Tris(2-carboxyethyl)phosphine (TCEP) was evaluated; this technique successfully lysed sperm cells and offered many advantages over current methods, including no lengthy proK digestion with DTT, room temperature processing with commercially available reagents, and more efficient lysis at physiological pH (~7-8) [14]. Although the TCEP procedure showed promising results, it still involved tube transfers, the lysate had to be further purified by spin column, and it produced DNA mixture profiles at similar rates as the traditional differential lysis procedure.

Pressure cycling technology (PCT), which uses hydrostatic pressure that alternates between ambient and high levels to induce stress and decrease the stability of cells, has also been explored within recent literature (Table 1) [15]. Subjecting a sexual assault sample to five minutes of pressure cycling at 20,000 psi in the presence of 0.4 N NaOH, followed by exposure to a second five-minute treatment at 95°C, resulted in the selective elution of epithelial and sperm cells, respectively, directly from a cotton swab within 20 minutes; this technique generated single-source DNA profiles for female:male DNA mixtures ranging from 1:1 to 5:1, but it still required multiple centrifugation steps and performed poorly with higher mixture ratios that are more commonly encountered in forensic casework [15]. While promising, PCT ultimately fails to reduce the time and labor commitments for sample processing.

Several studies have even explored the controversial notion of selectively degrading female DNA from lysed non-sperm cells using DNase I in an attempt to eliminate epithelial cell DNA in the sperm fraction (Table 1) [16,18,93]. Samples often have less contamination by female DNA after

this selective degradation technique, but they have not produced significantly more CODIS-eligible DNA profiles with actual casework samples and often perform similarly to standard differential methods [16,18,93]. Further, although this technique does significantly reduce non-sperm DNA carryover, studies with the commercial Erase Sperm Isolation kit reveal that it provides reduced sperm DNA recovery compared to the traditional differential technique – a problem for sexual assault samples which may already have low sperm counts at the outset [90]. While the selective degradation technique has yet to become widely adopted, it does show promise for implementation in the field. If sperm DNA recovery could be improved and if analysts could overlook the negative stigma of purposely destroying a non-probative portion of an evidentiary sample, then the obvious benefits in terms of analyst time could be a small, yet significant, step in the right direction. With all things being equal in terms of DNA profiling success rate, the reduction in cost by approximately $0.09 per sample and in time by an average of 1.1 hours per SAECK would be a noteworthy improvement that could lead to more cases being processed; this approach could be further enhanced given the potential for automation [18,94]. Nevertheless, more studies regarding potential premature lysis and therefore loss of sperm DNA alongside cost/benefit analyses are necessary; however, the development of other techniques that maintain the integrity of the female fraction while limiting the carryover of spermatozoa is still highly preferred, especially since the DNA profile from the female fraction serves as a control and can increase confidence in overall findings.

Ultimately, several modified differential extraction methods and improved cellular recovery techniques have shown some success, albeit limited. Currently, very few of the aforementioned methods have been widely adopted despite their potential advantages. While the field is often slow

to implement and adopt novel techniques, it is crucial to note that a given technique must significantly improve results before it will be considered. This is especially true in forensic DNA analysis, as both developmental and internal validations are required prior to implementation of a method [95]. Thus, copious research studies have heavily explored alternative techniques for handling sexual assault (and to a wider extent other potential mixture) samples in an effort to completely avoid and obviate differential extraction by more efficiently separating cellular components and reducing the potential for mixture DNA profile interpretation at the end of the workflow.

TABLE 1. Representative modified differential extraction procedures in the literature, adapted from Cotton & Fisher 2015 [30].

Article Title	Procedure Summary	Reference
DNA extraction from mixtures of body fluid using mild preferential lysis	Reduced washes and transfer steps to reduce loss of sperm for samples having lower amounts of sperm.	Wiegand et al. 1992
The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixture with semen	E-cell lysis at 70 °C, with a second digestion if E-cells remained un-lysed after first centrifugation step.	Yoshida et al. 1995
A physical method for separating spermatozoa from epithelial cells in sexual assault evidence	A mixture of E-cells and sperm cells was filtered through nylon mesh filters. The small sperm cells pass through and the E-cells are trapped.	Chen et al. 1998
Filtration based DNA preparation for sexual assault cases	E-cells in mixture lysed. Vacuum filtration separates E-cell DNA from sperm fraction.	Garvin et al. 2003
Sperm DNA extraction from mixed stains using the Differex system (+ 3 other titles)	Preferential lysis of mixture aided by organic/aqueous separation to achieve more pure sperm fraction.	Tereba et al. 2004 Tsukada et al. 2006 Valgren et al. 2008 Mudariki et al. 2013
Application of pressure cycling technology (PCT) in differential extraction	TCEP reducing agent added to mixed sample, pressure applied to preferentially lyse sperm cells.	Nori et al. 2011
DNA preparation from sexual assault cases by selective degradation of contaminating DNA from the victim	Use of DNase to remove female DNA from the sperm pellet prior to sperm cell lysis to reduce carryover of female DNA into the sperm fraction.	Garvin et al. 2009
Isolating DNA from sexual assault cases: A comparison of standard methods with a nuclease-based approach	Similar to above.	Garvin et al. 2012
Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence	Sodium hydroxide E-cell lysis followed by DNase digestions of E-cell DNA to isolate intact sperm cells for further manipulation	Hudlow et al. 2012

Alternate Techniques

Direct Amplification

More recent research has demonstrated the successful direct amplification (i.e., DNA extraction and amplification of a sample without DNA quantification) from semen samples for STR analysis [26]. While direct amplification techniques help to avoid the DNA loss commonly encountered with traditional DNA extraction and purification methods, they do not overcome the need to separate non-sperm and sperm cells prior to DNA extraction and STR amplification. Thus, direct

amplification of semen, although promising given its ability to obtain full DNA profiles with semen dilutions as low as 1:80, is not an adequate standalone technique for the processing of sexual assault samples [26]. Future research into direct methods which preferentially lyse sperm while leaving non-sperm cells intact would be needed for this technique to take hold. Instead, current research has shifted focus to the total separation of cell types up front to minimize and mitigate the issues that DNA mixture profiles can pose later in the workflow.

Microscopic Methods

Alternative techniques such as laser capture microdissection (LCM), micromanipulation, and optical trapping use light microscopy to visually locate and physically separate a specific cell of interest based on various physical properties. LCM combines either an IR or UV spectrum laser beam with a light microscope to target and isolate specific cells [22,27,96]. With LCM, when spermatozoa are microscopically visualized, a laser is activated and melts a portion of a thin plastic film suspended above the slide, trapping the sperm cell(s) for subsequent ejection into a collection tube [22,96]. While this technique shows promise, many limitations have prevented its widespread implementation. The shortest published analysis time from sample preparation to collection was approximately 140 minutes, demonstrating that this highly manual technique can be both laborious and time-consuming [19,22,23,27,97]. The SPERM HY-LITER™ method has also been developed, which combines fluorescence in situ hybridization (FISH) with LCM using Y chromosome-specific probes to automate the initial identification of sperm cells on a slide; however, LCM in general is not very amenable to automation and suffers from relatively high instrumentation costs [27,83,98,99]. Ultimately, the time intensive and potentially degradative sample preparation coupled with poor yields is perhaps the biggest problem with LCM.

Micromanipulation is very similar to LCM in that it utilizes a microscope to visualize target cells; however, instead of an IR or UV laser, it incorporates a Tungsten needle coupled with water-soluble adhesive to hover over targeted cells and aspirate them prior to their ejection into a tube [100,101]. This technique merely represents a simpler version of LCM and therefore suffers from the same disadvantages, with the added downside that there has yet to be an automated version reported in the literature.

Representing a slightly more complicated concept than LCM and micromanipulation, the use of an optical trap to suspend and maneuver, or "tweeze," target cells has been explored since the technique's development in 1985 [23,102,103]. By projecting a 1064 nm laser beam through an immersion objective lens, a focused optical tweezer can be formed; dielectric particles (e.g., cells) can be trapped or suspended within the focal point and subsequently maneuvered via manipulation and movement of this beam [23,102,103]. For this method, individual cells can be moved and concentrated to a specific area on a slide, where they can subsequently be collected via capillary action and transferred to a microcentrifuge tube for downstream analysis. Several studies have looked at the application of this technique to sperm cells [23,104,105], and a more recent study obtained complete DNA profiles when tweezing only 50 spermatozoa [23]. While this technique is relatively fast (approximately 40 minutes), requires minimal sample preparation and analyst training, and can produce single-source male DNA profiles with a 67% success rate, it still lacks high throughput capability and leads to an approximate 15% cell loss from trapping to DNA quantification [23].

Overall, techniques involving the use of microscopes and laser beams lend themselves to potentially high instrumentation costs and lengthy required operator times, rendering their widespread adoption in the field unlikely. Optical tweezers have perhaps the best potential to overcome these limitations, but additional studies concerning optimization, use with reconstituted samples, and validation are required.

Filtration Methods

Although most commonly employed in microfluidic settings, there have been many publications that harness the unique size, density, and other cellular properties of spermatozoa to filter and isolate them. This is unsurprising, as the traditional differential method has exploited the stability of sperm cell heads and the ability to centrifugally pellet spermatozoa since 1985 [85]. In 2003, Garvin et al. created a vacuum filtration system that enabled the DNA from lysed non-sperm cells to pass through a membrane while trapping intact spermatozoa [106]. Although this was a simple technique that utilized the large size of intact spermatozoa compared to DNA and other cellular debris, it was ultimately inefficient with old or degraded cells and was prone to clogging in the presence of excess epithelial cells [106]. A similar vacuum filtration method which could be performed in only 30 seconds was reported by Nakagawa et al. in 2022, but also experienced filter clogging purportedly from vaginal mucus and intact epithelial cells [107]. These limitations render this technique unlikely to be adopted, as an excess of female epithelial cells is commonly encountered with sexual assault samples. However, recent development of a filtration method (SpermX™), which utilizes a nanofiber membrane to trap sperm after two rounds of non-sperm cell lysis, has demonstrated great potential. This technique has reported trapping capability of 25 – >2.7 million sperm cells and demonstrates high sperm recovery when utilizing as little as 0.4 µL

of semen; however, it is still relatively time-consuming, sperm recovery drops substantially with lower volumes and when semen is mixed with epithelial cells, and approximately 5-15% of the total epithelial DNA recovered can be trapped within the nanofiber membrane itself [108,109]. Further, it should be noted that comparison of this technique to the standard differential resulted in 3 to 6-fold more male DNA in sperm fractions compared to the traditional differential extraction, but there was also an additional non-sperm lysis step employed which brings into question which aspect of the SpermX™ technique contributed to improved results [109].

Flow Cytometry & FACS Analysis

Flow cytometry is a commonly explored and utilized technique for identification of specific cell types, whereby a laser produces light scatter and excites fluorescent signals from cells based on their morphology and other physical properties (e.g., their ability to bind fluorescently tagged antibodies). Flow cytometers that have the added ability to retain cell populations that have been sorted based on differential fluorescence are referred to as Fluorescence Activated Cell Sorting (FACS) instruments [110,111]. In recent literature, flow cytometry and FACS analysis have been used to determine binding affinity between cells and ligands, quantify the number of antigens on a cell surface, and even to separate sperm and vaginal cells based on ploidy, major histocompatibility class, and cytokeratin expression [67,78,112–114]. Overall, this approach offers a highly specific and sensitive method that is able to analyze thousands of cells per second and provide a potential future method for sorting and analyzing forensically relevant cell types within mixture samples [27,115,116].

Despite their potential, however, flow cytometry and FACS have yet to be widely adopted in forensic science. This is likely due to relatively high instrumentation costs, better suitability to samples with lower cell concentrations, and the requirement of several wash steps - which bring the inevitable loss of cells [27,111]. In addition, separation based on morphology and other physical characteristics works well with fresh, living cells but may ultimately be inefficient with older, degraded, or eluted cells (i.e., those that are often encountered in forensic casework). FACS also requires efficient antibodies or other ligands to label the cells which, as discussed later, may pose additional problems. Even further, a 2016 survey given to research labs across academia, pharma, and biotech revealed that although FACS is one of the most popular techniques for cell isolation, it still suffers from major limitations such as poor efficiency/low recovery, poor reliability, and the need for large quantities of starting material [117]. These downsides have ultimately prevented implementation of this technique within the forensic science community, especially since cell isolation techniques need to excel in efficiency or throughput, purity, and recovery in order to be utilized for casework samples [99,117].

Given the many limitations and shortcomings of the aforementioned techniques, forensic scientists have increasingly turned to other means of targeting and isolating cells. Antibodies, which are often used during flow cytometry/FACS to label and detect cells, have received significant attention in recent literature. Rooted in natural affinity and specificity, antibodies should in theory be able to accomplish cell sorting for the purposes of separating cell mixtures prior to downstream amplification; therefore, the following section will comprehensively review antibodies and their application to cell isolation for sexual assault samples.

PROPOSED METHODOLOGY

Antibodies and Cell Isolation

Introduction

Antibodies are soluble proteins produced by plasma cells to mark foreign substances for destruction and facilitate their removal [118,119]. They can be divided into five different classes/isotypes, ranging in size from ~140 – 900 kDa, and they are commonly referred to as immunoglobulins (Ig) [118,120]. The base shape of all antibodies is typically composed of two heavy and two light chains, each containing constant and variable regions, which come together via disulfide bonds to yield their commonly described Y-shape [118,120]. Because antibodies are produced as a natural immune response to foreign substances, they are often highly specific. This specificity is bestowed upon antibodies by subtle sequence differences that code for the variable regions on both chains, which combine at the terminal end of the antigen-binding fragment (F_{ab}) to produce a unique antigen binding site (paratope); this paratope is a molecular complement of the epitope present on the targeted antigen [118–121].

Many individual forces contribute to how an antibody binds with its target antigen, consequently determining its affinity. This includes the weak, non-specific, non-covalent interactions that join an antibody to its antigen, as well as the electrostatic interactions between molecules with opposite charges, short-range van der Waals attractions between temporary dipoles (i.e., partial charges), hydrogen bonding, hydrophobic interactions between nonpolar molecules, and attraction of a temporary dipole to an oppositely charged ion. Although individually weak, the combination of these forces alongside shape complementarity can culminate in a very strong interaction [119,122]. While antibody-antigen binding is reversible, an antibody with a high affinity for its target will

have a dissociation rate that is slow enough to be utilized for many biological and laboratory purposes.

Since the natural production of antibodies occurs within the body as an immune response, techniques for discovery and development of antibodies for laboratory use have typically involved live organisms. In general, a host animal is injected with a specific target antigen to induce host B cells to produce antibodies against the foreign substance, which are subsequently retrieved from the host animal's antiserum. This is the general process for the production of polyclonal antibodies, which are a collection of antibodies that are specific to a target but have several different paratopes corresponding to numerous antigen epitopes; hence, polyclonal antibodies are naturally occurring antibodies [121,122]. Monoclonal antibodies, which are specific to only a single epitope on a target antigen, are produced by combining the B cells obtained from an immunized host animal with immortal myeloma cells to produce hybridoma cells [121,123]. These hybridoma cells are then separated and allowed to proliferate before being screened for a single antibody of choice [121,123]. As one can imagine, the labor and time required to identify and develop novel antibodies can be quite intensive, especially if they are monoclonal. Moreover, the decision to develop monoclonal or polyclonal antibodies is dependent upon whether it is more important to have a singular strong affinity for a target or a combination of affinities at multiple sites on a single target.

Applications in Forensic Science

Antibodies have enjoyed widespread use in laboratories since the 1970s, with techniques such as Western blotting and other immunoassays designed to detect a protein of interest for applications

such as disease therapy and diagnostics [123]. Antibodies have also historically been used in forensic serology to identify and confirm the presence of certain body fluids (e.g., Abacus Diagnostics® [ABA] and RSID™ cards for detection of blood, semen, urine, and saliva) [124–127]. Several early versions of commercial STR amplification kits for human identification even incorporated a modified *Taq* polymerase that included an antibody that would bind *Taq* and prevent activity until a certain temperature was reached [128].

The ability of antibodies to bind specific antigens makes them a suitable candidate for targeted cell selection of individual cell types, especially given that forensically relevant cells have distinctly different functions and thus often possess entirely different physical characteristics and protein expression patterns. Unsurprisingly, much of the recent literature on cell isolation within forensic science has focused on antibody-mediated methods. Whether attached to a paramagnetic or plastic bead, antibodies are commonly employed for immunoprecipitation (IP) assays designed to pull a target away from the remainder of the sample [123,129]. As noted in the following discussion, there are many variations of this technique.

While a portion of the most recent antibody literature discusses the use of protamine antibodies to isolate sperm DNA and mucosal epithelial antibodies (e.g., anti-Cytokeratin 4) to isolate buccal and vaginal epithelial cells, these targets have received little attention compared to sperm cells [36,40,66,130]. This is likely due to the prevalence of sperm cell antibodies that have been characterized in the reproductive health literature. More than 900 seminal fluid proteins and >6,000 sperm cell proteins have been described, and several have been identified as potential targets for antibody-mediated cell isolation for forensic samples (Table 2) [17,27,30,66,131–139]. Further,

several seminal proteins have been successfully targeted using antibodies for serological screening of biological evidence, as described above for body fluid identification. Consequently, the forensic community seems quite comfortable with the prospect of using antibodies for sperm cell isolation.

TABLE 2. Top-ranked sperm-specific antibody candidates [130–133].

Sperm Antibody	Species Specificity	Target Antigen Location	Expression Level
Intra-Acrosomal Protein Antibody (SP-10)	Human, mice	Acrosome, sperm head	High
Sperm associated antigen (SPAG 8/Sperm membrane protein 1)	Human	Acrosome and testis	High
Sperm Adhesion Molecule 1 (PH-20/SPAM-1)	Human, mice	Acrosome, epididymis, sperm head	High
A Kinase Anchoring Protein 3 (AKAP3)	Human, mice, rat	Acrosome and tail of sperm, cytoplasm	High
Cysteine-rich secretory protein 2 (CRISP2)	Human, mouse, rat, chicken, dog	Acrosomal cap, testis and epididymis	High
Motile sperm domain containing 3 (MOSPD3)	Human, mice, rat	Distributed through the sperm, but concentrated on head and tail	High
Disintegrin and metalloproteinase domain-containing protein 2 (ADAM2)	Human, mouse, rat, chicken, dog	Sperm surface protein	High
Zona pellucida receptor protein 2 (ZP2)	Human	Acrosomal cap	High
Zona pellucida receptor protein (ZP1)	Human	Acrosomal cap-coded by the female helps bind sperm to egg	High
Sperm associated antigen 9 (SPAG9)	Human	Acrosomal cap-associated with infertility	High
Sperm agglutination antigen-1 SAGA-1	Human, mice	Epididymis and multiple locations along the sperm cell	High
Sperm acrosome membrane-associated protein (SPACA-1/SAMP32)	Human, mice	Acrosome, sperm head, membrane protein localized in the equatorial segment of spermatozoa	High
Sperm associated antigen 6 (SPAG6)	Human	Tail-associated with infertility	High
Human epididymis-specific protein 5 (CD52)	Human, mouse, rat	Male reproductive track, specifically the epididymis	High
Anti-angiotensin-converting enzyme	Human, mouse, rat	Type-1 angiotensin II receptor on the spermatozoa located on tails, neck and mid-piece and flagellums of the sperm	High

In the early 2000s, independent studies demonstrated that antibodies could be used to target and enrich for spermatozoa from mixed cell populations. Monoclonal antibodies against the testicular isoform of angiotensin-converting enzyme (tACE) present on the neck, midpiece, and flagellum of spermatozoa were attached to magnetic beads for the isolation of almost 100% of sperm cells when 1,000,000 spermatozoa were mixed with 1 mL buccal epithelial cells; however, it was limited to swabs stored in PBS and was unsuccessful when tested with older vaginal swabs [139]. Others showed that antibodies against NUH-2, which inactivates spermatozoa and is found primarily on the tail region, was able to capture approximately 80% of target cells when covalently attached to magnetic beads [138]. While both of these studies were limited, they served as promising, proof of concept data demonstrating the potential use of antibodies for capture of forensically relevant cells. Since then, many studies have focused on various sperm antibodies coupled to magnetic or plastic beads for separating sperm from non-sperm cells within the typical microcentrifuge tube environment. Miltenyi Biotec has even recently commercialized a magnetic cell sorting kit for sperm isolation, which utilizes antibodies targeting cluster of differentiation 52 (CD52 or CAMPATH-1 antigen, *see Table 2*) and is marketed for forensic applications [140,141].

Although ostensibly simple in theory, many factors must be considered when selecting antibodies for successfully targeting and isolating spermatozoa. Antibodies must specifically and efficiently bind spermatozoa while demonstrating no cross-reactivity with other cells, and target antigens must be highly expressed on the cell's surface. In addition, it is crucial that these antibodies bind to areas of spermatozoa that are robust and remain after sample deposition, collection, and elution (e.g., sperm cell heads and acrosomal caps rather than sperm tails). Thus, antibody targets within the literature such as MHS-10/SP-10, MOSPD3, PH-20/SPAM-1, SPAG-8, AKAP3, CRISP2, and

CD52 have been heavily explored; these antigens all populate the acrosome or head regions of spermatozoa and are expressed at high levels (and therefore should persist for longer time periods and bind easily to the target antibodies) [17,25,130–133,138,142–144].

Various degrees of success have been demonstrated with antibodies for these target antigens. Unfortunately, however, there have also been many inconsistencies and reproducibility issues reported within the literature for the same antibodies. In 2002, Marshall et al. reported capturing almost 90% of sperm cells with MHS-10/SP-10 antibodies covalently coupled to magnetic beads [138], but subsequent studies in 2016 revealed that anti-SP-10 coupled to magnetic beads using a biotin-streptavidin interaction was inefficient for mixtures containing <10,000 spermatozoa per milliliter [17]. A study in 2014 successfully generated single-source male STR profiles from mixture samples containing at least 10,000 sperm cells per milliliter with MOSPD3 antibody coupled to paramagnetic beads via biotin-avidin [25], but other studies using biotin-streptavidin linkages have demonstrated non-specific binding of MOSPD3 to vaginal epithelial cells and DNA profiles with <50% of the expected male alleles [130,132]. In 2016, Zhao et al. used PH-20/SPAM-1 antibody to isolate spermatozoa from mixtures containing 1,000 sperm cells and 100,000 epithelial cells [17], but studies using anti-PH-20 within the Dawson Green laboratory at Virginia Commonwealth University resulted in sperm fractions containing only 37.66 ± 22% DNA from single-source semen samples and intermittent sperm fractions from mixture samples containing male DNA to female DNA ratios of >10:1 [130]. Finally, manufacturer generated studies using Miltenyi Biotec's MACSprep Forensic Sperm Microbead kit (CD52 antibody) reported an average increase in male DNA from 20 to 80% when testing 5:1 (HeLa cells:sperm cells) mixtures [140], but an external publication revealed a wide range of sperm recovery (approximately 5 – 85%) for

various semen dilutions and mixture samples, as well as reduced purity of sperm fractions when mixtures consisted of vaginal epithelial cells compared to buccal epithelial cells [145]. These inconsistencies could be due to many factors, including differences in solid support material and size (e.g., nanoscale magnetic beads or microscale plastic beads); method and specific location of conjugation between antibody and solid support (e.g., biotin-streptavidin linkage or covalent cross-linking); sample age and preparation (e.g., fresh, liquid samples or reconstituted, aged samples); types of cells present (e.g., buccal epithelial cells versus vaginal epithelial and endothelial cells); number of cells present in each sample tested; disparity in the antibody clonality, provenance, and/or lot number across studies; and the various binding and separation conditions that are present during the IP protocol (e.g., direct or indirect binding mechanism, pH and temperature, buffers, etc.).

Evidence of success in sperm cell isolation via antibodies, despite observed inconsistencies and reproducibility issues, is enough to warrant further investigation into the optimization and implementation of this technique within forensic DNA analysis. Continued research in this area is especially critical when considering antibody-mediated cell separation utilizes instrumentation and reagents already commonly employed within the field, is relatively cheap, and has the potential to be automated.

Microfluidic Methods

Introduction

Microfluidics, a term that broadly refers to the manipulation of liquids on the microscale, has gained substantial interest and attention in a variety of scientific fields since the early 1990s,

especially for the development of micro total analysis systems (μTAS) or lab-on-a-chip devices [146–148]. By taking advantage of several well-established concepts from physical chemistry and engineering, microfluidics and microdevices have enabled more precise liquid handling alongside an improved reaction efficiency. Microfluidics utilizes a multitude of channel and device designs to control liquid movement and accomplish mixing, metering, aliquoting, and reagent storage (among many other processes). One of the more complicated and crucial components of microdevice design is the choice of valving, which can involve mechanisms as simplistic as tape or as complicated as elastomeric diaphragms; regardless of the mechanism chosen, effective valving is perhaps the most vital consideration for the integration of the various modular processes mentioned above [149–152]. Aside from valving, both sample introduction and application of sufficient force for liquid propulsion throughout a microdevice can add time, complexity, and cost to its fabrication and operation.

Both the development and implementation of microdevices have primarily stemmed from the desire to perform laboratory processes in a manner that is much cheaper and faster than those on the macroscale; μTAS/lab-on-a-chip devices promise the possibility of creating sample-in-answer-out capabilities whereby an entire laboratory workflow can be conducted in a single microscale environment [148,153,154]. This concept ultimately generates many other advantages over traditional macroscale, in tube methods. Because smaller volumes of samples and reagents are utilized, costs and consumption can be greatly reduced. The closed environment of microdevices also minimizes the possibility of contamination and avoids the manual transfer steps inherent to many in vitro processes. Even further, the portability of microdevices has been especially appealing for forensic scientists, as they could be taken to crime scenes or used at hospitals and

borders. Examples of commercially available μTAS/lab-on-a-chip devices within the forensic science community include the RapidHIT™ ID by Applied Biosystems™ and the ANDE® Rapid DNA System [155–160]. While these have the ability to produce STR profiles from reference buccal swabs within hours, they have yet to be designed or widely implemented for other evidentiary samples, including mixed cell samples that require cell separation. Further, adoption of such devices that incorporate the entire forensic DNA workflow has been stifled by the extensive validation, personnel training, and peer review requirements imparted on the community.

As with any instrument or accessory, the materials used to fabricate microdevices are extremely important and highly dependent upon their intended application. Earlier microdevices were composed of glass and plastics such as PMMA or PDMS, making their fabrication difficult, expensive, and/or time-consuming [130,147,161–164]. These devices were commonly made by bonding together multiple layers of etched glass and/or plastics using physical or chemical means. While development of these models was driven by reusability and familiarity, glass proved especially difficult to implement in biological fields because of its tendency to adhere or bind to biological substances such as cells, DNA, and enzymes [162]. Although some studies initially took advantage of the binding of cells and DNA to silica as a means of cell isolation and DNA extraction, a lot of research on alternate materials for microdevice fabrication quickly ensued [97,165]. These studies have been critical for continued use of microdevices for cell sorting applications given that over 200 non-specific binding sites have been noted within some glass microdevice designs [166].

Rather than attempting to overcome the limitations of glass substrates by using expensive, complicated passivation and functionalization techniques, researchers have turned to cheaper and disposable polymeric materials. While low production costs are especially appealing to the forensic DNA community when considering the number of samples currently processed and backlogged, the development of a disposable microdevice is also greatly preferred because of its ability to prevent contamination. Perhaps the most promising microdevice research on cheaper plastic materials has focused on polyethylene terephthalate (PeT). PeT devices are assembled from several layers of "off-the-shelf" transparency film (i.e., plastic overhead projector sheets) through a simple print-cut-laminate fabrication process [133,152,162,167–169]. Several layers of clear PeT (some with printed black toner or dyed black) are cut into the proposed microdevice design by laser ablation, sandwiched around heat sensitive adhesive (HSA) layers, and bonded together using a laminator [133,152,162,167,170]. This print-cut-laminate technique can be easily performed with a CO_2 laser cutter and simple laminator, making production not only cheap but also easily scalable. Even further, PeT provides a smoother surface that allows molecules to move more freely than on other plastics, making it more suitable for the movement of biological samples without the need for surface modifications [162,169].

The development of centrifugally driven microdevices has served to increase the interest of the forensic science community. By taking advantage of centrifugal force, the liquid propulsion and flow within a microdevice can be actuated and precisely controlled without the need for bulky external hardware such as pumps [147,149,150,152,168,169,171,172]. Not only does this significantly reduce the cost of a device, but it also simplifies microdevice platform design and minimizes the size and footprint of the device, increasing the possibility of miniaturized

portability. Precise liquid control has been improved recently with the incorporation of a normally-closed toner (or "laser actuated") valve that is easily opened with a laser to allow for the movement of liquid to a downstream chamber [133,171,173]; these valves are also resealable, which helps control or prevent liquid backflow in the microdevice [152]. Irradiation of a plastic layer made of dark material (e.g., black toner printed onto PeT) leads to absorption of thermal energy, which allows for the eventual opening of a toner/laser actuated valve [163,171,173]. By changing the focus of the laser and irradiating a wider area in order to fuse the material, this valve can then be resealed [152]. Although printing black toner onto PeT has been successful in terms of laser actuated valving, the use of bPeT that is inherently black throughout the entire polymer reduces valving errors as the black pigment is within the sheet itself rather than simply printed onto the surface (i.e., the entire thickness of the sheet is subjected to absorption and ablation instead of the exterior, toner surface) [152].

Modular Approaches for Forensic Science

As previously mentioned, there are two commercially available μTAS/lab-on-a-chip devices approved for use in the forensic science community; however, some forensically relevant microfluidic research has focused more exclusively on building simpler, serially integrated modular microdevices that limit the number of processes incorporated rather than including modules that cover the entire DNA workflow. Developing a device that performs only a segment of the workflow is more enticing and easily implementable, as this could eliminate the need to validate new methods for the more delicate downstream procedures for amplicon detection and separation, as well as profile interpretation. Thus, separate modular microfluidic techniques for DNA extraction, DNA quantification, STR amplification, and even cell separation have been

copiously investigated [153,162,168,169,174–185]. Whether used alone or as simple, limited integrated microdevices, this approach serves to alleviate the need for hands-on processing while also reducing time, sample, and reagent consumption. Further, with an efficient, accurate cell separation module, complex mixture interpretation could be avoided.

DNA Isolation

DNA extraction is one of the most important steps in the forensic workflow; unfortunately, it can also be tedious and time-consuming. Thus, several microfluidic methods for DNA isolation have been investigated to provide an automated and resource efficient alternative to current techniques.

As previously mentioned, early microdevices were composed of glass (or silica). While initial studies took advantage of the properties of silica, research on alternate materials for microdevice fabrication eventually ensued, which necessitated the use of other extraction techniques [97,165]. Solid phase DNA extraction (SPE) is one of the most common approaches used in the forensic DNA community; however, early microdevices struggled to implement conventional SPE while keeping the microdevice design simple and capable of integration with downstream processes. The development of on-chip extraction methods that removed or eliminated SPE reagents was especially critical for subsequent PCR amplification since they are incompatible with and can inhibit PCR [153,180,186–188]. In addition, microdevice SPE proved difficult and inconsistent because of uneven packing of the solid phase combined with prolonged sample and reagent loading times [161]. Thus, studies began utilizing other methods, such as enzymatic DNA liberation, to provide a more rapid “extraction” process that was compatible with subsequent steps of the forensic DNA workflow.

The thermophilic enzyme EA1 has been heavily investigated for cell lysis and DNA liberation. Activation of EA1 at 75°C enables cell lysis and protein degradation, while its subsequent degradation at 95°C prevents the enzyme from inhibiting downstream processes; this enzyme has been successfully utilized in several forensic studies for DNA preparation prior to STR amplification, both within a microcentrifuge tube and microfluidic environment [66,130–133,147,161,189]. This enzymatic DNA preparation technique avoids transfer and centrifugation steps, eliminates the use of hazardous and inhibitory reagents, reduces manual labor, and makes the integration of DNA "extraction" onto the microdevice a simple task. In fact, this enzymatic digestion method has been successfully implemented alongside IR-PCR within a centrifugally driven microdevice; this device was capable of producing multiplexed STR amplicons from reference buccal swabs in only 45 minutes [147].

Sedimentation & Acoustic Trapping

Given the predominance of SAECK backlogs across the U.S., many studies have shifted focus to the isolation and separation of specific cell populations prior to lysis. Some of the earliest cell isolation studies involving microdevices exploited differences in physical characteristics between spermatozoa and epithelial cells. For example, Horsman et al. used a glass microdevice that allowed epithelial cells to aggregate, sediment, and then adhere to the silica surface while sperm cells migrated to a downstream chamber [97]. Although this method was somewhat effective, it still failed to completely isolate male and female fractions and it relied on gravity-driven flow that was extremely slow. A similar but slightly more complicated approach exploited hydrodynamic effect and cell size differences to force specific cell populations into separate chambers [99]. This

technique only took approximately 30 minutes but was inefficient and prone to clogging of the microdevice channels.

Acoustic trapping is another recent technique that has been recently explored for cell isolation in microfluidic devices. Termed acoustic differential extraction (ADE), this method uses a vibrating piezoelectric transducer to generate a standing acoustic wave within a microdevice that enables non-sperm cells to move into a separate chamber; this wave is strong enough to trap individual sperm cells because of their size, shape, density, and compressibility [19,184]. Because this technique takes advantage of so many physical characteristics, it works best with cell types that exhibit common properties across replicates. Thus, ADE is well suited for targeting and trapping spermatozoa because they possess relatively uniform physical characteristics across human males, while epithelial cells tend to take on a variety of shapes and sizes [190,191]. Unfortunately, although this technique takes only one hour to successfully separate female epithelial-sperm cell mixtures (from ratios as large as 40:1), it has only been demonstrated on fresh samples within microfluidic environments and has proven difficult to integrate with other workflow modules [19] (James Landers, personal communication).

Oligosaccharide Cell Capture

Microdevices have even been used to isolate specific cells based on affinity capture. In a 2018 study, a simple microdevice coated with the oligosaccharide Sialyl-Lewis X (SLeX), which is a major carbohydrate ligand present on the zona pellucida that facilitates sperm-egg binding, was developed to selectively capture spermatozoa from a mixed cell sample [166]. While this technique demonstrated 70-90% sperm cell capture efficiency and captured spermatozoa with various rare

morphologies, it still required 80 minutes for cell separation alone and involved several washing and incubation steps [166]. Even further, this microdevice was driven by large pumping mechanisms, was composed of glass, worked poorly with cotton substrates, and experienced some non-specific capture of epithelial cells [166]. Overall, this technique demonstrated that a naturally occurring attraction to carbohydrate ligand(s) could potentially be exploited for the isolation of spermatozoa; however, future studies are needed to achieve more efficient binding and elimination of epithelial cell capture.

Sexual Assault PeT Microdevice Platform

As previously noted, there has been significant research on microdevices for forensic DNA analysis, especially in an effort to achieve cell isolation for sexual assault sample processing. For years, the Landers (University of Virginia) and Dawson Green (Virginia Commonwealth University) research laboratories have explored the use of disposable PeT microdevices and antibody-bead mediated separation of spermatozoa and epithelial cells in an attempt to develop a modular approach that is more amenable to the forensic workflow. These microdevices incorporate fluid propulsion by centrifugal force and are capable of performing sample elution, antibody-mediated cell separation, DNA liberation, and custom multiplex STR amplification. Ongoing research has developed and evaluated an antibody-mediated sperm cell capture chemistry for use with this rotationally-driven microdevice platform, and successful attainment of resulting STR profiles has been reported using both the AmpFlSTR™ Identifiler™ (Identifiler; ThermoFisher Scientific, Waltham, MA) and PowerPlex® Fusion 5C (Promega, Madison, WI) chemistries [133]. These devices warrant further investigation because they show great promise for implementation

into the forensic DNA workflow due to their small footprint, automation capabilities, easy print-cut-laminate fabrication, and low cost (approximately $0.50 per microchip).

Bead-Mediated Antibody Cell Capture Chemistry

The bead-mediated antibody cell capture chemistry referenced above utilizes a biotin labeled antibody that is conjugated to a 200 μm streptavidin-coated polystyrene bead; this biotin-streptavidin linkage is used because it is the strongest known noncovalent interaction, making it essentially irreversible [66,130–133,192–194]. The antibody-bound bead is then incubated with a mixed cell sample containing semen and vaginal epithelial cells for 35 minutes, after which cells bound to the antibody-coated beads can be pelleted via centrifugation [66,130–132]. For the microcentrifuge tube assay, unbound cells are manually removed to a separate tube via pipette transfer of the supernatant; however, in the PeT microdevice, valve opening and spinning allows for the unbound cells to be moved to a separate chamber in a semi-automated fashion [133].

While the majority of antibodies evaluated thus far have targeted sperm cells (e.g., SPAG8, CRISP2, MOSPD3, PH-20/SPAM-1, and AKAP3), those that target buccal and other mucosal epithelial cells (e.g., CK4 and Laminin-1) have also been explored as a way to separate contributor fractions. In previous studies, SPAG8 and CRISP2 outperformed other sperm antibodies by retaining 70.09% and 52.61% of the total semen DNA, respectively, within the bound fraction of semen samples [132,133]. When considering the expected percentages of sperm (88%) and non-sperm (12%) cells within semen and correcting for ploidy, theoretical yields from semen should produce approximately 80% of DNA originating from spermatozoa and 20% from non-sperm contributions [24,28,30,48]. Thus, results obtained with the SPAG-8 and CRISP2 antibodies were

close to the expected values. When used to separate semen:vaginal cell mixtures in microcentrifuge tubes, PH-20 and AKAP3 generated average male:female ratios ~10:1 in the bound fraction and CK4 yielded average male:female ratios ~9:1 in the unbound fraction. This was ideal given that profiles presenting these ratios typically show a low or undetectable minor contributor and can therefore often be interpreted as single-source STR profiles [83,133,195]. Not only do these results demonstrate the ability of this assay to produce enriched male STR profiles that require minimal back-end mixture interpretation, but they also provide evidence that the CK4 antibody may be useful for isolating the female (epithelial cell) fraction of a sexual assault sample regardless of the male cell type(s) present.

When testing seminal fluid samples in a simple cell separation microdevice, incubation with SPAG8-coated beads captured 39% more sperm cell DNA than incubation without beads and showed significant improvement compared to a traditional differential lysis and DNA extraction technique within microcentrifuge tubes [133]. Unfortunately, cell separation using PH-20 on mock postcoital cell samples in a more recent PeT cell separation microdevice module resulted in mixtures in both the bound and unbound fractions; however, in these samples the male profile was enriched by 142% in the bound fraction [133]. Overall, while these results are promising, they clearly demonstrate the need for further optimization of the STR amplification chemistry, as well as the antibody-mediated cell capture chemistry, in order to assure that all spermatozoa are bound to the antibody-coated beads retained in the sperm fraction.

Microdevice Materials & Hardware Design

All hardware for the sexual assault microdevice platform was designed to promote ease of use and minimization of its footprint. Initially, microdevices were composed of PMMA and utilized mechanical valves in combination with centrifugal force to control liquid flow; however, issues with reproducibility of device fabrication, clogging of channels, and device complexity related to mechanical valving led to exploration of alternative materials.

Recent transition to a credit card-sized microdevice composed of five layers of PeT has significantly reduced fabrication costs and hardware complexity. These microdevices are now quickly and easily constructed via the previously described print-cut-laminate technique using overhead transparency sheets, AutoCAD software (Autodesk, Inc.), a VersaLaser® 3.50 CO_2 laser ablation system, and a commercially available thermal laminator (Apache AL 13P12) [133]. Heat sensitive adhesive (HSA) is fused to PeT and sandwiched between the outermost PeT and innermost toner-coated PeT layers on either side, bonding the microdevice together when laminated (Figure 1). The toner-coated middle layer enables laser actuated valves to be utilized for liquid movement to subsequent downstream chambers on the microdevice. Since these valves are only opened when irradiated by an IR laser that burns an opening in the material, valving is now more quickly achieved and more amenable to automation [133,163,171,173].

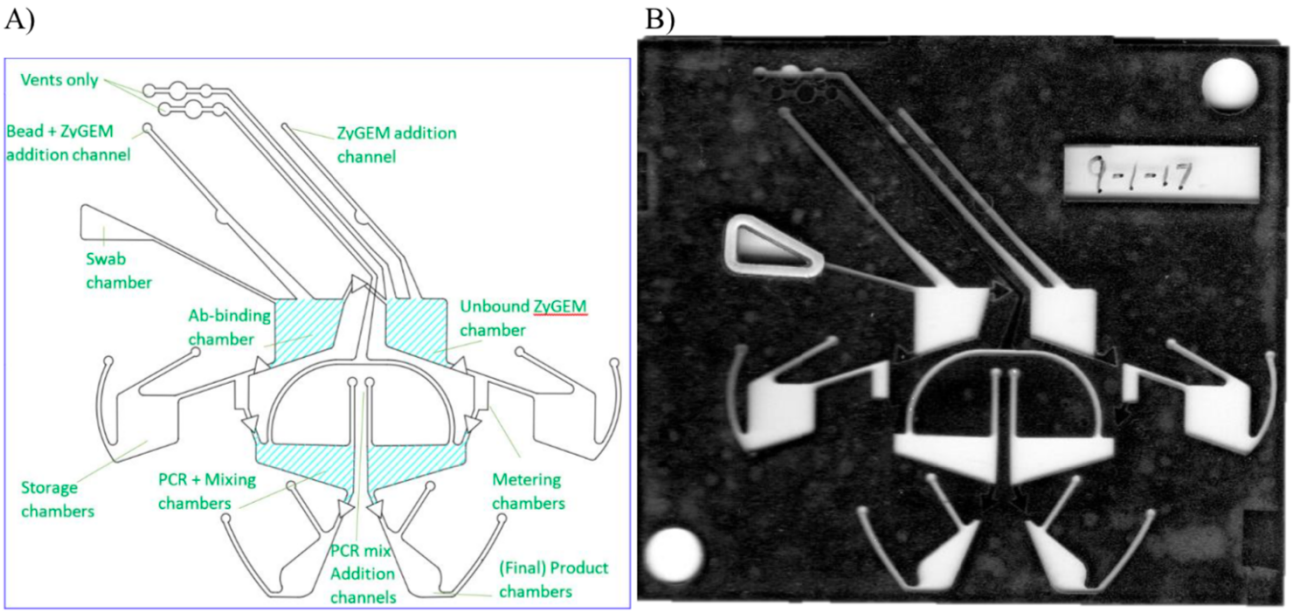

FIGURE 1 – Design of rotationally-driven sexual assault microdevice. (A) This microdevice utilizes an antibody-polystyrene bead complex to bind sperm cells in the antibody-binding chamber. After the laser valve is opened and a brief spin step, unbound cells are moved into a separate adjacent chamber and then both fractions are treated with a thermal enzymatic reaction for cell lysis. After cell lysis, the platform rotates to change the center of rotation, another laser valve is opened, and a brief spin allows for the liberated DNA from each fraction to fill the corresponding metering chambers, with the excess sent to corresponding storage chambers. This is followed by a third valve opening, a spin into the PCR mixing chambers, and a short mixing protocol where PCR reagents are added prior to movement to the PCR product chambers where thermal cycling commences. After PCR, products can be pipetted off of the microdevice and prepared offline for CE analysis. A △ indicates the location of the laser-tap valves. (B) The microdevices are constructed from layers of PeT transparency sheets, heat sensitive adhesive, and black printer toner Adapted from Dawson Cruz 2019 [133].

Although the updated PeT microdevice still achieves liquid movement via centrifugation, the accompanying hardware platform also required modification. Incorporation of a different spin motor (i.e., stepper motor) enabled more fine-tuned control over centrifugation parameters that can be managed via laptop software, significantly improving upon the original platform, which utilized a simple dial and voltage regulator [133]. In addition, shaking/mixing can be performed on top of traditional spinning, which should improve antibody-binding and allow for more homogenized

DNA liberation and PCR reactions. Further, the use of a Peltier clamp for heating/cooling and servo motors for altering microdevice orientation enables more efficient temperature cycling and precise control of liquid flow, respectively (Figure 2).

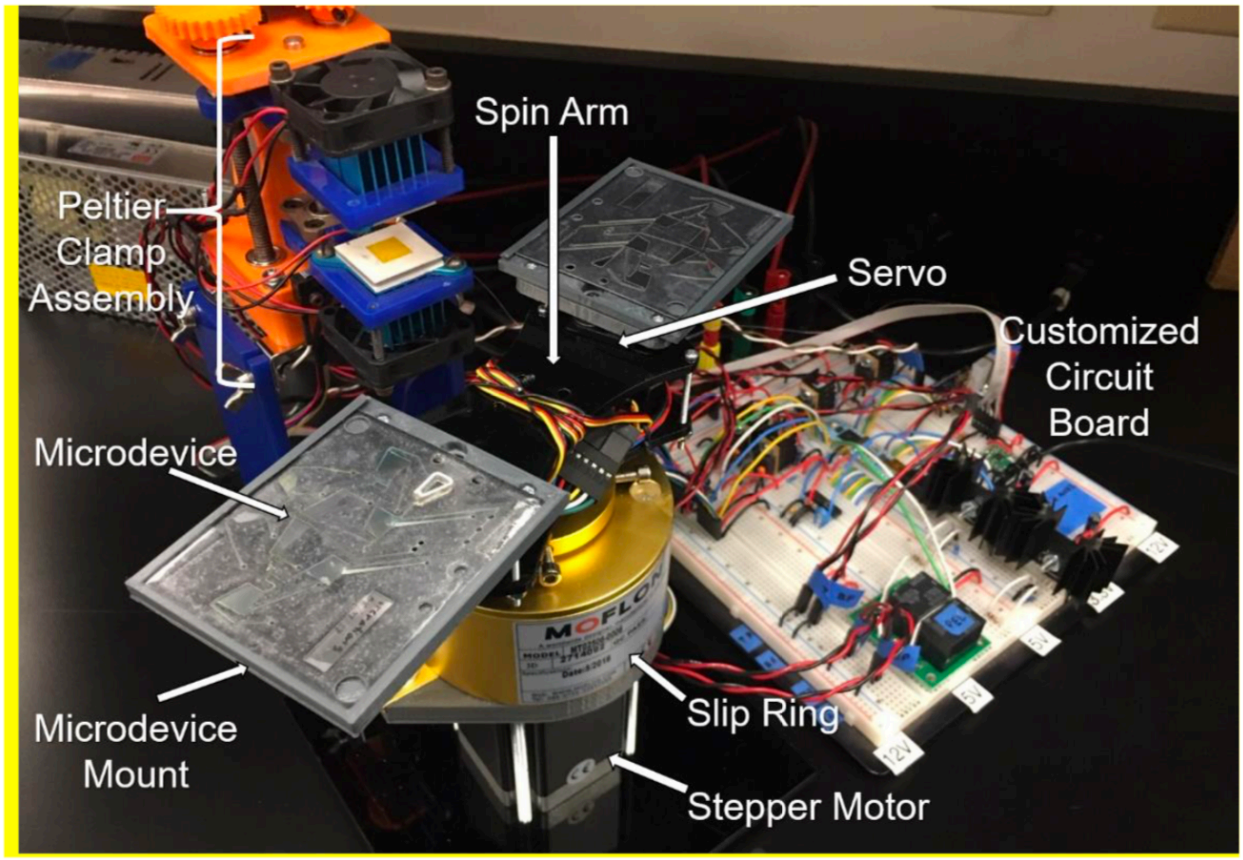

FIGURE 2 – Sexual assault microdevice platform hardware (v3). Microdevices are inserted into their mounts, which are connected via rotating servos to the spin arm. The spin arm is rotated through a slip ring via an electromagnetic stepper motor. Heating and cooling are performed via a Peltier clamp assembly, consisting of two sets (upper and lower) of Peltier chips, heat sinks, and fans. All components are connected to a circuit board hub, which also connects to a controller PC via USB. Not pictured: laser diode mount assembly used for laser/tap-valve activation. Adapted from Dawson Cruz 2019 [133].

All modifications to date for the sexual assault microdevice platform enable processing of samples from elution to STR amplification, omitting the DNA quantification step. Samples are added to the swab chamber and subsequently spun into the bound chamber, where antibody-mediated cell

separation occurs (Figure 1) [133]. After incubation with antibody-conjugated polystyrene beads, a laser actuated valve connecting the bound and unbound chambers is opened, and the supernatant containing all unbound cells is transferred via centrifugation [133]. Because the channels connecting chambers are smaller than 200 microns (~100 μm in diameter), bead-bound cells are unable to move into the next chamber. All subsequent processing for each fraction occurs in tandem.

Once bound and unbound fractions are separated, DNA is liberated via thermostable enzyme EA1. Earlier studies used the *prep*GEM™ Saliva and *forensic*GEM™ kits (microGEM™, Charlottesville, VA) with the addition of 1M DTT to promote lysis of spermatozoa, while a more recent evaluation also added 0.1% SDS to increase cell lysis efficiency [66,130–132]. Because this DNA liberation assay only requires a five-minute incubation at 75°C and no tube transfers, it is easily achieved either in a manual microcentrifuge tube environment or within the described microdevice.

Upon completion of cell lysis and DNA liberation in the microdevice, bound and unbound fractions are spun into their separate, respective amplification chambers. Amplification is then accomplished in approximately 45 minutes using attached Peltier clamps and an eight-microliter reaction mix consisting of Identifiler™ primers, 2X KAPA 2G Fast Multiplex PCR Mix, KAPA 2G Fast Hot Start polymerase, and Pfu Ultra HF polymerase [147]. More recent studies have explored the use of PowerPlex® Fusion 5C primers, but further optimization is needed to improve profiling results [133]. Regardless of the primers used, STR amplicons are subsequently removed from the microdevice for separation and detection using traditional capillary electrophoresis (CE). Although CE has been accomplished on microdevices, this sexual assault PeT microdevice

specifically avoids its incorporation because developmental and internal validations of new separation and detection techniques are extremely cumbersome due to the potential impact on profile interpretation. The continued use of traditional CE will enable labs to more easily and quickly adopt this sexual assault microdevice within the current workflow while eliminating the most labor-intensive and time-consuming steps.

Challenges & Limitations

Although promising results have been obtained thus far (as noted above), there are still many challenges and limitations to this approach that must be overcome prior to its adoption within the field. Issues which are pervasive when the assays are performed in a tube, such as cellular recovery and subsequent lysis, will likely also translate to the microdevice environment; therefore, such topics must be addressed and mitigated.

Cell Recovery from Substrate

Since the recovery of cellular material from an evidentiary substrate (e.g., cotton swab or fabric cutting) is a crucial step for efficient DNA extraction, many studies have tried to improve upon the elution of cells from various materials prior to differential cell lysis [67,196–198]. This issue can become exacerbated within the microfluidic environment, given the reduced volumes and faster sample processing times.

Evaluations of various enzymatic methods, alternative detergents and buffers, as well as substrate type have been performed. In a 2006 study, the Landers research lab at UVA employed a cellulase enzyme cocktail to degrade cotton fibers and reduce entanglement of cells within the swab,

enhancing the elution of sperm and epithelial cells [196]. Although a promising idea, sperm cell recovery with this technique varied widely for fresh samples and quickly dropped below 10% when evaluating samples that had been dried for four days, so it failed to truly improve upon traditional elution from cotton swabs [196]. Even further, this technique involved a four-hour incubation, which is longer than some traditional differential incubations and thus fails to save time [196]. In a different study, Orchid Cellmark's SpermElution© method recovered more than twice the number of spermatozoa compared to a typical water elution from cotton and polyester swabs, as well as several fabric types [197], and more sperm cells beyond 96 hours since intercourse [198]. However, despite this success, only ~70% of sperm cells were recovered and, consequently, male DNA carried over into the epithelial cell fraction [197,198].

Considering that the poor recovery efficiency of body fluids and cells from cotton swabs is a well-known issue, several studies have evaluated alternative substrates for sample collection as a means of improving both cell recovery and DNA extraction efficiency. The properties that make cotton swabs perfect substrates for sample collection (e.g., absorbency) also typically cause them to exhibit poor release of cells [15]. In early studies, it was concluded that the amount of DNA collected from a swab was inversely related to the density of its fibers [199,200]. Given that cellulose fibers are very tightly wound around the shaft of a cotton swab, it is no surprise that cellular material is inefficient at escaping once captured. Thus, several studies have evaluated swabs made of materials such as foam, nylon flock, and rayon [201,202]. VCU studies involving an earlier version of a PMMA microdevice found that DNA yields from buccal epithelial cells released from foam swabs in tube and in the microdevice were eight and four times higher, respectively, than those obtained when using cotton swabs [147]. In other studies, nylon flock

swabs have proven more effective at releasing cells, providing higher DNA yields than samples eluted from cotton swabs, but the field has yet to widely adopt the use of non-cotton swab materials due to cost and tradition [202].

Cell Lysis & DNA Recovery

Aside from cellular recovery and release, subsequent lysis of those cells is also a critical process that must be optimized. Considering that spermatozoa within sexual assault samples are typically vastly outnumbered by vaginal epithelial cells, and that cellular release from swabs is relatively inefficient, it is crucial all cells eluted from a sample are lysed so that sufficient DNA can be recovered. Further, the reduced sample volumes used within the sexual assault microdevice makes sufficient cell lysis even more essential.

Previous studies within our research group evaluated ten microliters of a 1:2 semen dilution, ten microliters from half a vaginal swab eluted in 200 µL 1X PBS (essentially 1/40 of a vaginal swab), and a combination thereof for mixture samples [66,130–132]. Based on average sperm cell counts, one could expect to retrieve ~250,000 – 750,000 spermatozoa (825 – 2,475 ng DNA) from these samples [24–26,28,44,203–205]. When considering average human DNA yields obtained from whole vaginal swabs and correcting for the amount of sample used in this research, the average expected DNA yield could range from 150 – 157 ng (which would correspond to approximately ~22,500 – 23,750 vaginal epithelial cells when assuming the absence of cell-free DNA) [206]. Although this would be more than sufficient DNA for a commercially available STR amplification kit, when enzymatic liberation assays were used (modified *prep*GEM™ and *forensic*GEM™ master mixes), DNA yields were orders of magnitude below the expected quantities and STR

profiles often exhibited dropout [130,132], indicating that not all cells were being lysed. Although addition of SDS increased DNA yields, quantities were still significantly lower than expected and STR profiles remained largely unchanged [132]. Thus, further optimization of this liberation assay or exploration of other potential cell lysis techniques is necessary to improve lysis of all cells.

Ideally, alternate lysis methods should maintain the same efficiency (e.g., no tube-to-tube transfers or wash steps) as the currently employed technique, avoid inhibitory reagents, and utilize relatively small reaction volumes (~25 µL). Additionally, they should be simple, quick, and involve non-proprietary reagents to assist with future licensing and commercialization. Several lysis methods already exist that could potentially improve upon the results seen thus far with modified *prep*GEM™ and *forensic*GEM™ chemistries. The *forensic*GEM™ Sperm kit from microGEM is an obvious alternative, as it incorporates an additional enzyme cocktail known as *Acrosolv* to preferentially lyse sperm at a lower optimal temperature than the EA1 enzyme. Casework Direct (Promega™) is a commercial kit designed for the lysis of sperm cells via 1-thiolglycerol instead of DTT, and it has already been implemented in forensic science for Y-screening and direct amplification (Table 3) [207–209]. SwabSolution™ by Promega™ is another promising, proprietary avenue that has been explored for the direct amplification of semen samples and only requires one incubation with no tube transfers (Table 3) [26,210]. Alternatively, some non-proprietary cell lysis solutions, such as alkaline lysis and NP-40 buffer, could be explored for semen-vaginal samples (Table 3). Alkaline lysis is a relatively quick and cheap method that utilizes sodium hydroxide to disrupt plasma membranes, denature nucleases, and preserve DNA [211–213]. Another non-proprietary cell lysis buffer involves nonyl phenoxypolyethoxylethanol (NP-40). NP-40, a non-ionic detergent often used alongside Tris and NaCl, has already been used

successfully on crude blood samples and shows promise for sperm cell lysis (Table 3) [214,215]. Although both commercially available (e.g., Casework Direct and Swab Solution) and non-proprietary (e.g., alkaline lysis and NP-40) techniques could potentially be used after the antibody-bead mediated assay, smaller reaction volumes (for inclusion in a microdevice) and shorter incubation times need to be explored.

Aside from commercially available kits/reagents and common lysis buffers, the exploitation of proteins and other molecules that promote the natural decondensation of spermatozoa within the human vagina could provide suitable results. The contraceptive literature has frequently utilized protein disulfide isomerase A3 (PDIA3) [216], lysolecithin (a hydrolysis product of membrane phospholipids) [217,218], glutathione (GSH) [219,220], heparin [220,221], and/or glutathione-S-transferase omega 2 (GSTO2) [222] for acrosomal cap removal and sperm cell decondensation for the purposes of intracytoplasmic sperm injection (ICSI) (Table 3). These components are not only naturally occurring, and thus non-hazardous to DNA, but they have also successfully lysed spermatozoa within 30 minutes to 1 hour. Although there has yet to be any research on the implementation of these reagents for extracting DNA from spermatozoa for forensic purposes, a combination of these substances could allow for the plasma and acrosomal membranes to be removed, thereby permitting sperm nucleus decondensation and DNA release.

TABLE 3. Potential alternative, direct-to-amplification cell lysis techniques within the literature.

Commercial Kits			
Cell Lysis Technique	**Pros**	**Cons**	**Key References**
SwabSolution™	- Stand-alone reagent - Direct amplification - No additional purification - Previous use for spermatozoa	- Proprietary - Large volumes - Wash steps	Promega Tobe et al. 2017
Casework Direct Kit	- Direct amplification - No purification - No wash steps - Previous use for spermatozoa	- Proprietary - Available only as kit - Large volumes	Promega Loten et al. 208 Hakim et al. 2019
Lysis Buffers and Other Additives			
Cell Lysis Technique	**Pros**	**Cons**	**Key References**
NP-40	- Non-proprietary - Quick - Cost-effective	- Incubation on ice - Large volumes - Wash steps - Tube transfers - No reported use on spermatozoa	Invitrogen Ji H. 2018 Zhang et al. 2010
Alkaline Lysis	- Non-proprietary - Quick, cheap, single tube - Previous use for spermatozoa	- Large volumes	Dissing et al. 1995 Klintschar and Neuhuber 2000 Rudbeck and Dissing 1998
Nuclease Based Approach/Erase Kit	- Potentially non-proprietary - Previous use for spermatozoa	- Time - Large volumes - Tube transfers - Kit (Erase Sperm)	Garvin et al. 2009
Katilius Cell Lysis Solution	- Non-proprietary - Previous use for spermatozoa	- DTT inhibits PCR - Large volumes	Katilius et al. 2018
Natural Sperm Decondensation Approaches			
Cell Lysis Technique	**Pros**	**Cons**	**Key References**
GSH – TX/LL and PDIA3	- Stand-alone reagents - Non-proprietary - Natural mechanism	- No forensic research - Wash steps - Unfavorable 0°C incubation	Li et al. 2014 Morozumi et al. 2006 Seita et al. 2009 Zambrano et al. 2017 Lee et al. 2015
GSH – Heparin and PDIA3	- Stand-alone reagents - Non-proprietary - Natural mechanism	- No forensic research - Heparin can inhibit PCR	Li et al. 2014 Cheng et al. 2009 Romanato et al. 2003 Julianelli et al. 2012 Canel et al. 2017 Pipolo et al. 2018 Sanchez et al. 2013 Hamilton et al. 2019

Chemistry Optimization

While cell recovery and lysis are critical issues to consider, the ability of the antibody-mediated cell separation technique to effectively and consistently isolate target cells is perhaps the most important aspect to optimize. As noted above, results from previous studies have suggested that not all target cells are being captured by the antibody-conjugated beads, as ≥30% of target cell DNA has escaped into the wrong fractions and major male STR profiles have been observed within both bound *and* unbound fractions of mixture samples separated using this approach [66,130,132,133]. Thus, modifications to antibody-bead and antibody-cell binding protocols may be needed to improve target cell recovery.

Unfortunately, there has been no single, optimized antibody binding protocol reported in the literature. Instead, each published study has incorporated a unique combination of temperature, time, antibody concentration, and solid phase support (among other factors) [17,25,122,138,139,223]. Recent studies in the Dawson Green lab at VCU have therefore explored various modifications to the binding of antibodies to both polystyrene beads and target cells in an attempt to identify the optimal protocol for antibody-mediated separation of spermatozoa. Overall, results suggested that addition of FcR blocker during cell binding, use of FcR blocker alongside indirect cell binding (whereby antibody is incubated with target cells prior to bead addition), and implementation of a 95°C incubation after DNA liberation could increase the DNA yield within bound fractions of semen samples [132]. Data from this evaluation was promising and indicated potential avenues for improved antibody-mediated cell separation; however, extremely low DNA quantities, poor STR profiles, and inconsistent replicate results necessitate further exploration of these modifications. Further, additional considerations such as antibody concentration

[122,223,224] and solid phase support material [129,223] should be investigated. For example, research within the Dawson Green laboratory has used 0.167 µg antibody per sample regardless of cellular input, but other studies report using anywhere from 0.5 – 10 µg antibody per 10^4 – 10^7 cells [225,226]. Thus, it is apparent from the literature that the appropriate antibody load depends on the specific antibody and target, highlighting the need to perform an antibody titration to determine the optimal load for this study. Further, finding the antibody load which provides enough binding sites to account for sperm cell variation across donors while avoiding too many excess antibodies is especially critical for indirect binding assays, as any free antibodies within solution could preferentially conjugate to beads and prevent all cell-bound antibodies from binding.

Alternative bead materials and sizes may also prove beneficial given that the current 200 µm polystyrene beads tend to pellet via gravity, non-specifically trapping cells at the bottom of the microcentrifuge tube and in the corners of microdevice chambers. Further, the large size of the polystyrene beads currently utilized makes it difficult to fully homogenize them when mixed with the cell sample. Paramagnetic beads ranging from nanometers to micrometers in size have been commonly employed throughout the literature for antibody-mediated cell separation [17,25,138,223]. Exploration of these beads, albeit more difficult and expensive to transition to the microdevice platform, could provide much more surface area for cell binding, as well as easier homogenization and manipulation during the assay. They would also remain suspended in solution until attracted by a magnet, circumventing the issue of non-specific cell trapping currently encountered with the 200 µm polystyrene beads. Even further, the use of smaller streptavidin-

coated polystyrene beads (or the coating of microcentrifuge tube and microdevice surfaces) could prove beneficial by providing a greater surface area-to-volume ratio for binding.

Alternatively, the use of hollow glass microspheres (e.g., Microbubbles by Akadeum Life Sciences, Inc.) could alleviate the aforementioned issues of homogenization and non-specific cell trapping while also avoiding the use of magnetic fields. Because they are hollow, these Microbubbles are less dense than the surrounding media (and cells), causing them (and any bound targets) to rise in solution [227]. This relatively new concept has been coined buoyancy activated cell sorting (BACS) and has already been utilized to achieve 90% separation efficiency with CD4+ cells from whole blood samples in less than five minutes [228]. Use of these solid supports would prevent non-specific trapping of epithelial cells, as they would float to the top of the microcentrifuge tube instead of settling to the tapered bottom. Although Microbubbles (~5-15 μm) are much smaller than our currently employed polystyrene beads and would thus require a redesign of the microdevice architecture, it would still be cheaper than employing magnetic fields. Headspace (i.e., height) in the microdevice chamber(s) could potentially be increased to allow Microbubbles (and attached cells) to rise while unbound cells sediment, which would mainly require the addition of microdevice layers.

PRELIMINARY RESULTS

In preparation for the research proposed herein, some preliminary studies have been performed to address the aforementioned challenges of the current sexual assault microdevice. Performance of various detergent additives for sperm cell lysis have been evaluated along with their potential to inhibit downstream PCR reactions. Additionally, cellular input (as it relates to antibody

concentration/binding sites) has been optimized, and a new DNA liberation assay has been explored.

Initial comparisons of DNA quantification results from samples treated with the modified *prep*GEM™ chemistry revealed a three-fold increase in the sperm/bound fraction (n = 3, p = 0.09) and a two-fold increase in the non-sperm/unbound fraction (n = 3, p = 0.40) when compared to a traditional differential extraction [131]. Although not significant, these results demonstrated that the modified *prep*GEM™ technique was suitable for use within both the microcentrifuge tube and microdevice environments, as sufficient DNA could be obtained without any need for further purification. Subsequent transition to *forensic*GEM™ Sperm (microGEM™) for DNA liberation with semen-containing samples drastically improved DNA yields compared to the modified *prep*GEM™ method. This kit utilizes the same EA1 enzyme as previously described alongside a cocktail of mesophilic enzymes (termed *Acrosolv*), which specifically lyse spermatozoa heads and eliminate the need for additional reducing agents such as DTT [229–231]. Testing of semen samples with *forensic*GEM™ Sperm showed a statistically significantly higher average yield of 757 ng DNA compared to 57.7 ng DNA for the previously implemented method (n = 6; $p = 0.049$), which indicates the ability of this kit to sufficiently lyse sperm cells and obtain DNA quantities closer to those theoretically expected based on known cell input (Figure 3). Utilization of this method also corrected the quantification issues seen with DTT, and it only added approximately ten minutes to the previously used protocol, which is minimal considering the advantages in terms of DNA yield.

Once an improved DNA liberation technique was identified, studies shifted to the preparation of mixture samples that more closely mimicked 1:1 male-to-female DNA ratios to better evaluate the results of antibody-mediated cell separation. Previous sample preparations (described above) involved excess spermatozoa compared to vaginal epithelial cells, resulting in major male STR profiles in both bound and unbound fractions and making it difficult to determine assay efficiency.

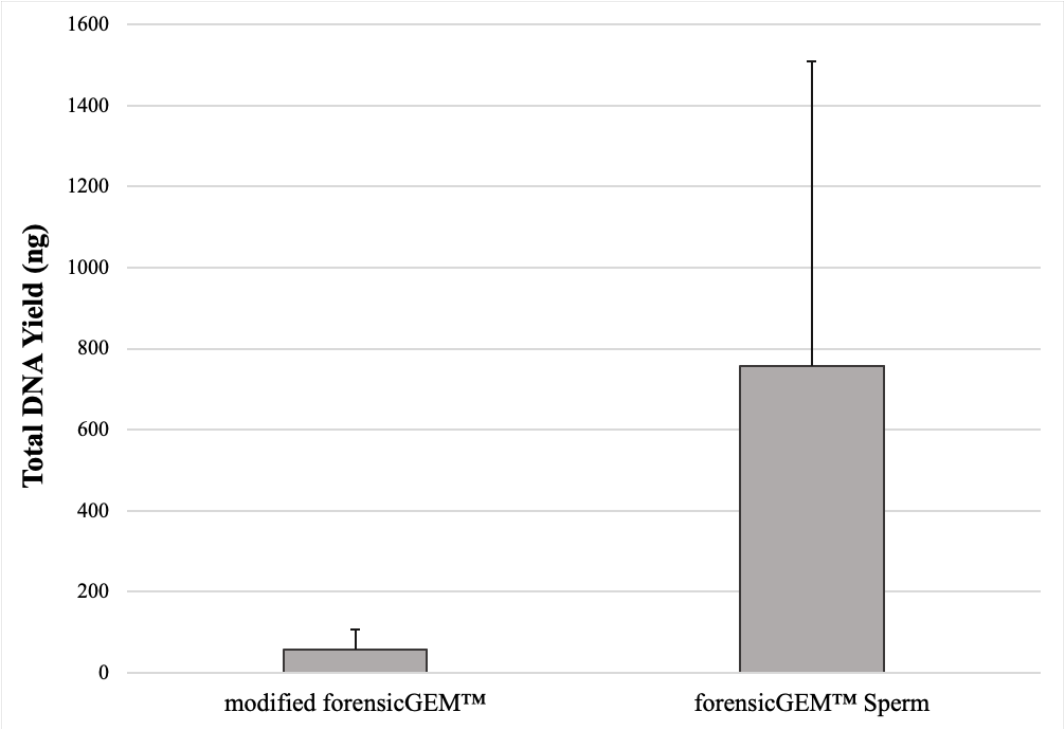

FIGURE 3 – Total DNA yield from 1:2 semen dilutions (n = 6) processed with the modified *forensic*GEM™ and *forensic*GEM™ Sperm DNA liberation chemistries. These results revealed the *forensic*GEM™ Sperm kit could produce significantly higher ($p = 0.049$) DNA yields than the modified *forensic*GEM™ technique.

Evaluation of male-to-female DNA ratios after quantification and male-to-female STR peak height ratios after amplicon separation and detection revealed that combining ten microliters of 1:40 semen (i.e., ~12,500 – 37,500 spermatozoa) with ten microliters of vaginal swab eluate (prepared as previously described) could produce approximate 1:1 mixtures on average (Figure 4).

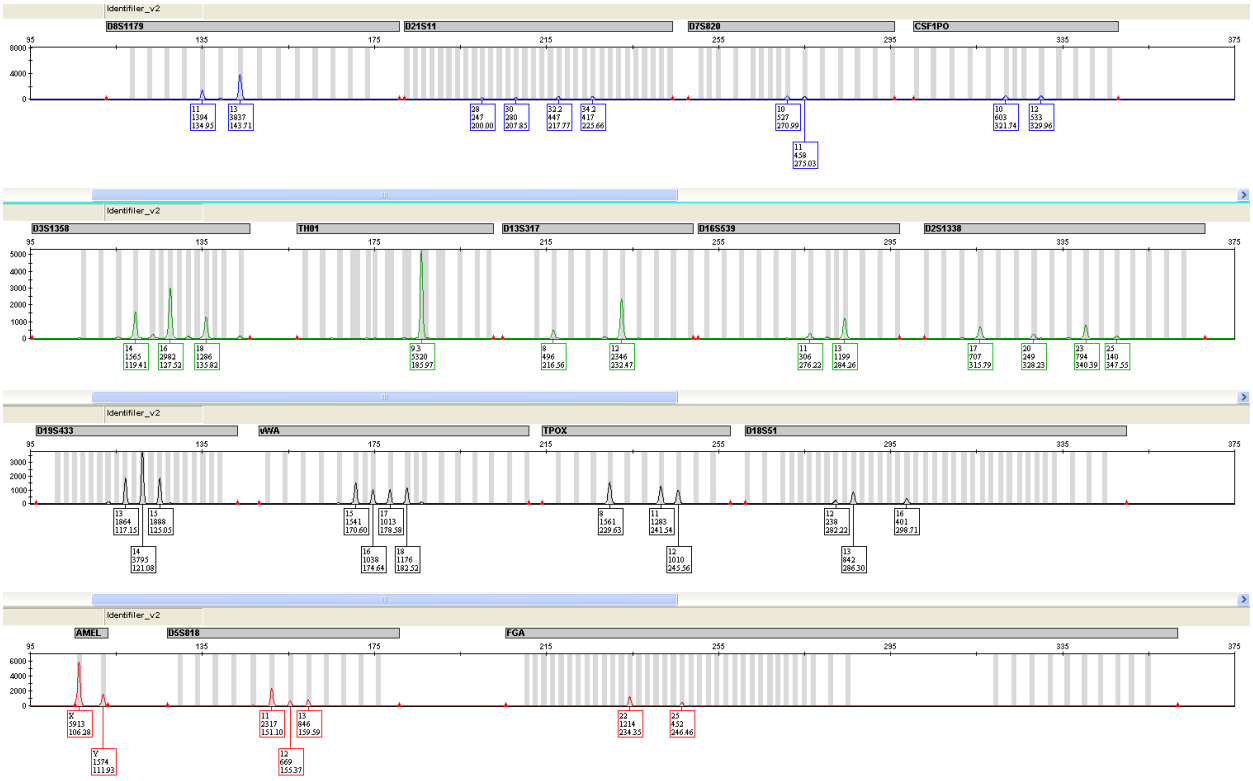

FIGURE 4 – Representative Identifiler™ electropherogram of unseparated semen-vaginal mixture samples that was prepared by combining 10 µL 1:40 semen with 10 µL vaginal cell eluate eluted from half of a swab in 200 µL PBS. On average, the male:female STR peak height ratio was approximately 1.25:1 when taking into account loci that had no shared alleles. Overall, this sample preparation method produced male:female STR ratios of ~1:1 (n = 3).

Unfortunately, implementation of this sample preparation method with antibody-mediated cell separation and DNA liberation via *forensic*GEM™ Sperm resulted in DNA yields lower than expected and incomplete STR profiles despite apparent adequate DNA input. Subsequent investigation into this issue determined there was an inhibitor(s) in the assay that impacted STR amplification, the effects of which may have been exacerbated by lower cellular input and therefore fewer sample dilutions prior to downstream processes. Buccal samples (prepared using the same elution method as previously described for vaginal swabs) were spiked with individual components of the *forensic*GEM™ Sperm and antibody-mediated cell separation assays in an attempt to identify the culprit of the inhibition. Buccal samples extracted using the QIAamp® DNA

Investigator kit (QIAGEN) served as controls. Results revealed that the polystyrene beads and PH-20 antibodies used for the sexual assault separation chemistry did not inhibit STR amplification, which was important given these are critical to the assay; however, the individual presence of AKAP3 antibodies, DTT, *Acrosolv*, and *forensic*GEM EA1 enzyme resulted in STR profiles that were either severely inhibited or absent altogether. The inhibition mechanism of AKAP3 antibodies was not further explored, but the stock concentration was lower than that of the PH-20 antibodies, meaning the original solution (and thus the potential inhibitor) was not as diluted when added to samples. When a secondary purification procedure was performed on all inhibited samples (via Centri-Sep columns from Prince Separations, Adelphia, NJ), full STR profiles were resolved. Further, the addition of a 95°C step at the end of DNA liberation ameliorated some of the inhibition. Re-evaluation of samples with an aliquot of freshly autoclaved 18 mΩ water improved results for samples containing the *forensic*GEM™ enzyme, revealing it should not normally cause inhibition and indicating its extreme sensitivity to water quality. It should be noted that initial studies with *forensic*GEM™ Sperm did not produce these complications, but it was believed that reducing cellular input caused samples to be diluted less (and sometimes even concentrated), increasing the potential for inhibition. Although *forensic*GEM™ Sperm adequately lyses spermatozoa and newer STR amplification kits such as PowerPlex® Fusion 5C have shown the potential to overcome its inhibitory nature, it is necessary to investigate other avenues for cell lysis to avoid the need for sample purification; this is especially critical for transition of this assay into a microdevice platform, where adding subsequent purification steps to remove inhibitors can severely complicate the device. Development of a non-proprietary alternative cell lysis or DNA liberation technique would also benefit the eventual commercialization of the sexual assault microdevice platform.

SUMMARY

The inefficiency of sexual assault sample processing continues to plague the forensic DNA community. Although backlogs have largely been tackled across the United States and many technological advancements have made the forensic DNA analysis workflow quicker, techniques for handling sexual assault samples remain time-consuming, tedious, and inefficient at separating cellular fractions from the victim and perpetrator – ultimately hindering the ability of forensic analysts to keep pace and consistently resolve cases. In order to overcome these limitations, a more efficient technique for separating non-sperm and sperm contributions within sexual assault samples is needed. Alternative, direct-to-PCR cell lysis techniques could reduce sample processing times associated costs, as well as potential DNA loss, in comparison to current methodology. Further, utilization of antibodies to specifically target and pull sperm cells away from the rest of the sample has shown promise throughout the literature, but studies which assess forensically relevant samples and small volumes is sorely needed. Automation of such techniques on platforms, such as centrifugal microdevices, would additionally reduce cost, time, and variability – providing the forensic DNA community with a more efficient means of processing sexual assault samples.

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CHAPTER TWO:

EVALUATION OF ALTERNATIVE SPERM LYSIS TECHNIQUES FOR DIRECT-TO-PCR SEXUAL ASSAULT SAMPLE PROCESSING: IMPACT(S) ON DNA QUANTIFICATION AND STR PROFILING

This chapter was published as two articles in the Journal of Forensic Sciences (2020 and 2022):

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Schellhammer SK, Hudson BC, Cox JO, Dawson Green T. Alternative direct-to-amplification sperm cell lysis techniques for sexual assault sample processing. J Forensic Sci. 2022;00:1–11. https://doi.org/10.1111/1556-4029.15027

ABSTRACT

The prevalence of sexual assault cases and increasing sensitivity of DNA analysis methods have resulted in sexual assault kit backlogs in the United States. Although a traditional DNA extraction and purification utilizing detergents, proteinase K, and DTT has been the primary technique for lysing sperm cell fractions from these samples, it is labor-intensive and inefficient regarding time and sperm DNA recovery. Further, the forensic DNA community has recently explored Y-screening, direct amplification, and direct cell lysis assays that omit purification but employ reducing agents to lyse spermatozoa. Thus, this study examined the impact of residual DTT on downstream processes involving fluorescent dyes to see if it must be removed prior to downstream processes. In addition, seven alternative sperm cell lysis techniques which avoided DTT were evaluated to identify a method that could efficiently lyse sperm and consistently generate high quality profiles in reduced time, labor, and cost. When DTT remained in extracts, DNA yields of multiple targets were artificially increased and the estimated male:female DNA ratios were consequently impacted, regardless of the kit utilized. DNA quantification also demonstrated all alternative methods performed comparably to the control method of *forensic*GEM™ Sperm ($p >$ 0.06), while STR profile analysis revealed that unpurified lysates from Casework Direct, alkaline, and NP-40 techniques produced DNA profiles with acceptable mean STR peak heights and interlocus balance. Ultimately, based on the data reported herein, alkaline lysis is the recommended alternative sperm lysis approach given its ability to generate high quality profiles, save time, and decrease the cost per reaction when compared to traditional sperm cell lysis methods.

KEYWORDS: forensic genetics, sexual assault, spermatozoa, dithiothreitol (DTT), quantification, polymerase chain reaction (PCR), direct amplification, fluorescent dyes, cell lysis, alkaline, differential lysis

INTRODUCTION

Modern advances in DNA technology have led to more efficient processing of forensic samples with highly discriminatory results [1–4]. However, unfortunately, sexual assaults are still committed at a rate with which forensic scientists are unable to keep pace – approximately 430,000 victims of rape and sexual assault each year on average in the United States [5,6]. Due to both the abundance of sexual assaults and the time required to process their associated samples in forensic laboratories, backlogs remain a persistent issue. Even further, although sexual assault evidence collection kit backlogs have largely been tackled over the past few years, legislation requiring the submission and testing of all collected kits is likely generating future backlogs [7].

The most common form of probative biological evidence encountered in sexual assault cases is semen. Semen contains spermatozoa, which are morphologically different from somatic cells; sperm cell heads possess a plasma membrane, acrosome, and nuclear cap to protect the nucleus, as well as a midpiece and tail [8]. Moreover, protamines replace 85% of the histones around sperm DNA and form many disulfide bonds that enable tight coiling, reducing the sperm cell nucleus to anywhere from 1/7-1/20th that of a somatic cell [9–12].

In addition to spermatozoa, vaginal epithelial cells are also commonly encountered in sexual assault samples. Due to the nature of how these samples are deposited and the sites from which they are collected, there is often an overwhelming number of (epithelial) cells from the female contributor compared to those from the male contributor in sexual assault evidence. This frequently results in an imbalanced mixture DNA profile and/or a masked male DNA profile [13]. However, the secondary DNA contributor in an STR profile is often undetectable when using

traditional capillary electrophoresis if present at a level ≤ 1/10th that of the primary DNA contributor, a situation which is often encountered in sexual assault samples due to their intimate nature [13–15]. To circumvent this and other general issues experienced with mixture samples, a differential cell lysis is typically performed for sexual assault evidence as a way to physically separate sperm from epithelial cells. With these methods, differences in both morphology and susceptibility to lysis reagents are exploited in order to enrich for the male contributor and prevent downstream complicated mixture profiles [16,17]. Given the increased sensitivity of forensic DNA kits and the fact that a single spermatozoon contains 3.3 picograms (pg) of DNA (which is half the amount within diploid epithelial cells), full DNA profiles can now be obtained from as few as 50 sperm cells [18,19]; this makes the ability to both retain as many sperm cells as possible (without contaminating non-sperm cells) *and* efficiently lyse all of them very crucial for male DNA profile generation.

The difference in DNA packaging and morphology of sperm versus non-sperm cells necessitates strong reducing agents for cell lysis and subsequent access to sperm DNA. Because it reduces the disulfide bonds present in both sperm heads and sperm-specific protamines that bind and tightly coil the sperm DNA, dithiothreitol (DTT) is typically employed for cell lysis and DNA extraction from the sperm fraction of semen-containing sexual assault samples [11,17,20]. Unfortunately, the strong reducing power of DTT is not limited to the disulfide bonds within sperm cell heads; it also has the potential to interact with many components commonly found in forensic biology protocols, even after it has reduced sperm heads and subsequently become oxidized.

While traditional sperm cell fraction lysis methods which use DTT are mostly viable, the techniques are ultimately inefficient, laborious, time-consuming, and often require multiple tube-to-tube transfers [16,17,21–23]. Thus, a cell lysis method that considerably decreases processing time while reducing the risk for contamination and sample loss is needed so that labs can process samples of this nature more efficiently and effectively, potentially providing a step towards reduction of the existing sexual assault backlogs. Since a significant portion of DNA can be lost during traditional extraction and purification [13,24–26], it may be beneficial for laboratories to move towards direct amplification – a method that adds the sample lysate directly to a multiplex STR amplification reaction, skipping the DNA extraction, purification, and quantification steps – for streamlining the workflows of both low template samples and evidence from higher volume crimes [27]. Because this method bypasses quantification, many labs have only adopted it for processing of reference samples, which are exempt from the FBI Quality Assurance Standard 9.4 requiring human DNA quantification [28]. However, direct amplification has recently been explored in the literature for rapid screening of some forensic evidence samples from higher volume crimes, such as those seen in sexual assault cases [27,29]. Several prominent studies have even developed and tested microdevices for processing sexual assault samples in an effort to increase fractional separation efficiency and reduce time [30–34]. The goal of maintaining simplicity with these microdevices usually manifests in the implementation of a direct cell lysis assay, rather than conventional DNA extraction and purification [35–37]. Regardless of the approach, processing of these samples always requires the addition of DTT or another reducing agent to sufficiently lyse any sperm cells present. Because there is no wash step, this results in carryover of these chemicals to downstream processes in the workflow – necessitating the need to evaluate the potential effect(s) on downstream analyses.

The issues outlined above have also spurred an influx of research on the use of alternate cell lysis techniques which avoid DTT altogether [11,23,38–45] – including those that may be amenable to quantitative and end-point PCR *without* subsequent purification [29,46]. Such methods encompass alkaline-based cell lysis and the use of nonyl phenoxypolyethoxylethanol (NP-40) cell lysis buffer, both of which are non-proprietary chemical approaches that have been reported to disrupt cell membranes from a variety of cell types, including sperm cells [43,47–51]. Alkaline lysis solutions exert a strong denaturing effect on proteins and are an efficient means of protein solubilization due to the ionization of certain amino acids. This technique utilizes sodium hydroxide (NaOH) to disrupt plasma membranes, denature nucleases, and preserve the DNA, while the subsequent addition of Tris-HCl neutralizes the lysate and enhances stability [47,48,52]. On the other hand, NP-40 cell lysis buffer is a mild, non-ionic detergent commonly used for DNA extraction and purification in other, non-forensic applications [53,54]. NP-40 has also been successfully used for direct amplification of crude blood samples and is believed to have potential for various sample types (including semen) [51].

Alternatively, there are proprietary, commercially available direct-to-PCR kits that can be used to lyse cells commonly associated with sexual assault samples without disrupting downstream PCR processes. For example, Promega's™ Casework Direct utilizes 1-thioglycerol in place of DTT for successful lysis of sperm cells. This kit has produced reliable profiles from a variety of forensically relevant samples and has already been implemented in forensic science for Y-screening and autosomal STR profiling [55–58]. Another proprietary reagent from Promega™ that has been investigated for direct-to-PCR amplification of semen samples is SwabSolution™. This lysis reagent requires only a single incubation step, has zero tube transfers, and has been used to reliably

produce high quality STR profiles in previous studies [27,29]. There are also enzymatic lysis kits which are marketed for direct-to-PCR applications. More specifically, microGEM™ produces two kits (prepGEM™ and *forensic*GEM™) which utilize the thermostable enzyme EA1 to degrade proteins and lyse non-sperm cells [59–61]. Further, the company's *forensic*GEM™ Sperm kit can be used to lyse sperm with the addition of an enzyme cocktail called *Acrosolv*, which has a lower optimum temperature than EA1 and can subsequently be degraded by it [60]. Kits such as these are promising avenues for alternative, direct-to-PCR lysis within forensics because they occur in a single tube, only require short incubation steps, and degrade proteins which typically interfere with downstream processes.

In addition to the non-proprietary and commercial cell lysis techniques above, natural sperm decondensation approaches could potentially be used to expose the DNA within semen samples. Published studies on intracytoplasmic sperm injection (ICSI) employing this approach have revealed the use of Triton X-100 (TX), glutathione (GSH), HEPES buffer, and heparin for sperm cell decondensation and the removal of acrosomal caps in such a way that resembles the natural fertilization process [62–65]. Although there has yet to be any research on the implementation of these reagents for extracting DNA from spermatozoa for forensic applications, a combination of these reagents could allow for the plasma and acrosomal membranes to be easily removed, thereby permitting the sperm nucleus to be decondensed and the nuclear material to be quickly released.

Ultimately, the development of alternative cell lysis techniques which omit purification could prove beneficial in tackling the current sexual assault case backlog by saving both time and costs. Thus, this study was completed in two parts: Initially, the impact(s) of DTT on quantification

results from commercial qPCR kits frequently used in forensic casework were evaluated to see whether this chemical could be implemented for more direct-to-PCR applications without adverse effects. Subsequently – in an effort to identify a faster, inexpensive, more efficient process for sperm cell lysis that could be easily implemented into the current forensic DNA workflow – several non-traditional cell lysis techniques were evaluated and compared to identify the best performing method based on DNA yields, quality of resulting STR profiles, as well as cost and time requirements.

MATERIALS & METHODS

Sample Collection and Preparation

For the DTT study, semen samples from five donors were collected in accordance with the university-approved Institutional Review Board (IRB) protocol HM20002942 and were diluted by volume 1:2 in 1X phosphate-buffered saline (PBS) (pH 7.4) (Quality Biological; Gaithersburg, MD).

For evaluation of the seven alternative lysis approaches, semen was collected from ten anonymous donors in accordance with the university-approved IRB protocol HM20002931 and was diluted 1:10 by volume in 1X PBS. Fisherbrand™ PurSwab foam swabs (Fisher Scientific; Hampton, NH) were dipped into the semen dilutions (absorbing approximately 80 µL) and were allowed to dry overnight at room temperature; multiple dilutions and swabs were prepared per donor to accommodate all methods and replicates tested. Once dry, the swabs were cut into twelfths and stored at 4°C. Subsequent testing for all cell lysis methods utilized $1/12^{th}$ of a foam swab (equivalent to approximately 0.67 µL of neat semen) for each donor in triplicate.

Cell Lysis and DNA Liberation

QIAamp® DNA Investigator kit

First, to generate control extracts for the DTT study in which DTT was not present, 5.0 µl of each 1:2 semen sample was processed in duplicate using the QIAamp® DNA Investigator kit (QIAGEN, Hilden, Germany) and following the manufacturer recommended protocol for sexual assault samples, but omitting the portion for lysis and removal of the epithelial fraction [66]. The maximum recommended elution volume of 50 µL was used. Lysates were stored at 4°C until further processing.

*prep*GEM™ + DTT

A modified version of the *prep*GEM™ Saliva kit (microGEM™, Charlottesville, VA) was used to provide DNA samples containing DTT. One microliter *prep*GEM™ enzyme, 10.0 µL 10X Blue Buffer, and 9.0 µL 1.0 M DTT were added to 5.0 µL of the 1:2 semen sample, and the reaction was brought up to 100 µL with HyPure Molecular Biology Grade (MBG) Water (GE Healthcare Life Sciences; Marlborough, MA). Reactions were heated at 75°C for five minutes on a ProFlex™ PCR Dual 96-well PCR system (ProFlex™; Thermo Fisher Scientific, Waltham, MA), and resulting lysates were stored at 4°C until further processing.

*forensic*GEM™ Sperm

The *forensic*GEM™ Sperm kit (microGEM™) served as the control direct-to-amplification cell lysis method for the alternative lysis techniques evaluation. For this method, 2.0 µL *forensic*GEM™ enzyme, 10 µL Acrosolv, and 10 µL 10x Orange+ Buffer were added to each semen sample. The reactions were brought up to 100 µL with HyPure MBG water, placed onto the

ProFlex™, and incubated as follows: 52°C for ten minutes, 75°C for three minutes, and 95°C for three minutes.

SwabSolution™

Modified versions of the SwabSolution™ and proteinase K method described by Tobe et al. were assessed in this study to determine the appropriate incubation time for optimal lysis [29]. Samples were incubated in 23 µL of SwabSolution™ (Promega™) and 2.0 µL Proteinase K (Thermo Fisher Scientific; Waltham, MA) at 70°C for either 15, 30, or 60 minutes [29,67]. Lysates were stored at 4°C until further processing. The best performing reaction time was used in all subsequent tests.

Casework Direct System

Modified versions of the manufacturer recommended protocol for the Casework Direct System (Promega™) were tested in an attempt to reduce the total reaction volume. Semen swab cuttings were incubated for 30 minutes at 70°C in either 25, 50, or 100 µL of Casework Direct solution (Promega™) containing 0.125 µL, 0.25 µL, and 0.5 µL 1-thioglycercol (Promega™), respectively [55]. After incubation, swabs were placed in a spin basket and centrifuged for five minutes at 10,000 *x g* to maximize liquid recovery. Lysates were stored at 4°C until further processing. The best performing reaction condition was used in all subsequent tests.

NP-40 Cell Lysis Buffer

Three different strengths of NP-40 cell lysis buffer (Thermo Fisher Scientific) were evaluated to ensure optimal cell lysis while also minimizing PCR inhibition. Semen swab cuttings were submerged in 25 µL of either 1%, 0.75%, or 0.5% NP-40 lysis buffer (diluted in MBG water). All

reactions were incubated on ice for 30 minutes with vortexing every ten minutes, as recommended by the manufacturer protocol [68]. Swabs were transferred to spin baskets and centrifuged at 13,000 *x g* for ten minutes. Lysates were stored at -20°C until further processing. The best performing reaction strength was used in all subsequent tests.

Alkaline Lysis

Semen-soaked swab cuttings were incubated in 16 µL of 1X PBS and 4.0 µL of 1M NaOH (Thermo Fisher Scientific) at 75°C for five minutes [47,48]. Following incubation, 4.0 µL of 1M Tris-HCl (Invitrogen; Carlsbad, CA) were added to neutralize the lysate and the samples were briefly vortexed. The swabs were then transferred to a spin basket and centrifuged for five minutes at 13,000 *x g*. Lysates were stored at -20°C until further processing.

HEPES Buffer + Triton X-100 (HTX)

In an attempt to mimic the female body's approach for sperm cell decondensation and lysis, semen samples were subjected to two different "natural sperm decondensation" assays. The first assay utilized a stock solution of HEPES buffer (Sigma Aldrich; St. Louis, MO) containing 0.04% Triton X-100 (Thermo Fisher Scientific). For this approach, semen samples were submerged in 25 µL of HEPES/Triton X-100 (HTX) solution and vortexed for one minute [62]. Swab cuttings were then placed in spin baskets and centrifuged for three minutes at 17,000 *x g*. Lysates were stored at 4°C in foil until further processing to prevent reactive-oxygen species formation [69].

Modified HTF Medium + Glutathione + Heparin (HGH)

The second "natural sperm decondensation" assay consisted of a stock solution of modified HTF medium (Irvine Scientific; Santa Ana, CA) containing 10 mmol/L glutathione (Sigma Aldrich) and 46 μmol/L heparin (Sigma Aldrich) [65,70]. Semen-soaked swab cuttings were incubated in 25 μL of the HTF/glutathione/heparin (HGH) solution at 37°C for either 15, 30, or 60 minutes [65,70]. Lysates were stored at 4°C until further processing. The best performing reaction time was used in all subsequent tests.

Microscopy

Microscopic analysis was performed on all samples evaluated with the alternative lysis approaches. Prior to and following cell lysis, Kernechtrot Picroindigocarmine Stain (KPICS) was applied to each sample to visually gauge the effectiveness of each method. For this, two microliters of pre- and post-lysis sample were spotted onto a microscope slide and stained with one drop of Kernechtrot stain (Serological Research Institute; Richmond, CA) and one drop of Picroindigocarmine stain (Serological Research Institute). Sperm cells were then visualized under a Micromaster microscope (Thermo Fisher Scientific) using 400x magnification. Each sperm slide was scored using a 0 – 4+ scale with "0" indicating that no sperm were identified, "1+" indicating there was a single spermatozoon observed in some fields, "2+" indicating 1-5 sperm were observed in most fields, "3+" indicating 5-10 sperm were observed in most fields, and "4+" meaning more than 10 sperm were observed in all fields; for each sample, ten different fields-of-view were scored to produce a mean score.

DNA Quantification

In order to evaluate the effect(s) of DTT on real-time PCR (qPCR), as well as the total amount of DNA obtained from semen samples after each alternative lysis method, all resulting lysates were quantified on an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific).

Investigator® Quantiplex HYres

All lysates were quantified with the Investigator® Quantiplex HYres Kit (QIAGEN) and 7500 System SDS version 1.4 (Thermo Fisher Scientific) following manufacturer recommendations, with modifications for half-volume reactions [71]. This included 5.75 µL Reaction Mix FQ, 5.75 µL Primer Mix IC FQ, and 1.0 µL template DNA per sample. However, given that SwabSolution™ and Casework Direct kit components are known to inhibit real-time PCR (qPCR), an additional 2.0 µL of 5X AmpSolution™ (Promega™) were added to these lysate groups (and corresponding standards) to ensure accurate results [55,67]. Additionally, for the DTT study, a separate set of DNA standards was spiked with the appropriate volume of 1M DTT to produce a final concentration of 0.09 M DTT (which was present in the modified *prep*GEM™ samples); each standard was quantified in duplicate. An automatic threshold and baseline were used to analyze the results for all targets.

Prior to analysis of DNA quantity, qualitative metrics for amplification and component plots were assessed to identify any potential signs of inhibition. Amplification plots were examined for a sigmoidal shape consisting of geometric, linear, and plateau phases, with the plots crossing the cycle threshold (C_q) during the exponential phase; the C_q for samples typically falls between 20 – 30, while the acceptable C_q for the internal positive control (IPC) is between 27 – 31.

Multicomponent plots were examined for any deviation from the expected fluorescent signals of the three amplification targets, which should remain flat for the first 15 – 20 cycles prior to exponential growth of the PCR product. Additionally, the passive reference dye signal was examined for any deviation from a flat, consistent curve throughout the entirety of the assay.

Total DNA yields were calculated by multiplying the appropriate target's concentration by the elution/sample volume. For the DTT evaluation, a Mann-Whitney U test was performed to compare the DNA yields for each target from modified *prep*GEM™ samples to those from QIAamp® DNA Investigator samples ($\alpha = 0.05$). Since the sperm count and therefore DNA yield in semen is highly variable, the median and range DNA yield (rather than average and standard deviation) for each target were also calculated for modified *prep*GEM™ and QIAamp® DNA Investigator samples; this evaluation was deemed more appropriate when considering the number of donors and replicates analyzed for this particular study. Male:human DNA ratios were calculated by dividing the Y-target quantity by the human target quantity for each individual sample.

For the alternative lysis technique evaluation, which assessed more semen donors and replicates compared to the DTT study, the mean and standard deviation for each experimental group were calculated and compared. If a potential outlier was observed, the Grubb's outlier test was performed by subtracting the mean from the suspected outlier value and dividing by the standard deviation. If the G_{test} was greater than the $G_{critical}$, the outlier was confirmed and removed. For the lysis methods that had multiple conditions tested (e.g., SwabSolution™, Casework Direct, NP-40, and HGH), an ANOVA was performed to compare the DNA yields of the control method

(*forensic*GEM™ Sperm) to the three conditions tested of that given method ($\alpha = 0.05$). Any significant differences were further identified using a Tukey HSD test in order to establish which condition(s) to select for downstream analysis. Once the best performing conditions were identified, an ANOVA was then performed to compare all cell lysis methods to one another, and any significant differences were further identified using a Tukey HSD test.

Quantifiler™ Trio

Lysates containing residual DTT were also evaluated using the Quantifiler™ Trio DNA Quantification kit (Thermo Fisher Scientific) and the HID Real-Time PCR Analysis Software version 1.2 (Thermo Fisher Scientific) following manufacturer recommendations, but modified for half-volume reactions [72]. Thus, the reactions included 4.0 µL Quantifiler® Trio Primer Mix, 5.0 µL Quantifiler® THP PCR Reaction Mix, and 2.0 µL template DNA per sample. Again, a separate set of DNA standards was spiked with 1M DTT following the same method as described above for Investigator® Quantiplex HYres, keeping the final concentration of DTT at 0.09 M. Due to the large normalized reporter signals (ΔR_n) for samples containing DTT, the software had to extrapolate curves that never crossed the C_q in order to produce an estimated DNA quantity. In the rare event that a quantity was unable to be determined, that sample was not factored into the data. An automatic threshold and baseline were used to analyze the results for all targets. Total DNA yields were calculated by multiplying the appropriate target's concentration by the elution/sample volume. Additionally, amplification and multicomponent plots were examined as described above for Investigator® Quantiplex HYres.

For data from Quantifiler™ Trio, a Mann-Whitney U test was performed to compare the DNA yields for each of the three targets from modified *prep*GEM™ samples to those from QIAamp® DNA Investigator samples ($\alpha = 0.05$); this approach followed the same reasoning as described above for the DTT study samples quantified with Investigator® Quantiplex HYres. For each individual sample, a percent increase in DNA yield was reported for the samples lysed with modified *prep*GEM™ versus those extracted with QIAamp® DNA Investigator. This was calculated by dividing the difference between the *prep*GEM™ and QIAamp® DNA Investigator yields by the QIAamp® DNA Investigator yield.

STR Amplification

Two samples containing residual DTT and all lysates from the alternative lysis approaches were amplified using the Promega™ PowerPlex® Fusion 5C System with a template DNA input of 0.25 ng and following manufacturer recommendations, but with half-volume reactions [73]. Thus, each reaction consisted of 2.5 µL sample (at 0.1 ng/µL), 2.5 µL PowerPlex® Fusion 5x Master Mix, 2.5 µL PowerPlex® Fusion 5x Primer Pair Mix, and 5.0 µL amplification-grade water. Amplification was carried out using the ProFlex™ and the following parameters: 96°C for one minute, 30 cycles (94°C for ten seconds; 59°C for one minute; 72°C for 30 seconds), and a 60°C hold for 45 minutes.

Capillary Electrophoresis and Data Analysis

STR amplicons were separated on an ABI® 3130 Genetic Analyzer (Thermo Fisher Scientific) using Data Collection software v3.1 (Thermo Fisher Scientific). One microliter of amplified sample or allelic ladder was added to 0.3 µL WEN ILS 500 (Promega™) and 9.7 µL Hi-Di™ Formamide (Thermo Fisher Scientific), except for samples lysed using the SwabSolution™ and

Casework Direct methods (for which 0.5 µL of each sample was added to 0.3 µL WEN ILS 500 and 10.2 µL Hi-Di™ Formamide). Injection parameters followed manufacturer recommendations and included a 36 cm capillary array (Thermo Fisher Scientific), POP-4® polymer (Thermo Fisher Scientific), and a 3kV 5s injection. Results were analyzed with GeneMapper™ software v4.1 (Thermo Fisher Scientific) following manufacturer recommendations and an analytical threshold of 100 RFU [74].

Profiles were qualitatively and quantitatively evaluated for any signs of inhibition (e.g., interlocus imbalance and allelic dropout). Mean peak heights were calculated by finding the average peak height of all observed STR alleles across all sample profiles obtained for each lysis method. To account for homozygosity, the peak heights for homozygous alleles were halved to represent each of the assumed two copies of the allele at that locus. Based on our laboratory's internal validation for PowerPlex® Fusion 5C, the ideal mean peak height for the target input was expected to be ~1645 RFU (B. Hudson, personal communication, Nov. 2020). The coefficient of variation (CV) for locus peak height:total peak height (LPH:TPH) ratios for each locus of the entire DNA profile was calculated to estimate interlocus balance, excluding the Amelogenin and DYS391 loci (since one is not an STR locus and the other is a single-copy locus, respectively). The CV was calculated by first determining the LPH:TPH ratio by dividing the sum of a locus's peak height by the sum of the peak heights at all loci; this was repeated for each locus. The standard deviation of all LPH:TPH ratios was then calculated and divided by the mean of those ratios to calculate the CV. Based on the available literature, ideal CV values when using this method are ≤ 0.35, which signifies the peak heights at any given locus vary no more than 35% from peak heights at other loci within the same DNA profile [75]. For the mean peak heights, the mean percentage of STR

alleles detected, and the mean CV of LPH:TPH, an ANOVA was performed to compare the results of the control method to the six additional lysis methods evaluated ($\alpha = 0.05$). If the ANOVA resulted in a $p < 0.05$, a Tukey HSD test was performed to identify where the significant differences occurred.

RESULTS & DISCUSSION

Evaluation of Residual DTT

Investigator® Quantiplex HYres

For the samples with DTT quantified using Investigator® Quantiplex HYres, all component plots (Figure 1A) of the male target dye (cyanine 5 or Cy5) exhibited a steadily increasing signal. The other target dyes showed the expected sigmoidal signal curves, while the passive reference dye (ROX) showed a characteristic flat curve. The amplification plots (Figure 1B) for all samples with DTT revealed a corresponding steep amplification curve for the male target (Cy5 dye) with C_q of 2.9 – 3.3 for experimental samples ($C_q = 3.3$ for the reagent blank), while the human and IPC targets crossed at values consistent with uninhibited samples ($C_q \sim 23 – 27$). These very low C_q values for the male target resulted in statistically higher calculated male quantities for all samples containing DTT compared to those without DTT ($p = 0.00056$, Table 1). On the other hand, a Mann-Whitney U test comparing the modified *prep*GEM™ and QIAamp® DNA Investigator samples revealed no significant differences in the true mean human DNA yield ($p = 0.73$), demonstrating the impact on reported male DNA yields was likely due to residual DTT rather than DNA extraction method (Table 1). The male:human DNA ratio was calculated for all samples with and without DTT and then averaged to better display the impact of these results on the estimation of male DNA in a sample (Table 1). The presence of DTT within a sample lysate used for

quantification with Investigator® Quantiplex HYres resulted in a greater than 150,000,000-fold increase in the male:human ratio on average (which is theoretically impossible). Although not a true reproducibility study, other research using the modified *prep*GEM™ method within our laboratory has shown similar results (data not shown).

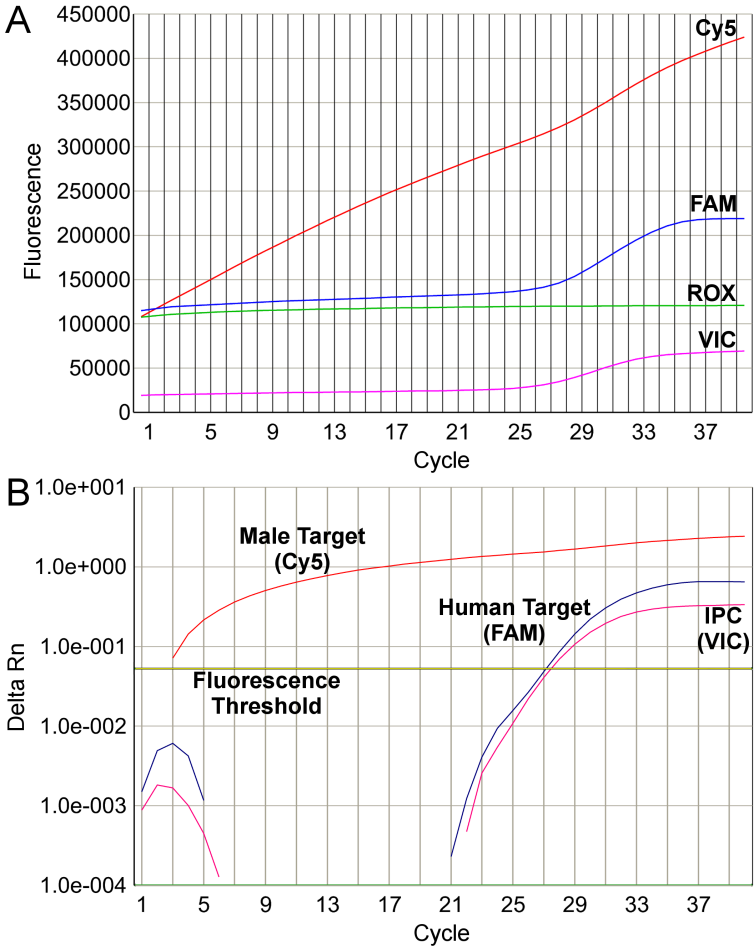

FIGURE 1 – Representative Investigator® Quantiplex HYres component (A) and amplification (B) plots for a sample containing DTT. With DTT present in the sample, the Cy5 component (red) demonstrated an atypical line rather than the expected curve (A), and the amplification curve (B) crossed the threshold after 2–4 cycles compared to the other targets crossing at ~27 cycles.

TABLE 1. Comparison of Investigator® Quantiplex HYres quantification results of DNA samples with or without DTT

DNA Preparation Method (n = 5)	Male DNA Yield (ng)*		Human DNA Yield (ng)†		Average Male:Human DNA Ratio
	Median	Range	Median	Range	
QIAamp® DNA Investigator (DTT filtered out)	128.25	24.90, 765.0	127.8	25.95, 696.5	1.13 : 1
***prep*GEM™ (DTT present)**	8.055e8	7.740e8, 9.260e8	86.60	21.80, 812.0	3.91e6 : 1

*$p = 0.00056$

†$p = 0.73$

All comparisons are between QIAamp® DNA Investigator and *prep*GEM™

To confirm the above observations were due to the presence of DTT rather than other differences in the DNA extraction methods used, a set of DNA standards was spiked with DTT and subjected to DNA quantification with Investigator® Quantiplex HYres. Average human target estimated quantities were close to the expected values for each DNA standard, while the average male quantity was estimated at approximately 8,000,000 ng/µL (average C_q = 3.2) regardless of the standard concentration (Table 2). Amplification and multicomponent plots exhibited the same trends as those from DNA samples extracted using the modified *prep*GEM™ method. Therefore, since DTT clearly confounds the results of the Investigator® Quantiplex HYres assay, Y-screening methods should not be used in conjunction with this kit when DTT remains in the sample. In order to use quantification for Y-screening, DNA quantification kits must be able to accurately quantify both the male and total human DNA within a sample.

TABLE 2. Investigator® Quantiplex HYres quantification results for DNA standards spiked with DTT

DNA Standard (n = 2)	Expected DNA Concentration (ng/µL)	Average DNA Concentration (ng/µL)	
		Human	Male
Standard 1	20.00	17.1100 ± 1.4284	$9.56e^6 \pm 7.71e^5$
Standard 2	5.000	5.0750 ± 0.5162	$8.16e^6 \pm 6.22e^5$
Standard 3	1.250	1.1600 ± 0.1697	$7.61e^6 \pm 5.09e^5$
Standard 4	0.3125	0.2520 ± 0.0170	$7.79e^6 \pm 5.59e^5$
Standard 5	0.0781	0.0649 ± 0.0068	$8.19e^6 \pm 7.64e^5$
Standard 6	0.0195	0.0160 ± 0.0018	$7.94e^6 \pm 1.18e^6$
Standard 7	0.0049	0.0048 ± 0.0002	$7.06e^6 \pm 3.04e^5$

Our results suggest that DTT was interacting with the Cy5 dye, as only the male target for Investigator® Quantiplex HYres was affected. An increase in fluorescent signal in the Cy5 channel indicated that DTT either breaks the Cy5 reporter away from the Scorpions® primer or degrades the quencher dye, both of which would disrupt Förster resonance energy transfer (FRET) and generate more fluorescence. While the ability of strong thiols (e.g., glutathione and DTT) to reduce and degrade azobenzenes (i.e., black hole quencher dyes or non-fluorescent quencher dyes) has been reported in the literature, that was unlikely the case here because other fluorescent dyes accompanied by the same quenchers were not impacted [76–78]. Thus, it is apparent the interaction between DTT and Cy5 is more likely, which would not be surprising, as studies in nanotechnology and molecular imaging have used DTT to specifically displace some cyanine dyes from nanoparticle surfaces [79]. In fact, an evaluation of the effects of strong reducing agents on cyanine dyes demonstrated that DTT causes Cy5 to have a shorter initial lifetime, increased frequency of blinking, and longer off-blink events [80]. Because fluorescence lifetime is the average time before a dye emits a photon after absorbing one, this translates to a quicker oncoming and shorter-lived fluorescent signal [81]. Our results demonstrated maximized fluorescence after the first few cycles of qPCR, which persisted throughout subsequent cycles (Figure 1B). Based on this observation, it

is likely that the presence of DTT immediately caused the vast majority of Cy5 dye molecules to become liberated from the Scorpions® primers. Subsequently, the detection stage of qPCR was short enough to capture the brief initial lifetime and on-blink event of Cy5, while the following cycles were long enough for the extended off-blink caused by DTT to commence before Cy5 was excited again during the next detection stage.

Although not evaluated in this specific study, other cyanine dyes such as SYBR Green I, PicoGreen, or Quasar 670 could be impacted in the same way as Cy5. For example, Quasar 670 within the PowerQuant® System (Promega™) is a cyanine dye used to label the kit's degradation target. Implementation of a Y-screening protocol involving a direct lysis method (meaning no subsequent purification step) with DTT followed by quantification with this kit could be expected to produce a drastic increase in the fluorescent signal from this target, as has been previously observed (Sorenson Forensics, personal communication, Dec. 2018). Because Quasar 670 has essentially the same structure and serves as a direct replacement for Cy5 [81,82], it is not surprising that DTT causes a similar result in both cases. Previous reports demonstrate a comparable interaction between DTT and SYBR Green I, showing increased background fluorescence and shallower amplification curves when both were present during qPCR [83]. Because certain structures and interactions promote fluorescence [84,85], DTT likely would have a similar effect on the PicoGreen dye, as it has similar function and structure (only differing by one R group) to SYBR Green I [86].

Quantifiler™ Trio

In addition to the aforementioned cyanine dyes, other fluorescent dyes were also impacted by DTT. The ability of DTT to cause the Mustang Purple® passive reference dye within the Quantifiler™ HP Kit to drop to a signal of close to zero has been previously reported [87]. This present study evaluated whether the same observation held true with the newer Quantifiler™ Trio kit, which also utilizes the Mustang Purple® passive reference dye. After quantification of the semen DNA extracts with Quantifiler™ Trio, the mean and range DNA yield was calculated using each of the kit's targets (small autosomal, large autosomal, and Y) for samples containing DTT and samples with DTT removed, as well as the average percent increase in yield for modified *prep*GEM™ samples compared to QIAamp® DNA Investigator samples to better display an overall trend in the data (Table 3). A Mann-Whitney U test revealed that the true mean DNA yield for modified *prep*GEM™ samples was significantly higher than the true mean DNA yield for QIAamp® DNA Investigator samples for the large autosomal target ($p = 0.038$), but no significant differences were observed in the true mean DNA yield for the small autosomal and Y targets ($p = 0.214$ and $p = 0.098$, respectively) (Table 3). In addition, when DTT remained in the extract, the average DNA yield for the small autosomal target increased 1.1-fold, the average DNA yield for the large autosomal target increased greater than 9.5-fold, and the average DNA yield for the male (Y) target increased 1.3-fold when compared to samples without DTT (Table 3).

TABLE 3. Comparison of Quantifiler™ Trio quantification results of DNA samples with or without DTT

DNA Preparation Method (n = 5)	Total DNA Yield (ng)						*prep*GEM™ Yield Increase over QIAamp® Investigator		
	Small Autosomal*		Large Autosomal†		Y‡		Small Autosomal	Large Autosomal	Y
	Median	Range	Median	Range	Median	Range			
QIAamp® DNA Investigator (DTT filtered out)	167.6	41.63, 1034	170.8	46.83, 991.2	176.9	41.61, 908.3	112.2 ± 131.4%	957.5 ± 399.9%	133.3 ± 63.77%
***prep*GEM™ (DTT present)**	285.0	29.84, 1564	1213	0.000, 1531	352.2	46.62, 1767			

*$p = 0.214$
†$p = 0.038$
‡$p = 0.098$
All comparisons are between QIAamp® DNA Investigator and *prep*GEM™

This overestimation (and inaccurate quantification) of DNA for samples containing DTT correlated with a simultaneous drop in the passive reference dye (Mustang Purple® or MP) within these samples compared to the DNA samples without DTT in the same run (Figure 2), which is concordant with the results obtained in a previous study using Quantifiler™ HP [87]. To confirm the observations above were due to the presence of DTT instead of other differences in the DNA extraction methods, a set of spiked DNA standards was also evaluated and subjected to quantification using Quantifiler™ Trio. The average DNA quantities for all targets were greatly overestimated for DNA standards spiked with DTT compared to their expected values, some more than others but with no clear pattern (Table 4). In addition, these DTT-spiked DNA standards also exhibited a decreased passive reference dye signal similar to that observed for *prep*GEM™ samples (data not shown). It is posited that the interaction between DTT and the passive reference dye causes the dye to either be quenched or lose its fluorescence by some other mechanism. Since Mustang Purple® is a proprietary dye, there is no way to further explore a link between DTT and its diminished signal. Regardless of the actual mechanism, these results demonstrate the

overestimation of DNA quantity within samples containing DTT; this means that not enough DNA would be added to downstream STR amplification, causing casework results to be negatively impacted and sample processing time and costs to soar. Further, the overestimation of the male target quantity (more than the small autosomal quantity) would also yield an overestimated male:female ratio, rendering it unacceptable for Y-screening purposes.

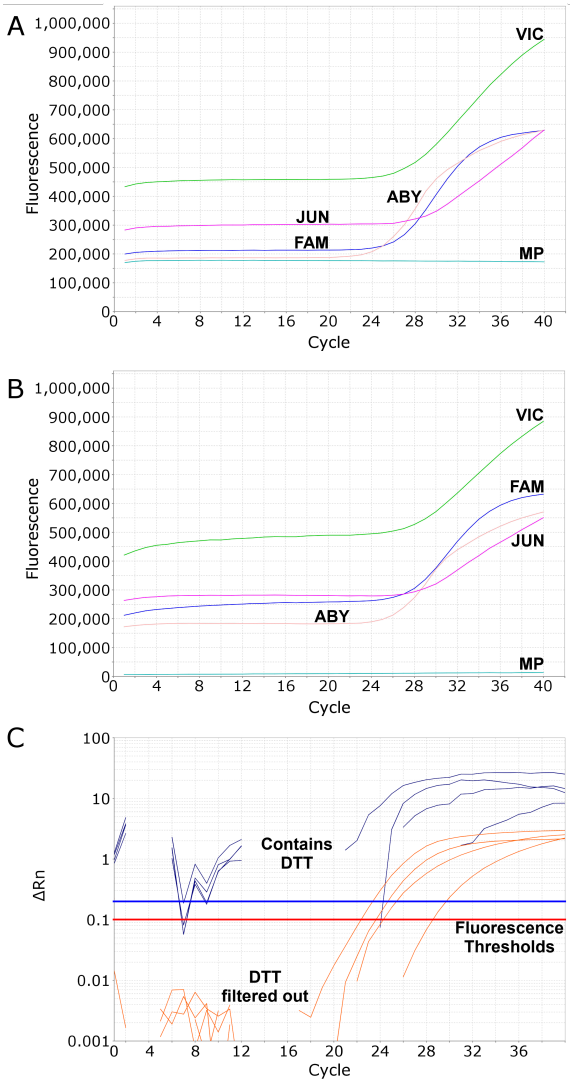

FIGURE 2 – Representative component plots for a sample with DTT filtered out (A) and a sample containing DTT (B) quantified using the Quantifiler™ Trio DNA Quantification kit, as well as an amplification plot showing one of each such sample (C). With residual DTT in the extract, a noticeable drop in the passive reference dye signal was observed compared to samples where DTT had been removed, resulting in inaccurate comparison to the unaffected standards and overestimation of DNA quantity. Samples containing DTT had higher ΔRn values for all targets, preventing the amplification curves from crossing their respective fluorescence thresholds during the exponential phase.

TABLE 4. Quantifiler™ Trio quantification results for DNA standards spiked with DTT

DNA Standard (n = 2)	Expected DNA Concentration (ng/µL)	Average DNA Concentration (ng/µL)		
		Small Autosomal	Large Autosomal	Y
Standard 1	50.00	99.75*	661.44 ± 704.05	88.79*
Standard 2	5.000	1451.09 ± 2011.06	470.11 ± 124.77	631.74 ± 866.49
Standard 3	0.5000	0.49 ± 0.13	10.28 ± 13.14	0.53 ± 0.04
Standard 4	0.0050	0.54 ± 0.73	16.71 ± 23.55	0.28 ± 0.36
Standard 5	0.0005	0.11 ± 0.14	0.46 ± 0.62	0.04 ± 0.05

*One of duplicates "undetermined," so no average produced.

While the fluorescence signal for the passive reference dye was reduced dramatically to values of ~8,000 – 11,000 RFU (as opposed to the standard 150,000 – 180,000 RFU signal), the corresponding quantification results were not necessarily affected proportionately, as one would expect them to be much larger due to the increased signal magnitude (Figure 2, Table 3). These observations stemmed from the altered shape of the amplification curves combined with extremely large ΔR_n for samples containing DTT (Figure 2C). These samples had ΔR_n values up to 100-fold higher than those without DTT due to reduced passive reference signals, resulting in the amplification curves either crossing the threshold at the onset of their fluorescence signal or not at all. Since ΔR_n is calculated by subtracting the magnitude of fluorescence generated by the passive reference dye from that of the reporter dye at each cycle, and since the threshold is automatically chosen based on the ΔR_n amplification plot, this leads to inaccurate quantities for samples whose curves do not cross during the exponential amplification phase [72]. Thus, the software was unable to accurately calculate a C_q for samples containing DTT and instead extrapolated from the curves to give seemingly normal, albeit still inaccurate, quantification values. Amplification curves for all targets in the kit are not identical, so the impact of this phenomenon depended on the specific target, as demonstrated by the exaggerated large autosomal values compared to the small autosomal values (Table 3). Regardless of the magnitude or specific target, the adverse effect of

residual DTT within DNA extracts was obvious and further demonstrated that its use in Y-screening and direct amplification methods could lead to reduced accuracy and reliability of the results. Further, DTT's impact on Quantifiler™ Trio is even more concerning than its impact on Investigator® Quantiplex HYres, as the reported quantities, although exaggerated, were still plausible. Therefore, these values could easily be mistaken as correct without raising any "red" flags or interruptions to the workflow.

STR Analysis

Another dye impacted by DTT is the TMR reporter dye used to label the internal positive/PCR control (IPC) within the PowerQuant® system (Sorensen Forensics, personal communication, Dec. 2018). TMR is a rhodamine dye that is commonly used as the yellow channel dye within Promega™ multiplex STR kits to label amplicons for detection with capillary electrophoresis; therefore, two *prep*GEM™ samples were evaluated for potential impacts of DTT on the PowerPlex® Fusion 5C System results. However, in this study, STR profiles of samples prepared with *prep*GEM™ (with DTT) were complete and exhibited no quality issues when amplified using the PowerPlex® Fusion 5C System, potentially demonstrating the ability of this amplification kit to overcome inhibitors or, alternatively, the miniscule amount of residual DTT within the amplification reaction (data not shown). In fact, only one unusual observation was noted for these samples: After amplification, the samples appeared cloudy. This opacity could have been precipitated DTT, as this is not typically observed in samples amplified with this kit.

Evaluation of Alternative Lysis Methods

Method Optimization

Based on quantification data, sample processing times, and assay volumes, the 30-minute incubation time for SwabSolution™, the 25 µL reaction volume for Casework Direct, the 0.5% NP-40 cell lysis buffer concentration, and the 15-minute incubation time for HGH cell lysis were selected as the best conditions for each method (data not shown). Thus, these conditions were used for all downstream analyses.

Microscopy

Based upon microscopic visualization of the lysates, the Casework Direct and alkaline lysis techniques were the only methods that resulted in the complete lysis of sperm cells (i.e., mean score of "0"). Samples lysed with NP-40 and HTX methods resulted in a mean score of "3+," while samples lysed using *forensic*GEM™ Sperm resulted in a mean score of "1+" (data not shown). Additionally, as expected, those samples lysed with SwabSolution™ and HGH methods exhibited a decrease in the number of sperm visualized as the incubation time increased (data not shown).

DNA Quantification

A significant difference in DNA yields was observed across all cell lysis methods tested (p = 0.0037). Although the *forensic*GEM™ Sperm, alkaline, and HGH cell lysis groups produced the highest mean DNA yields, subsequent statistical analysis with Tukey HSD revealed that only the differences between the HTX and the HGH cell lysis groups were significant. HTX cell lysis samples produced DNA yields that were as much as 13% lower than those produced by HGH cell

lysis ($p = 0.0377$, Figure 3). No other significant differences in DNA yields were revealed between any other experimental groups ($p > 0.06$, Figure 3).

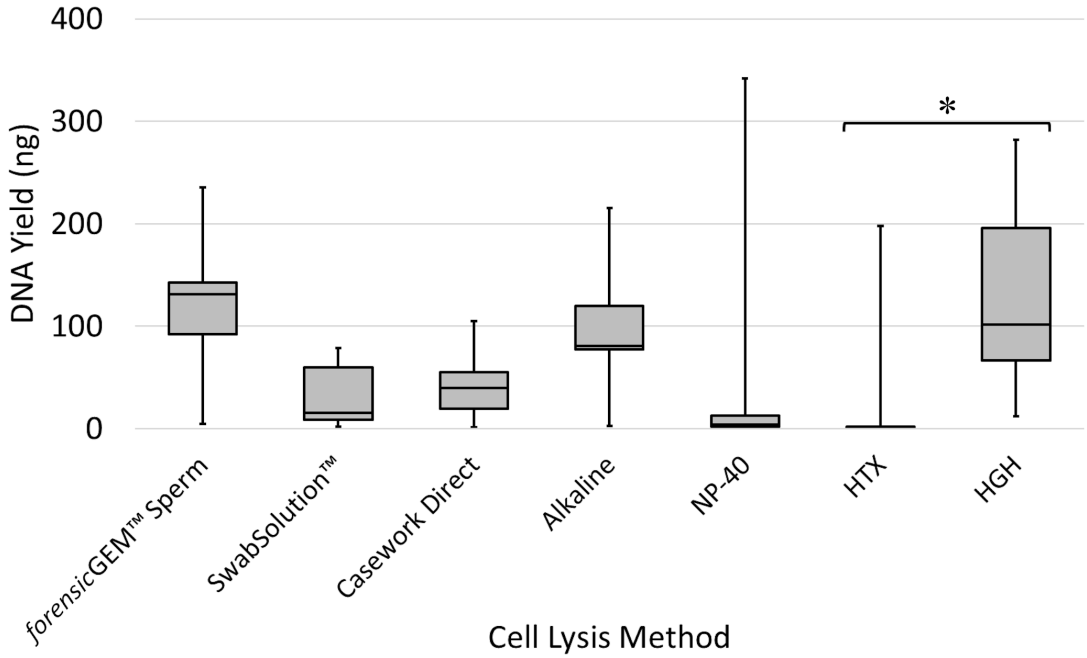

FIGURE 3 – Mean DNA yields obtained from real-time PCR (qPCR) comparing *forensic*GEM™ Sperm ($n = 9$) to six alternative cell lysis methods ($n = 10$). Significant decreases in DNA yield were observed between HTX cell lysis and HGH cell lysis groups ($p < 0.05$). No other significant differences were observed.

Additionally, quantification results for all samples showed qPCR component plots with the expected sigmoidal curves for each target dye, as well as a characteristic flat curve for the passive reference dye. The amplification plots revealed a characteristic trend with samples crossing the threshold during the exponential phase, where those samples with higher DNA concentrations crossed the threshold at an earlier cycle than those with lower DNA concentrations, as expected. However, under certain lysis conditions, the IPC curves crossed the threshold at a later cycle than expected ($C_q \geq 29$), which is consistent with inhibition and often results in the underestimation of DNA concentration [88–90]. This phenomenon occurred most notably in those semen samples lysed with SwabSolution™ and Casework Direct, which was anticipated given that the addition of

the 5X AmpSolution™ generated a lower efficiency reaction by inherently diluting the other master mix components. Additionally, 20% of the donor samples processed with alkaline cell lysis and 70% of the donor samples lysed using the HTX and HGH methods displayed delayed IPC values; however, because the IPC was not delayed in every sample and was only noted in the non-proprietary experimental groups when a new quantification kit lot was utilized, these unexpected observations could be due to donor differences or lot-to-lot variation among the reaction components.

STR Analysis

STR profiles were analyzed and compared across all donors for each lysis method, with the goal of identifying the best performing technique(s) – that which was most likely to achieve full STR profiles and generate STR profiles of equal or higher quality than the control method (*forensic*GEM™ Sperm). The HTX cell lysis sample profiles displayed a significant reduction in the percentage of STR alleles detected compared to all other lysis methods by as much as 48% ($p < 0.00005$, Figure 4A). No significant differences were observed between any of the other alternative methods and the control, and all other lysis methods produced profiles with > 90% of expected STR alleles detected (Figure 4A).

In addition to profile completeness, STR allele peak heights for each lysis method tested were expected to be consistent with those previously observed in the laboratory's internal validation (B. Hudson, personal communication, Nov. 2020) and within the dynamic range of the instrument (up to ~8,200 RFU) [91], as well as at or above those observed from the control method (*forensic*GEM™ Sperm). Although not significant, the Casework Direct, alkaline, and NP-40 cell

lysis methods generated mean STR allele peak heights that were slightly higher than those obtained from samples processed using *forensic*GEM™ Sperm ($p > 0.1$, Figure 4B). Further, the alkaline and NP-40 cell lysis groups had more samples reach or exceed the expected mean STR allele peak height than all other methods tested (Figure 4B). Significant decreases in mean peak height were observed with HTX samples when compared to alkaline ($p = 0.0009$), Casework Direct ($p = 0.0108$), and NP-40 cell lysis groups ($p = 0.0029$). Additionally, mean peak heights from those samples processed using the HGH method were significantly lower than those processed with alkaline lysis ($p = 0.0216$). It is important to note that, overall, peak heights were likely lower than the validated mean due to differences in sample preparation; all DNA samples amplified for the internal validation were those that had been purified after cell lysis.

Minimal variation in STR allele peak heights across all loci within a single STR profile (i.e., interlocus balance) is essential, as mixture profile deconvolution relies on the assumption that balance is obtained and is consistent across the entire sample profile. Adequate interlocus balance assures that all allele peaks are sufficiently above the analytical threshold so that allelic dropout is avoided and true homozygosity can be confidently determined, and it enables analysts to distinguish peaks from one contributor versus another. All lysis methods explored in this study exhibited comparable interlocus balance to the control method except the HTX cell lysis group, which displayed a significant increase in CV (i.e., worsened interlocus balance) versus all other methods ($p < 0.00005$, Figure 4C). While not significantly different, it should be noted that samples processed with the alkaline lysis method produced a mean CV lower than that of the control group and lower than the optimal value of 0.35 ($p = 0.99$, Figure 4C).

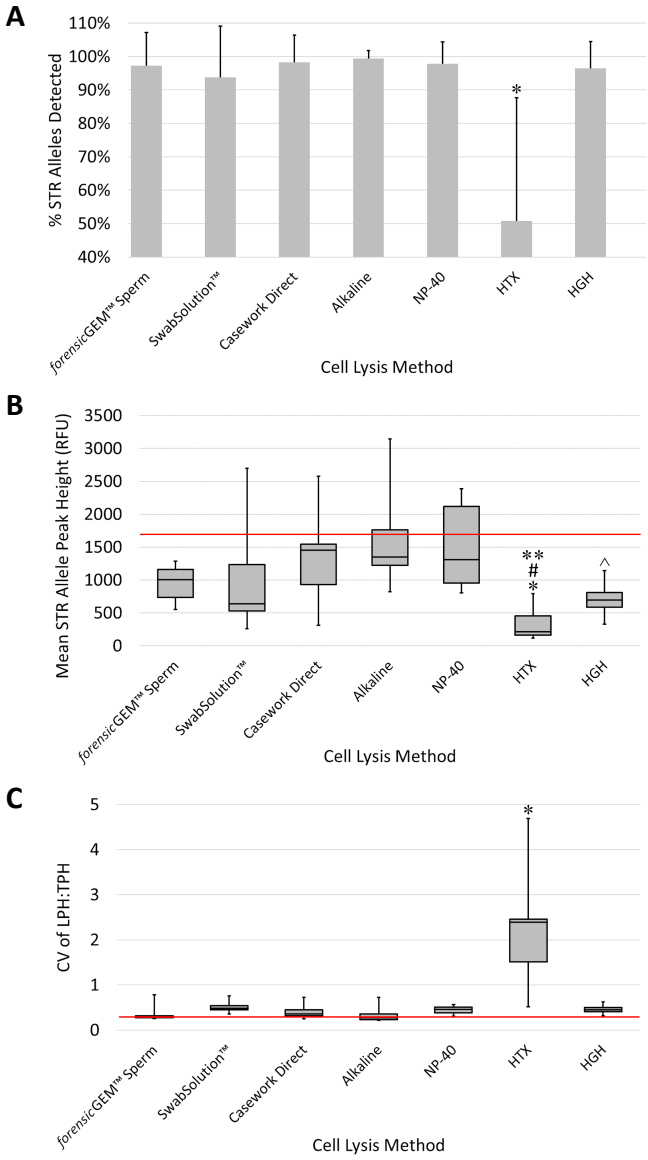

FIGURE 4 – STR profile analysis of lysates from each alternative method. (A) Percentage of STR alleles detected for samples processed with each cell lysis method (n = 10) revealed that HTX cell lysis produced significantly fewer STR alleles than all other methods ($p < 0.00005$), but no other significant differences were observed. (B) Mean STR allele peak heights for samples processed with each of the seven cell lysis methods (n = 10), with the red line representing the expected mean peak height as reported in our laboratory's internal validation of the PowerPlex® Fusion 5C kit (B. Hudson, personal communication, Nov. 2020). HTX lysates exhibited profiles with significantly lower peak heights compared to those from alkaline (*$p = 0.0009$), Casework Direct (#$p = 0.0108$), and NP-40 (**$p = 0.0029$) methods. Additionally, STR profiles HGH lysates exhibited significantly lower peak heights than samples processed with alkaline cell lysis (^$p = 0.0216$). Although not significantly different, STR profiles Casework Direct, alkaline, and NP-40 lysates exhibited mean peak heights that were slightly higher than the control group. (C) Mean interlocus balance (CV of LPH:TPH) for samples processed with the seven alternative lysis methods (n = 10), with the optimal CV (≤ 0.35) represented by the red line [75]. STR profiles HTX lysates exhibited significantly higher CV (i.e., worse interlocus balance) than all other lysis methods tested ($p < 0.00005$). STR profiles from samples lysed using all other methods exhibited CV below or slightly above 0.35, indicating a relatively balanced DNA profile.

When STR profiles from semen processed with each cell lysis method were evaluated, no method significantly or consistently outperformed the control method (*forensic*GEM™ Sperm) across every metric examined; however, three of the alternative cell lysis methods exhibited positive improvements in *some* metrics when compared to the control (Table 5), making them the most suitable alternative sperm lysis methods tested. Lysates from Casework Direct, alkaline lysis, and NP-40 cell lysis buffer techniques all exhibited similar or higher percentages of STR allele detection, slightly higher mean STR allele peak heights (some higher than expected based on our internal validation), and comparable or better interlocus balance (Figure 5). Notably, samples processed using the HTX cell lysis method consistently performed more poorly than all other sample groups, resulting in lower peak heights, poor interlocus balance, and substantial allelic drop out (Figure 6).

TABLE 5. Summary of quantitative STR data for samples lysed with each alternative method (n = 10)

Alternative Lysis Method	Peak Height (RFU)	Interlocus Balance (CV of LPH:TPH)	STR Alleles Detected (%)
***forensic*GEM™ Sperm**	941.98 ± 385.18	0.386 ± 0.24	97%
SwabSolution™	999.15 ± 958.35	0.499 ± 0.21	94%
Casework Direct	1388.04 ± 807.84	0.389 ± 0.14	98%
Alkaline Lysis	1590.64 ± 817.17	0.326 ± 0.17	99%
NP-40 Lysis Buffer	1538.06 ± 787.84	0.454 ± 0.14	98%
Natural Decondensation – HTX	406.61 ± 305.33	1.861 ± 1.32	51%
Natural Decondensation – HGH	705.00 ± 364.23	0.457 ± 0.11	97%

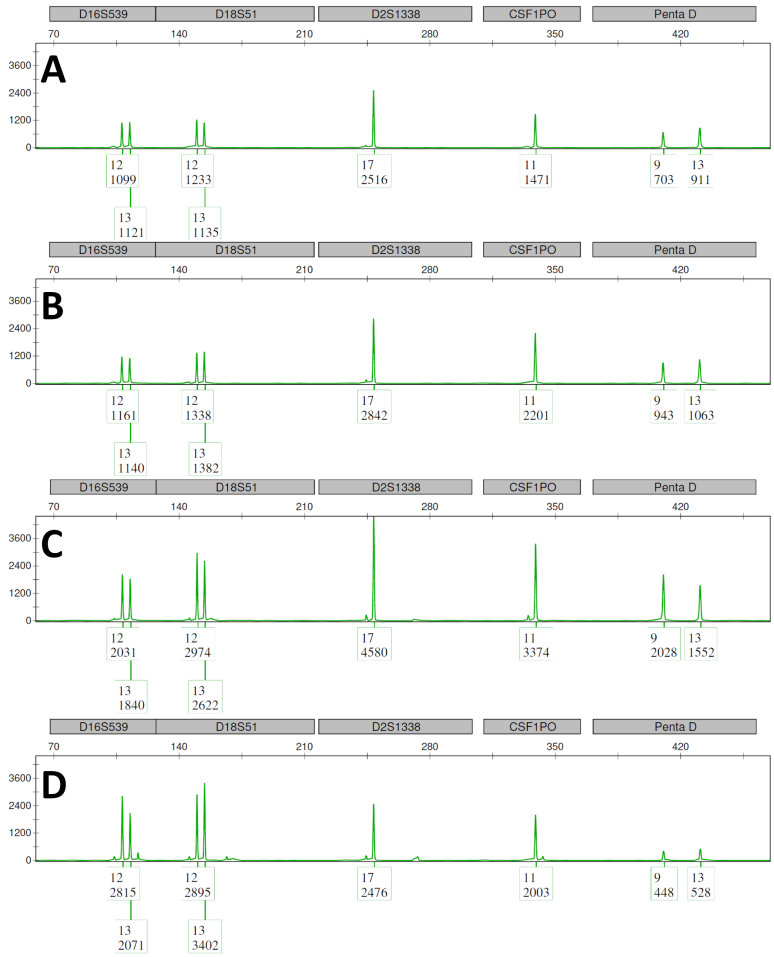

FIGURE 5 – The green channel of representative electropherograms displaying samples lysed using the *forensic*GEM™ Sperm (A), Casework Direct (B), alkaline (C), and NP-40 (D) methods. An increase in mean STR allele peak heights, as well as similar interlocus balance, was observed for samples lysed with the alternative methods compared to the control (*forensic*GEM™ Sperm).

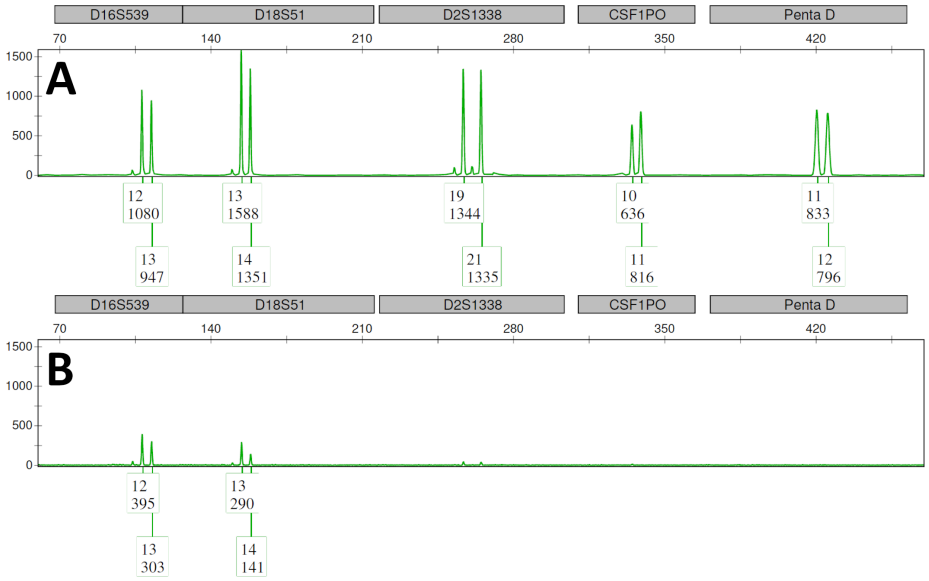

FIGURE 6 – The green channel of representative electropherograms comparing samples lysed with the control method of *forensic*GEM™ Sperm (A) to those lysed using HTX (B). Samples lysed with HTX exhibited allelic drop-out, diminished peak heights, and ski-slope.

CONCLUSIONS

Overall, the results of this study demonstrate the negative impact of DTT on multiple fluorescent dyes. While most dyes presumed to be impacted are from the cyanine family (e.g., Cy5, Quasar 670, and SYBR Green I), our research confirms that additional dyes such as Mustang Purple® are also affected. Although previous reports have demonstrated DTT's impact on the rhodamine dye TMR (Sorensen Forensics, personal communication, Dec. 2018), our evaluation of this dye within PowerPlex® Fusion 5C revealed that either newer kits may be able to overcome this problem or the amount of residual DTT within the amplification reaction was small enough to be ineffective. All aforementioned molecules, albeit from multiple dye families and various excitation/emission wavelengths, possess a common tertiary amine structure (i.e., nitrogen-containing functional group or heterocycle) that carries a positive charge [81,82,86,92,93]. We posit that this shared structure may either directly react with DTT or indirectly make other functional group(s) on the dye more susceptible to reaction(s) with DTT. Evaluation of the data from this study and the available

literature suggests that results obtained from the Quantifiler™ HP, Quantifiler™ Trio, PowerQuant®, and Investigator® Quantiplex HYres quantification kits are impacted by the presence of residual DTT in sample DNA extracts [37; Sorensen Forensics, personal communication, Dec. 2018]. In addition, protocols using SYBR Green I (and therefore potentially PicoGreen) are also likely similarly impacted [83,86].

Our results demonstrate the need to carefully review the shapes of the component and amplification curves, as well as the signal of the passive reference dye, prior to moving forward with resulting quantification values. Additionally, relying on the quality flags alone is not recommended, as the flag for a bad passive reference signal was only triggered in three samples containing DTT in this study despite severely reduced signals in each sample. Thus, Y-screening, direct amplification, and direct cell lysis assays used in conjunction with DTT should be implemented cautiously, as residual DTT has the ability to result in overestimated and unreliable quantification values.

Alternatively, and ideally, other sperm cell lysis reagents should be implemented for use with downstream forensic DNA procedures. Within this study, seven alternative sperm lysis methods that obviate the need for both DTT and purification were evaluated. When STR profiles from semen processed with each cell lysis method were evaluated, no method significantly or consistently outperformed the control method (*forensic*GEM™ Sperm) across every metric examined; however, lysates from Casework Direct, alkaline lysis, and NP-40 cell lysis buffer techniques all exhibited similar or higher percentages of STR allele detection, slightly higher mean STR allele peak heights (some higher than expected based on our internal validation), and

comparable or better interlocus balance (Figure 5). It should be noted that, although not shown here, the same semen lysates were also amplified using a specific input *volume* rather than a targeted DNA amount to evaluate situations in which quantification may not be performed (e.g., direct amplification and microfluidic devices). When STR profiles from each lysis method were evaluated using this approach, results were consistent with those obtained in the studies reported herein when a specific DNA input of 0.25 ng was amplified, but lysates from the HGH method exhibited higher quality results; HTX lysates still performed poorly (data not shown).

Because the ability to tackle the sexual assault kit backlog is impacted by more than just STR profile quality, one must consider additional factors prior to the implementation of new lysis techniques (such as the time required for hands-on and overall processing, as well as cost); therefore, these factors were also examined. Assuming a sample size of 20, the estimated hands-on time for the sperm lysis portion of a traditional differential cell lysis and purification (e.g., a standard Qiagen® DNA extraction) is ~90 minutes, while the total processing time is ~180 minutes (Table 6**)** [66]. Not only is this a manual, time-consuming process, but it also includes proprietary components, is the most expensive per reaction (compared to alternative methods in this study), and presents a number of challenges when attempting to integrate onto an automated platform (Table 6) [94,95]. While *forensic*GEM™ Sperm offers a fast alternative that is easily automatable, it is the most expensive method of those tested during this study and it contains proprietary components. Of the additional alternative lysis approaches examined herein, SwabSolution™ and Casework Direct also include proprietary components, which inherently makes them more expensive to implement in forensic laboratories (Table 6). Alternatively, all other methods that were explored in this study have an approximate cost of less than one dollar per reaction; they also

only require 30 – 50 minutes of hands-on time and 40 – 85 minutes of total processing time (n = 20) (Table 6), which is advantageous over traditional methods. Finally, the NP-40 and HTX cell lysis techniques tested herein demonstrated other issues that could limit their widespread adoption. The NP-40 lysis buffer technique requires an on-ice (~4°C) incubation that can be difficult to achieve in an automated, miniaturized format, while cell lysis using HTX solution without downstream DNA purification resulted in inhibited STR profiles to a level that may substantially complicate profile interpretation.

TABLE 6. Summary of additional factors to consider when identifying alternative lysis method(s) for future implementation

Alternative Lysis Method	Cost (per reaction)*	Hands-on Time (mins)†	Total Processing Time (mins) †	Ownership	Potential Issue(s) with Automation
Standard Qiagen	$5.60	90	180	Proprietary	Large volumes, wash steps, silica filtration
***forensic*GEM™ Sperm**	$4.30	30	50	Proprietary	None
SwabSolution™	$0.144	45	75	Proprietary	None
Casework Direct	$0.625	50	85	Proprietary	None
Alkaline Lysis	$0.321	45	55	Non-Proprietary	None
NP-40 Lysis Buffer	$0.007	50	85	Non-Proprietary	Incubation on ice
Natural Decondensation – HTX	$0.018	30	40	Non-Proprietary	CE inhibition
Natural Decondensation – HGH	$0.031	30	50	Non-Proprietary	None

*All costs are approximations based on current available pricing for VCU.
†All times are approximations (n = 20) based on internally validated protocols.

Ultimately, based on the DNA quantity and STR profile quality obtained in the studies herein, as well as the time and cost considerations, DTT should be avoided in lysis methods which omit

purification. Additionally, the alkaline lysis method is proposed as the best alternative sperm cell lysis technique for sexual assault samples after the traditional, more "gentle" epithelial cell lysis and cell separation steps have been performed. Not only does this technique produce reliable qPCR results, but it could also be used for direct amplification of semen, which may be needed for microdevice-based, rapid casework processing. Further, it should be noted that preliminary evaluation of alkaline lysis with vaginal swab eluates and mock sexual assault samples indicated its ability to lyse epithelial cells as well as sperm cells (data not shown); therefore, it could potentially be used for lysis and direct amplification of numerous sample types. Overall, alkaline lysis offers a quick, low-cost, non-proprietary option that consistently produces high quality STR typing results without the requirement of lysate purification.

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CHAPTER THREE:

A NOVEL SPERMATOZOA ISOLATION TECHNIQUE USING BUOYANCY ACTIVATED CELL SORTING (BACS) AND PH-20 ANTIBODY

ABSTRACT

The inefficiency of sexual assault sample processing continues to plague the forensic DNA community. Although backlogs have largely been tackled across the United States and many technological advancements have made the forensic DNA analysis workflow quicker, techniques for handling sexual assault samples remain time-consuming, tedious, and inefficient at separating cellular fractions from the victim and perpetrator – ultimately hindering the ability of forensic analysts to keep pace and consistently resolve cases. Thus, this study aimed to build upon recent literature and develop an antibody-mediated sperm cell isolation technique that was faster and more efficient than traditional differential lysis, as well as potentially automatable. Polyclonal PH-20 antibodies were coupled to buoyant Microbubbles utilizing either indirect or direct binding order to achieve buoyancy activated cell sorting (BACS) within semen and semen-vaginal mixture eluates. Microscopy revealed sperm-antibody-Microbubble complexes after binding, with attachment via multiple regions of sperm cells. Human DNA quantification results demonstrated the ability of indirect and direct assays to retain 57.2 ± 19% and 58.0 ± 15% of seminal DNA, respectively, within antibody-bound fractions. M:F ratios in STR profiles were improved 2.10 ± 0.43 and 2.76 ± 0.92-fold ($p = 0.041$) for indirect and direct antibody-bound fractions, respectively, when compared to unseparated/untreated mixture controls. While this study revealed statistically (and practically) significant enrichment of sperm cells within forensically relevant samples when utilizing the direct binding assay, further evaluation of the proportion of DNA within the supernatant/"subnatant" of samples highlighted the impact of residual "subnatant" within antibody-bound fractions and indicated that DNase treatment and/or automation of this technique are necessary to optimize fractional separation. Overall, this study developed a novel, small-volume, BACS technique for sperm which could accomplish binding, fractional separation, and cell lysis in under 45 minutes. Future studies should evaluate the implementation of DNase treatment to reduce non-sperm DNA carryover, as well as automation of this technique to eliminate manual separation of fractions (and thus analyst variability).

KEYWORDS: forensic genetics, sexual assault, sperm, PH-20 antibody, Microbubbles, buoyancy activated cell sorting (BACS)

INTRODUCTION

The true extent of backlogged sexual assault evidence collection kits (SAECKs) is difficult to determine; however, the National Institute of Justice estimated a backlog of ~350,000 cases nationwide in 2015 [1]. Despite recent efforts aimed at tackling this backlog across the United States, new legislation in many states mandating the submission and testing of all collected SAECKs is attributing to persistent backlogs. Further, despite a reduction in violent crime since 2018, the most recent Criminal Victimization report from the Bureau of Justice Statistics notes that sexual assault and rape still comprise approximately 22% of victimizations reported to police [2] – further highlighting the prevalence of sexual assaults and their associated evidence within the criminal justice system each year.

While the sheer number of sexual assault cases and their associated evidence are staggering and hampering, there are additional factors within a forensic DNA laboratory that contribute to backlog generation. Perhaps the most notable factor is the inability of scientists to keep pace with submission and testing due to manual, time-consuming, and inefficient sexual assault sample processing techniques. Traditionally, and even still today, sexual assault samples have been processed with a technique known as differential cell lysis (or differential extraction), which was developed by Gill et al. in 1985 [3]. Briefly, this method involves the use of proteinase K (proK) and sodium dodecyl sulfate (SDS) to initially lyse epithelial cells, followed by centrifugation and removal of the supernatant containing DNA alongside cellular components from the lysed cells – leaving behind pelleted, intact spermatozoa. After several washes, the remaining sperm cells are lysed using proK, SDS, and a reducing agent (e.g., DTT) that will break disulfide bonds present within the head of sperm cells and release the DNA. This procedure ultimately splits an evidentiary

sample into two fractions – non-sperm and sperm – that are processed in tandem for the remainder of the DNA workflow. Although commonly employed, this technique suffers from many limitations. Inefficient separation of sperm and non-sperm cells can stem from the presence of old or degraded sperm that are susceptible to premature lysis, excess female epithelial cells that remain intact and within the sperm fraction, sperm loss due to repeated wash steps, or analyst skill level (i.e., poor manual pipetting technique) [4]. Differential extraction is also labor intensive, time-consuming, and still often results in mixture DNA profiles that require lengthy interpretation and review [3,5–7]. Further, although it has been accomplished to some degree [8], this technique is inherently difficult to fully automate – preventing further reduction of hands-on time and the ability to disencumber analysts.

These limitations have spurred copious research into modifications to the differential lysis technique to improve efficiency, reduce time, and minimize carryover of cells between non-sperm and sperm fractions [5,7,9–11]. Several alternative methods which attempt to separate sperm and non-sperm fractions using other means (e.g., manual microscopic sorting via morphology and/or differential staining, levitation and optical tweezers, filtration, and flow cytometry/FACS/DEPArray) have also been explored [4,12–17]. Regardless of the approach, the ultimate measure of success for sexual assault sample processing techniques relates to the ability to quickly retain as much sperm DNA as possible while minimizing non-sperm DNA carryover – the most ideal result being a fast, efficient procedure that produces a sperm fraction containing only DNA from the male perpetrator and a non-sperm fraction containing only DNA from the victim.

Despite the limited success observed with proposed modified differential lysis techniques – such as those incorporating additional lysis and/or wash steps – they still often suffer from reduced purity of sperm and non-sperm fractions, and many are unduly laborious. On the other hand, methods which incorporate a DNase treatment to minimize non-sperm carryover in sperm fractions (e.g., PTC Erase Sperm Isolation kit) often suffer from poor sperm DNA recovery despite enhanced purity of their respective fractions [11,18–21].

Recently, immunoprecipitation assays that incorporate a sperm-specific antibody coupled to a solid support for sperm targeting and isolation have been heavily explored. Techniques utilizing antibodies, which are familiar to forensic DNA analysts due to historical body fluid identification methods, have the potential to more specifically enrich for sperm cells given that they rely on high specificity and affinity to a particular target rather than non-specific density and centrifugation. The possible targets for antibody-mediated sperm isolation are also seemingly endless, with >900 seminal fluid proteins and >6,000 sperm cell proteins already described throughout the available literature [4,10,22–29]. In fact, recent studies have revealed the ability of various sperm-specific antibodies to bind and enrich for sperm within sperm-epithelial cell mixture samples. Recovery of ≥80% of sperm cells using antibodies against the testicular isoform of angiotensin-converting enzyme (tACE) [24], NUH-2 [23], MHS-10/SP-10 [23], MOSPD3 [30], and PH-20/SPAM-1 [29] has been reported. Miltenyi Biotec has even marketed a sperm isolation kit for forensic samples which targets CD52. Despite many published studies and purported success of various sperm-specific antibodies, this technique has yet to be adopted within the field. There are many reasons for this, including irreproducibility, manual techniques which involve numerous wash steps and/or large volumes, as well as a general lack of published studies demonstrating the applicability of

these antibodies for sperm cell isolation from forensically relevant samples (e.g., compromised samples, samples containing relatively few sperm cells, reconstituted samples). Many of the aforementioned studies exhibited reduced sperm recovery when applied to dried samples, casework samples, and even samples containing vaginal rather than buccal epithelial cells. Further, these publications failed to present data which are most applicable to the forensic DNA workflow, such as DNA quantification results, STR profiles, M:F ratio improvement, and relevant details about the non-sperm fraction (a sample which serves as an important control in many sexual assault cases).

The general success of sperm-specific antibodies noted throughout the literature, as well as the limitations and forensic questions that remain, prompted further investigation into the use of sperm-specific antibodies for forensically relevant sperm cell capture and isolation. Thus, this study aimed to develop and optimize an in-tube, antibody-mediated sperm cell isolation technique which could efficiently separate sperm and non-sperm cells while incorporating smaller reaction volumes and reduced assay time – aiming to make this technique more amenable to implementation within the forensic DNA workflow. To accomplish this, the antibody targeting Protein hyaluronidase 20/sperm adhesion molecule 1 (PH-20/SPAM-1) was utilized. This protein has already been successfully used in fundamental studies for sperm cell isolation from liquid semen [29].

PH-20 is a glycosyl phosphatidylinositol (GPI)-anchored protein on spermatozoa [31–33]. The utility of this protein for targeting and isolation of sperm cells within sexual assault samples is apparent, as it has been reported to migrate from the posterior head plasma membrane to the inner

acrosomal membrane (IAM) [31,32], and studies have demonstrated its presence across approximately 85% of the sperm surface (i.e., head, midpiece, and tail regions) [34]. The presence of PH-20 on all regions of sperm is critical, as it will not only provide multiple binding sites on a single sperm cell, but it will also afford the opportunity to bind sperm tails (and thus isolate associated mitochondria) and give increased probability of binding degraded or otherwise compromised sperm cells.

The type of solid support implemented during cell immunoprecipitation can also provide variability in success and determine the feasibility of the technique, and each type of support inherently brings its own benefits and limitations. For example, agarose and polystyrene beads can be used to isolate cells and are generally cheaper than paramagnetic beads, but paramagnetic beads are often available in smaller diameters, require slightly less manual manipulation (e.g., segregation of beads to the side of a tube via magnetic fields), and often result in higher purity [35,36]. On the other hand, paramagnetic beads require a magnet for isolation of antibody-bound fractions – a stipulation that can prove to be expensive – and the reported compatibility with downstream DNA extraction varies with magnetic bead type [37]. Recently, buoyancy activated cell sorting (BACS) has emerged as a new, gentler technique for target cell isolation. For this technique, hollow, glass microspheres which are less dense than the surrounding media are mixed with antibodies and cells; over time (or, alternatively, due to centrifugation) these beads and any bound cells rise to the top of solution (Figure 1) [38]. This behavior can be explained by the interaction of gravity ($F_g = \frac{4}{3}\pi a^3 \rho_{Microbubble} g$), buoyant ($F_b = \frac{4}{3}\pi a^3 \rho_{liquid} g$), and drag ($F_d = 6\pi\mu a v$) forces. As time passes, these forces act upon the cells and Microbubbles, causing them to settle or rise within solution at a rate described by the terminal velocity equation ($v =$

$\frac{2}{9}\frac{(\rho_{liquid}-\rho_{Microbubble})ga^2}{\mu}$). This technique, albeit relatively new, has already been utilized to achieve 90% separation efficiency with CD4+ cells from whole blood samples in less than five minutes [39]. Use of these solid supports could theoretically limit non-specific trapping of epithelial cells within sexual assault samples, as the antibody-bead-target cell complexes would float to the top of the solution while unbound non-target cells would pellet.

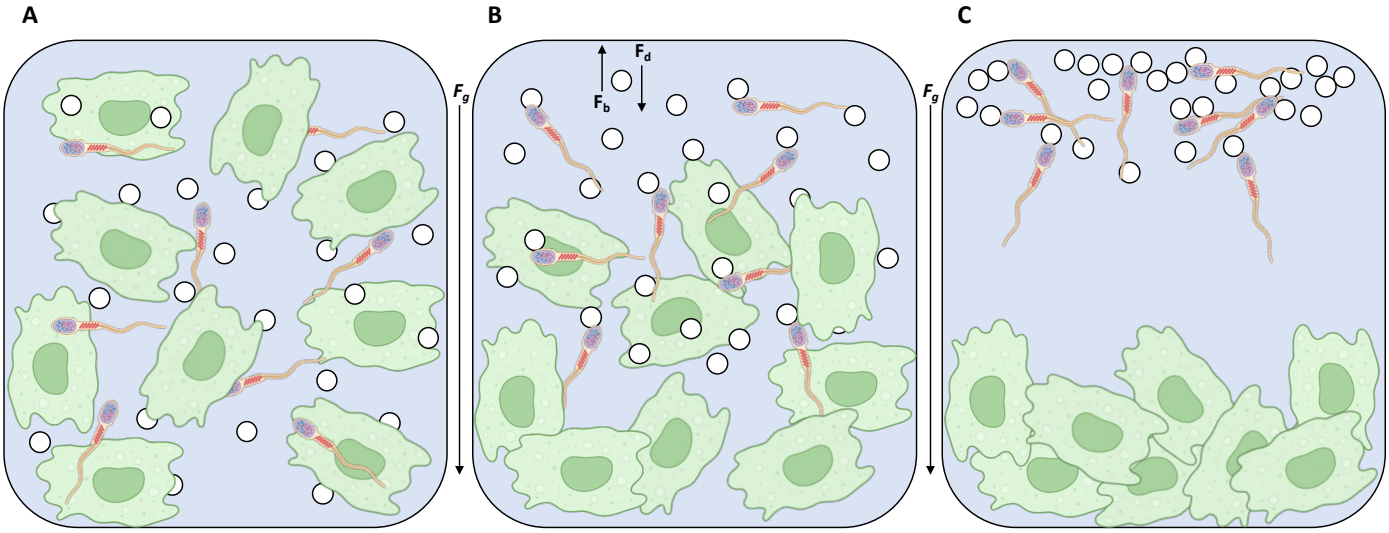

FIGURE 1 – Depiction of the behavior of antibody-Microbubble complexes (white), sperm (pink/purple), and epithelial cells (green) within a sample. After homogenization, all components within the sample are evenly distributed (A). However, as time passes, the gravity force, buoyant force, and drag force act upon the cells and Microbubbles (B). Eventually, the Microbubbles (and cells attached to them via antibody-mediated binding) rise to the top of solution while unbound cells pellet to the bottom (C). (Figure created in part using BioRender.com)

MATERIALS & METHODS

Sample Collection & Preparation

Semen samples and vaginal swabs were collected from ten and one anonymous donor, respectively, following the university-approved Institutional Review Board (IRB) protocol HM20002931. Semen was diluted 1:60 by volume using Gibco™ 1X Dulbecco's phosphate-buffered saline (DPBS) (Fisher Scientific; Waltham, MA). Cells were eluted from vaginal swabs

by submerging a half-swab cutting in 200 µL DPBS and incubating at 37ºC for two hours, with brief vortexing every 15 minutes. Because one vaginal donor was used for the mixture studies, cells were eluted from a total of six separate swabs and eluates were combined to ensure homogeneity of cellular material across samples.

All samples tested within this study were dried onto foam swabs prior to elution and antibody-mediated cell separation. Samples were prepared in a total volume of 80 µL, as previous studies have demonstrated that foam swabs are capable of absorbing this volume [40]. For semen swabs, Fisherbrand™ PurSwab foam swabs (Fisher Scientific) were dipped into tubes containing 30 µL of 1:60 semen and 50 µL of DPBS. Mixture samples were prepared by combining semen dilutions and vaginal swab eluates in proportions described below in order to produce approximate 1:1 M:F ratios in resulting STR profiles, as has been demonstrated within our lab (data not shown). This involved dipping foam swabs into tubes containing 30 µL of 1:60 semen combined with 50 µL of vaginal eluate. This was repeated for each semen donor to generate ten unique mixture swabs. It should be noted that a single vaginal donor was utilized in an attempt to assess the efficiency of this assay with various sperm cell counts rather than various sperm *and* non-sperm cell counts. Multiple swabs were prepared to accommodate all experimental testing, as described below. Swabs were allowed to absorb the samples prior to drying overnight at room temperature. Once dry, swabs were cut into fourths (vertically, to generate equal portions) and stored at 4 ºC until testing; all swabs were tested within 1-2 months of preparation.

All subsequent testing utilized $1/4^{th}$ of a foam swab, which was expected to contain approximately 6,000 – 19,000 sperm cells based on the literature and the semen dilutions used herein [41–47].

Samples were eluted from swab cuttings by combining into a new tube with 20 µL of DPBS containing 0.5% BSA (Sigma Aldrich; St. Louis, MO) and incubating at room temperature for five minutes with brief vortexing every minute.

Overall, semen and semen-vaginal mixture samples were assessed throughout this study. Semen and mixture samples were separated into antibody-bound and unbound fractions, but additional mixture samples not subjected to antibody-binding and separation (i.e., "unseparated" mixtures) were also assessed as controls.

Microscopy

Prior to sample preparation, all semen dilutions and vaginal eluates were evaluated using Kernechtrot Picroindigocarmine Stain (KPICS) to confirm the presence of intact sperm and non-sperm cells. Briefly, ten microliters of each semen and vaginal sample were spotted onto microscope slides, dried, and stained with one drop Kernechrot stain (Serological Research Institute (SERI); Richmond, CA) and one drop of Picroindigocarmine stain (SERI). Following incubation, cells were visualized at 400X and 1000X magnification on a Micromaster microscope (Thermo Fisher Scientific). This procedure was also performed on semen samples after the antibody-Microbubble binding to visualize conjugation of sperm cells to antibody-Microbubble complexes.

Additional staining of semen after antibody-Microbubble binding was performed using Trypan Blue. This was performed to assess whether drying and fixation processes utilized for KPICS (described above) impacted the sperm to antibody-Microbubble complex binding. Briefly,

processed semen samples were combined with ten microliters of 0.4% Trypan Blue (Invitrogen; Waltham, MA), triturated via pipetting for five seconds, and then spotted onto a microscope slide. Visualization of intact cells followed the same procedure as described above for KPICS.

Antibody-Mediated Cell Isolation

All studies for the antibody-mediated sperm cell isolation assay utilized streptavidin-coated Microbubbles (Akadeum Life Sciences; Ann Arbor, MI) and biotin-conjugated, rabbit anti-human polyclonal PH-20/SPAM-1 antibody (#LS-C829922-100 [aa36-490], in PBS; Lifespan Biosciences; Seattle, WA). Throughout this study, customary wash steps after antibody binding were omitted in an attempt to minimize sample loss and assay time.

Direct Binding

Antibody-mediated cell isolation assays were performed using two different binding protocols. The first protocol involved direct order binding (referred to as "direct binding"), where biotin-conjugated PH-20 antibodies were initially incubated with one microliter streptavidin-coated Microbubbles and Akadeum separation buffer in a total of 41 µL on a tube revolver (Thermo Fisher Scientific; Waltham, MA). Mixing during binding was achieved using "reciprocating mode" for five minutes at room temperature. Subsequently, swab eluates (~19 µL) were added to the antibody-Microbubble mixture and incubated on a tube revolver with "reciprocating mode" for 20 minutes at room temperature. Samples were then centrifuged at 400 *x g* for five minutes. Following centrifugation, tubes were placed on the tube rotator, rotated 90º (Figure 2), and allowed to sit for five minutes to allow the buoyant Microbubbles to rise to the top sidewall. Due to the nature of the buoyant Microbubbles and their tendency to drag with the meniscus of the liquid, not all of the

"subnatant" was removed; this was a precaution to avoid inadvertent removal of cell-Microbubble complexes, as the force of adhesion between Microbubbles nearest the liquid fraction (i.e., those experiencing more contact with the "subnatant") is reduced compared to the force of adhesion for Microbubbles closest to the air-liquid interface (Figure 3). Thus, 42 µL of the "subnatant" were then removed and kept as the "unbound" fraction, leaving behind the antibody-bound ("bound") fraction. When doing this, it was ensured that the visible cell pellet at the bottom of the tube was retrieved, and residual Microbubbles were avoided. The bound fraction was then brought to 42 µL using Akadeum separation buffer, making sure to resuspend any Microbubbles along the tube walls.

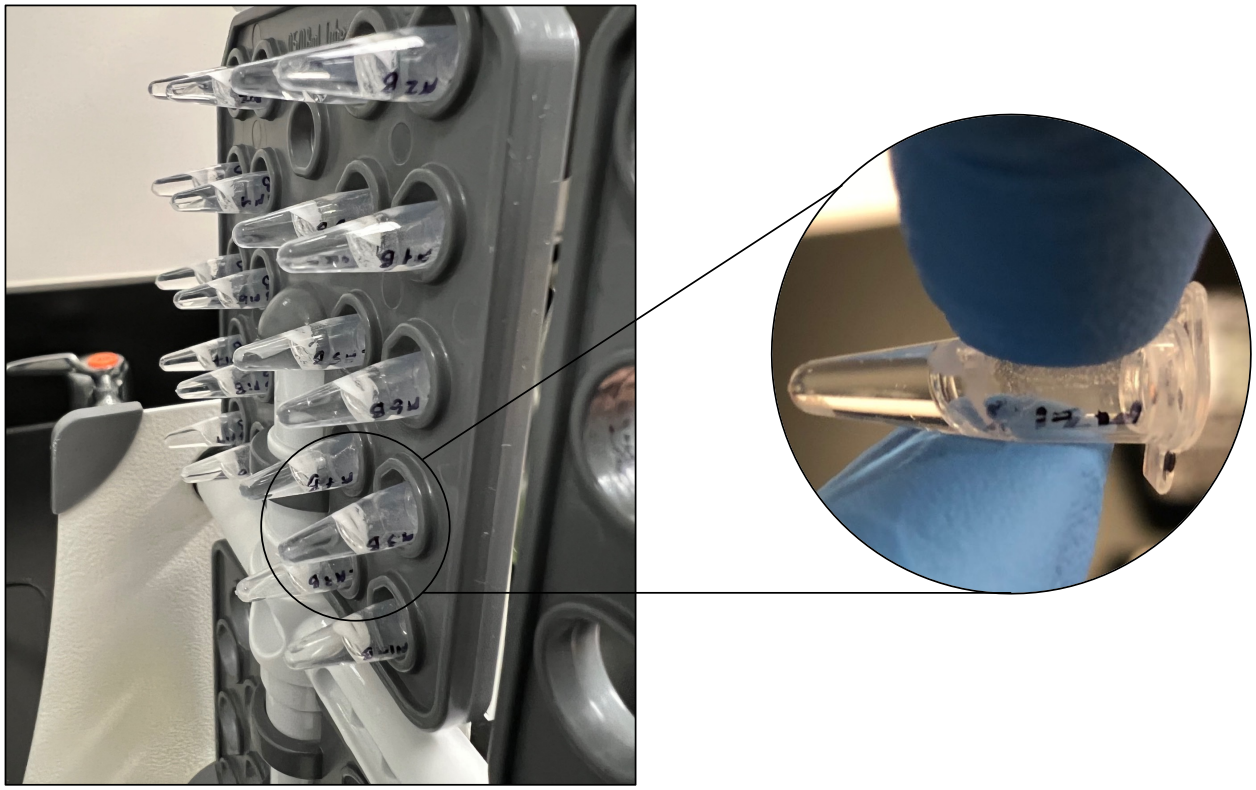

FIGURE 2 – Placement of sample tubes in the tube revolver at 90 degrees after binding and centrifugation. After five minutes, the Microbubbles can be observed along the top sidewall of the tube, while a visible cell pellet is at the bottom of the tube. The cell pellet and liquid fraction can be removed by inserting a pipette into the "subnatant" below the Microbubble complexes.

Indirect Binding

Indirect order binding (referred to as "indirect binding") was also assessed, as studies have reported the ability of this technique to work better in situations where the target molecule is present in relatively low amounts [35–37]. This involved incubating biotin-conjugated antibodies with eluted cells, followed by subsequent addition of streptavidin-coated Microbubbles. Thus, eluted samples (~19 µL) were combined with PH-20 antibodies and Akadeum separation buffer in a total volume of 59 µL on a tube revolver; binding involved "reciprocating mode" for 20 minutes at room temperature. Subsequently, one microliter of streptavidin-coated Microbubbles was added to the sample-antibody mixture and incubated on a tube revolver with "reciprocating mode" for five minutes at room temperature. The remainder of the protocol followed that of direct binding above.

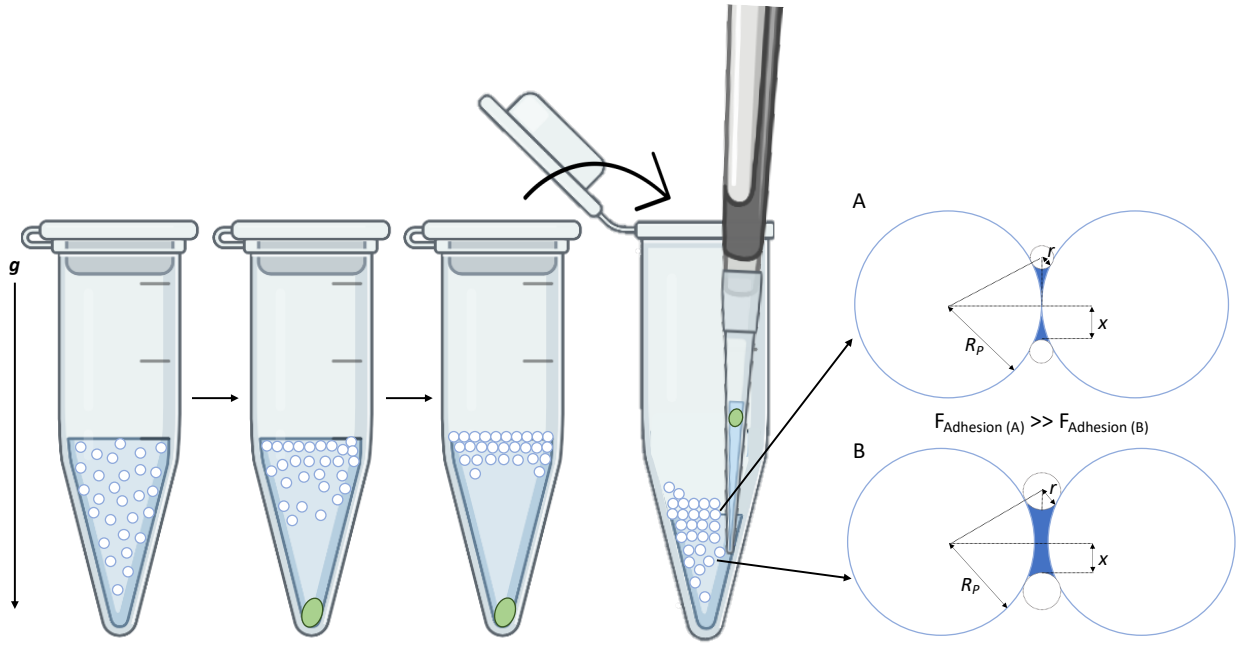

FIGURE 3 – Impact of gravity and "subnatant" removal on Microbubbles. Over time (or with centrifugation), Microbubbles rise and form a compact, crystallized layer due to their density compared to that of most solutions. As the "subnatant" (i.e., liquid fraction beneath the Microbubbles) is removed, some Microbubbles tend to drag with the meniscus of the liquid and have the potential to be inadvertently pipetted. This can be explained by the difference in adhesion force acting on Microbubble complexes closer to the air interface (A) compared to that of Microbubbles which have more contact with liquid (B), where the adhesion force of the former is much greater and prevents their reconstitution. (Figure created in part using BioRender.com)

PH-20/SPAM-1 Antibody Titration

To determine the optimal antibody load (μg) per microliter of Microbubbles for the cell binding assay, a titration was performed. For this, one microliter of streptavidin Microbubbles was utilized with either 0, 0.10, 0.25, 0.50, 0.65, 0.825, 1.0, 1.5, 2.0, or 2.5 μg of biotin-conjugated PH-20 antibody for sperm cell isolation from eluted semen samples (n = 3, in singlicate). This was performed using both direct and indirect order of binding (as described above). Binding capability was assessed after cell separation, lysis, and human DNA quantification. Human DNA yields were calculated by taking the mean percentage of total DNA isolated from bound and unbound fractions. The antibody load for each binding order that retained the highest percentage of seminal DNA was deemed optimal and carried forward to all subsequent testing. After optimal antibody load determination, additional semen donors and replicates (n = 10, in triplicate) were assessed to better characterize the percentage of seminal DNA retained in antibody-bound fractions.

Evaluation of DNA Provenance

Additional mixture samples were assessed for the proportion of DNA originating from cell pellet and supernatant/"subnatant" fractions before and after their subjection to Microbubbles. Elution from swab cuttings was the same as described above. One set of eluted mixtures was centrifuged at 400 *x g* for five minutes prior to removal of the entire supernatant. The cell pellet was then resuspended in 19 μL DPBS and subjected to alkaline lysis (as described below), while the supernatant was carried forward to downstream DNA quantification and STR amplification without being subjected to cell lysis. To assess the impact, if any, of Microbubbles on cell integrity, a second set of eluted mixtures was subjected to the direct binding assay described above (absent PH-20 antibodies). After binding, samples were centrifuged at 400 *x g* for five minutes, and tubes

were placed on the tube revolver and turned 90º to allow Microbubbles to rise. Five microliters of the "subnatant" were removed and carried forward to downstream processes without further treatment, while the remainder of the sample was subjected to alkaline lysis as described below.

Cell Lysis & DNA Liberation

Cell lysis/DNA liberation for all resulting "bound" and "unbound" fractions, as well as unseparated mixture controls, was performed according to the alkaline lysis method described by Schellhammer et al. [40], with a final volume of 60 µL.

DNA Quantification

Human and male DNA from all resulting DNA extracts was quantified using the Investigator® Quantiplex HYres kit (QIAGEN; Hilden, Germany) on the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific). Manufacturer recommendations were followed, with modifications for half-volume reactions. Thus, 4.5 µL Reaction Mix, 4.5 µL Primer Mix IC YQ, and 1.0 µL template DNA were combined in each well, and thermal cycling conditions involved: 95 ºC for three minutes, followed by 40 cycles of 95ºC for five seconds and 60ºC for 35 seconds. Resulting data were analyzed using Sequence Detection System (SDS) software v1.4 (Applied Biosystems™), with automatic baseline and threshold settings for each target.

To identify any possible signs of inhibition, assessment of qualitative metrics in resulting amplification and component plots was conducted as previously described by Hudson et al. [48]. In addition, several quantitative metrics were assessed, as follows. Total and fractional human DNA yields for each sample were calculated by multiplying the human target's concentration by

the sample volume; this was repeated for the male target. To determine the percentage of human and male DNA in each fraction, the fractional DNA yield was divided by the total DNA yield (i.e., the sum of DNA yields in bound and unbound fractions) and multiplied by 100. The mean and standard deviation for each experimental group were then calculated, and all comparisons were assessed using a Student's *t*-test ($\alpha = 0.05$). Considering the proportions of sperm (~88%) and non-sperm (~12%) contributions within normal semen, and correcting for ploidy, the theoretically expected percentage of seminal DNA originating from sperm cells was 80% [10,41,42,49]. For DNA provenance assessment within mixture samples, the cell pellet versus supernatant/"subnatant" DNA proportion was determined. The supernatant/"subnatant" DNA yield was divided by the total DNA yield (i.e., the sum of DNA yields in supernatant/"subnatant" and cell pellet fractions) and multiplied by 100; this was repeated for the cell pellet DNA yield, and a Student's *t*-test was conducted to determine statistical significance ($\alpha = 0.05$). Male-to-female (M:F) ratios in unseparated, bound fraction, unbound fraction, supernatant/"subnatant" DNA, and cell pellet DNA samples were calculated by dividing the male DNA concentration by the difference between the human and male DNA concentrations; these values were then averaged for each experimental group to determine the mean M:F ratio, and a Student's *t*-test was conducted to determine statistical significance ($\alpha = 0.05$).

STR Amplification

All experimental samples, as well as known female and male reference samples, were amplified using the Promega™ PowerPlex® Fusion 6C System with a template DNA input of 0.25 ng following manufacturer recommendations, with the only modification being half-volume reactions; therefore, each reaction included 5.0 µL sample (at 0.05 ng/µL), 2.5 µL PowerPlex®

Fusion 5X Master Mix, 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix, and 2.5 µL amplification-grade water. Thermal cycling was conducted on the ProFlex™ 3x32-well PCR System (Applied Biosystems™) following manufacturer-recommended parameters [50].

Capillary Electrophoresis & Data Analysis

Resulting STR amplicons were separated using an Applied Biosystems™ 3500 Genetic Analyzer and Data Collection software v4 (Thermo Fisher Scientific) following manufacturer recommendations. This involved combining one microliter of sample or allelic ladder with 0.5 µL WEN ILS 500 (Promega™) and 9.5 µL Hi-Di™ Formamide (Thermo Fisher Scientific) in each well on the plate. Injection parameters also followed manufacturer recommendations and included a 36 cm capillary array (Thermo Fisher Scientific), POP-4® polymer (Thermo Fisher Scientific), and a 1.2 kV 15 second injection. Resulting STR profiles were then analyzed with GeneMapper™ *ID-X* software v1.6 (Thermo Fisher Scientific) following manufacturer settings and an analytical threshold of 150 RFU [50].

Single-source profiles from semen samples were qualitatively and quantitatively assessed for signs of inhibition (e.g., allelic dropout and interlocus imbalance). M:F ratios within unseparated controls, bound fractions, unbound fractions, and supernatant/"subnatant" DNA fractions from mixture samples were calculated by dividing the total peak height for male alleles by the total peak height for female alleles at each locus where there was no allele sharing between donors. These ratios were then averaged across all loci within a single sample, as well as across all samples within an experimental group (e.g., all ten antibody-bound fractions). A Student's *t*-test was conducted to compare mean M:F ratios across bound fractions and unseparated controls ($\alpha = 0.05$).

The M:F ratio fold improvement for bound fractions compared to unseparated controls was then determined by dividing the mean M:F ratio within a bound fraction by the mean M:F ratio in its associated unseparated control (e.g., M:F for Mixture 1 bound fraction ÷ M:F for unseparated Mixture 1). The mean fold improvement was then calculated by averaging the M:F fold improvement across all ten mixture samples. A Student's *t*-test was conducted to compare mean M:F ratio fold improvement for bound fractions and unseparated controls ($\alpha = 0.05$). Bound fractions were additionally assessed for the number of male contributor alleles that were recovered (i.e., detected above analytical threshold) compared to their corresponding unseparated controls; these numbers were then averaged to obtain the mean number of male alleles recovered for each experimental group.

RESULTS & DISCUSSION

PH-20 Antibody Titration

Semen samples (n = 3) were subjected to indirect and direct antibody binding protocols with varying loads of PH-20 antibody. Results demonstrated differences in binding capability between indirect and direct binding protocols across antibody loads (Figure 4). The general trendline observed was consistent with that expected of an antibody titration, where maximum signal or detection of the target is reached and subsequently followed by a dip in target detection due to increased background (or non-specific binding) [51,52]. Although variation was relatively high across the titration (likely due to low sample size and sperm count variability across donors), the optimal antibody load for indirect binding was determined to be 0.65 µg (retaining 69.15 ± 20.6% of total DNA), while 0.825 µg antibody was deemed the optimal load for direct binding (retaining

80.57 ± 19.3% of total DNA). Moving forward, all indirect and direct binding protocols utilized these loads of PH-20 antibody.

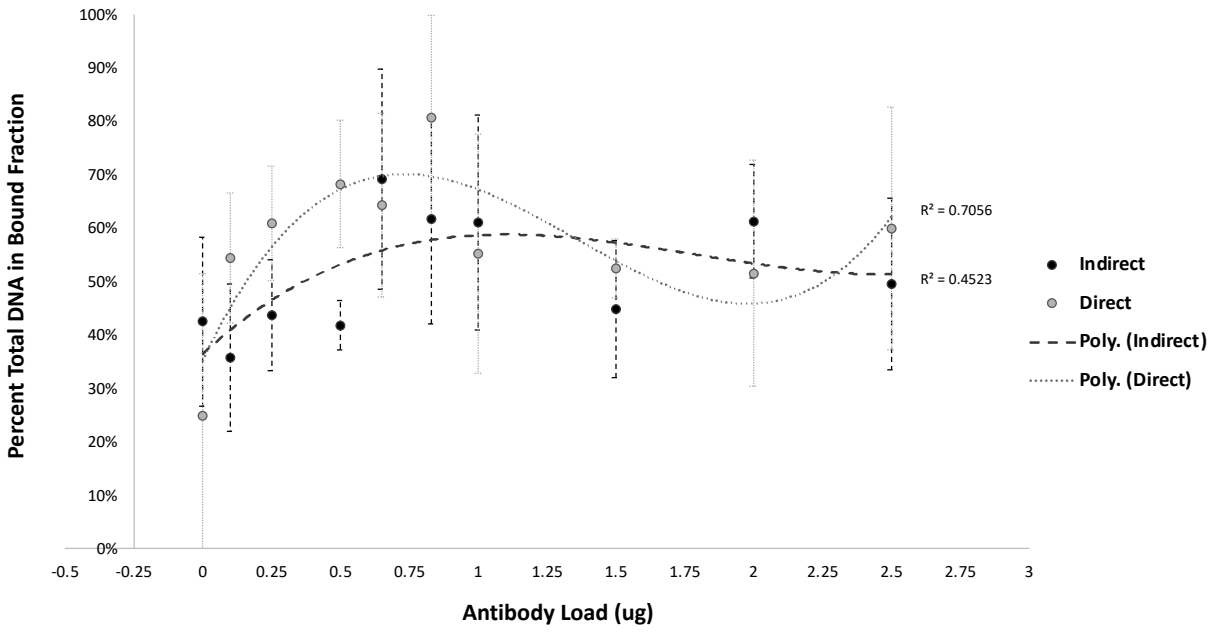

FIGURE 4 – Percentage of total DNA retained in antibody-bound fractions for semen (n = 3) when utilizing various antibody loads for indirect and direct binding protocols. All antibody loads experienced relatively high standard deviation; however, the optimal load for indirect binding was 0.65 µg, while the optimal load for direct binding was 0.825 µg. These amounts retained 69.15 ± 20.6% and 80.57 ± 19.3% of total DNA, respectively. Second order polynomial ("Poly.") trendlines demonstrated the relationship between antibody load and percentage of DNA retained in bound fractions.

Evaluation of Sperm Binding in Semen Samples

Following determination of optimal PH-20 load for indirect and direct binding, single-source semen eluates (n = 10, in triplicate) were subjected to the antibody-mediated sperm cell isolation assays. Microscopic evaluation of antibody-bound fractions demonstrated the presence of some free-floating sperm (i.e., sperm not conjugated to antibody-coated Microbubbles), as well as many sperm-antibody-Microbubble complexes, regardless of whether a fixed or non-fixed cell staining

technique was implemented (Figure 5). More importantly, sperm were attached to antibody-Microbubble complexes via head *and* tail regions, confirming the presence of PH-20 antigen across each section of sperm [34]. Although one of the reported limitations of using immunoprecipitation for forensically relevant cells involves the possible alteration of cell surface proteins and thus negative impacts on efficient antibody binding, these results indicate that it is still possible for reconstituted/eluted sperm to bind PH-20 antibody.

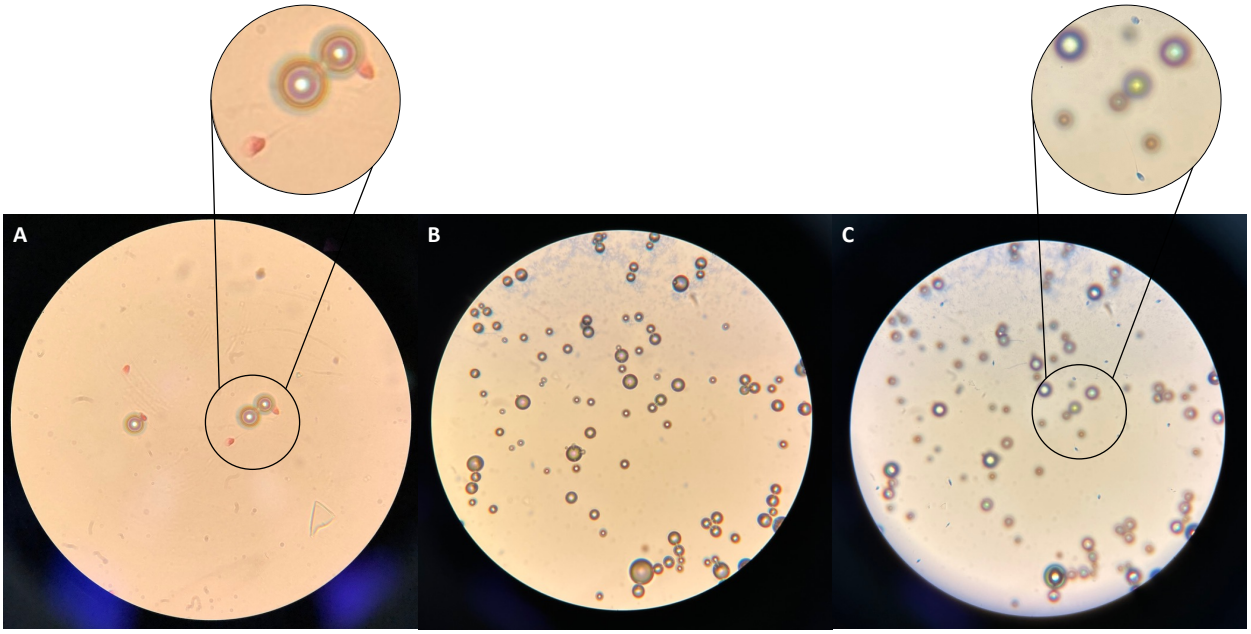

FIGURE 5 – Representative micrographs of antibody-bound fractions from semen samples that were subjected to the PH-20/Microbubble assay. Fixed staining with KPICS (1000X magnification) revealed sperm-antibody-Microbubble complexes (A). Non-fixed staining with trypan blue (400X magnification) also demonstrated sperm binding to antibody-Microbubble complexes, where floating Microbubbles were in focus (B) at a different depth of field compared to sperm (C). Both staining methods indicated sperm binding via head and tail regions, as well as the presence of free-floating sperm (i.e., sperm not attached to antibody-Microbubble complexes).

Assessment of the percentage of total seminal DNA retained within antibody-bound fractions revealed similar binding capabilities regardless of indirect or direct binding order (p = 0.86). Indirect and direct binding protocols were able to retain 57.2 ± 19.2% and 58.0 ± 15.1% of DNA within antibody-bound fractions, respectively (Figure 6). Although the theoretically expected

percentage of seminal DNA associated with sperm is approximately 80% [10,41,42,53], these results indicate the ability to retain *most* sperm within semen samples when using these assays. Further, deviations from the theoretical expectation could be due to many reasons, including sperm count variation across donors, age of semen aliquots, damage to sperm, and other biological differences across donors. The percentage of DNA retained in each antibody-bound fraction was also plotted against the total DNA within each sample to determine whether the total DNA present in a sample impacted the antibody binding efficiency (Figure 7). No relationship was observed between the percentage of DNA retained in either indirect (Figure 7A) or direct (Figure 7B) bound fractions and the total DNA within the semen sample, indicating that sperm binding ability with this assay was not impacted by the binding order or the amount of cellular material present (which is ideal, as the amount of cellular material within a forensic sample can vary greatly).

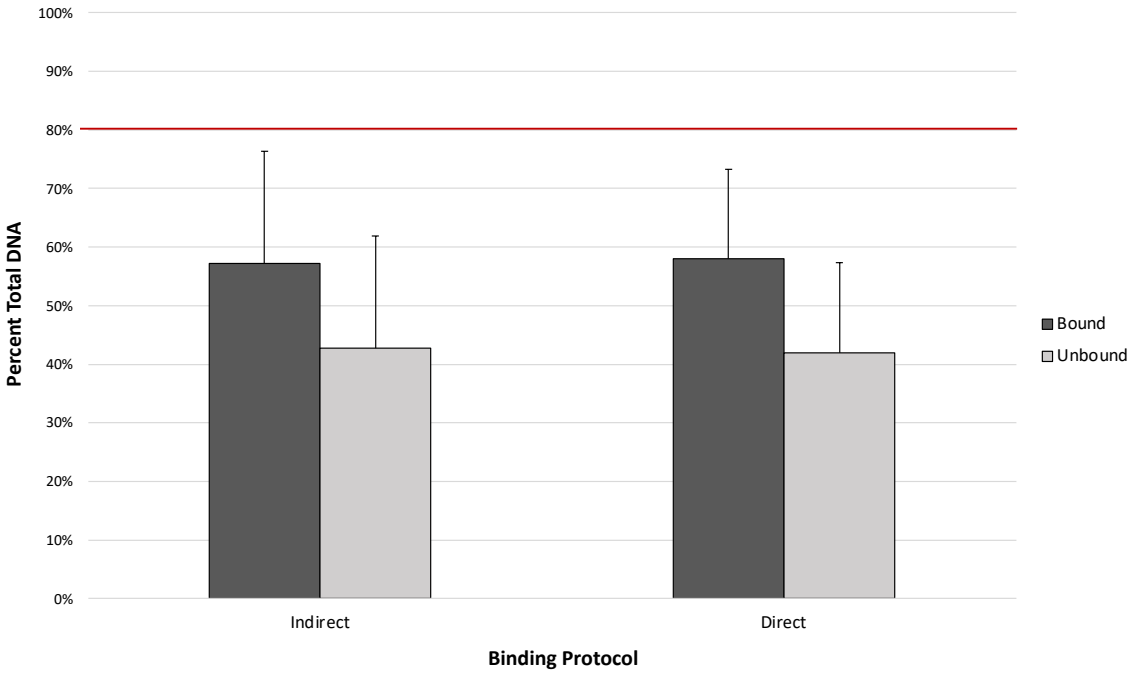

FIGURE 6 – Percentage of total DNA retained in antibody-bound and unbound fractions after treatment of semen eluates (n = 10, in triplicate) with the indirect and direct antibody-Microbubble assays. Both protocols performed similarly, retaining 57.2 ± 19.2% (indirect binding) and 58.0 ± 15.1% (direct binding) of DNA within antibody-bound fractions (p = 0.86). The red line indicates the theoretically expected percentage of seminal DNA associated with sperm.

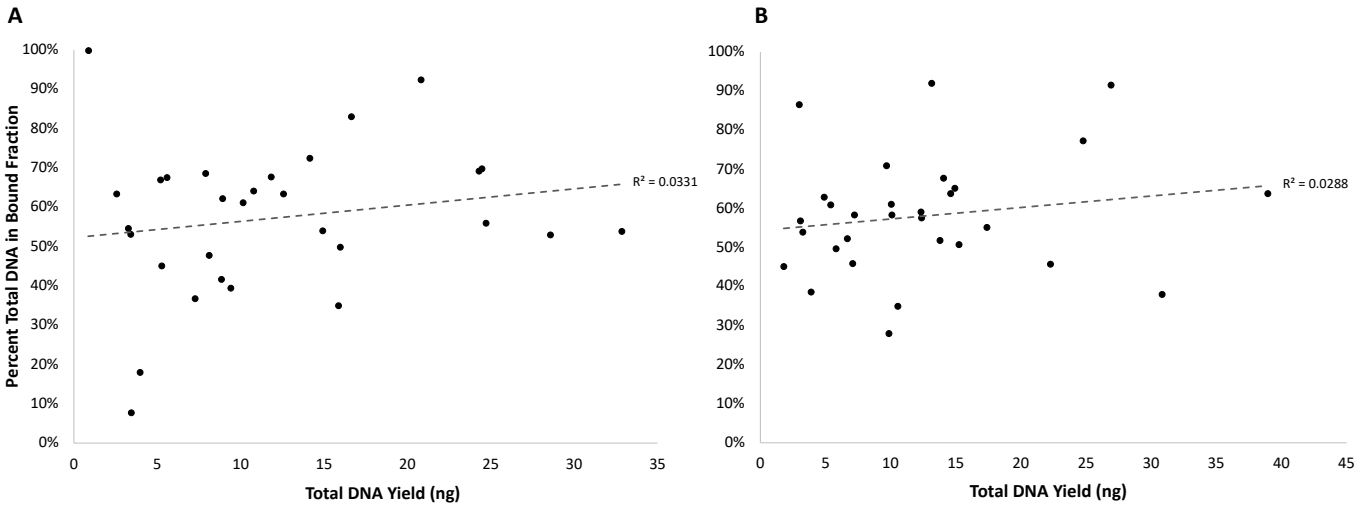

FIGURE 7 – Percentage of total DNA retained in antibody-bound fractions for each semen sample when using indirect (A) and direct (B) binding order compared to the total DNA obtained from each sample. Regardless of binding order, there was no observed relationship between percentage of seminal DNA retained and the total DNA obtained.

In an effort to improve and further optimize sperm cell binding using this assay, various binding protocol modifications were assessed, including the type of mixing during incubation, incubation time, and buffer composition. However, none of the alterations led to improved sperm binding (Supplemental Figure S1). Ultimately, despite binding efficiency of both assays for semen eluates, the true metric of success relates to the ability of each antibody assay to retain sperm cells while simultaneously *not* retaining non-sperm cells; therefore, an assessment of semen-vaginal mixtures was necessary.

Evaluation of Sperm Binding in Mixture Samples

To assess sperm binding and separation efficiency, these assays were also applied to mixture samples (n = 10). The percentage of total and male DNA retained in antibody-bound and unbound fractions was calculated (Figure 8). Although indirect and direct binding protocols retained similar proportions of total DNA (37.6 ± 9.3% and 38.7 ± 10.2%, respectively; $p = 0.80$), direct binding

appeared to retain more *male* DNA in bound fractions than indirect binding (53.0 ± 12% versus 49.3 ± 13%; $p = 0.52$). While not statistically significant, this difference in retainment of male DNA could make a practical difference in STR profiling.

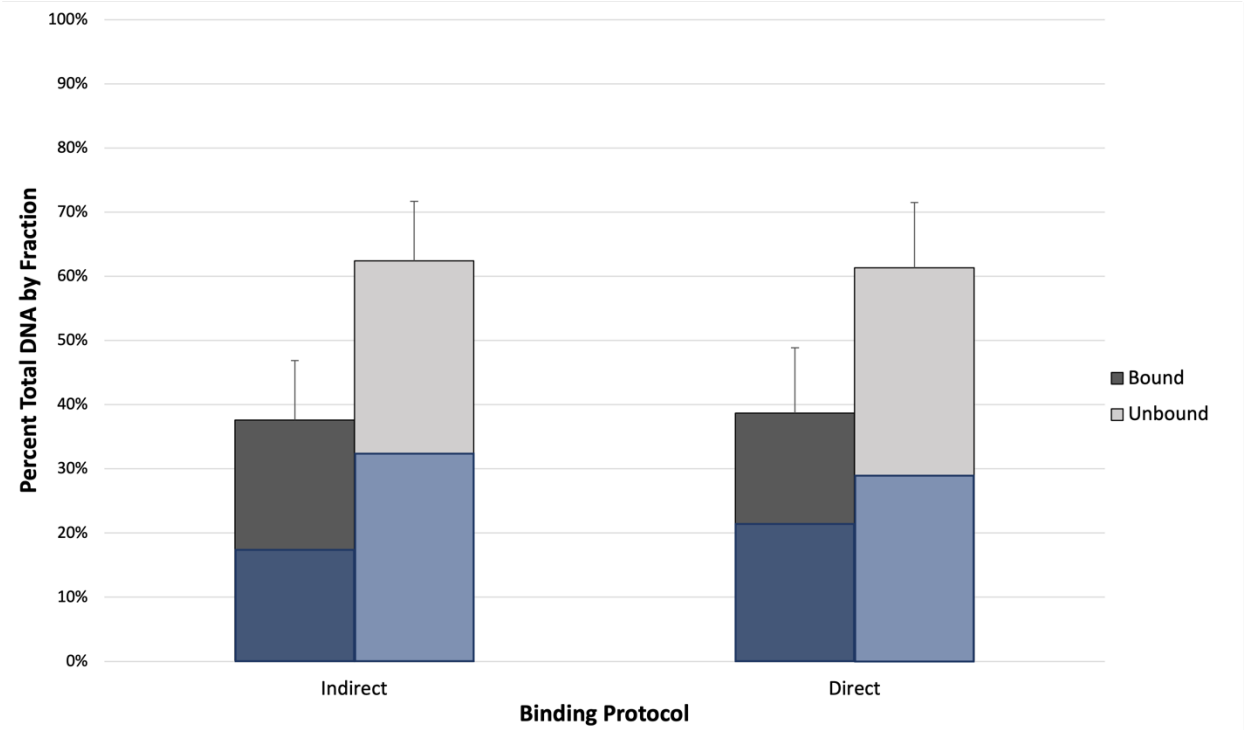

FIGURE 8 – Percentage of total DNA retained in antibody-bound and unbound fractions for mixture samples (n = 10) processed with the indirect and direct binding assays, with the blue overlays depicting the proportion of each fraction that was male DNA. Indirect and direct binding protocols retained similar percentages of total DNA (37.6 ± 9.3% and 38.7 ± 10.2%, respectively; $p = 0.80$); however, the direct binding protocol retained slightly more male DNA in antibody-bound fractions (53.0 ± 12% versus 49.3 ± 13%; $p = 0.52$).

M:F ratios were assessed from DNA quantification values as well as from subsequent STR profiling data. On average, unseparated mixture controls demonstrated a M:F ratio of 1:2.30 at quantification; however, antibody-bound fractions from the indirect assay demonstrated a mean M:F ratio of 1.18:1, while antibody-bound fractions from the direct assay produced a mean M:F ratio of 1.46:1 (Table 1) – both of which were statistically significant improvements over the

unseparated controls ($p = 0.02$ and $p = 0.01$, respectively). It should be noted that mean M:F ratios in unbound fractions for the indirect and direct assay were 1:1.79 and 1:2.01, respectively, at quantification (data not shown); this indirectly demonstrates the enrichment of sperm within antibody-Microbubble fractions, but also indicates possible carryover of sperm into unbound fractions. Although STR profiles of unbound fractions exhibited mean M:F ratios of 1:1 and 1.50:1 (data not shown), mean M:F ratios within STR profiles for antibody-bound fractions when using the indirect and direct assays were 2.13:1 and 2.52:1, respectively (Table 1). When comparing these ratios to those within unseparated mixture controls, indirect binding produced a mean 2.10 ± 0.43-fold improvement in the M:F ratio in the bound fraction; direct binding produced a statistically significant 2.76 ± 0.92-fold improvement in M:F ratio on average ($p = 0.041$), indicating that it was capable of enriching for sperm cells more consistently. Results also revealed the ability of the antibody assays to recover male contributor associated alleles within bound fraction STR profiles; on average, 0.2 – 0.4 unshared male contributor alleles were recovered in bound fractions when compared to their associated unseparated controls. This recovery of male contributor alleles, albeit small, could potentially lead to greater likelihood ratios for inclusion of perpetrators and thus assist with case resolution. Overall, these data clearly demonstrate the ability of these assays to enrich for sperm and – importantly – the potential to simplify mixture profile deconvolution and thereby reduce STR profile interpretation time at the end of the forensic DNA workflow (Figure 9).

TABLE 1. Male-to-female (M:F) ratios after DNA quantification and STR profiling in unseparated controls and antibody-bound fractions from semen-vaginal mixtures.

Sample	DNA Quantification			STR Profile Analysis				
	M:F Ratio			M:F Ratio			M:F Fold Improvement (B/Unseparated)	
	Unseparated	Indirect*	Direct**	Unseparated	Indirect	Direct†	Indirect	Direct
Mixture 1	1 : 1.20	1.12 : 1	1.44 : 1	2.61 : 1	4.52 : 1	8.37 : 1	1.73	3.21
Mixture 2	1 : 3.91	2.00 : 1	1 : 2.08	1 : 1.08	1.78 : 1	3.03 : 1	2.79	4.76
Mixture 3	1 : 2.79	1 : 1.63	1.71 : 1	1 : 1.71	1.38 : 1	2.14 : 1	2.36	3.65
Mixture 4	1 : 2.49	1 : 1.47	1.04 : 1	1 : 1.69	1.28 : 1	1.17 : 1	2.17	1.99
Mixture 5	1 : 1.45	1.03 : 1	1.30 : 1	2.28 : 1	3.73 : 1	5.44 : 1	1.64	2.38
Mixture 6	1 : 2.52	1 : 1.30	1.14 : 1	1 : 1.19	1.64 : 1	2.03 : 1	1.94	2.41
Mixture 7	1 : 6.35	1 : 2.72	1 : 1.63	1 : 2.65	1 : 1.20	1 : 1.44	2.21	1.84
Mixture 8	1 : 1.65	2.58 : 1	2.47 : 1	2.12 : 1	3.28 : 1	4.14 : 1	1.54	1.95
Mixture 9	1 : 2.03	2.45 : 1	3.93 : 1	1.17 : 1	2.26 : 1	2.79 : 1	1.93	2.39
Mixture 10	1 : 6.07	1 : 3.60	1 : 2.03	1 : 5.12	1 : 1.90	1 : 1.72	2.69	2.97
AVERAGE	**1 : 2.30**	**1.18 : 1**	**1.46 : 1**	**1.17 : 1**	**2.13 : 1**	**2.52 : 1**	**2.10 ± 0.43**	**2.76 ± 0.92**

All comparisons between unseparated controls and either indirect or direct binding.
*$p = 0.02$
**$p = 0.01$
†$p = 0.041$

An assessment of resulting STR profiles revealed several other interesting observations. First, the ability of the indirect and direct antibody assays used herein to efficiently enrich for sperm cells was related to the number of sperm present within the original sample. For example, unseparated mixture controls demonstrating a clear major male contributor also demonstrated higher-fold improvement in M:F ratios after separation, highlighting the ability of antibody-mediated assays to better perform when the target cell is in higher proportions within a sample. This phenomenon is expected and has been well documented throughout previous literature involving antibody-mediated sperm cell isolation [29,30,54].

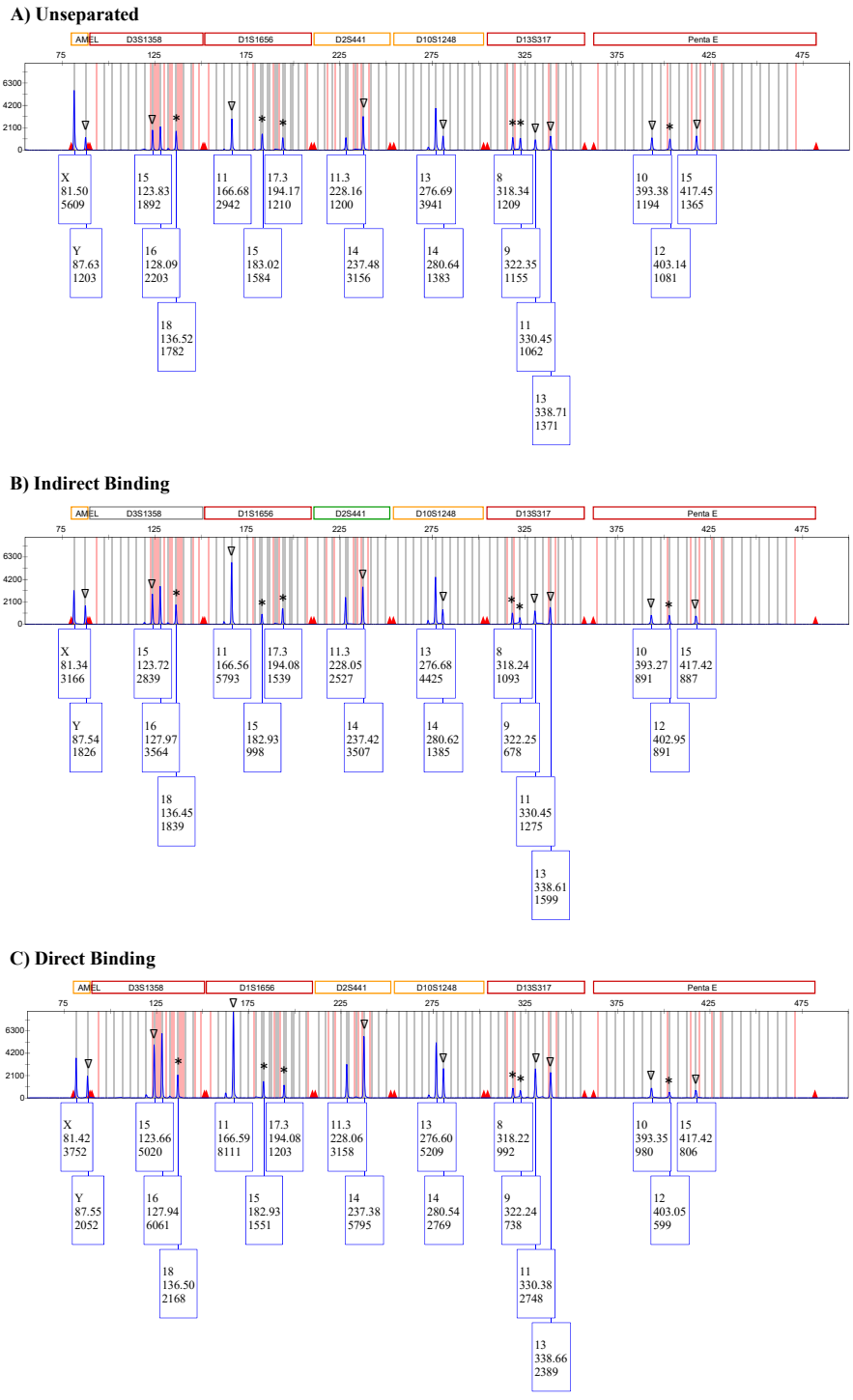

FIGURE 9 – Representative blue channel electropherograms for PH-20/Microbubble binding, which demonstrates the mean enrichment of the male component in antibody-bound fractions. The unseparated mixture control exhibited a M:F ratio of approximately 1:1 (A). The indirect binding protocol resulted in a 1.9-fold improvement in the M:F ratio (B), while the direct binding protocol resulted in a 2.4-fold improvement (C). Unshared female contributor alleles are denoted by an asterisk, while unshared male alleles are denoted by ∇.

Indirect and Direct Binding Protocol Modifications

Although STR profiling results demonstrated the ability of both the indirect and direct binding assays to enrich for sperm (and thus male contributor DNA), additional studies were performed in an effort to improve sperm cell retention *and* reduce retainment/carryover of non-sperm DNA. First, although there was an increased risk of inadvertent Microbubble removal due to their tendency to drag with the meniscus of the liquid fraction (Figure 2), removal of more "subnatant" was explored to determine whether resulting M:F ratios were being impacted by residual DNA left behind in the "subnatant" of antibody-bound fractions. Additionally, 2-fold and 3-fold PH-20 antibody load (and a concomitant increase in Microbubbles) were implemented in an attempt to bind and recover more sperm, as it was believed the presence of non-sperm cells (e.g., vaginal epithelial cells) within mixture samples reduced the availability of and interaction with binding sites between antibodies and sperm.

Overall, regardless of the protocol modification implemented, no improvement in sperm cell binding and concomitant M:F ratios in antibody-bound fractions was observed. In fact, all modifications led to reduced sperm enrichment and M:F ratio fold improvements compared to the original protocol. Removal of more "subnatant" revealed the possible carryover of free-floating sperm into unbound fractions, while increased proportions of PH-20 antibody and Microbubbles during the assay indicated increased potential for non-specific binding and/or reduced sperm binding efficiency (Supplemental Figure S2). This was exemplified by mean M:F ratios of approximately 1:2.40 and 1:2.00 regardless of binding order for 2-fold and 3-fold loads of antibody and Microbubbles, respectively (data not shown). The majority of antibody-bound fractions

exhibited equal or worsened M:F ratios with these protocol modifications, indicating that sperm binding was negatively impacted.

Assessment of DNA Provenance in Mixture Samples

Unseparated mixture controls were also assessed for DNA provenance (i.e., the proportion of DNA originating from the cellular/cell pellet fraction versus the supernatant/"subnatant" fraction) before and after antibody-Microbubble assay treatment. The purpose of this assessment was two-fold: 1) To evaluate the impact, if any, the Microbubbles had on the integrity of cells within samples, and 2) To determine the proportion of cellular/cell pellet versus supernatant/"subnatant" DNA within the samples utilized throughout this study and characterize the impact of this on resulting STR profiles. Fortunately, human DNA quantification results indicated there was no significant change in DNA provenance within eluted mixtures (n = 10) after subjection to the antibody-Microbubble assay when compared to those that were untreated ($p = 0.90$) (Figure 10). This is a critical finding that supports the gentle nature of BACS with Microbubbles, and it demonstrates that cell integrity is not impacted by this assay.

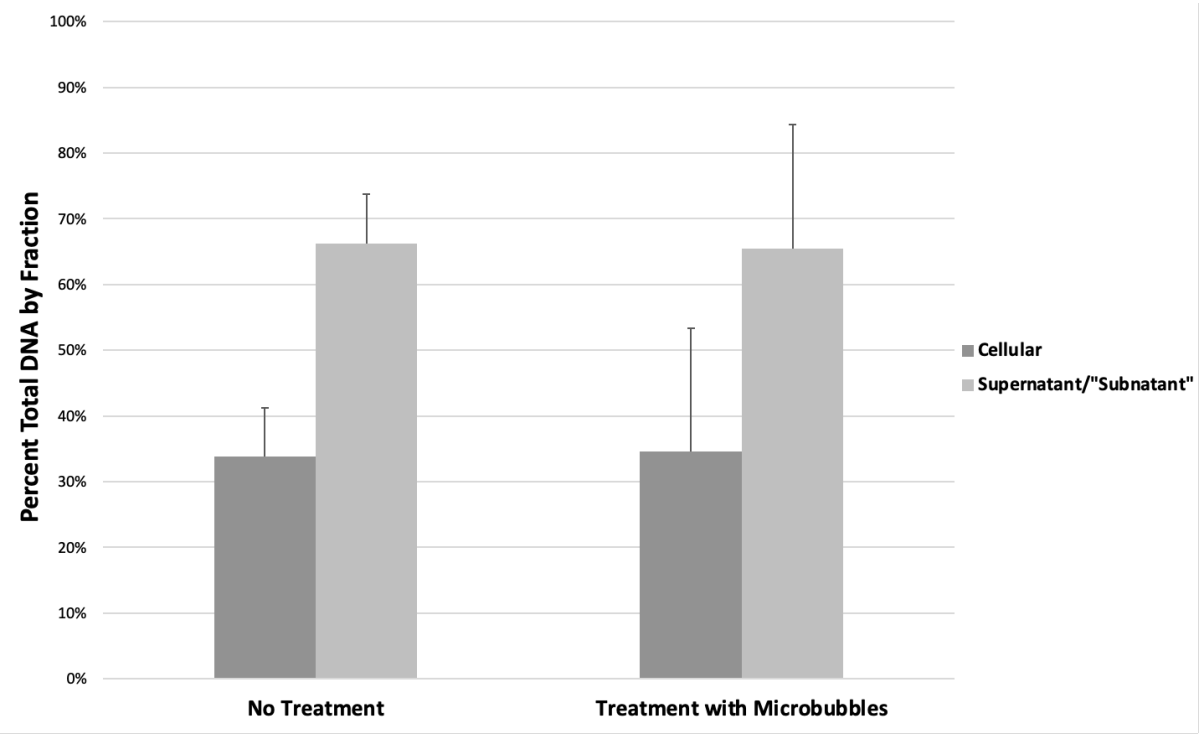

FIGURE 10 – Percentage of total DNA associated with cellular/cell pellet versus supernatant/"subnatant" fractions for mixture samples before and after subjection to the Microbubble assay (n = 10). No significant difference was observed between treatments ($p = 0.90$).

However, analysis of resulting STR profiles from "subnatant" fractions revealed M:F ratios ranging from 1:3.03 to 1:14.08 (mean M:F ratio of 1:6.53; n = 9) (Figure 11), with one profile exhibiting a single-source DNA profile of the female contributor. Additionally, five samples exhibited M:F ratios greater than 1:10 and five profiles only contained two loci with the expected unshared male contributor alleles above the analytical threshold. These data were critical in revealing that any leftover "subnatant" after antibody binding during this assay was likely to contain DNA stemming mostly from the female contributor – which could originate from smaller cells (e.g., leukocytes) or cell fragments that fail to pellet at 400 *x g*, or even be cell-free/pure DNA. Not only does this highlight the necessity of removing all "subnatant" after our PH-20/Microbubble assay, but it also indicates that the M:F ratios obtained in antibody-bound fractions were confounded by the amount of female DNA in the "subnatant" that was left behind.

To combat this issue, future studies should evaluate removal of all "subnatant" (ideally in an automated fashion to avoid tedious manual pipetting that may vary from analyst to analyst), the implementation of a DNase treatment prior to sperm lysis to remove contaminating non-sperm DNA, and/or a wash step prior to sperm cell lysis. In fact, previous studies using PH-20 antibody for sperm cell isolation have already demonstrated the ability of a DNase treatment to eliminate female contributor alleles in antibody-bound fractions [29].

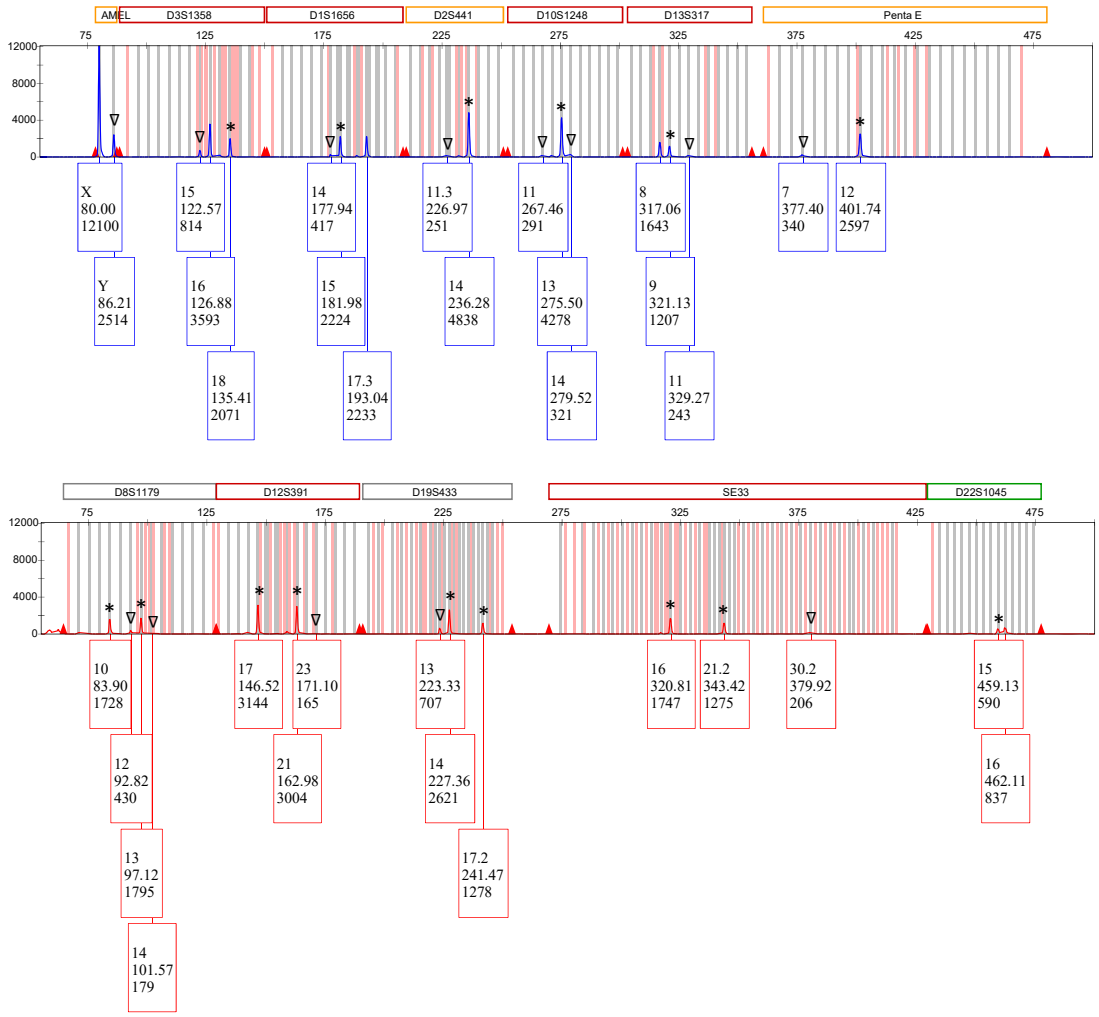

FIGURE 11 – Representative blue and red channel electropherograms from "subnatant" fractions of mixture samples that were processed with antibody-free Microbubbles. Major female STR profiles were obtained (with a mean M:F of 1:6.5), signifying that any residual "subnatant" within processed mixture samples has the potential to negate the enrichment of sperm cells with PH-20 antibody. Unshared female contributor alleles are denoted by an asterisk, while unshared male alleles are denoted by ∇.

CONCLUSIONS

Traditional processing of sexual assault samples is notoriously time-consuming, labor intensive, and inefficient at fully separating perpetrator and victim (i.e., sperm and non-sperm) fractions. These downfalls have contributed to SAECK backlogs, reduced statistical power of perpetrator/suspect inclusion within resulting STR profiles, and fewer case resolutions [4,55,56]. Naturally, much of the recent research within the field has been dedicated to tackling these issues through various modifications to the traditional differential extraction procedure to reduce time and increase efficiency [5,7,9–11]. Alternately, cell separation and isolation techniques such as filtration and FACS have been explored as a way to improve sperm cell recovery while reducing non-sperm cell carryover [14–16,57,58], whereas others have explored and implemented probabilistic genotyping software to more quickly and reliably deconvolute the inevitable complex DNA mixture profiles at the end of the workflow [59–62]. Most notably for this current work, a substantial body of research has been dedicated to the development of immunoprecipitation assays whereby sperm-specific antibodies can be attached to solid phase supports and thus be used to pull sperm away from the rest of the sample.

Although there have been many promising publications on the use of sperm-specific antibodies for sexual assault sample processing, many focus on fresh/liquid samples, assess only samples containing high sperm counts, and often fail to report metrics that are applicable to forensic DNA analysis. Thus, this study aimed to develop and optimize an immunoprecipitation assay for sperm cell isolation using PH-20 antibody and buoyant Microbubbles with emphasis on lower sperm input, samples that have been dried and reconstituted/eluted, and metrics which directly relate to STR profiling (which is the culmination of forensic DNA analysis).

Overall, this study demonstrated the ability of PH-20 antibody and BACS using Akadeum Microbubbles to separate sperm from non-sperm cells within sexual assault samples. Using the method described herein, 57.2 ± 19% and 58.0 ± 15% of seminal DNA was retained when implementing indirect and direct binding assays, respectively. Although this is less than the theoretical maximum of 80% when considering proportions of sperm and non-sperm cells within normal semen, sperm cell counts within this study were relatively low. Nonetheless, this assay was still capable of capturing enough sperm cells to generate full STR profiles from the male contributor of mixture samples. Even further, microscopic, human quantification, and STR profiling results demonstrated that this assay was capable of binding and isolating sperm cells with potentially degraded or otherwise altered cell surfaces.

Indirect and direct antibody assays revealed the ability to enrich for sperm in bound fractions, as well as recover unshared male contributor alleles which were not detected in corresponding unseparated controls. Based on the results herein, optimal sperm binding within both semen and semen-vaginal mixture samples can be accomplished with direct binding, as this retained the highest percentage of total seminal DNA and resulted in statistically (and practically) significant M:F ratio fold improvement for mixture samples. In fact, 80% of antibody-bound fractions exhibited major male contributor profiles to some degree. Overall, this study developed a novel, small-volume (~60 µL total) assay that can accomplish sample elution, antibody-binding, fractional separation, and cell lysis within 45 minutes; this considerably reduces hands-on and overall processing time when compared to the traditional differential lysis procedure without the requirement of new equipment, allowing easy implementation into the existing forensic workflow.

Future evaluations of this technique with additional mixture samples, alternative *or* additional antibodies to further enhance sperm binding, removal of all "subnatant," careful removal of the Microbubble portion rather than the "subnatant," and even the addition of a DNase treatment on antibody-bound fractions prior to sperm lysis should be conducted to see if further optimization can be achieved. Additionally, as is true with any antibody assay, an evaluation and comparison of assay efficiency across preparations/lots of antibodies should be evaluated to determine reproducibility and establish quality control parameters – one of many steps that would be needed for developmental validation of this technique and eventual implementation with casework samples. Finally, automation of this assay should be explored, as the density difference between cell-antibody-Microbubble complexes and unbound cells could be exploited within centrifugally-driven microdevice platforms for more efficient and complete separation of antibody-bound and unbound fractions (which, in turn, would further reduce processing time and minimize the dependency of assay success on analyst skill level).

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SUPPLEMENTAL FIGURES

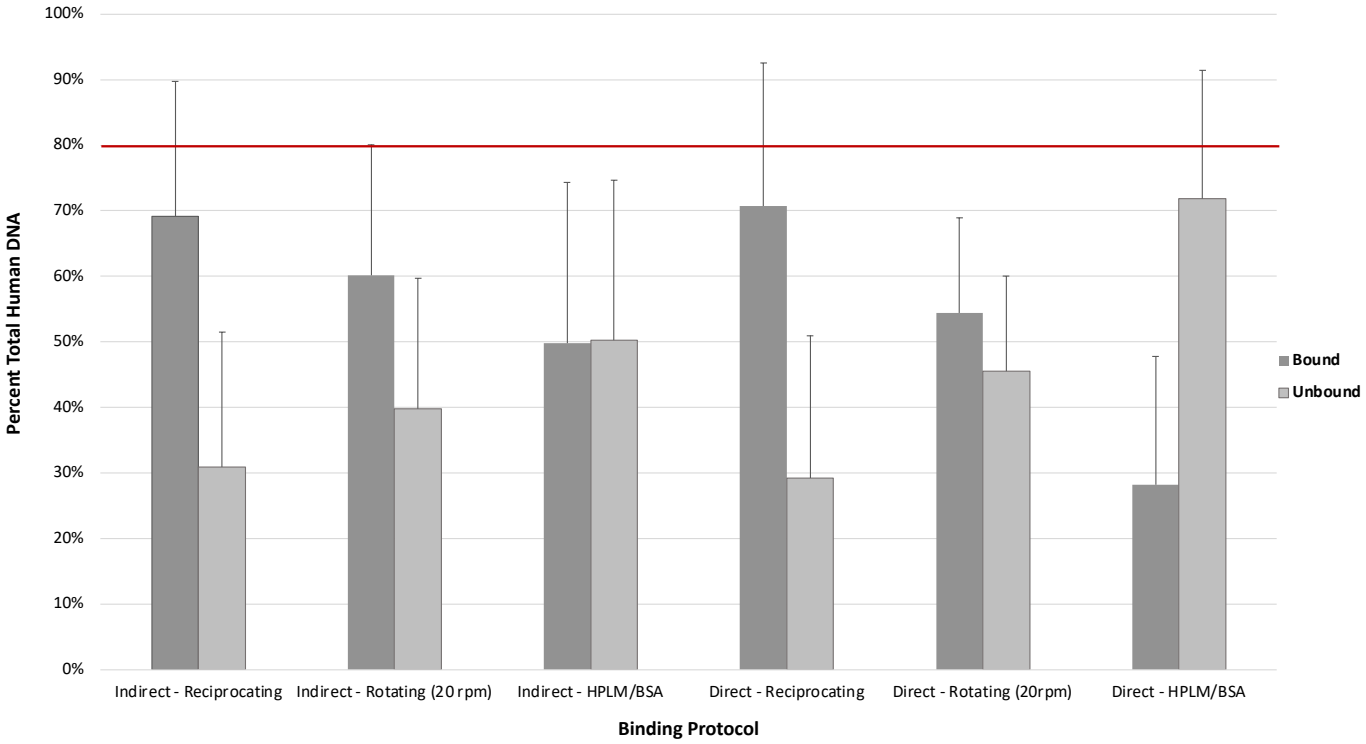

FIGURE S1 - Percentage of total DNA retained in antibody-bound and unbound fractions after treatment of semen eluates when implementing various binding protocol modifications (reciprocating versus rotating modes, n = 3; HPLM/BSA media, n = 10). The red line indicates the theoretically expected percentage of seminal DNA associated with sperm. Regardless of the modification tested, the original indirect and direct protocols (which utilized reciprocating mode) retained the highest mean percentage of seminal DNA.

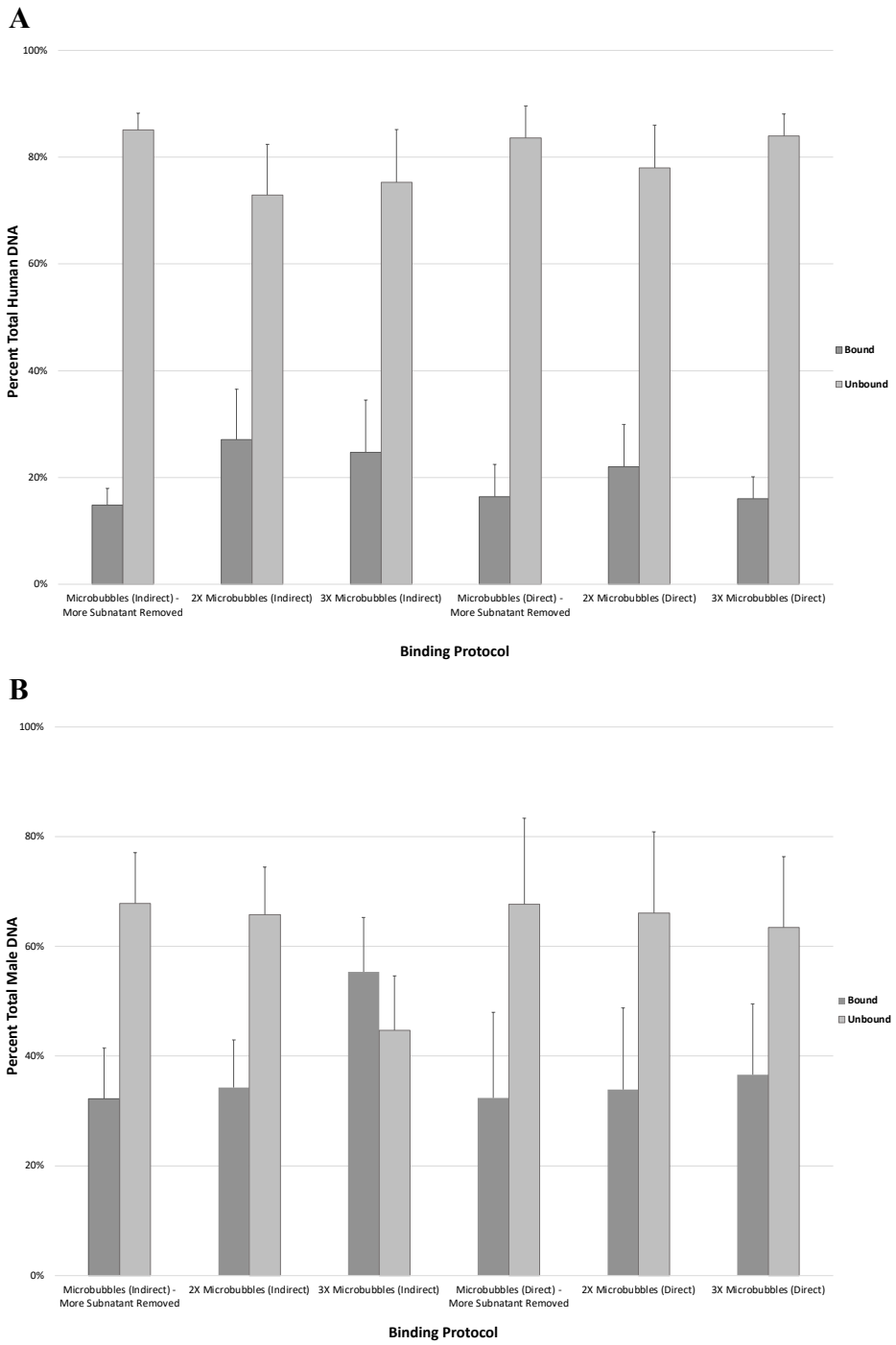

FIGURE S2 – Percentage of total DNA (A) and total male DNA (B) retained in antibody-bound and unbound fractions after treatment of mixture eluates when implementing various binding protocol modifications. Removal of more "subnatant" was accompanied by recovery of less total DNA without a change in the recovery of male DNA. Increased PH-20 and Microbubble loads during binding resulted in recovery of less total DNA when compared to original protocols but appeared to recover an equal or higher percentage of male DNA for indirect binding.

CHAPTER FOUR:

CHARACTERIZATION OF BUOYANCY ACTIVATED CELL SORTING (BACS) WITH MICROBUBBLES ON A CENTRIFUGAL MICRODEVICE

ABSTRACT

Cell sorting, or the targeting and isolation of specific cells from a heterogenous sample, is a critical and prominent technique that has been used for numerous biological applications. Although this procedure is ubiquitous, methods for its automation remain limited and expensive. The recent advent of buoyancy activated cell sorting (BACS) has provided a unique opportunity for the automation of immunoprecipitation and cell isolation, as this method takes advantage of gravitational, buoyancy, and drag forces to accomplish fractional separation of target and non-target cells. Given this, minimal excessive force and external hardware are required to accomplish separation, and centrifugation can be applied to accelerate the process. While this technique has shown great promise in-tube, there is no available literature exploring its potential for automation. Thus, this study explored the behavior and movement of buoyant microbeads (e.g., Microbubbles) within a centrifugal microdevice, as fluid propulsion within such a device is accomplished by the same forces required to achieve BACS. An antibody-mediated sperm isolation assay was then transitioned onto this microdevice, and the ability to accomplish automated cell sorting within sexual assault samples was evaluated. Ultimately, dye studies and microscopic observations demonstrated the ability to achieve homogenization of Microbubbles throughout the microdevice chamber, as well as the capability of controlling the rate and position of bead aggregation. Further, it was possible to retain most Microbubbles within their original chamber, even after opening a normally closed valve via laser ablation and spinning the solution into an adjacent chamber. Although antibody-bound fractions of mixture samples processed on this microdevice exhibited only minor enrichment of sperm cells, this study served as proof of concept for the automation of BACS. Future studies should evaluate architectural modifications and faster spin speeds to ensure more efficient fractional separation and reduce carryover of Microbubbles. Ultimately, the microdevice described herein is amenable to multiple assays and could provide automation of cell targeting for biomedical, diagnostic, and forensic applications. This technique could inherently increase the efficiency of fractional separation in a relatively cheap and quick fashion.

KEYWORDS: buoyancy activated cell sorting (BACS), Microbubbles, centrifugal microfluidics, microdevice, sperm, PH-20/SPAM-1

INTRODUCTION

Cell sorting, or the process of isolating and separating a specific cell type, has historically been used throughout the biological sciences to obtain a homogenous sample population. While cells can be sorted based on various properties, perhaps one of the most common means of achieving isolation is by targeting specific regions or molecules on a cell surface. Antibodies raised against unique proteins or other antigens have improved cell isolation specificity, especially for cell types which have other biological properties (e.g., density, surface charge, deformability, lysis susceptibility) that are similar to other, non-target cell populations. Not only has this been applied for disease diagnostics and therapy, but it has also been heavily explored for forensic applications.

Although fluorescence activated cell sorting (FACS) is a more common technique for applying antibodies to isolate cell types within a sample, immunoprecipitation methods have gained a lot of attention and popularity within recent decades. Such methods rely on the conjugation of an antibody-bound solid support to target cells, forming a complex which can then be physically pulled away from the rest of the sample by centrifugation, size filtration, or magnetic fields. Magnetic activated cell sorting (MACS) is a prominent immunoprecipitation method which has enjoyed widespread use throughout the scientific community. Not only do paramagnetic beads typically achieve higher purity, but they also provide an opportunity for automation [1,2]. A magnet can be affixed to a tube rack, liquid handling robot, or column, enabling specific positioning of the target cells to a region of the tube or well that can be avoided when removing the rest of the sample. Although MACS provides advantages of reproducibility and automation, and although there are several commercial kits available, this technique is still relatively expensive; thus, other solid supports have been developed in recent years.

Hollow glass microspheres (e.g., Akadeum Microbubbles) have emerged as a new and promising solid support type for cell sorting applications. Because they are hollow, these beads are less dense than the surrounding media, causing them to rise in solution [3]. Separation of buoyant Microbubbles can be passively achieved due to the interaction of gravitational, buoyant, and drag forces acting together on the beads. Alternatively, centrifugation can be applied to hasten the process. Because most cells easily pellet with centrifugal force, these beads are intriguing for cell sorting applications; centrifugal force causes these beads (and any attached cells) to rise, while unattached/non-target cells pellet. In comparison to dense agarose and polystyrene beads, this method could theoretically reduce non-specific trapping of cells since they would not settle at the tapered bottom of microcentrifuge tubes or wells. Additionally, no external hardware (e.g., a magnet) is required to accomplish separation, reducing costs and limiting the use of additional forces that could negatively impact antibody-cell interactions. This relatively new concept has been coined buoyancy activated cell sorting (BACS), and it has already been utilized to achieve 90% separation efficiency with CD4+ cells from whole blood samples [4]. Although this method is marketed for applications of negative selection, whereby non-target cells are bound and removed, our research group has recently explored the use of BACS for positive selection and subsequent processing of target *and* non-target cell fractions (*see Chapter 3*).

While BACS serves as a promising avenue for cell sorting, the principles and concepts governing this method also make achieving efficient separation of target and non-target fractions inherently difficult. Negative selection is relatively simple to accomplish when the non-target cell population does not need to be kept, as the buoyant microbeads that have risen to the top of solution can be removed and discarded via vacuum aspiration [5]. However, when both target and non-target cell

populations need to be analyzed, this technique can be problematic; removal of the risen bead-bound fraction via pipetting is difficult, and the loss of a portion of this fraction within the pipette tip is possible. Alternatively, removing the liquid fraction beneath the beads (i.e., the "subnatant") is not a menial task. For example, the direction of gravity can be manipulated to force Microbubbles to the top sidewall of a tube, allowing one to go underneath them and remove the "subnatant," (*see Chapter 3*) [3]; however, the efficiency of this technique is highly dependent upon analyst skill. Further, because of the nature of these Microbubbles and the differing adhesion forces acting upon them when in various degrees of contact with solution, it is nearly impossible to manually remove the entire "subnatant" without inadvertently removing some of the Microbubbles as well. Thus, in order to serve as an efficient cell isolation technique, a means for automating BACS is desperately needed.

Given that BACS relies upon gravity and centrifugation to accomplish fractional separation, an obvious potential avenue for automation of this technique is centrifugal microfluidics. While microfluidics in general refers to the manipulation of liquids on the microscale, centrifugal microfluidics takes advantage of centrifugal force and device architecture to control liquid propulsion – obviating the need for bulky and potentially expensive external pumps [6–8]. Theoretically, the behavior of buoyant microbeads within a centrifugal microdevice should be similar to that of an in-tube environment; however, there are no studies within the available literature that have explored this concept. Thus, the research described herein aimed to demonstrate and characterize the behavior of Microbubbles within a centrifugal microdevice, with the goal of providing proof of concept for the automation of BACS. It is believed that these Microbubbles can be homogenized, congregated, and separated by utilizing centrifugal force and specific

microdevice architecture (Figure 1). Additionally, although Microbubbles that are surrounded by more solution experience smaller adhesion forces than those closer to the air-liquid interface and are thus more likely to be removed with the trailing edge of the liquid phase (Figure 1), this study evaluated the ability of device architecture and spin parameters to minimize the transfer of Microbubbles to subsequent chambers within the microdevice. After evaluation of fluidic movement and Microbubble behavior, a PH-20 antibody-mediated sperm cell isolation assay was preliminarily explored to determine the feasibility of this technique for automating immunoprecipitation assays for cell sorting.

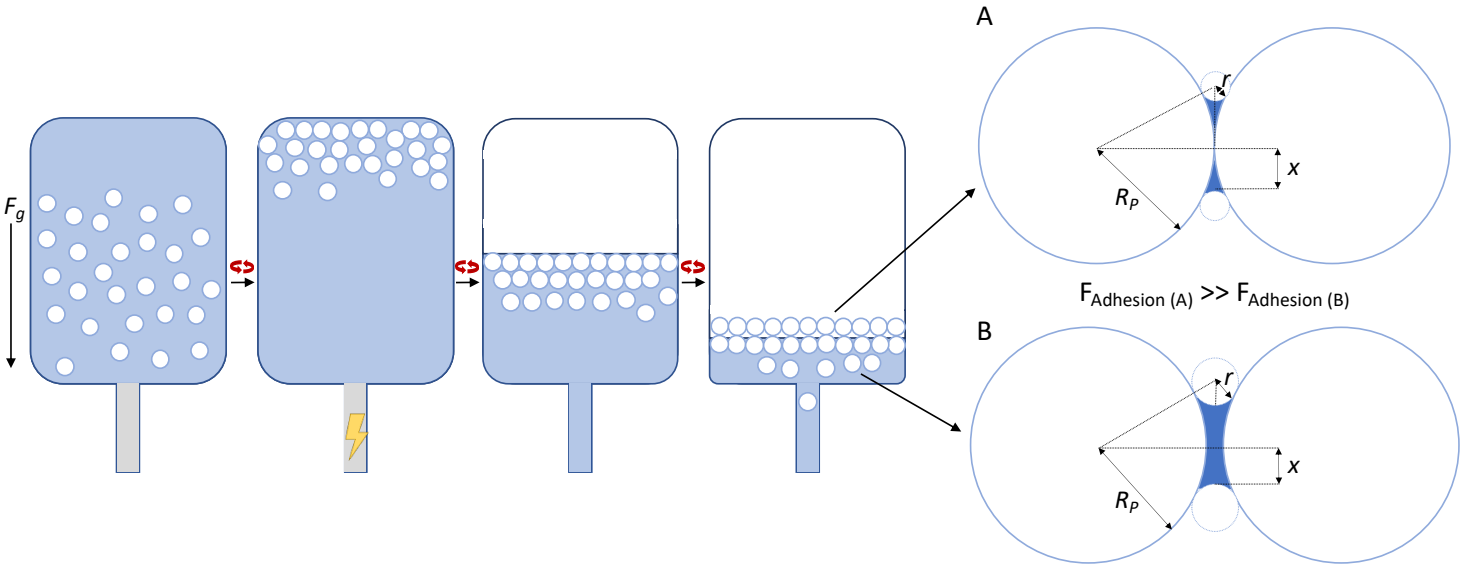

FIGURE 1 – Impact of gravity (F_g), centrifugation, and "subnatant" removal on Microbubbles within a microdevice chamber. Microbubbles rise and form a compact, crystallized layer due to their density compared to that of most solutions, a phenomenon which can be hastened by centrifugal force. The "subnatant" (i.e., liquid fraction beneath the Microbubbles) can be removed into a subsequent chamber by opening a valve (ϟ) and spinning. As this happens, most Microbubbles are forced to the trailing edge of the liquid, while others closest to the liquid fraction tend to drag with the meniscus and have the potential to move into the adjacent microdevice chamber. This can be explained by the difference in the force of adhesion acting on the Microbubble complexes closer to the air interface (A) compared to that on the Microbubbles which have more contact with the liquid (B), where the force of adhesion between the former is much greater and prevents their movement with bulk flow.

MATERIALS & METHODS

Sample Collection & Preparation

Liquid semen and vaginal swabs were collected from ten and one anonymous donor(s), respectively, following the university-approved Institutional Review Board (IRB) protocol HM20002931. Semen was diluted 1:60 by volume with Gibco™ 1X Dulbecco's phosphate-buffered saline (DPBS) (Fisher Scientific; Waltham, MA). To elute cells from vaginal swabs, a half-swab cutting was emerged in 200 µL DPBS and incubated at 37ºC for two hours, with brief vortexing every 15 minutes. To ensure homogeneity of vaginal cellular material across all mixture samples, cells were eluted from a total of four vaginal swabs and resulting eluates were combined.

All samples tested within this study were dried onto foam swabs prior to elution and antibody-mediated cell separation. Samples were prepared in a total volume of 80 µL, and foam swabs were dipped into the solution. Semen-vaginal mixture samples were prepared by combining 30 µL of 1:60 semen with 50 µL of vaginal eluate, as this method has produced approximate 1:1 M:F ratios in resulting STR profiles within our lab when fractional separation does not occur (data not shown). This was repeated for each semen donor to generate ten unique mixture swabs. A single vaginal donor was utilized in an attempt to assess the efficiency of this assay with various sperm cell counts rather than various sperm *and* non-sperm cell counts. Swabs were allowed to absorb the entire sample prior to drying overnight at room temperature. Once dry, swabs were cut into fourths and stored at 4 ºC until testing; all swabs were tested within 1-2 months of preparation.

All testing utilized 1/4th foam swab cuttings which, according to the available literature and the semen dilutions used herein, were expected to contain approximately 6,000 – 19,000 sperm cells

[9–15]. Mixture samples were separated into antibody-bound and unbound fractions, but additional mixture samples not subjected to antibody-binding and separation (i.e., “unseparated” mixtures) were also assessed as controls.

Microdevice Fabrication & Hardware

Microdevices used for these studies consisted of five polyethylene terephthalate (PeT) layers: two exterior layers of clear, 101.6 µm PeT film; two layers of clear, 101.6 µm PeT with an affixed 50.8 µm heat-sensitive adhesive (HSA; EL-7970-39, Adhesives Research, Inc.; Glen Rock, PA, USA) to enable adhesion; and a middle layer of 75 µm black PeT (bPeT; Lumirror* X30, Toray Industries, Inc.; Chuo-ku, Tokyo, Japan) to facilitate valving. The previously described print-cut-laminate (PCL) method was employed for device fabrication [16]. Microfluidic architecture was designed using AutoCAD® LT 2022 (Autodesk®, Inc.; San Rafael, CA, USA) and cut using a VersaLASER® 3.50 CO_2 laser platform (VLS3.50; Universal® Laser Systems; Scottsdale, AZ, USA). Prior to assembly, all layers were sterilized as follows: washing for 30 minutes in molecular biology-grade water (MBG H_2O) on an orbital mixer, drying at room temperature, wiping with 70% isopropanol followed by MBG H_2O, and drying at room temperature. Each five-layer device was then bonded using an Apache AL 13P12 laminator at 379ºF (~192ºC). Layer order was as follows: clear PeT, clear PeT/HSA, bPeT, clear PeT/HAS, and clear PeT. In addition, 2.0 mm thick polymethyl methacrylate (PMMA) pieces were laser ablated and affixed to the exterior of the microdevice using 55.8 µm pressure sensitive adhesive (PSA) (ARcare 7876; Adhesives Research, Inc.) to provide chamber depth and volume for the swab input and lysate recovery modules. The bPeT layer in each device provided a valving mechanism that could be actuated, or opened, by

firing a 700 mW 638 nm laser diode (L638P700M; Thorlabs, Inc., Newton, NJ, USA) that was positioned ~15 mm above the device [17].

The final device architecture consisted of modules for swab insertion, sample elution, antibody binding and lysis (i.e., bound chamber), supernatant transfer and lysis (i.e., unbound chamber), and lysate recovery (Figure 2).

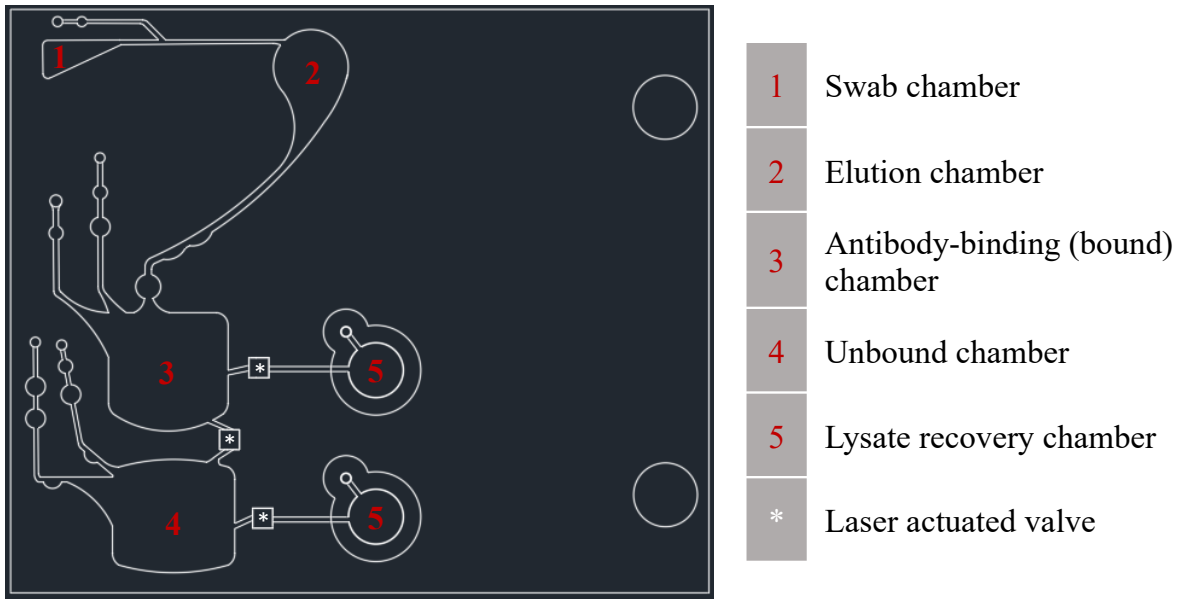

FIGURE 2 – Final microdevice architecture for antibody-Microbubble mediated cell isolation. A swab cutting can be inserted into the swab chamber (1), and centrifugation of the device can force eluate into the elution chamber (2). A change in center of rotation followed by spinning forces the sample into the antibody-binding/bound chamber (3), where antibody/Microbubble binding can take place. Upon opening a laser actuated valve (*) between the two primary chambers, "subnatant" can be spun into the unbound chamber (4). Cell lysis can take place in both chambers in tandem, and lysates can ultimately be spun into the side-by-side recovery chambers (5) for retrieval.

Fluidic propulsion within the microdevice was controlled using spinning/centrifugation. Microdevices were inserted into custom mounts, which are connected to a central spin arm via rotating servos (Hitec RCD; San Diego, CA, USA). The spin arm rotates through a slip ring (MOFLON Technology Co., Ltd.; Shajing, Shenzhen, China) by use of an electromagnetic stepper

motor (Pololu Corporation; Las Vegas, NV, USA). A Peltier clamp assembly comprised of two sets of 1" x 1" Peltiers, heat sinks, and fans (SUNON; Kaohsiung City, Taiwan) was used to perform heating steps; the microdevice was sandwiched in between this assembly during heating. All centrifugation, change in center of rotation, and heating functions were controlled using a terminal interface with a custom command menu within Propeller Tool software (Parallax, Inc.; Rocklin, CA, USA).

Fluidics/Dye Studies

Dyes studies were performed to evaluate fluid movement and control throughout the microdevice using 20mM Erioglaucine, 20mM Tartrazine, and 10mM Allura Red dyes (Sigma-Aldrich; St. Louis, MO, USA) prepared in MBG H_2O. Individual unit operations were performed prior to implementation of the entire differential cell lysis workflow to demonstrate fluidic movement and control (as described below). All sample, Microbubble, and buffer volumes were the same as described below for the antibody-binding assay.

Microscopy

The behavior of the Akadeum Microbubbles within the microdevice was assessed via microscopy. To accomplish this, one microliter streptavidin-coated Microbubbles was combined with nine microliters Akadeum separation buffer. Thirty-two microliters DPBS was added to the swab chamber, the swab chamber was capped, and the solution was then transferred to the elution chamber. The pre-mixed Microbubble/buffer solution was then added to the antibody-binding chamber, the microdevice was rotated, and all fluid was transferred into the antibody-binding chamber via spinning. The behavior of the Microbubbles was then assessed via changes in device

orientation (0 – 90°), as well as centrifugation at various speeds (500-1600 rpm); this was observed directly in the microdevice using brightfield microscopy at 100X, 200X, and 400X magnification on a Micromaster microscope (Thermo Fisher Scientific).

Antibody-Mediated Cell Isolation

The antibody-mediated sperm isolation assay utilized biotin-conjugated, rabbit anti-human polyclonal PH-20/SPAM-1 antibody (#LS-C829922-100 [aa36-490], in PBS; Lifespan Biosciences; Seattle, WA) alongside streptavidin-coated Microbubbles (Akadeum Life Sciences; Ann Arbor, MI) ranging in diameter from 5 – 15 µm. Antibody-mediated cell isolation was performed as previously optimized and described (*see Chapter* 3). Direct order binding occurred in a 0.2 mL tube, whereby 0.825 µg biotin-conjugated PH-20 antibodies were initially incubated with one microliter streptavidin-coated Microbubbles and Akadeum separation buffer in a total volume of 10 µL. Binding occurred for five minutes at room temperature on a tube revolver (Thermo Fisher Scientific; Waltham, MA) that was set to "reciprocating mode".

A mixture swab cutting was inserted into the swab chamber of the centrifugal microdevice alongside 32 µL DPBS. The swab cutting was then briefly teased with a pipette tip and the swab chamber was capped with clear PeT. The microdevice was then inserted into its mount, and agitation of the wetted swab was conducted by "shaking" for 180 steps at 100 rpm for 30 cycles. Centrifugation of the microdevice at 1600 rpm for ten seconds transferred the swab eluate to the elution chamber, and the antibody-Microbubble solution (prepared as described above) was loaded into the antibody-binding chamber of the microdevice via pipetting. Next, the microdevice was rotated 90° and spun at 1600 rpm for ten seconds to force the eluate and any residual antibody-

Microbubble solution into the adjacent antibody-binding chamber. Antibody-conjugated bead-to-cell binding was then conducted at room temperature for 20 minutes, with changes in orientation of the microdevice (from portrait to landscape, in 45° and 90° increments; covering all axes and Euler angles) every minute to ensure adequate mixing. The microdevice was then spun for five minutes at 1600 rpm (~140-175 *x g*) to pellet any unbound cells and to force all Microbubble complexes to the trailing edge of the liquid within the binding chamber. Laser ablation was then used to open the (normally closed) bPeT valve between the antibody-binding and unbound chambers. Spinning of the unbound fraction, or the fraction not bound to Microbubbles, into the adjacent chamber was conducted at 900 rpm (~50 *x g*) for one second.

To accomplish cell lysis in resulting antibody-bound and unbound fractions, 4 µL 1M sodium hydroxide (NaOH) was added to the unbound chamber. Additionally, 21 µL DPBS was combined with 4 µL 1M NaOH and subsequently added to the antibody-binding chamber (for the bound fraction). The microdevice was then positioned within the Peltier clamp assembly and heated at 75ºC for five minutes.

Next, the normally closed bPeT valves (connecting antibody-bound and unbound chambers to their respective recovery chambers) were opened via laser ablation. Each lysate was transferred to its associated recovery chamber by spinning, aspirated using a pipette, and neutralized with 1M Tris-HCl (Invitrogen).

Preparation of Unseparated Controls

Unseparated mixtures were also processed to serve as a point of comparison for this technique. Mixture swab cuttings (prepared as described above) were eluted in 26.5 µL DPBS for five minutes with vortexing every minute, followed by the addition of 4 µL 1M NaOH. Samples were incubated at 75ºC for five minutes on the ProFlex™ 3x32-well PCR System (Applied Biosystems™), and resulting lysates were neutralized with 4 µL 1M Tris-HCl [18]. To reduce variability due to sampling, only unseparated controls originating from the same initial whole swab (as experimental samples) were evaluated.

DNA Quantification

Human and male DNA quantification was conducted with the Investigator® Quantiplex HYres kit (QIAGEN; Hilden, Germany) on the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific). A half-volume reaction was utilized; thus, 4.5 µL Reaction Mix, 4.5 µL Primer Mix IC YQ, and 1.0 µL template DNA were combined in each well, and thermal cycling conditions followed manufacturer recommendations [19]. Resulting data were analyzed using Sequence Detection System (SDS) software v1.4 (Applied Biosystems™), with automatic baseline and threshold settings for each target.

Amplification and component plots were evaluated as previously described by Hudson et al. to identify any possible signs of inhibition [20]. Male-to-female (M:F) ratios in unseparated controls, antibody-bound fractions, and unbound fractions were calculated by dividing the male DNA concentration by the estimated female DNA concentration (i.e., the difference between the human and male DNA concentrations); these values were then averaged for each experimental group to

determine the mean M:F ratio, and a Student's *t*-test was conducted to determine statistical significance ($\alpha = 0.05$). The M:F ratio fold improvement for antibody-bound fractions compared to unseparated controls was then determined by dividing the mean M:F ratio within an antibody-bound fraction by the mean M:F ratio in its associated control; the mean fold improvement was then calculated by averaging the M:F fold improvement for all ten mixture samples.

STR Amplification

All samples were amplified using the Promega™ PowerPlex® Fusion 6C System with a template DNA input of 0.25 ng following manufacturer recommendations at half-volume; thus, each reaction consisted of 5.0 µL sample (at 0.05 ng/µL), 2.5 µL PowerPlex® Fusion 5X Master Mix, 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix, and 2.5 µL amplification-grade water. Thermal cycling was conducted on the ProFlex™ 3x32-well PCR System following manufacturer-recommended parameters [21]. Samples which demonstrated inhibition and allelic dropout after STR profiling were subsequently amplified with an input *volume* of 2.5 µL (0.15 – 0.225 ng DNA) rather than a target DNA input.

Capillary Electrophoresis & Data Analysis

Amplicons were separated on an Applied Biosystems™ 3500 Genetic Analyzer using Data Collection software v4 (Thermo Fisher Scientific) and manufacturer recommendations. One microliter of sample or allelic ladder was combined with 0.5 µL WEN ILS 500 (Promega™) and 9.5 µL Hi-Di™ Formamide (Thermo Fisher Scientific). Injection parameters also followed manufacturer recommendations and included a 36 cm capillary array (Thermo Fisher Scientific), POP-4® polymer (Thermo Fisher Scientific), and a 1.2 kV 15 second injection. Resulting STR

profiles were analyzed with GeneMapper™ *ID-X* software v1.6 (Thermo Fisher Scientific) using manufacturer settings with an analytical threshold of 150 RFU [21].

STR profiles were qualitatively assessed for signs of inhibition (e.g., allelic dropout and poor interlocus balance). M:F ratios were calculated by dividing the total peak height for male alleles by the total peak height for female alleles at each locus where there was no allele sharing between donors. Mean M:F ratios were then calculated by averaging across all loci within a profile, as well as across all profiles within an experimental group. A Student's *t*-test was conducted to compare mean M:F ratios across antibody-bound fractions and unseparated controls ($\alpha = 0.05$).

The M:F ratio fold improvement for antibody-bound fractions was then determined by dividing the mean M:F ratio within an antibody-bound fraction by the mean M:F ratio in its associated unseparated control; the mean fold improvement was then calculated by averaging the M:F fold improvement for all ten mixture samples.

RESULTS & DISCUSSION

Microbubble Behavior in a Centrifugal Microdevice

In order to characterize the behavior and movement of the buoyant Microbubbles within the microdevice architecture, several dye studies and microscopic evaluations were performed. The dye study with Microbubbles demonstrated that fluidic movement could be controlled with minimal loss and backflow of liquid, even after a heating step (Figure 3). Fluid was successfully transferred from the swab chamber to all downstream chambers, "lysis reagents" were added through inlets, and valves could be opened to enable precise control of liquid transfer when desired.

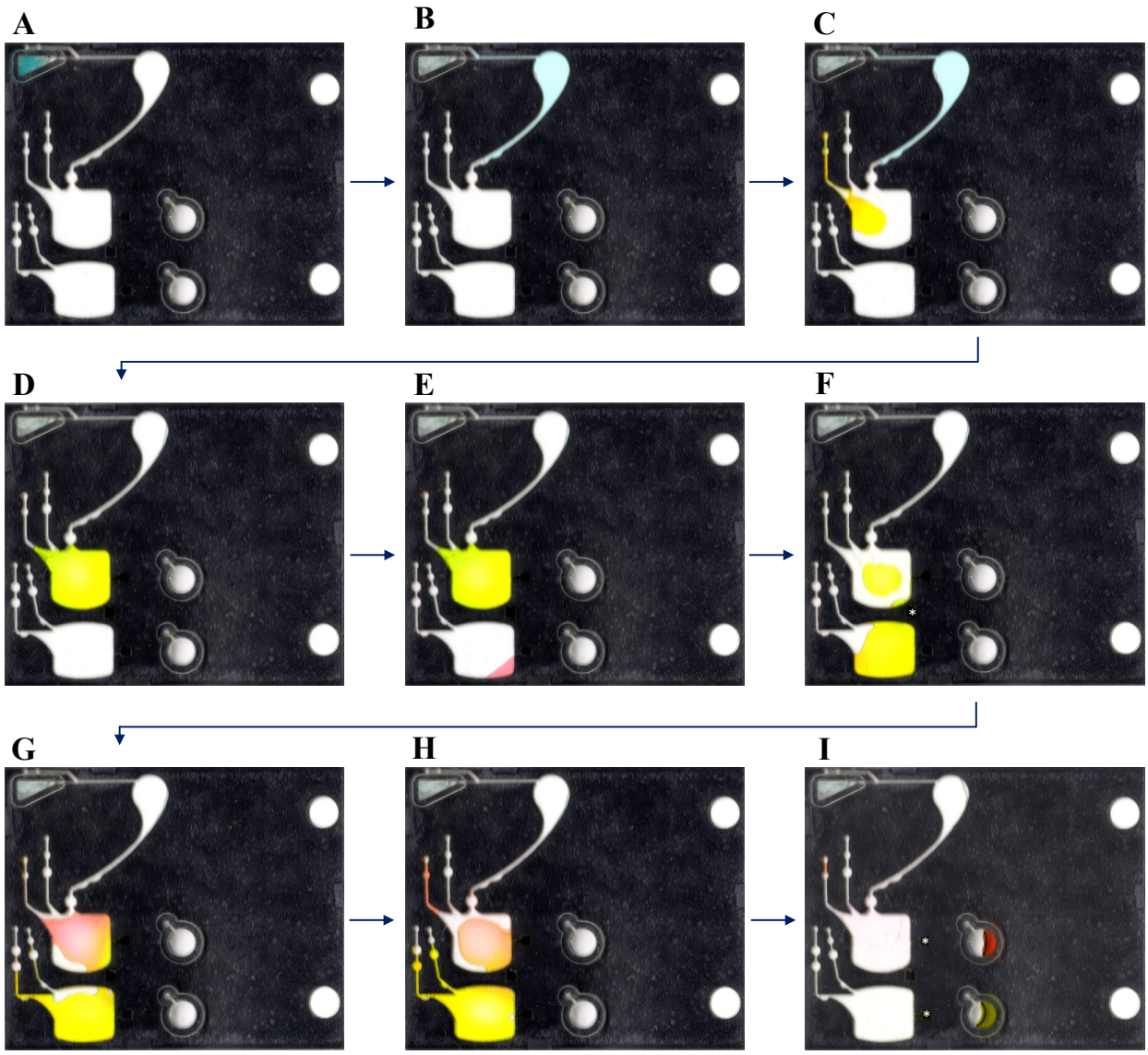

FIGURE 3 – Fluidic movement throughout the microdevice. A swab with liquid (blue) is inserted into the swab chamber, which is then capped (A). Centrifugation forces liquid to move into the elution chamber (B). Antibody-conjugated Microbubbles (yellow) can then be added to the antibody-binding chamber (C), after which change in center of rotation and centrifugation forces all liquid into the antibody-binding chamber (D). Lysis solution (red-orange) can be added to the downstream chamber (E). Opening of a normally closed valve via laser ablation and spinning of the supernatant into the downstream chamber then accomplishes fractional separation (F). Sperm lysis master mix (red-orange) is then added to the antibody-binding chamber (G), the device is heated (H), valves are opened, and both resulting fractions are spun into recovery chambers (I) to facilitate aspiration/removal from the microdevice. Valves are denoted by white asterisks.

The ability to mix or homogenize the Microbubbles within the microdevice chamber was also observed via microscopy. It was discovered that frequent change in the orientation of the device (and thus manipulation of gravitational force) could sufficiently mix the Microbubbles and fluid within the antibody-binding chamber (Figure 4A-B). This was intuitive, as the Microbubbles are less dense than most solutions and thus their movement is driven by an interaction between gravity and buoyant forces. By selectively controlling the orientation of the device (x, y, and z axes; all Euler angles) in consistent intervals, the Microbubbles can be homogenized throughout the chamber of the microdevice without the need for additional forces; this is beneficial for antibody-binding applications, as most in-tube formats utilize gentle rotation for mixing to prevent adverse impacts on antibody-target interactions. Further, the speed at which these Microbubbles are able to traverse the chamber without added force is advantageous over other solid supports such as paramagnetic beads, and almost serves as an intermediate between diffusion and vortexing.

After demonstrating the ability to mix Microbubbles within the microdevice, centrifugation was performed to assess whether positioning of the Microbubbles within the device could be controlled. Upon spinning the microdevice, Microbubbles congregated toward the trailing edge of the fluid within the microdevice chamber (Figure 4C-D). In fact, most of the Microbubbles formed a compact mass at the air-liquid interface (Figure 4D), with some even exiting the liquid meniscus and sticking to the portion of the chamber occupied by air. As the Microbubbles encountered more of the fluid, their compact nature lessened and some even remained suspended/mixed. This observation was consistent with the theory previously outlined (Figure 1), where the adhesion forces of Microbubbles nearest the air-liquid interface are stronger than those between Microbubbles which are surrounded by more liquid. On the other hand, microscopic evaluation of

the end of the chamber farthest from the center of rotation (i.e., near the normally closed valve) revealed negligible Microbubbles (Figure 4E). This was a promising observation, as it demonstrated that almost all Microbubbles (and any attached molecules) could be forced to the side of the microdevice chamber which was directly opposite the valve connecting it to a subsequent chamber; this provides an opportunity to achieve maximum separation between Microbubble-bound complexes and any unbound molecules, as those would be pelleted with centrifugal force.

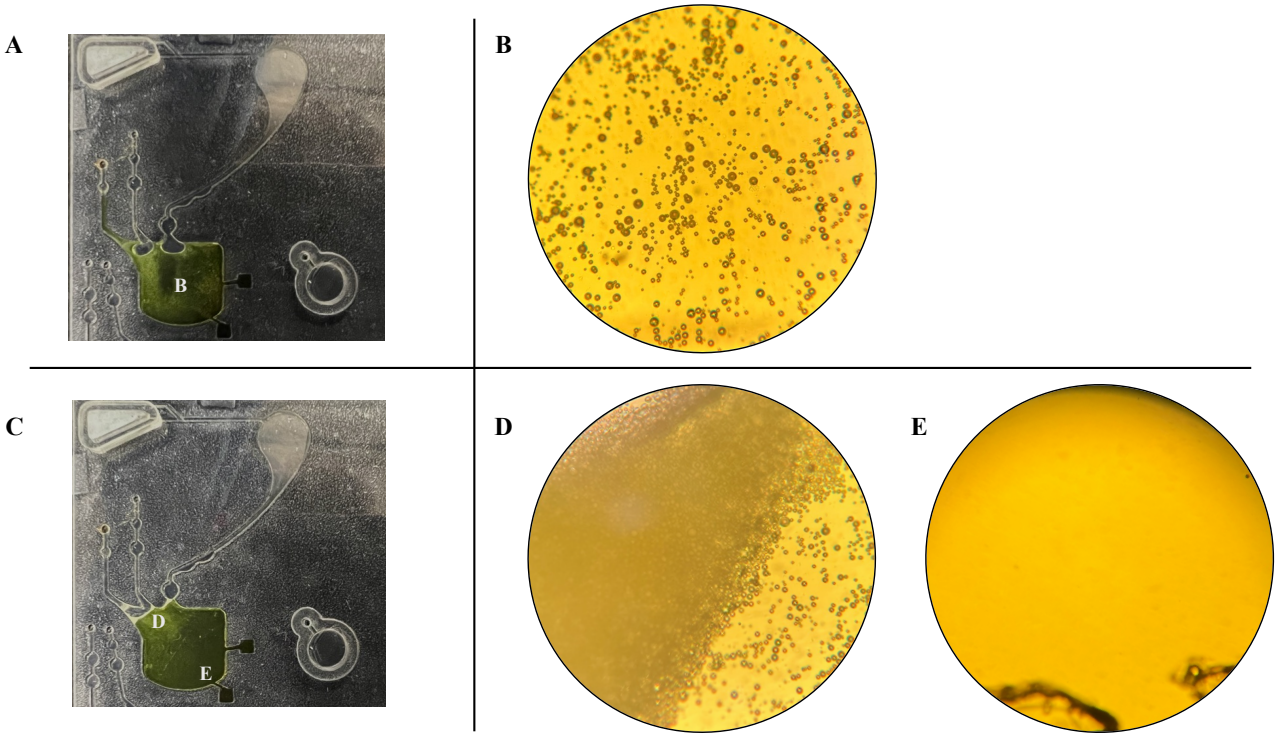

FIGURE 4 – Assessment of the movement and mixing of buoyant Microbubbles within a centrifugal microdevice. Several changes in orientation enabled mixing of Microbubbles within the antibody-binding chamber (A), and homogenization of the Microbubbles was accomplished (B). After centrifugation (C), most Microbubbles were forced to the trailing edge of the liquid and formed a compact conglomerate (D). On the other hand, only a few Microbubbles were observed at the opposite end of the chamber near the normally closed valve (E). All micrographs were obtained using 400X magnification.

Next, the movement of Microbubbles after valve opening and transfer of the liquid into the adjacent unbound chamber (Figure 2) was assessed. This was a critical evaluation, as the ability to efficiently separate the Microbubble and liquid fractions would be necessary for an antibody-mediated cell isolation assay. Upon valve opening and centrifugation, most of the liquid was transferred into the unbound chamber (Figure 5). Observation of the antibody-binding chamber revealed many Microbubbles (Figure 5A), some within the residual fluid and some outside of it. On the other hand, microscopic evaluation of fluid transferred into the downstream microdevice chamber revealed many fewer Microbubbles (Figure 5B). In a perfect result, one would be able to retain all Microbubbles within the initial chamber and transfer all fluid into the downstream chamber; however, this is likely impossible due to the adhesion forces acting on Microbubbles that experience more contact with the fluid within the chamber, as well as the density difference once the Microbubbles are in contact with air (Figure 1). While minimization of Microbubble transfer could be achieved by architecture and spin speed optimization, some degree of Microbubbles will always drag with the trailing edge of the fluid as it leaves the chamber due to smaller forces of adhesion between Microbubbles that are surrounded by more liquid.

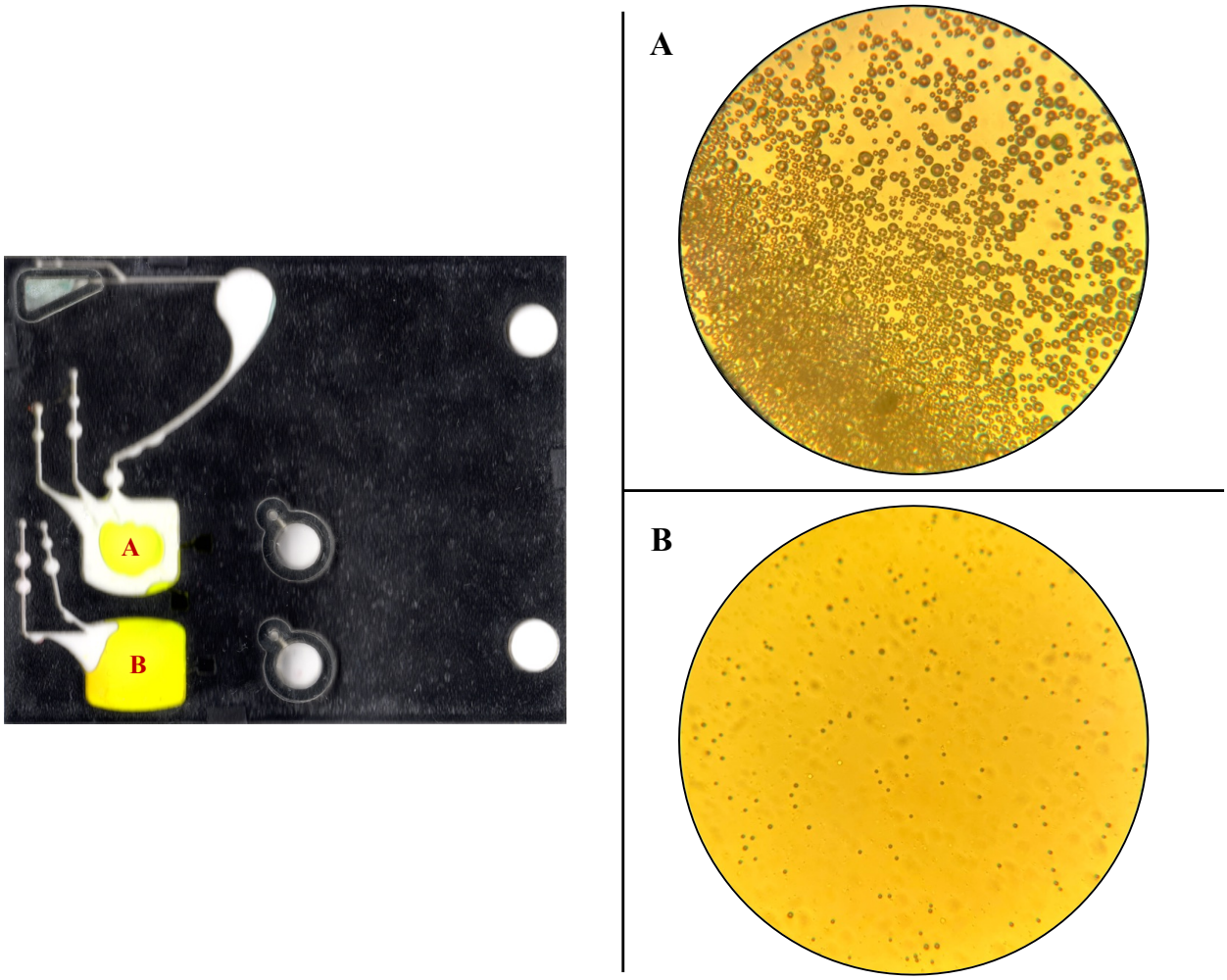

FIGURE 5 – Secondary movement of buoyant Microbubbles within a centrifugal microdevice after opening a laser actuated valve. The majority of the Microbubbles remained within the antibody-binding chamber (A), while some traveled with the fluid into the adjacent unbound chamber during centrifugation (B). All micrographs were obtained using 400X magnification.

Microscopic evaluation of the valving architecture within the microdevice further demonstrated the ability of the Microbubbles to enter the valve and traverse the laser ablated hole connecting the separate chambers (Figure 6). Some Microbubbles were even observed along the edge of the hole, signifying that they may adhere to it to some degree (Figure 6C). Observation of this valving architecture also demonstrated the circular shape and ~130-150 µm diameter of laser ablated openings – something that, to our knowledge, has not yet been documented.

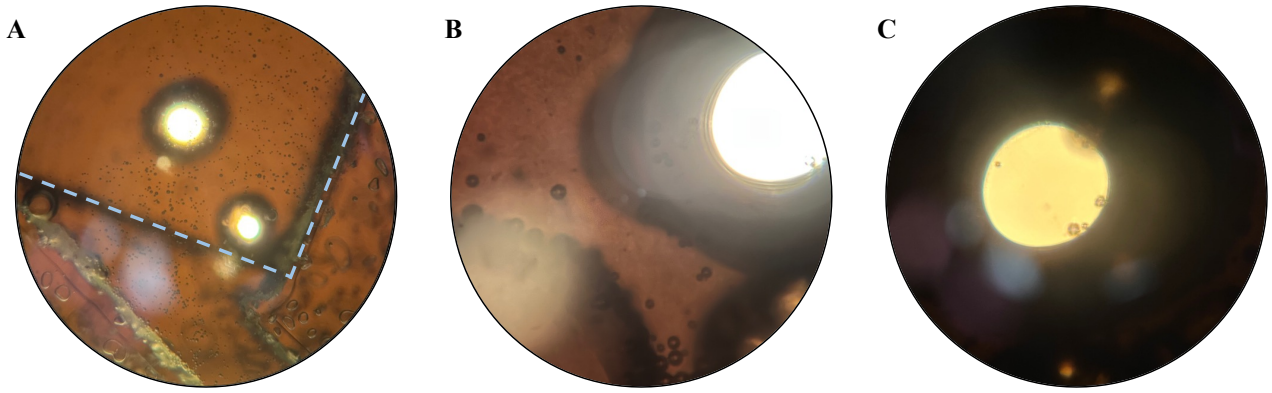

FIGURE 6 – Microbubbles within the valve architecture of the centrifugal microdevice at 100X (A), 200X (B), and 400X (C) magnification. Microbubbles (5 – 15 µm) were observed within the channel leading into the valve, as well as the valving architecture itself (A, B) – the perimeter of which is denoted by light blue dashes. In addition, some Microbubbles were observed hovering within the hole created to open the laser actuated valve (C), demonstrating that it is possible for Microbubbles to travel through valves into adjacent chambers.

Assessment of Microdevice Architecture Modifications

A subsequent preliminary evaluation of changes to the microdevice architecture used for this study has recently demonstrated increased potential for fractional separation. This modified device contains the same modules as the original design but utilizes 1.5 mm thick attachments (Figure 7); incorporation of these accessories was intended to add depth and maximize the distance between the top of the antibody-binding chamber and the valves leading to the unbound chamber. Initial dye studies, performed as previously described, demonstrated the ability to control fluidic movement throughout the device, with less fluid backflow in comparison to the original design (Figure 8). Additionally, all of the "subnatant" was transferred to the unbound chamber, demonstrating the potential to avoid complications from residual cell-free DNA.

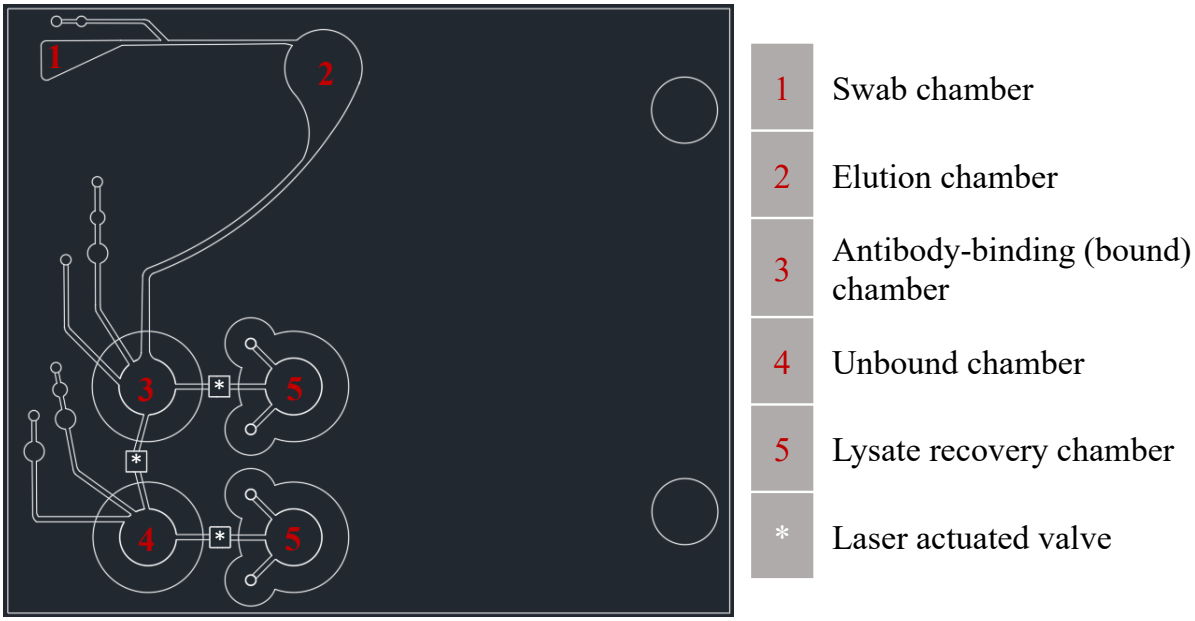

FIGURE 7 – Modified microdevice architecture for antibody-Microbubble mediated cell isolation which has PMMA attachments for increased chamber depth. A swab cutting can be inserted into the swab chamber (1), and centrifugation of the device can force eluate into the elution chamber (2). A change in center of rotation followed by spinning forces the sample into the antibody-binding/bound chamber (3), where antibody/Microbubble binding can take place. Upon opening a laser actuated valve (*) between the two primary chambers, “subnatant” can be spun into the unbound chamber (4). Cell lysis can take place in both chambers in tandem, and lysates can ultimately be spun into the side-by-side recovery chambers (5) for retrieval.

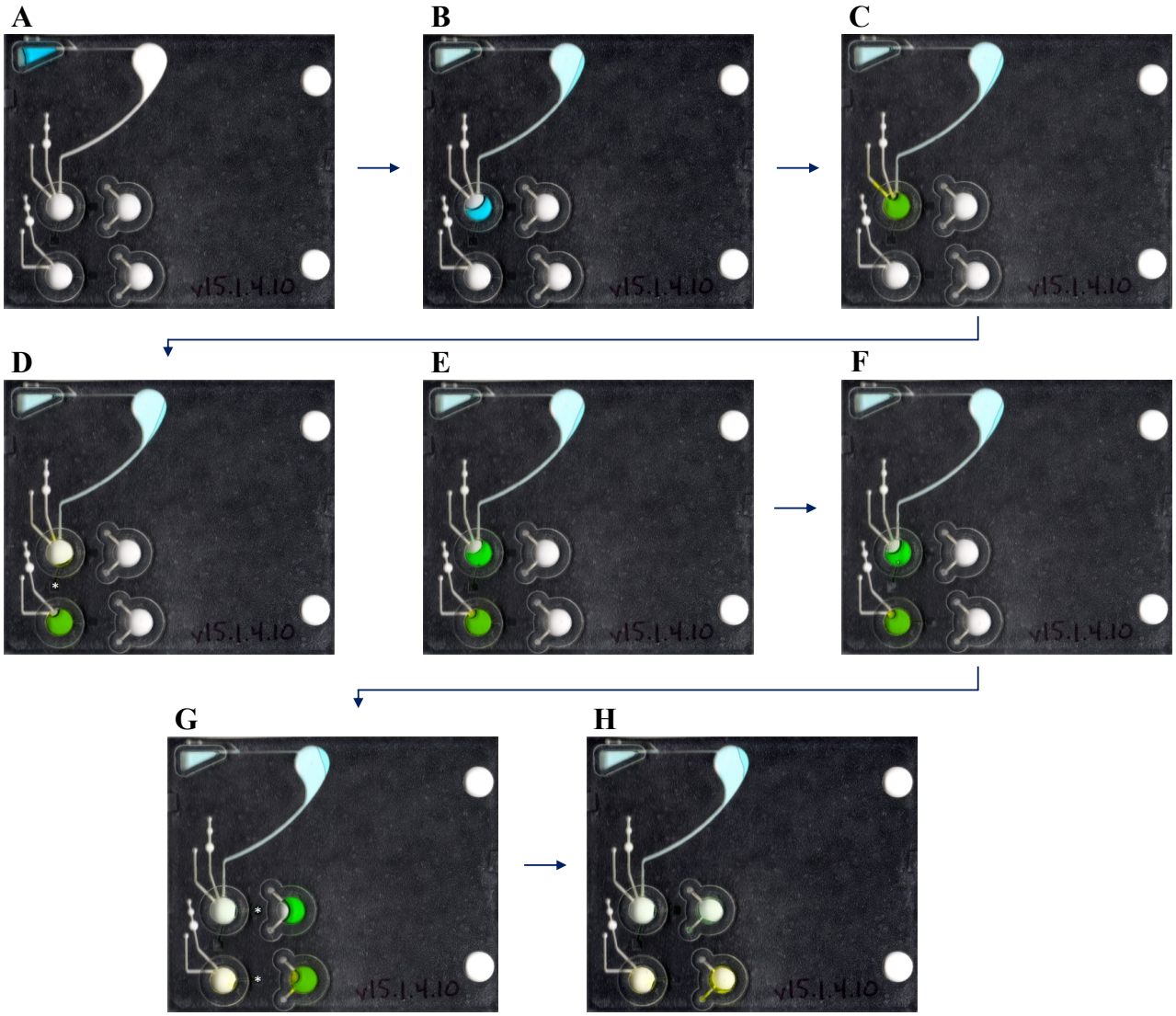

FIGURE 8 – Fluidic movement throughout the microdevice with PMMA attachments for added depth of chambers. A swab with eluant (blue) is inserted into the swab chamber and closed off (A). Centrifugation forces eluate into the elution chamber (B). Subsequent change in center of rotation and centrifugation forces all liquid into the antibody-binding chamber (B). Antibody-conjugated Microbubbles (yellow) can then be added to the antibody-binding chamber (C), and mixing can take place by frequent change in orientation of the device. Opening of a normally closed valve (*) via laser ablation and spinning of the supernatant into the downstream chamber then accomplishes fractional separation (D). Lysis master mix is then added to the antibody-binding chamber (E), the device is heated (F), valves to each recovery chamber are opened, and both resulting fractions are spun into their respective recovery chambers (G) to facilitate aspiration/removal from the microdevice (H).

Subsequent microscopic evaluation of the microdevice chambers after valve opening and liquid transfer also demonstrated the superior ability of this design to retain Microbubbles within the antibody-binding chamber (Figure 9A). The liquid within the adjacent unbound chamber still contained some Microbubbles, but fewer in comparison to the original microdevice design, and much fewer than those retained in the antibody-binding chamber (Figure 9B). Although this design exhibited major improvements in retaining Microbubbles in comparison to the initial microdevice, application of heating steps for lysis brought additional difficulties. The added depth of the chamber in comparison to the inlets and valves created a drastic difference in capillary pressure, which caused evaporation and fluid loss through inlets upon heating of the device during lysis. Future studies should focus on optimizing the architecture of this microdevice to accommodate heating steps required for lysis, as it shows great promise for maximum retainment of Microbubbles within the antibody-binding chamber.

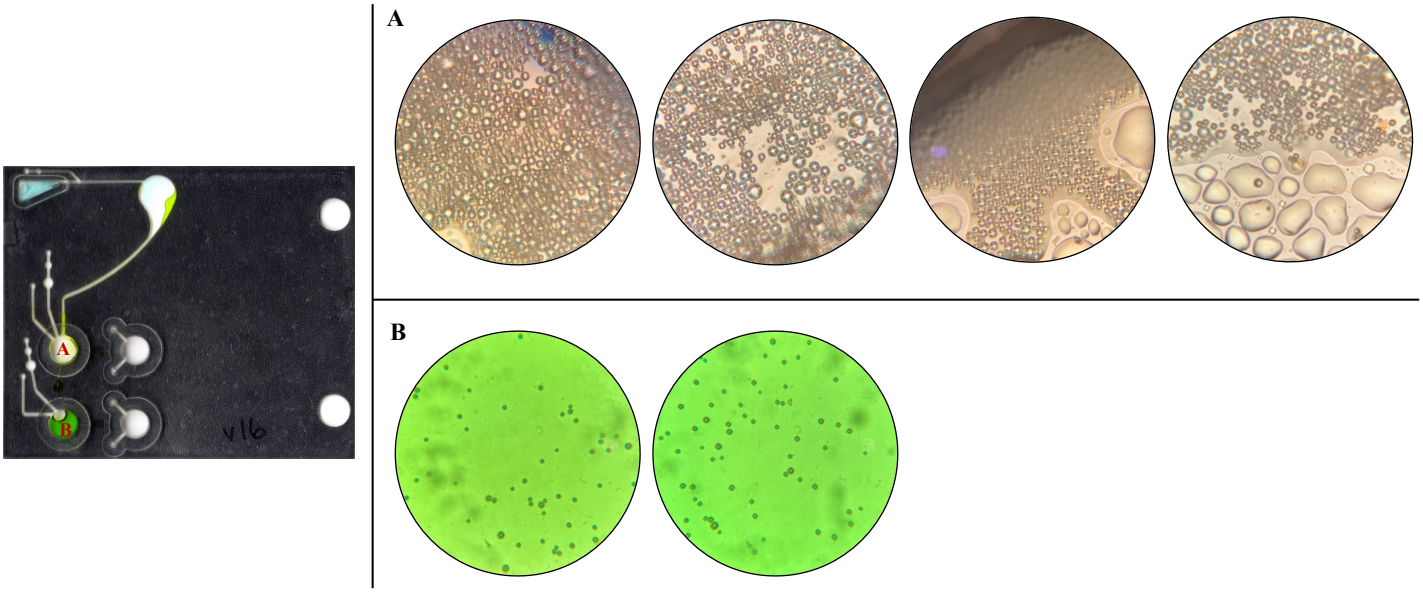

FIGURE 9 – Secondary movement of buoyant Microbubbles within a centrifugal microdevice equipped with PMMA attachments. After the transfer of fluid into a subsequent chamber through a laser actuated valve, the majority of the Microbubbles remained within the original chamber (A), while a few traveled with the fluid into the adjacent chamber (B). All micrographs were obtained using 400X magnification.

Buoyancy activated cell sorting with PH-20 and Microbubbles

In order to determine if the transfer of a minor portion of Microbubbles into the unbound chamber would practically impact attempts to isolate target cells from a mixed cell population, preliminary evaluation of the initial microdevice architecture with an antibody-mediated sperm cell isolation assay was performed. Ten semen-vaginal mixture swab cuttings were tested, from swab insertion to fractional separation and lysis, entirely on the proposed microdevice platform. DNA quantification revealed mean M:F ratios of 1:2.84 in antibody-bound fractions, which exhibited a 1.4 ± 0.77-fold improvement over unseparated mixture controls ($p = 0.29$) (Table 1). On the other hand, the mean M:F ratio for unbound fractions was 1:7.65. These results indicated minor enrichment of male DNA contributions (i.e., sperm cells) within antibody-bound fractions; however, DNA quantification results do not always directly translate to STR profiling; thus, samples were subsequently subjected to STR amplification and capillary electrophoresis.

TABLE 1. Male-to-female (M:F) ratios after DNA quantification and STR profiling in unseparated controls, antibody-bound fractions, and unbound fractions from semen-vaginal mixtures treated with the microfluidic antibody-mediated separation assay.

Sample	DNA Quantification			STR Profile Analysis			
	M:F Ratio			M:F Ratio			M:F Fold-Improvement (B/Unseparated)
	Unseparated	Bound Fraction	Unbound Fraction	Unseparated	Bound Fraction	Unbound Fraction	
Mixture 1	1 : 2.00	1.16 : 1	1 : 2.49	1.38 : 1	1.94 : 1	1.05 : 1	1.41
Mixture 2	1 : 4.27	1 : 1.91	1 : 6.74	1 : 1.87	1 : 1.66	1 : 4.14	1.13
Mixture 3	1 : 5.36	1 : 6.16	1 : 25.86	1 : 2.17	1 : 4.07	1 : 9.83	0.53
Mixture 4	1 : 9.90	1 : 3.95	1 : 11.60	1 : 5.47	1 : 4.84	1 : 4.45	1.13
Mixture 5	1 : 4.73	1 : 3.73	1 : 9.59	1 : 2.02	1 : 2.64	1 : 3.44	0.76
Mixture 6	1 : 4.27	1 : 2.00	1 : 6.26	1 : 1.79	1 : 1.35	1 : 2.53	1.33
Mixture 7	1 : 8.30	1 : 17.51	1 : 9.31	1 : 5.08	1 : 6.48	1 : 7.98	0.78
Mixture 8	1 : 2.47	1 : 3.44	1 : 5.89	1 : 1.34	1 : 1.93	1 : 3.25	0.70
Mixture 9	1 : 4.53	1 : 4.05	1 : 22.35	1 : 2.23	1 : 2.89	1 : 4.20	0.77
Mixture 10	1 : 15.09	1 : 19.23	1 : 21.60	1 : 7.23	1 : 8.51	1 : 12.83	0.85
AVERAGE	**1 : 4.38**	**1 : 2.84**	**1 : 7.65**	**1 : 1.95**	**1 : 1.90**	**1 : 3.28**	**0.94 ± 0.29**

Unfortunately, sperm enrichment was not as drastic in resulting STR profiles. Instead, antibody-bound fractions exhibited mean M:F ratios that were similar to those within profiles of unseparated

controls, with a 0.94 ± 0.29-fold M:F ratio improvement (Table 1). Unbound fractions *did* contain STR profiles that showed enrichment of female DNA contributions, but there was no concomitant enrichment of sperm cells within antibody-Microbubble fractions. While some individual mixture samples showed minor improvements in M:F ratios, others exhibited worsened M:F ratios that were indicative of sperm cell carryover into unbound fractions. Although the inefficiency of this microdevice assay could be due to poor antibody-cell binding, in-tube results within our lab have demonstrated the ability of this procedure to enrich for sperm cells to a higher degree than exhibited herein (*see Chapter 3*). Nevertheless, this microfluidic environment is inherently different than an in-tube environment; therefore, binding protocol modifications and optimization should be explored to investigate whether binding and sperm enrichment can be improved within this architecture.

Alternatively, the transfer of some Microbubbles into the unbound chamber alongside the liquid portion could also be responsible for sperm carryover and poor M:F ratios within resulting antibody-bound fractions. Additionally, the retention of some liquid within the antibody-binding chamber after valve opening and spinning further complicates resulting M:F ratios, as unbound cells and cell-free DNA could be present and left behind with the bound fraction.

Finally, it should also be noted that DNA concentrations within some resulting antibody-bound fractions were lower than expected, as well as lower than encountered within previous in-tube studies (data not shown; *see Chapter 3*). Because of this, these samples were not as diluted and thus more neat lysate was added to STR amplification in order to achieve the desired DNA input. Even though evaluation of internal PCR controls (IPCs) during DNA quantification gave no

indication of inhibition, initial STR profiling results for these samples exhibited poor interlocus balance of allele peak heights (i.e., ski slope) and allelic dropout. However, this was easily resolved by using a maximum input of 2.5 µL sample lysate (i.e., 0.15 – 0.225 ng DNA) for STR amplification. Not only does this reveal the possibility of alkaline lysates to inhibit STR amplification at certain concentrations, but it also highlights the necessity to either adjust final lysate volume on the microdevice to increase DNA concentrations or, alternatively, to meter ≤ 2.5 µL when moving toward a more automated microdevice that includes STR amplification modules. Ensuring maximum retention of sperm cells within antibody-Microbubble fractions would also mitigate the possibility of inhibition during STR amplification, as DNA concentrations would proportionately increase.

CONCLUSIONS

The ability to effectively target and isolate certain cells within a sample is critical not only for sexual assault sample processing in the forensic DNA community, but also for biomedical applications such as red blood cell removal and circulating tumor cell detection. Although fluorescence detection and paramagnetic beads have been the primary means of accomplishing this, a newer technique called buoyancy activated cell sorting (BACS) has shown great promise, as well as potential for automation. Because BACS relies on density differences to accomplish fractional separation, centrifugal microfluidics could be the perfect means of automating such a technique in a way that is cheaper than implementing magnetic fields. Thus, this study aimed to assess and characterize the behavior of buoyant Microbubbles within a centrifugal microdevice, as well as to preliminarily evaluate the transition of an antibody-mediated sperm cell isolation assay onto this microfluidic format.

Microscopic evaluation of the centrifugal microdevice described herein demonstrated both the ability to mix and homogenize Microbubbles, as well as to control the movement and positioning of the Microbubbles via centrifugation. Further, although assessment of the laser ablated valve opening and fractions revealed the ability of some Microbubbles to move with the liquid fraction upon transfer into an adjacent unbound chamber, a clear majority of Microbubbles were retained within the original antibody-binding chamber. The proportion of Microbubbles retained in the antibody-binding chamber was further increased by adjusting the microdevice architecture to incorporate PMMA attachments for added chamber depth. Not only does this have major implications for automation of BACS, but it also serves as the first characterization of Microbubble behavior within a microfluidic environment.

Despite promising microscopic observations, preliminary transition of an antibody-mediated sperm cell isolation assay onto this microdevice did not yield efficient or consistent results. PH-20 antibody-bound fractions exhibited similar M:F ratios as unseparated mixture controls after STR profile generation, while unbound fractions indicated minor enrichment of female DNA contributions. Although not ideal, these results still signify the ability to perform such an assay on a centrifugal microdevice in an automated fashion, and future studies which optimize and improve upon this method could greatly impact the scientific community.

Overall, this study demonstrated the potential for combining BACS and centrifugal microfluidics to automate fractional separation for many applications, including circulating tumor cell detection for diagnostic assays, red blood cell removal for point-of-care, as well as isolation of forensically relevant cells (e.g., sperm, buccal epithelial cells, vaginal epithelial cells, and leukocytes) for

mixture DNA profile prevention and/or enrichment of minor cell populations. While some architecture optimization may be needed for specific applications, we believe this approach could be used to efficiently separate Microbubble-bound and unbound fractions. This study evaluated the application of this technique with a PH-20 antibody assay for sperm cell isolation, but it could be customized by the end user for various targets. In fact, Akadeum already markets specific kits for red blood cell and dead cell removal applications, meaning that the only hurdle to automation of these assays is microdevice architecture optimization; numerous other previously optimized antibody or aptamer assays could also be transitioned to this format. Ultimately, not only does this provide proof of concept for the pairing of BACS and microfluidics, but it also opens the door for cheap and quick automation of this technique for a wide variety of applications.

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CHAPTER FIVE:

EVALUATION OF AN IN-TUBE AND MICROFLUIDIC ASSAY FOR PROCESSING SEXUAL ASSAULT SAMPLES USING ENZYMATIC AND ALKALINE LYSIS

ABSTRACT

Sexual assault sample processing, despite recent funding and research efforts, remains time-consuming, laborious, and inefficient. Limitations of the traditional differential lysis procedure, combined with the prominence of sexual assaults each year, necessitate the development of a cheaper, quicker, and more robust method for separating victim and perpetrator contributions within sexual assault evidence so that analysts can keep pace with submissions and cases can be resolved in a timely manner. Thus, this study examined the use of a combined enzymatic and alkaline approach for differential cell lysis in-tube, as well as preliminary evaluation of this technique on a centrifugal microdevice platform – with the goal of developing a quick, cheap, and more efficient method. Quantification results for the in-tube assay revealed that 72.0 ± 18.3%, 15.8 ± 14.2%, and 46.4 ± 29.6% of total DNA was retained in sperm fractions for semen, vaginal, and semen-vaginal mixture eluates, respectively. STR analysis of mixture samples processed with this technique exhibited sperm fraction DNA profiles with mean M:F ratios of 1.74:1, which was a 3.01 ± 2.3-fold improvement in M:F ratios and led to the recovery of 5.90 ± 7.8 unshared male contributor alleles in sperm fractions that were otherwise undetected in unseparated controls. Transition of this technique onto a centrifugal microdevice demonstrated minor enrichment of male DNA contributions within the sperm fraction, with mean M:F ratios of 1.4:1. Although the microfluidic assay failed to exhibit the same level of sperm enrichment as the in-tube method, it generated non-sperm fractions trending toward major female DNA profiles. Overall, this study presented a modified differential lysis approach using *prep*GEM™ and sodium hydroxide treatments that can accomplish cell elution and fractional lysis within 25 minutes. Further, a centrifugal microdevice capable of pelleting and trapping intact cells was developed; this device has the potential to automate differential cell lysis and fractional separation, which would further reduce time, variability, and costs. Future studies should investigate alternative non-sperm cell lysis methods to enhance lysis efficiency and minimize inhibition, as well as design and protocol modifications for optimization of the microdevice platform.

KEYWORDS: forensic genetics, sexual assault, sperm, *prep*GEM, alkaline lysis, centrifugal microfluidics, microdevice

INTRODUCTION

Despite many technological and sensitivity improvements in forensic DNA analysis, the processing of sexual assault samples remains time-consuming and inefficient. This, combined with the fact that approximately 22% of violent crimes reported in 2021 were comprised of rape or sexual assault [1], has led to an ongoing sexual assault evidence collection kit (SAECK) backlog. Further, despite nationwide efforts to reduce such backlogs, legislation that requires the submission and processing of all collected SAECKs is compounding this issue – necessitating the development of sample processing techniques which are cheaper, quicker, and more efficient in order to offset the demands on laboratories.

Differential lysis has been the most widely accepted, traditionally used technique for separating the predominant cell types found in sexual assault samples to date. This method, which was originally described by Gill et al. in 1985, takes advantage of the difference in lysis susceptibility between non-sperm and sperm cells when exposed to certain reagents in order to accomplish fractional separation of victim and perpetrator DNA contributions [2]. Ideally, this technique would culminate in a sperm fraction containing only DNA from the male perpetrator, as well as a non-sperm fraction containing DNA from the lysed female vaginal epithelial cells; however, it often inefficiently isolates sperm and non-sperm cells due to many factors (e.g., the presence of old or degraded sperm cells that are susceptible to premature lysis, excess female epithelial cells that fail to completely lyse and remain within the sperm fraction, loss of sperm due to repeated wash steps, as well as poor and tedious manual pipetting technique) [3,4]. Further, it requires long incubations, relies heavily on manual pipetting and transfer steps, and is inherently difficult to automate.

Given these drawbacks, many modified techniques have been investigated and reported for handling sexual assault samples. Cotton and Fisher provided a summary of several modified techniques that have been explored [4], focusing on those which have attempted to reduce incubation times and minimize female DNA carryover. Some of the earliest modifications involved simple lysis condition adjustments, such as milder reagents and increased temperature, to avoid the unintended loss of sperm DNA and promote more efficient lysis of epithelial cells [5,6]. A second mild lysis step prior to sperm cell lysis has even reportedly improved male:female DNA ratios in sperm fractions by as much as 6-fold [7], as well as resulted in 5.5-fold reduction in non-sperm DNA carryover without reducing sperm DNA recovery [8]. Studies have also focused on the replacement of DTT for sperm lysis with TCEP or 1-thioglycerol in an attempt to reduce incubation times [9–12], as well as to avoid the effects of DTT on downstream processes if not removed via purification (i.e., for direct-to-amplification applications) [10–13]. Unfortunately, despite the reduced time and cost requirements, many of these techniques still result in mixtures and/or lead to incomplete male DNA profiles in sperm fractions; they also typically require a post-lysis purification step – which can lead to additional loss of DNA and adds time to the forensic DNA workflow – as well as hands-on processing that includes several tube transfers.

In order to drastically reduce sample processing times and costs, as well as minimize DNA loss, newer lysis methods that omit subsequent DNA purification of the lysate have been developed. These methods often utilize detergents and/or enzymes to break open membranes and denature or degrade proteins. The *prep*GEM™ and *forensic*GEM™ kits from microGEM (Charlottesville, VA) are prominent examples, which implement the thermophilic enzyme EA1 to accomplish cell lysis at 75°C in mere minutes. Many studies have applied these kits to forensically relevant

samples and have obtained usable DNA profiles without the need to further purify the resulting lysates [14–20]. While this method works for non-sperm cells, additional techniques have been reported for more robust cells such as sperm; *forensic*GEM™ Sperm utilizes an additional enzyme cocktail known as *Acrosolv* to accomplish sperm cell lysis at a lower temperature, followed by EA1 lysis to further degrade proteins as well as the *Acrosolv* itself [12]. On the other hand, additional sperm lysis techniques which do not require enzymes have also been explored. Notably, Schellhammer et al. investigated several direct-to-amplification techniques for sperm lysis involving commercial, "homebrew," and "natural decondensation" reagents (*see Chapter 2*). Results from this study found several candidate methods for this application which could produce usable STR profiles with reduced time, cost, and volumes; however, the method ultimately recommended was a five-minute incubation with sodium hydroxide at 75°C, followed by neutralization with Tris-HCl [12].

Not only could these direct-to-amplification methods reduce overall time and costs associated with processing sexual assault samples via traditional bench methodology (e.g., tube transfers, spin baskets, etc.), but they are also much more likely than traditional DNA extraction and purification to be compatible with microfluidic platforms. Microfluidics utilizes a multitude of channel and overall architectural designs to precisely control fluid movement and accomplish elution, mixing, metering, reagent release, and many other processes on a singular device. The transition of numerous biological techniques to a microfluidic environment has been heavily explored in the past decade for the reduction of sample and reagent consumption, contamination risk, and processing time. The development of centrifugal microdevices has even gained the interest of the biomedical and forensic science communities. By taking advantage of centrifugal force, which is

already commonly utilized on the macroscale within these fields, the liquid propulsion and flow within such a microdevice can be actuated and precisely controlled without the need for bulky external hardware such as pumps [15–18,21–24]. Not only does this significantly reduce cost, but it also simplifies microdevice platform design and minimizes the size and footprint of the device, increasing the possibility of miniaturized portability. Precise liquid control on these devices has been improved with the incorporation of a normally closed, laser actuated valve that is easily opened with a laser to allow for the movement of liquid to a downstream chamber [18,25]. Recent studies have also demonstrated that these valves can be reclosed, which can help further control or prevent liquid backflow within the microdevice [22].

Centrifugal microfluidics is also a heavily explored area for procedures that require cell isolation and sorting. Several microdevices have been designed for single cell isolation from a heterogeneous sample by the combined effects of centrifugal force and device architecture [21,26–29]. Such devices are appealing because they can manipulate small volumes in relatively short timeframes without requiring external pumps to accomplish liquid flow. Centrifugal devices have been described for the successful isolation of breast cancer [29], circulating tumor [30], and blood cells [31], and they also show promise for modified differential cell lysis. For the latter, a centrifugal microdevice containing two modular features – a cell trap which could capture pelleted sperm cells after non-sperm lysis and a separate chamber into which the supernatant could be transferred after valve opening – could automate the differential lysis procedure used for processing sexual assault samples. This approach would reduce manual pipetting, eliminate analyst-to-analyst variability, reduce opportunities for contamination, as well as decrease time and costs to the forensic laboratory.

Thus, the research herein attempted to address and overcome the previously outlined drawbacks of traditional differential lysis by exploring a consecutive enzymatic (*prep*GEM™) and alkaline approach. Use of these direct-to-amplification techniques, even in a traditional microcentrifuge tube environment, would ideally increase sample processing efficiency by more effectively lysing non-sperm cells, reducing non-sperm DNA carryover, retaining as many sperm cells as possible within the sperm fraction, as well as providing time and cost savings. Additionally, to further reduce time, manual processing, and variability, a centrifugal microdevice capable of trapping and retaining intact cells was also explored – with the added goal of providing an automated differential lysis technique for sexual assault sample processing.

MATERIALS & METHODS

Sample Collection & Preparation

Semen samples and vaginal swabs were collected from ten anonymous donors in accordance with the university-approved Institutional Review Board (IRB) protocol HM20002931. Semen was diluted 1:60 by volume using Gibco™ 1X Dulbecco's phosphate-buffered saline (DPBS) (Fisher Scientific; Waltham, MA). Cells were eluted from vaginal swabs by submerging a half-swab cutting in 200 µL DPBS and incubating at 37ºC for two hours, with brief vortexing every 15 minutes. All dilutions and eluates were stored at 4ºC.

Semen, vaginal, and semen-vaginal mixture samples were dried onto Fisherbrand™ PurSwab foam swabs (Fisher Scientific) prior to subsequent testing. All samples were prepared as indicated below in tubes. Subsequently, the foam swabs were dipped into the tubes and allowed to absorb the entire sample. For semen swabs, 30 µL of 1:60 semen was combined with 50 µL of DPBS.

Vaginal swabs were prepared similarly by combining 30 µL of DPBS and 50 µL of vaginal eluate. Mixtures were prepared by combining 30 µL of 1:60 semen with 50 µL of vaginal eluate. When processed without any fractional separation, this specific mixture preparation method has generated approximate 1:1 M:F ratios in resulting STR profiles, on average, within our lab (data not shown). For each sample, multiple swabs were prepared to accommodate all testing. Swabs were allowed to absorb the samples prior to drying overnight at room temperature. Once dry, swabs were cut into fourths and stored at 4ºC until testing; all swabs were tested within two months of preparation.

All subsequent testing utilized 1/4th of a foam swab, which should contain approximately 6,000 – 19,000 sperm cells based on the average sperm counts in normal semen and the dilutions used herein [32–37]. Samples were eluted from swab cuttings by combining into a new tube with 20 µL of DPBS and incubating at room temperature for five minutes, with brief vortexing every minute. Semen, vaginal, and mixture samples were subjected to differential cell lysis for this study; however, additional swab cuttings of each mixture sample were not subjected to differential lysis (i.e., "unseparated" mixtures) and assessed as untreated controls.

In-Tube Assay

Differential Cell Lysis

Semen, vaginal, and mixture sample eluates (~19 µL) were transferred to 0.2 mL PCR tubes prior to differential cell lysis. Enzymatic lysis of non-sperm cells was then performed using the *prep*GEM™ Universal kit (microGEM; Charlottesville, USA) (Figure 1). To accomplish this, 0.5 µL *prep*GEM™ enzyme, 5.0 µL 10X Blue buffer, and 25.5 µL HyPure Molecular Biology Grade

Water (MBG H_2O; GE Healthcare Life Sciences; Chicago, USA) were added to each sample. Samples were then incubated using the ProFlex™ 3x32-well PCR System (Applied Biosystems™) as follows: 75ºC for five minutes, then 95ºC for two minutes. A subsequent test of this method using 2X *prep*GEM™ enzyme (i.e., 1.0 µL instead of 0.5 µL) for non-sperm lysis was conducted, keeping all other steps and conditions the same.

Following non-sperm lysis, samples were centrifuged at 17,000 *x g* for five minutes to pellet intact sperm cells. The entire supernatant (50 µL) was then removed as the non-sperm fraction. The sperm pellet was then resuspended in 50 µL MBG H_2O. Alkaline lysis was performed on non-sperm and sperm fractions according to the method described by Schellhammer et al. [12] (Figure 1).

Additional samples of each mixture created were processed alongside differentially lysed samples to serve as an untreated, unseparated control for this technique. For this, the previous procedure was performed, but the removal of the non-sperm fraction after *prep*GEM™ lysis was omitted (i.e., no fractional separation occurred).

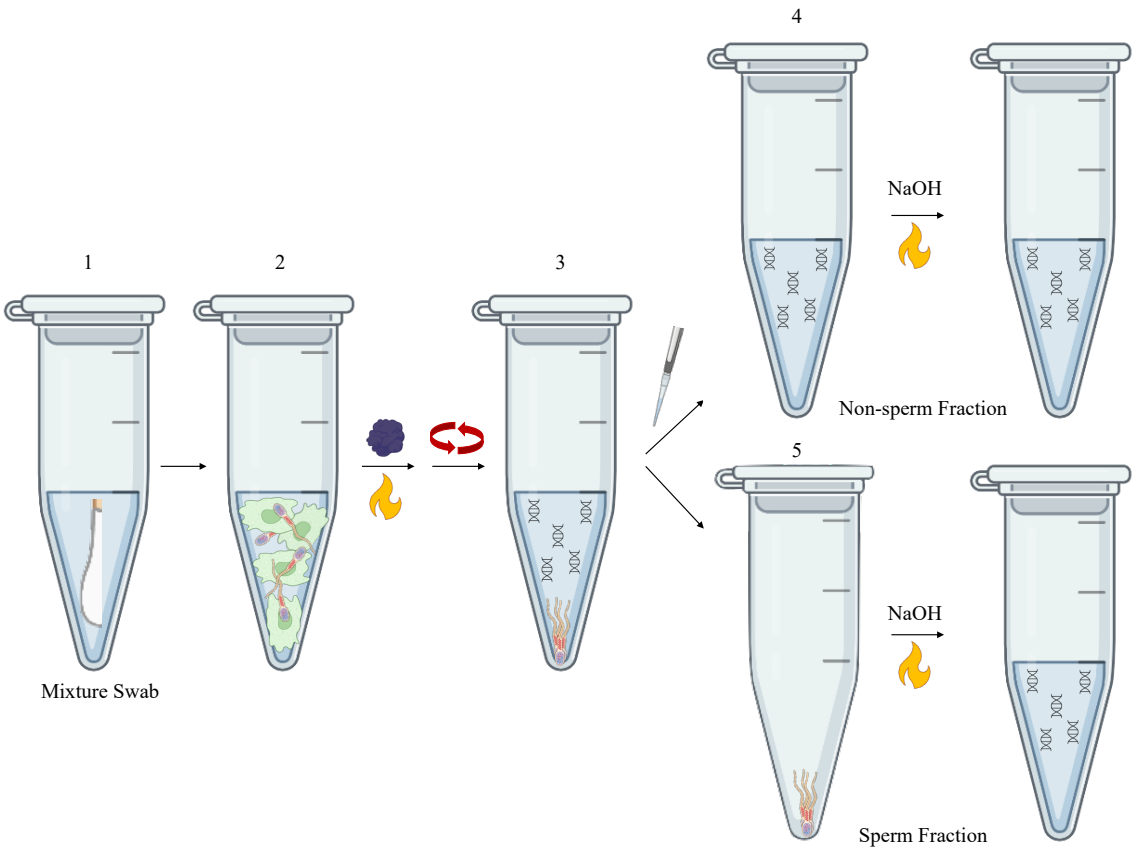

FIGURE 1 – The modified differential lysis technique using a combined enzymatic and alkaline approach. Cells are first eluted from a swab cutting (1). Enzymatic lysis of non-sperm cells is conducted using *prep*GEM™ (●) and heat (🔥) (2), and intact sperm cells are then pelleted via centrifugation (3). The entire supernatant is removed as the non-sperm fraction (4), leaving behind the pellet as the sperm fraction (5). Both non-sperm and sperm fractions are then subjected to alkaline solution to lyse all remaining cells. (Figure created in part using BioRender.com)

Microdevice Assay

Microdevice Fabrication & Hardware

The microdevice used for this study was comprised of polyethylene terephthalate (PeT) film and contained modules for swab insertion, sample elution, cell lysis and pelleting/trapping, and lysate recovery. Each microdevice consisted of five layers: two exterior layers of clear, 101.6 µm PeT; two layers of clear, 101.6 µm PeT with an affixed 50.8 µm heat sensitive adhesive (HSA; EL-7970-39, Adhesives Research, Inc.; Glen Rock, PA, USA) to enable adhesion; and a middle layer

of black, 75 µm PeT (bPeT; Lumirror* X30, Toray Industries, Inc.; Chuo-ku, Tokyo, Japan) to facilitate valving. Devices were fabricated according to the previously described print-cut-laminate (PCL) method [38]. Layer order was as follows: clear PeT, clear PeT/HSA, bPeT, clear PeT/HSA, and clear PeT. Microfluidic architecture was designed using AutoCAD LT software (Autodesk®, Inc.; San Rafael, CA, USA) and printed/ablated using a VersaLASER® 3.50 CO_2 laser platform (VLS3.50; Universal® Laser Systems; Scottsdale, AZ, USA). Prior to assembly, all layers were sterilized by the following procedure: a 30-minute wash in MBG H_2O on an orbital mixer, drying at room temperature, wiping with 70% isopropanol followed by MBG H_2O, and drying at room temperature. Each five-layer device was then bonded using an AL 13P12 laminator (Apache; Phoenix, AZ, USA) at 379ºF (or ~192ºC). Subsequently, 1.5 mm thick polymethyl methacrylate (PMMA) pieces were fabricated via laser ablation and affixed to the microdevice using 55.8 µm pressure sensitive adhesive (PSA) (ARcare 7876; Adhesives Research, Inc.) to provide depth for swab and recovery modules.

The bPeT layer in each microdevice provided normally closed valves to control fluid passage between channels in interior layers. When necessary, these valves were opened by firing a 700 mW 638 nm laser diode (L638P700M; Thorlabs, Inc., Newton, NJ, USA) positioned ~15 mm above the device [22].

All fluidic movement within the microdevice was controlled via centrifugal force. For this, microdevices were inserted into mounts, which are connected via rotating servos (Hitec RCD; San Diego, CA, USA) to a spin arm. The spin arm rotates through a slip ring (MOFLON Technology Co., Ltd.; Shajing, Shenzhen, China) via an electromagnetic stepper motor (Pololu Corporation;

Las Vegas, NV, USA). Additionally, heating and cooling were performed via Peltier clamp assembly, which consisted of two sets (upper and lower) of 1" x 1" Peltiers, heat sinks, and fans (SUNON; Kaohsiung City, Taiwan). All centrifugation, change in center of rotation, and heating steps were controlled using a serial terminal interface with custom commands within Propeller Tool software (Parallax, Inc.; Rocklin, CA, USA).

The final device architecture consisted of modules for swab insertion, sample elution, lysis and sperm cell pelleting (i.e., sperm chamber), supernatant transfer (i.e., non-sperm chamber), and lysate recovery (Figure 2).

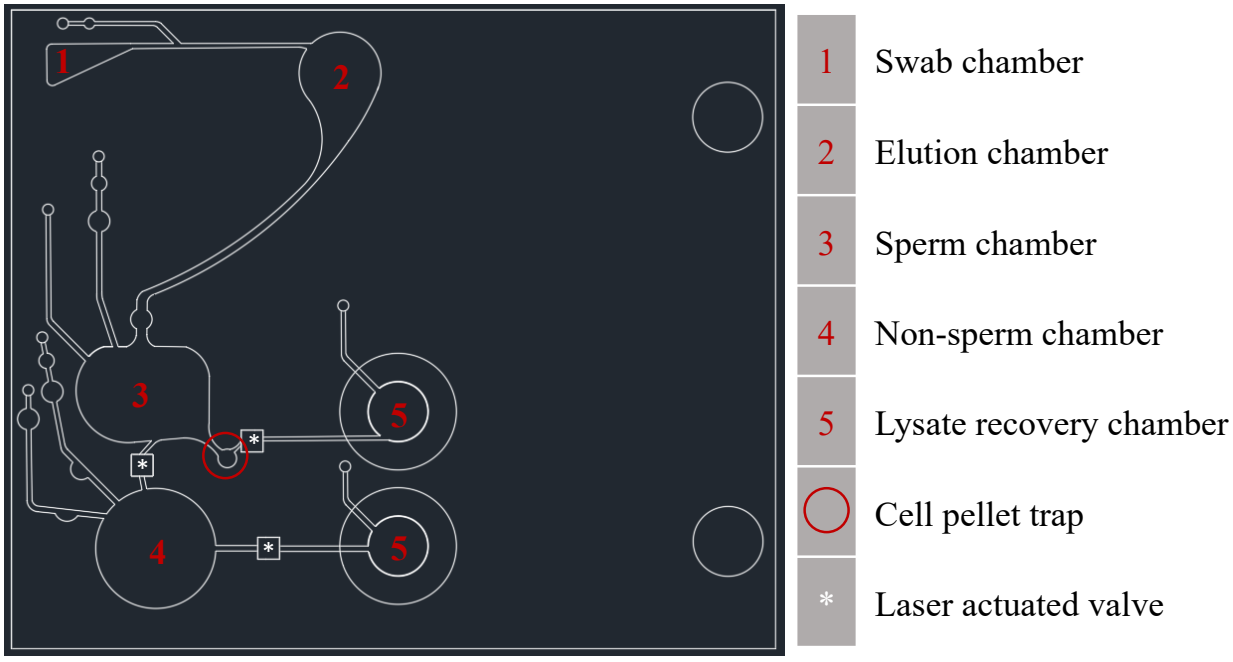

FIGURE 2 – Final microdevice architecture for differential cell lysis. A swab cutting can be inserted into the swab chamber (1), and centrifugation of the device can force eluate into the elution chamber (2). A change in center of rotation followed by spinning forces the sample into the sperm chamber (3), where non-sperm lysis and cell pelleting can take place (○). Upon opening a laser actuated valve (*) placed between the two primary chambers, supernatant can be spun into the non-sperm chamber (4). Ultimately, the remaining two laser actuated valves can be opened and lysates from each fraction can be spun into the side-by-side recovery chambers (5) for retrieval.

Fluidics/Dye Studies

Fluidic movement through the microdevice was initially assessed using 20mM Erioglaucine, 20mM Tartrazine, and 10mM Allura Red dyes (Sigma-Aldrich, St. Louis, MO, USA) (prepared in MBG H_2O). Individual unit operations were performed prior to implementation of the entire differential cell lysis workflow to demonstrate fluidic movement and control (as described below).

Microscopy

Prior to implementation of the differential cell lysis assay in the microdevice, pelleting and trapping of cells within liquid semen was assessed via microscopy. To accomplish this, 10 µL of neat semen were combined with 15 µL MBG H_2O. The entire sample was then added to the swab chamber, the swab chamber was capped, and the sample was transferred into the elution chamber via spinning at 1600 rpm for ten seconds. The angle of the microdevice was changed, and the sample was then spun into the sperm chamber using the same spin parameters. Next, cell pelleting was accomplished by centrifugation at 1600 rpm (~100 *x g*) for ten minutes. The supernatant and cell pellet fractions were then recovered, and cells were evaluated using Kernechtrot Picroindigocarmine Stain (KPICS). Briefly, each recovered fraction was spotted onto a microscope slide, dried, and stained with one drop of Kernechrot stain (Serological Research Institute (SERI); Richmond, CA) and one drop of Picroindigocarmine stain (SERI). Cells were visualized at 400X and 1000X magnification on a Micromaster microscope (Thermo Fisher Scientific). This assessment was repeated after each modification to the microdevice architecture, with the exception that DPBS was utilized instead of MBG H_2O for subsequent testing (as this is the eluant used for sample elution from swabs).

Differential Cell Lysis

Differential cell lysis was performed on the microdevice utilizing the proposed *prep*GEM™ and alkaline technique. Mixture swab cuttings were inserted into the swab chamber alongside 26.5 µL DPBS and teased for five seconds via pipette tip. The swab chamber was then capped using clear PeT, the device was inserted into its mount on the spin system, and agitation was conducted by "shaking" for 180 steps at 100 rpm for 30 cycles. Centrifugation at 1600 rpm for ten seconds was then performed to transfer the swab eluate to the elution chamber. Next, 3.5 µL *prep*GEM™ master mix (3.0 µL 10X Blue buffer + 0.5 µL *prep*GEM™ enzyme) were added to the sperm chamber, the device was rotated 90 degrees, and the entire swab eluate was transferred into the sperm chamber by spinning at 1600 rpm for ten seconds. The microdevice was then positioned within the Peltier clamp assembly, and non-sperm lysis was performed by heating at 75ºC for five minutes and 95ºC for 30 seconds. Following lysis, the microdevice was briefly mixed via shaking and the first round of cell pelleting was performed by spinning at 1600 rpm (~100 *x g*) for five minutes. Spinning was then performed for 30 seconds at a 0º angle and 30 seconds at a 90º angle to remove cell debris from the chamber walls, prior to a second round of cell pelleting by spinning for five minutes at 1600 rpm. At this point, the valve between the sperm and non-sperm chambers was opened via laser ablation, and the supernatant was transferred by spinning at 1000 rpm for ten seconds. Twenty-five microliters of alkaline lysis master mix (21 µL MBG H_2O + 4.0 µL 1M NaOH) were added to the sperm chamber. The microdevice was then positioned between the Peltier clamp assembly, and sperm lysis was performed by heating at 75ºC for five minutes. Valves to the lysate recovery chambers were then opened via laser ablation, and each fraction was spun (1000 rpm for ten seconds) into its corresponding recovery chamber. Each resulting non-sperm

and sperm fraction was aspirated via pipette, and sperm fraction eluates were neutralized by the addition of 4 µL 1M Tris-HCl (pH 7.5) (Invitrogen).

As with the in-tube assay, unseparated mixtures were processed in-tube alongside differentially lysed mixtures on the microdevice to serve as a point of comparison for this technique. For this, mixture swab cuttings were eluted in 26.5 µL DPBS for five minutes with vortexing every minute, then 3.5 µL *prep*GEM™ master mix (3.0 µL 10X Blue buffer + 0.5 µL *prep*GEM™ enzyme) were added. Samples were incubated at 75ºC for five minutes and 95ºC for two minutes to accomplish non-sperm lysis. Subsequently, 4 µL 1M NaOH were added, samples were incubated at 75ºC for five minutes, and then 4 µL 1M Tris-HCl were added to neutralize the lysate.

DNA Quantification

Human and male DNA quantities within all resulting lysates were determined using the Investigator® Quantiplex HYres kit (QIAGEN; Hilden, Germany) on the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific). Manufacturer recommendations were followed, with modifications for half-volume reactions. Thus, 4.5 µL Reaction Mix, 4.5 µL Primer Mix IC YQ, and 1.0 µL template DNA were combined in each well, and thermal cycling conditions involved: 95 ºC for three minutes, followed by 40 cycles of 95ºC for five seconds and 60ºC for 35 seconds. Resulting data were analyzed using Sequence Detection System (SDS) software v1.4 (Applied Biosystems™), with automatic baseline and threshold settings for each target.

To identify any possible signs of inhibition, an assessment of qualitative metrics in resulting amplification and component plots was conducted as previously described by Hudson et al. [13].

Additionally, several quantitative metrics were assessed. Total and fractional human DNA yields for each sample were calculated by multiplying the human target's concentration by the sample volume; this was repeated for the male target. To determine the percentage of human and male DNA in each fraction, the fractional DNA yield was divided by the total DNA yield (i.e., the sum of DNA yields in sperm and non-sperm fractions) and multiplied by 100. The mean and standard deviation for each experimental group were then calculated, and all comparisons were assessed using a Student's *t*-test ($\alpha = 0.05$). Given the reported proportions of sperm (~88%) and non-sperm (~12%) cells within normal semen, and upon correcting for ploidy, the theoretically expected percentage of seminal DNA originating from sperm cells was 80% [4,32,33,39]. Male-to-female (M:F) ratios in unseparated controls, sperm fractions, and non-sperm fractions from processed mixture samples were calculated by dividing the male DNA concentration by the difference between the human and male DNA concentrations. These values were then averaged for each experimental group to determine the mean M:F ratio, and a Student's *t*-test was conducted to determine statistical significance ($\alpha = 0.05$).

STR Amplification

All samples were amplified using the Promega™ PowerPlex® Fusion 6C System with a template DNA input of 0.25 ng following manufacturer recommendations, but with half-volume reactions; each reaction included 5.0 µL sample (at 0.05 ng/µL), 2.5 µL PowerPlex® Fusion 5X Master Mix, 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix, and 2.5 µL amplification-grade water. Thermal cycling was conducted on the ProFlex™ 3x32-well PCR System following manufacturer-recommended parameters [40].

Capillary Electrophoresis & Data Analysis

Resulting STR amplicons were separated using an Applied Biosystems™ 3500 Genetic Analyzer and Data Collection software v4 (Thermo Fisher Scientific) following manufacturer recommendations. One microliter of sample or allelic ladder was combined with 0.5 µL WEN ILS 500 (Promega™) and 9.5 µL Hi-Di™ Formamide (Thermo Fisher Scientific) in each well on the plate. Injection parameters also followed manufacturer recommendations and included a 36 cm capillary array (Thermo Fisher Scientific), POP-4® polymer (Thermo Fisher Scientific), and a 1.2 kV 15 second injection. Resulting STR profiles were analyzed with GeneMapper™ *ID-X* software v1.6 (Thermo Fisher Scientific) following manufacturer settings with an analytical threshold of 150 RFU [40].

M:F ratios within unseparated controls, as well as resulting sperm and non-sperm fractions from processed mixture samples, were calculated by dividing the total peak height for male alleles by the total peak height for female alleles at each locus where there was no allele sharing between donors. These ratios were then averaged across all loci within a single sample, as well as across all samples within an experimental group (e.g., all ten sperm fractions). A Student's *t*-test was conducted to compare mean M:F ratios across sperm fractions and unseparated controls ($\alpha = 0.05$).

The M:F ratio fold improvement for sperm fractions was then determined by dividing the mean M:F ratio within a sperm fraction by the mean M:F ratio in its associated unseparated control (e.g., M:F for Mixture 1 sperm fraction ÷ M:F for unseparated Mixture 1). The mean fold improvement was then calculated by averaging the M:F fold improvement for all ten mixture samples. Sperm fractions were additionally assessed for the number of male contributor alleles that were recovered

(i.e., detected above analytical threshold) compared to their corresponding unseparated controls; these numbers were then averaged to obtain the mean number of unshared male alleles recovered.

Additionally, STR profiles were assessed for interlocus balance using the coefficient of variation (CV of LPH:TPH) method [12,41]. For this, all unshared female contributor alleles in resulting non-sperm and sperm fractions were assessed, and the mean CV of LPH:TPH was determined for each experimental group. Mean peak heights of unshared female contributor alleles were also calculated, with corrections for homozygosity. These metrics were then compared across experimental groups and assays with a Student's *t*-test ($\alpha = 0.05$).

RESULTS & DISCUSSION

In-Tube Assay

DNA Quantification

Evaluation of semen samples revealed that 72.0 ± 18.3% of total DNA was retained in sperm fractions (Figure 3), which is close to theoretical expectation that 80% of total DNA within normal semen stems from sperm cells [4,32,33,39]. In addition, 15.8 ± 14.2% of total DNA was retained in sperm fractions for vaginal samples (Figure 3). Each of these data sets included an outlier as indicated by the Grubbs test; only 28.7% of total DNA was retained in the sperm fraction for one semen donor, while 54.4% of total DNA was retained in the sperm fraction for one vaginal donor. As expected, approximately half of the total DNA yield was retained in the sperm fraction and half in the non-sperm fraction for mixture samples. Overall, these results demonstrated the ability of this modified differential lysis technique to sufficiently lyse most non-sperm cells using

*prep*GEM™ while leaving behind intact sperm cells, which can then be lysed with more stringent techniques.

The percentage of total DNA retained in sperm fractions was then plotted against the DNA yield for the entire sample (i.e., the sum of DNA yields in non-sperm and sperm fractions) to determine whether there was a relationship between the percentage of DNA retained within sperm fractions and cellular input (Figure 4). No association was observed for semen *or* vaginal samples, as the percentage of DNA retained in sperm fractions was consistent regardless of the total DNA (Figure 3). This was promising, as it suggested that *prep*GEM™ is capable of handling (and lysing) a wide range of non-sperm cells within samples.

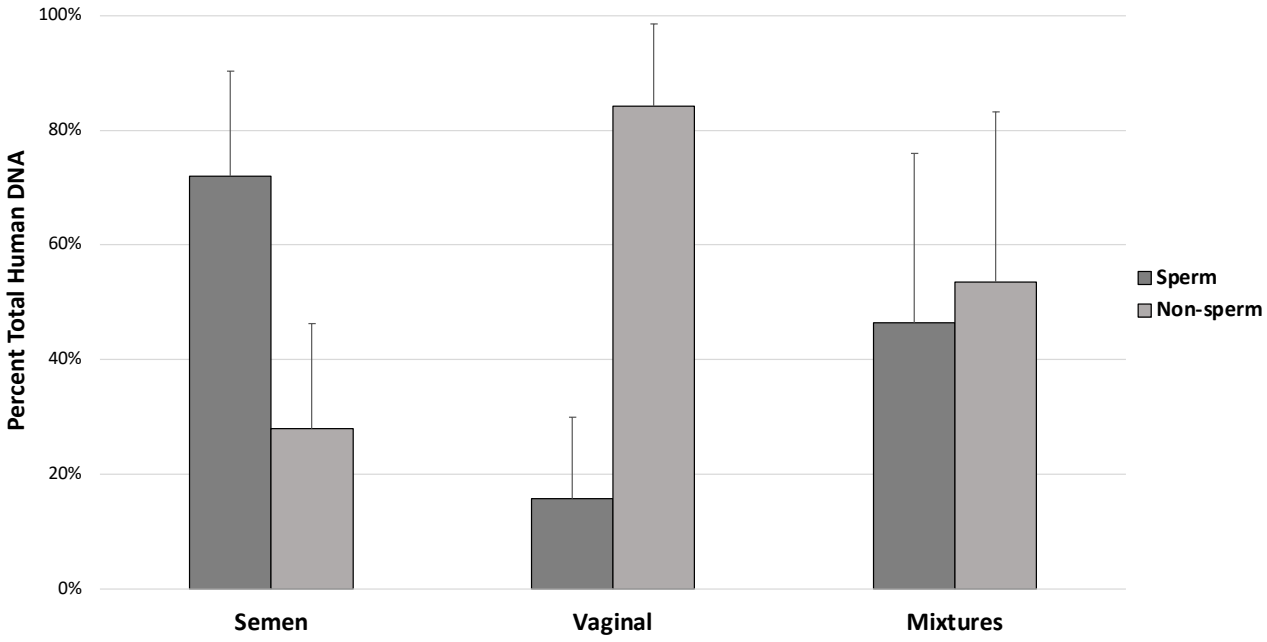

FIGURE 3 – Percentage of total DNA retained in sperm and non-sperm fractions after treatment of semen, vaginal, and mixture eluates (n = 10) with the *prep*GEM™/alkaline assay in-tube. This technique was able to retain 72.0 ± 18.3% of total DNA in sperm fractions for semen, while leaving behind only 15.8 ± 14.2% of total DNA in sperm fractions for vaginal eluates. As expected, approximately half of the total DNA yield was retained in the sperm fraction and half in the non-sperm fraction for mixture samples.

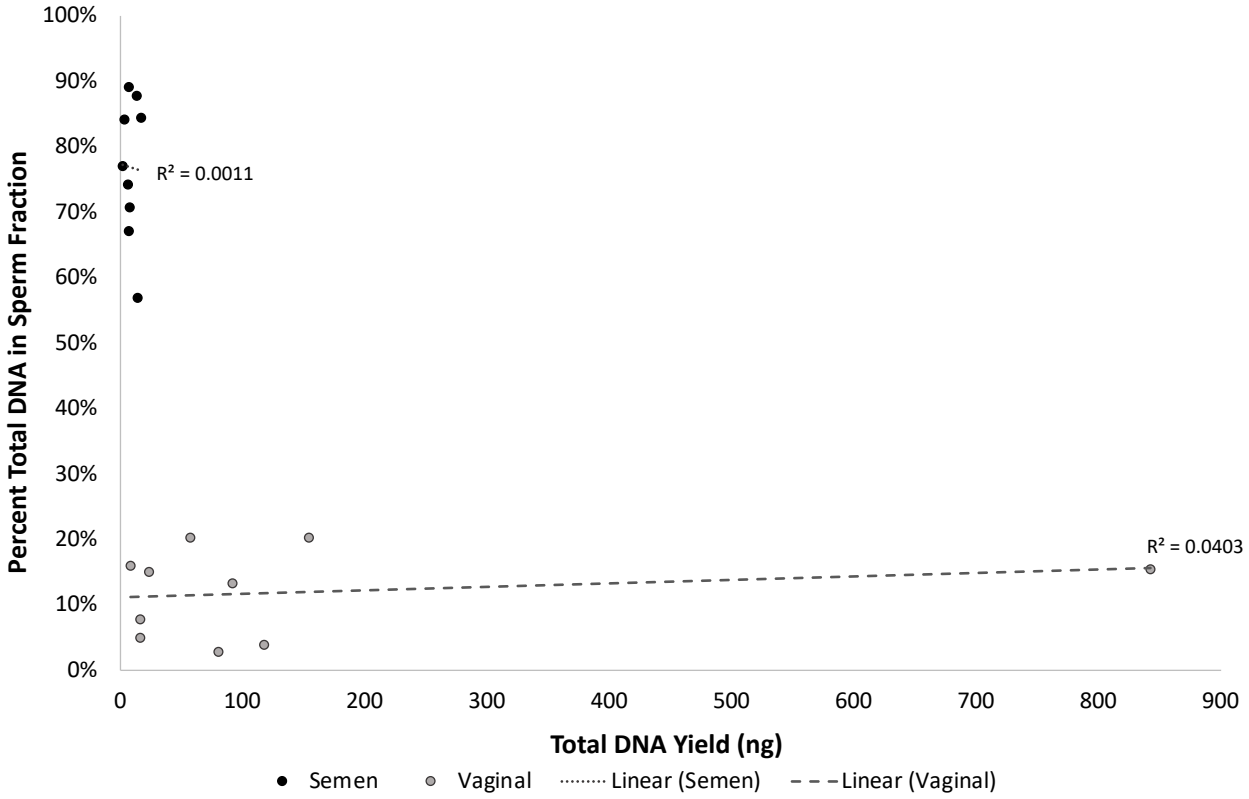

FIGURE 4 – Percentage of total DNA retained in sperm fractions for semen and vaginal eluates in-tube versus the total DNA yield. Linear trendlines indicated no correlation between cellular input (i.e., total DNA yield) and the percentage of DNA retained in sperm fractions.

After DNA quantification, male-to-female (M:F) ratios were also determined for mixture samples processed with this technique. Sperm fractions exhibited mean M:F ratios of 1:1.38; this was a drastic improvement in comparison to unseparated mixture controls, which exhibited mean M:F ratios of 1:8.02 (Table 1). In fact, the data revealed a 4.4 ± 2.8-fold improvement in the M:F ratios (p = 0.26) for sperm fractions when mixtures were subjected to the in-tube differential lysis technique described herein. Although not statistically significant, this improvement could make a practical and important difference in subsequent STR profiling results.

TABLE 1. Male-to-female (M:F) ratios after DNA quantification and STR profiling for unseparated controls, sperm fractions, and non-sperm fractions from differentially lysed mixture samples processed in-tube.

Sample	DNA Quantification			STR Profile Analysis			
	M:F Ratio			M:F Ratio			M:F Fold-Improvement (S/Unseparated)
	Unseparated	Sperm Fraction	Non-sperm Fraction	Unseparated	Sperm Fraction	Non-sperm Fraction	
Mixture 1	1 : 36.5	1 : 4.7	1 : 46.2	1 : 7.63	1 : 2.88	1 : 7.07	2.65
Mixture 2	1 : 41.7	1 : 6.2	1 : 64.9	1 : 3.71	1 : 3.44	1 : 33.74	1.08
Mixture 3	1 : 13.1	1 : 3.2	1 : 12.7	1 : 2.84	2.11 : 1	1 : 6.08	6.00
Mixture 4	1 : 71.8	1 : 29.8	1 : 175.2	1 : 12.10	1 : 8.47	1 : 51.05	1.43
Mixture 5	1 : 1.8	5.2 : 1	1 : 3.7	1.42 : 1	11.03 : 1	1 : 1.81	7.78
Mixture 6	1 : 13.1	1 : 6.7	1 : 37.8	1 : 2.90	1 : 1.20	1 : 13.51	2.42
Mixture 7	1 : 3.0	1 : 1.4	1 : 25.9	2.33 : 1	1.77 : 1	1 : 2.05	0.76
Mixture 8	1 : 38.5	1 : 13.8	1 : 76.8	1 : 23.54	1 : 6.27	1 : 23.86	3.76
Mixture 9	1 : 17.0	1 : 3.3	1 : 18.9	1 : 4.87	1 : 2.39	1 : 6.06	2.03
Mixture 10	1 : 14.6	1 : 11.2	1 : 15.1	1 : 7.25	1 : 3.33	1 : 6.36	2.17
AVERAGE	**1 : 8.02**	**1 : 1.38**	**1 : 16.88**	**1 : 1.88**	**1.74 : 1**	**1 : 5.46**	**3.01 ± 2.3**

STR Profiling

A similar assessment was also performed after STR profiling, as M:F ratios at the DNA quantification step do not necessarily translate to M:F ratios observed in subsequent STR profiles. This evaluation was deemed especially critical for determining applicability of the modified differential technique described herein, as STR profiles are the final product of the forensic DNA workflow and therefore the ultimate applicability of this technique will depend upon the results obtained at this step. STR profiles of sperm fractions from processed mixtures exhibited mean M:F ratios of 1.74:1, which was a 3.01 ± 2.3-fold improvement ($p = 0.26$) over the mean M:F ratio of 1:1.88 observed in STR profiles for unseparated mixture controls (Table 1, Figure 5). Although not statistically significant, this improvement in M:F ratios (and thus sperm cell enrichment) for resulting sperm fractions demonstrates a practical difference when using this technique. Non-sperm fractions exhibited a mean M:F ratio of 1:5.46, indirectly demonstrating the enrichment of male contributions in sperm fractions and highlighting the ability of this technique to generate non-sperm fractions with clear major female contributor profiles. This can be meaningful as it makes profile interpretation easier and provides more confidence in genotyping of the victim DNA profile (serving as an important control for the identification process).

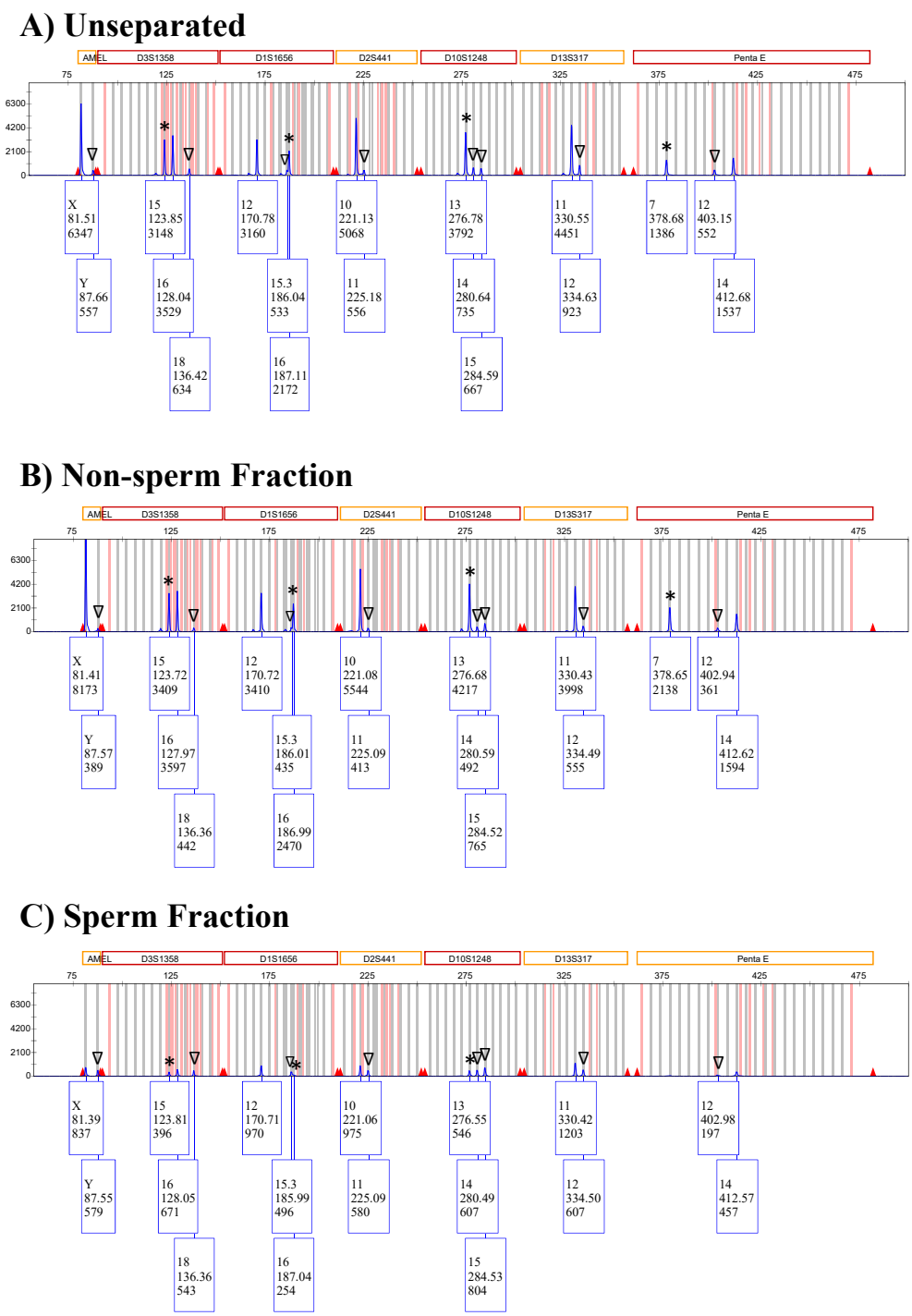

FIGURE 5 – Representative blue channel electropherograms for mixtures processed using the in-tube *prep*GEM™/alkaline differential lysis technique in-tube. Unseparated mixture controls exhibited a mean M:F ratio of 1:1.9 (A), while non-sperm fractions had a mean M:F ratio of 1:5.5 (B). Sperm enrichment was demonstrated in sperm fractions, with STR profiles experiencing a 3.0-fold M:F improvement over unseparated controls and a mean M:F of 1.7:1. Asterisks denote unshared female alleles and unshared male alleles are represented by ∇.

Overall, resulting STR profiles for sperm fractions demonstrated the ability of a combined enzymatic and alkaline technique to enrich for sperm cells. Not only was this apparent when comparing M:F ratios between sperm fractions and their corresponding unseparated controls, but it was also noticeable when considering the number of unshared male contributor alleles that were recovered in sperm fractions but were otherwise undetected in unseparated controls. In fact, 5.90 ± 7.8 unshared male contributor alleles were recovered in sperm fractions, with the most drastic improvement resulting in the recovery of 24 male alleles that were not observed in the unseparated control (i.e., nearly an entire STR profile). Further, the only sperm fractions that failed to recover additional male contributor alleles were samples that already exhibited full male STR profiles within their associated controls. Not only does this highlight the ability of this modified differential lysis technique to enrich for the male contributor within sperm fractions, but it also indicates that this assay could dramatically improve the recovery of male STR profiles from mixture samples with low male contributions *and* emphasizes the need to assess multiple metrics at STR profiling (other than mean M:F ratios) when determining whether a proposed technique is practically beneficial.

Protocol Modification (2X *prep*GEM enzyme)

While these results demonstrated the ability of a combined in-tube enzymatic and alkaline lysis technique to enrich for sperm within mock sexual assault samples, careful evaluation of sperm fractions also indicated that a portion of intact non-sperm cells may remain in sperm fractions after treatment with *prep*GEM™. Thus, additional studies were performed on semen, vaginal, and semen-vaginal mixtures with 2X *prep*GEM™ enzyme. DNA quantification of resulting samples revealed that 59.0 ± 18.7%, 19.7 ± 15.7%, and 30.2 ± 19.5% of DNA was retained in sperm

fractions for semen, vaginal, and mixture eluates, respectively, when utilizing 2X *prep*GEM™ enzyme (Supplemental Figure S1). A plot of the percent total DNA in sperm fractions relative to the total DNA yield within the initial sample once again indicated no apparent relationship between the DNA retained and the cellular input for semen and vaginal samples (Supplemental Figure S2). Overall, these DNA quantification results appeared to indicate that non-sperm cell lysis was *not* increased by additional *prep*GEM™ enzyme; however, premature sperm lysis and/or impact on sperm integrity (and thus pelleting) were potential ramifications, as the percentage of seminal DNA retained in sperm fractions was reduced in comparison to the original protocol. This translated to a reduced percentage of total DNA retained within sperm fractions for mixture samples. M:F ratios in resulting sperm fractions at the quantification step also decreased in comparison to the original protocol (1:3.01 versus 1:1.38) (Supplemental Table S1); however, sperm fractions still demonstrated a 3.80 ± 2.9-fold improvement ($p = 0.13$) in the M:F ratio when compared to unseparated mixture controls. Similarly, STR profiles from resulting sperm fractions exhibited a mean M:F ratio of 3.35:1, which was a 2.95 ± 2.1-fold improvement ($p = 0.25$) over the mean M:F ratio of 1:1.91 observed in unseparated controls (Supplemental Table S1). Further, non-sperm fractions displayed a mean M:F ratio of 1.70:1, which highlighted the possible premature lysis (and thus loss) of sperm cells when implementing additional *prep*GEM™ enzyme.

Microdevice Assay

Given the relative success of the in-tube differential cell lysis assay proposed herein, preliminary transition of this technique to a microfluidic format was pursued. Initial assessment of sperm cell pelleting within a preliminary microdevice (absent any lysis) was conducted to confirm the ability to pellet and trap intact cells. Microscopic evaluation of resulting sperm and non-sperm fractions

revealed that the majority of sperm cells could be pelleted *and* retained within the cell trap, even after transfer of the supernatant to a subsequent chamber (Figure 6). After an architectural modification of the microdevice to include larger chamber sizes (to accommodate the required volume for lysis), this assessment was repeated and revealed similar results; however, it should be noted that the number of sperm leaking into the downstream non-sperm chamber slightly increased with this modification (Figure 7). Despite the observed loss of sperm cells, this modified device architecture was carried forward and evaluated for differential cell lysis, as the clear majority of sperm were retained.

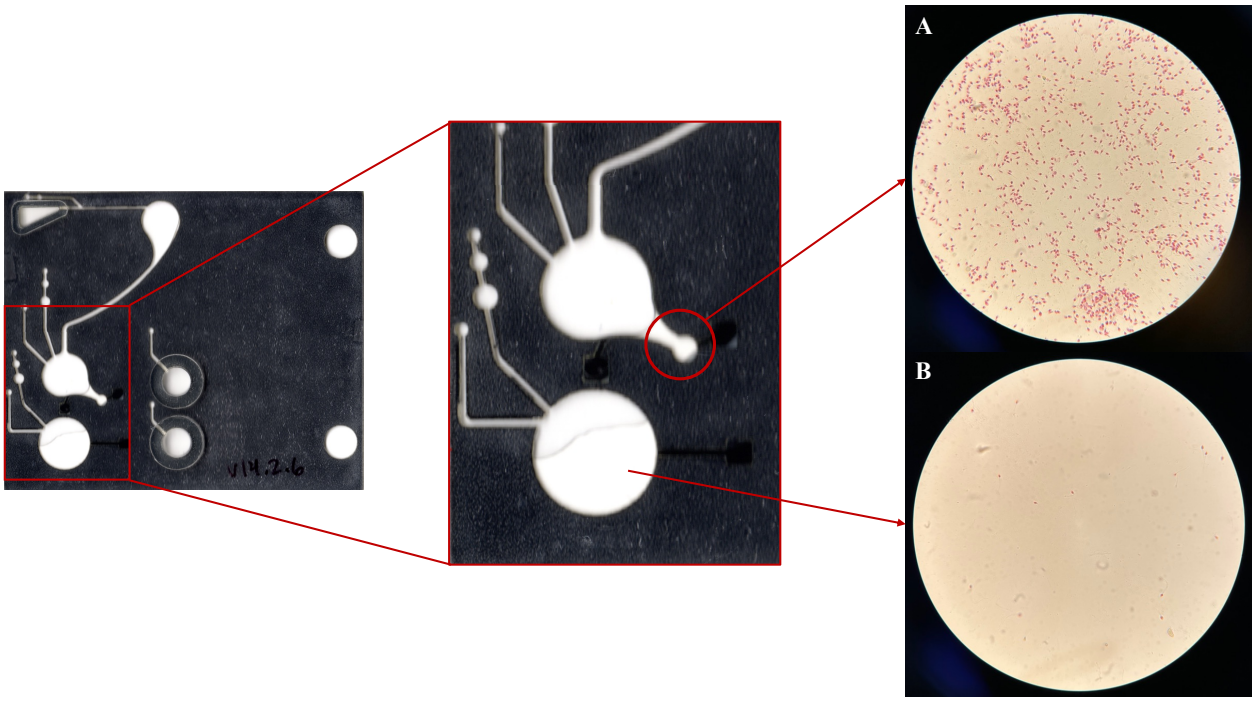

FIGURE 6 – Pelleting and fractional separation of sperm cells within a preliminary cell trap microdevice. The majority of sperm cells were retained within the cell pellet trap (○) and was subsequently recovered (A), while very few transferred to the non-sperm chamber upon valve opening and centrifugation (B). Micrographs were obtained using KPICS and 400X magnification.

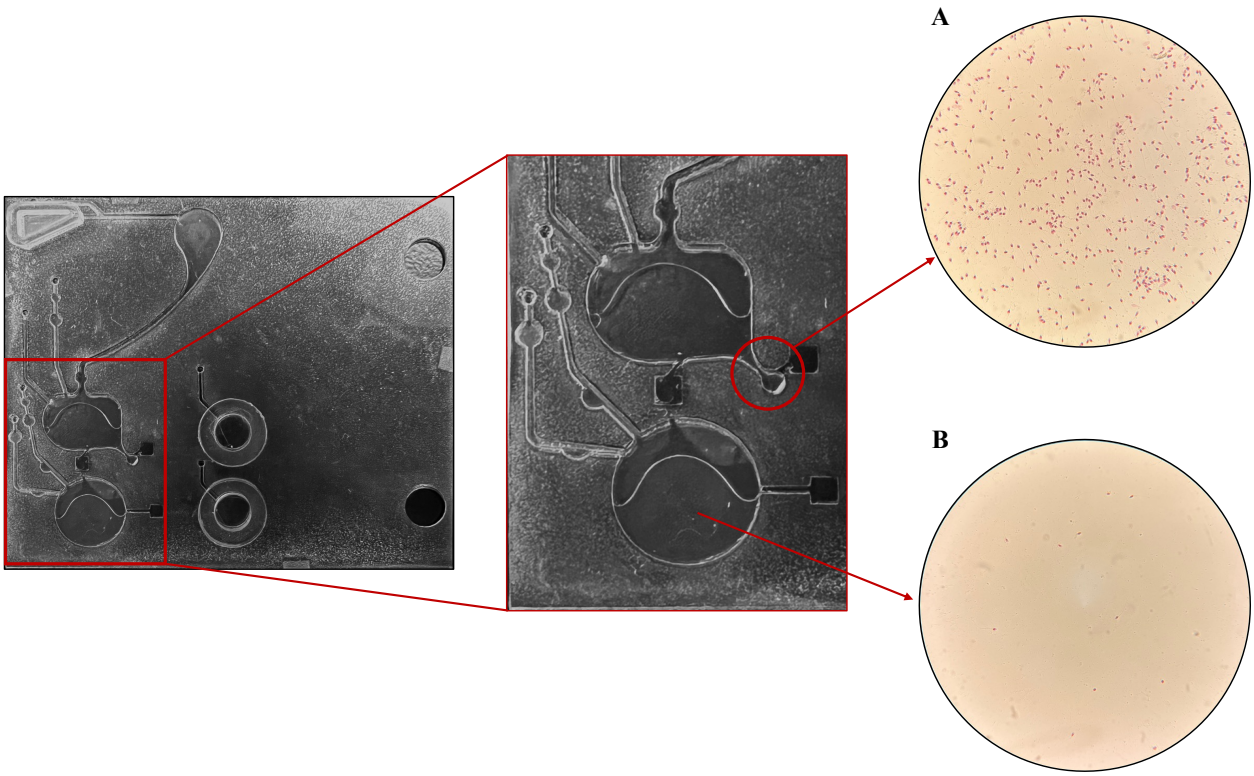

FIGURE 7 – Pelleting and fractional separation of sperm cells within the final microdevice, which includes enlarged sperm and non-sperm chambers. Again, the majority of sperm cells were retained within the trap (○) and subsequently recovered (A), while very few transferred to the non-sperm chamber upon valve opening and centrifugation (B). Micrographs were obtained using KPICS and 400X magnification.

Dye studies using the final microdevice architecture demonstrated the ability of fluidic movement to be controlled within the proposed design, with minimal fluid loss and backflow during spin and heating steps (Figure 8).

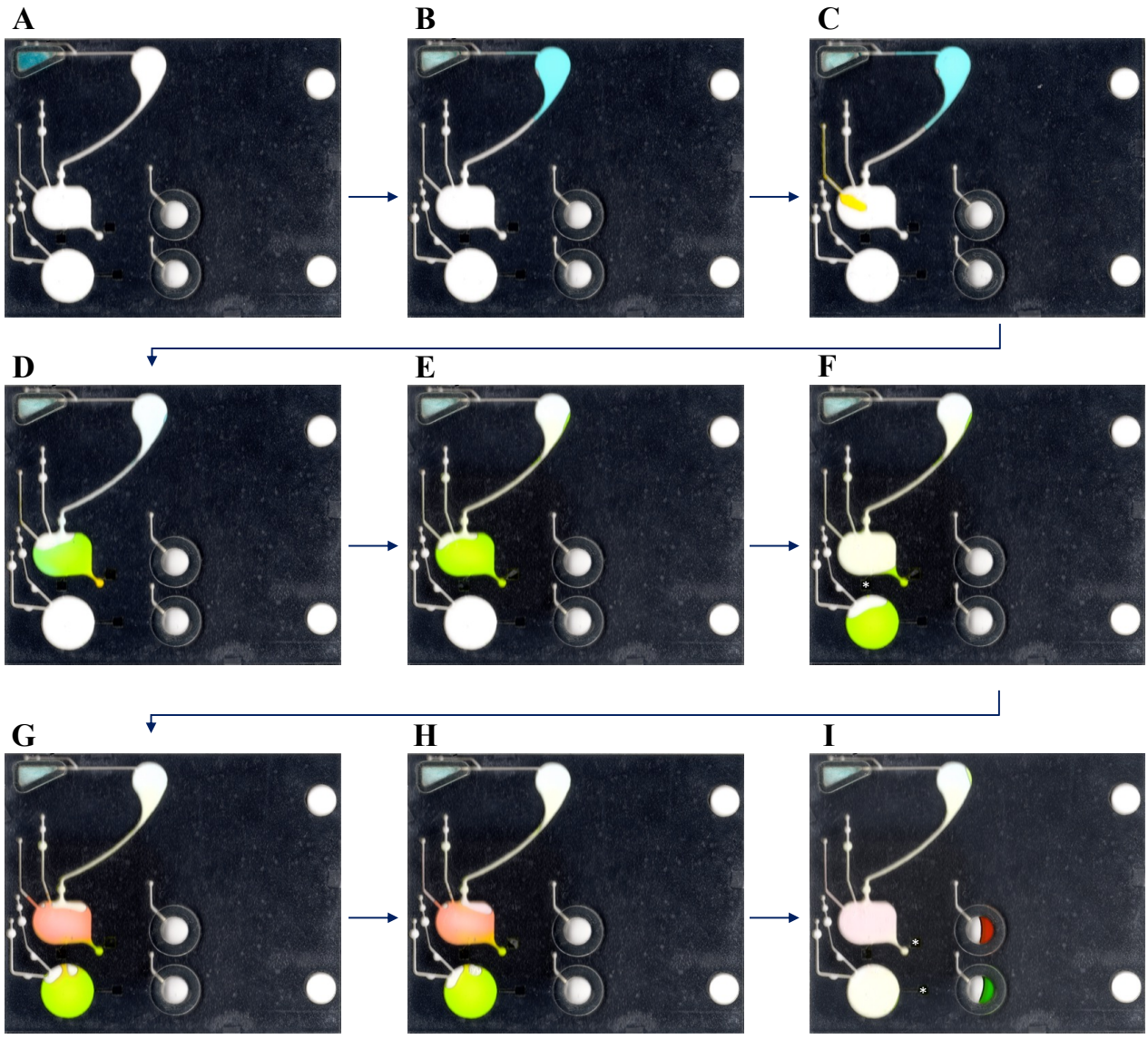

FIGURE 8 – Fluidic movement throughout the cell trap microdevice. A swab with liquid (blue) is inserted into the swab chamber, which is then capped (A). Centrifugation then forces liquid to move into the elution chamber (B). Non-sperm lysis master mix (yellow) can then be added to the sperm chamber (C), while a subsequent change in center of rotation and centrifugation forces all liquid into the sperm chamber (D). Following a heating step (E), opening of a normally closed valve via laser and spinning of the supernatant into the non-sperm chamber then accomplishes fractional separation (F). Sperm lysis master mix (red-orange) is then added to the sperm chamber (G), the device is heated (H), valves are opened, and both resulting fractions are spun into recovery chambers to facilitate aspiration/removal from the microdevice (I). Valves are denoted by white asterisks.

After demonstration of the ability to pellet cells and control fluidic movement, mixture swab cuttings were processed using the novel differential lysis technique completely on the microdevice platform. Human DNA quantification revealed that this technique was able to produce a 2.1 ± 0.96-fold M:F ratio improvement ($p = 0.30$) in resulting sperm fractions compared to unseparated controls. Although not statistically significant, this was a practical improvement, as mean M:F in unseparated mixture controls and sperm fractions were 1:4.57 and 1:2.03, respectively (Table 2). Further, M:F ratios in non-sperm fractions were almost halved in comparison to unseparated controls, indirectly demonstrating that sperm were being retained and enriched in sperm fractions.

Analysis of resulting STR profiles for unseparated mixtures, sperm fractions, and non-sperm fractions revealed a similar trend. Unseparated controls exhibited mean M:F ratios of approximately 1:1, while sperm and non-sperm fractions displayed mean M:F ratios of 1.4:1 and 1:2.95, respectively (Table 2). The M:F fold-improvement in resulting STR profiles was less drastic than predicted after DNA quantification (Figure 9), as sperm fractions only experienced a 1.04 ± 0.33-fold increase ($p = 0.73$, Table 1). Further, while some new unshared male alleles were observed after separation, only 1.5 ± 2.5 unshared male alleles were recovered in comparison to unseparated controls, which is lower than what was observed using the in-tube assay.

TABLE 2. Male-to-female (M:F) ratios after DNA quantification and STR profiling for unseparated controls, sperm fractions, and non-sperm fractions from mixture samples processed on the microdevice platform.

Sample	DNA Quantification			STR Profile Analysis			
	M:F Ratio			M:F Ratio			M:F Fold-Improvement (S/Unseparated)
	Unseparated	Sperm Fraction	Non-sperm Fraction	Unseparated	Sperm Fraction	Non-sperm Fraction	
Mixture 1	1 : 5.78	1 : 3.12	1 : 60.06	1 : 1.03	1 : 1.21	1 : 14.53	0.85
Mixture 2	1 : 23.60	1 : 8.34	1 : 73.31	1 : 1.62	1 : 1.50	1 : 9.78	1.08
Mixture 3	1 :7.04	1 : 4.61	1 : 28.13	1 : 1.83	1 : 2.44	1 : 12.02	0.75
Mixture 4	1 : 1.50	2.53 : 1	1 : 1.09	4.76 : 1	5.39 : 1	1.98 : 1	1.13
Mixture 5	1 : 1.59	1 : 1.55	1 : 4.38	1.06 : 1	1.44 : 1	1 : 2.68	1.36
Mixture 6	1 : 6.66	1 : 5.38	1 : 25.40	1 : 2.61	1 : 3.57	1 : 10.73	0.73
Mixture 7	1 : 3.43	1 : 1.38	1 : 39.90	2.60 : 1	4.63 : 1	1 : 1.92	1.78
Mixture 8	1 : 50.72	1 : 21.49	1 : 85.90	1 : 7.71	1 : 7.92	1 : 18.46	0.97
Mixture 9	1 : 35.03	1 : 11.49	1 : 73.47	1 : 8.02	1 : 7.82	1 : 25.48	1.03
Mixture 10	1 : 21.40	1 : 24.18	1 : 46.97	1 : 9.20	1 : 12.60	1 : 13.17	0.73
AVERAGE	**1 : 4.57**	**1 : 2.03**	**1 : 7.54**	**1.13 : 1**	**1.40 : 1**	**1 : 2.95**	**1.04 ± 0.33**

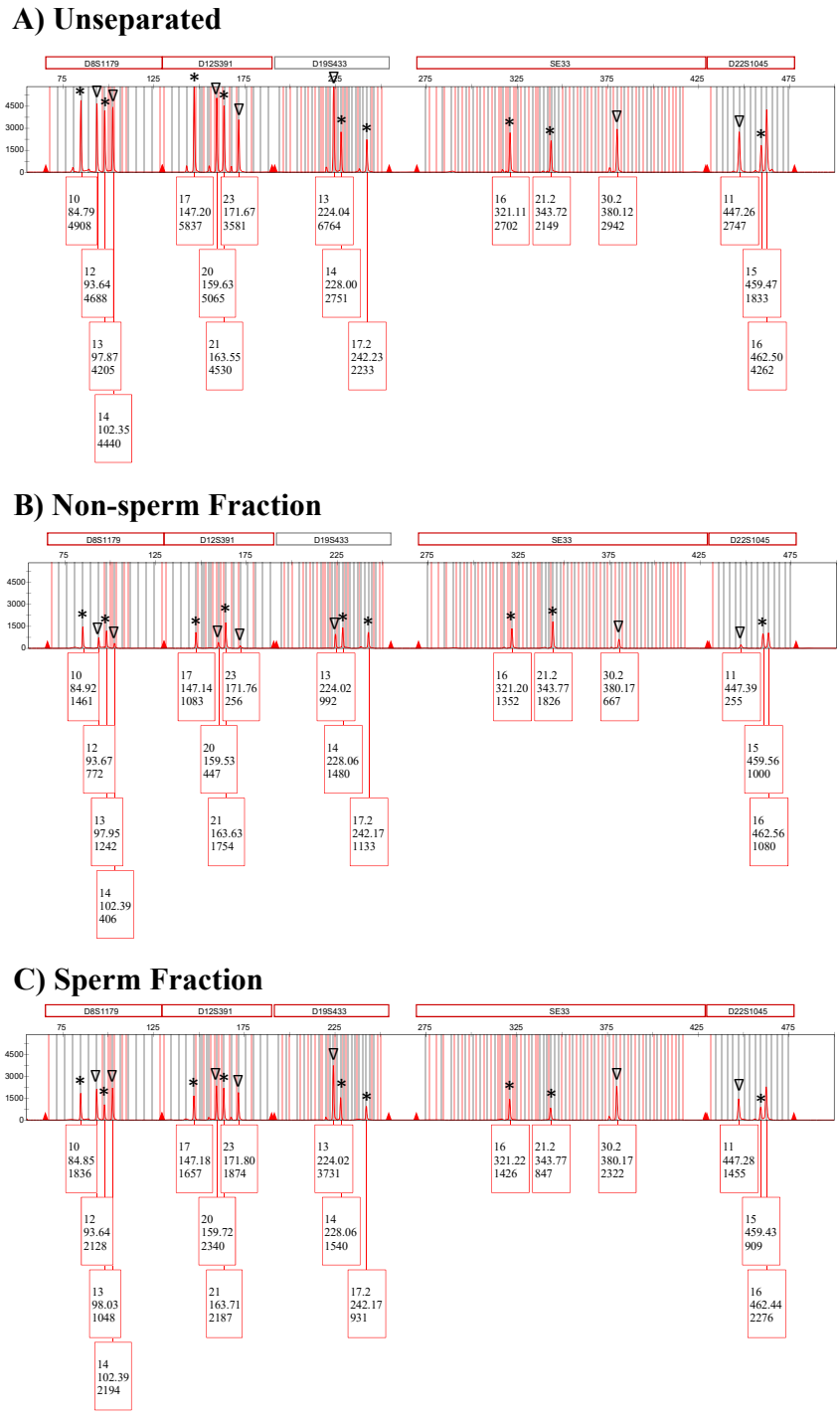

FIGURE 9 – Representative red channel electropherograms for mixtures processed on the microdevice. Unseparated controls exhibited a mean M:F ratio of ~1:1 (A), while non-sperm fractions had a mean M:F ratio of ~1:3 (B). M:F ratios in sperm fractions were ~1.5:1, demonstrating a 1.25 ± 0.76-fold M:F ratio improvement over unseparated controls and revealing the ability of this assay to enrich for sperm cells. Unshared female alleles are denoted by asterisks and unshared male alleles are denoted by ▽.

As noted, this differential lysis method did not generally perform as well as the in-tube assay when implemented on the proposed microdevice platform. Although cell pelleting was easily demonstrated in the microdevice with microscopy, the reduced efficiency of differential separation could be due to many reasons. First and foremost, the stepper motor utilized on the spinning platform in this study is incapable of generating enough rpm to match that of a standard centrifuge; when considering the rpm and distance from center of rotation for this microdevice, a relative centrifugal force of only ~100 *x g* was accomplished. In contrast, the in-tube assay utilized 17,000 *x g* for cell pelleting, which is magnitudes higher and thus capable of producing more stable cell pellets. In addition, the in-tube assay removed *all* supernatant from the resulting cell pellet after non-sperm lysis; however, the design of the microdevice left behind approximately 1-2 µL of supernatant (i.e., ~5% of total sample volume) to prevent inadvertent removal of the cell pellet. While one could hypothesize that the DNA within this residual supernatant (which could stem from lysed epithelial cells) possibly confounds resulting M:F ratios for the sperm fraction, comparison of DNA quantification and STR profiling results for non-sperm fractions points more toward inefficient non-sperm cell lysis as the culprit for poor fractional separation. The mean DNA yields obtained from non-sperm fractions were 176 ± 316.5 ng and 80.6 ± 85.66 ng ($p = 0.38$) for the in-tube and microdevice assays, respectively, signifying that fewer non-sperm cells were being lysed with *prep*GEM™ in the microdevice (data not shown). STR profile analysis of unshared female alleles within these fractions further corroborated this, as the mean peak height decreased from 2177 ± 1288 RFU to 1849 ± 1187 RFU when this assay was transitioned to the microdevice (data not shown). Given that sample preparation and DNA input for STR amplification were the same across these assays, it is likely that fewer non-sperm cells were being lysed on the microdevice and were thus trapped alongside intact sperm cells within the sperm chamber. This

notion was further supported by the increase in mean peak height for unshared female contributor alleles within sperm fractions from the microdevice assay in comparison to those obtained from the in-tube assay (1846 ± 1444 RFU versus 889.6 ± 606.6 RFU, respectively) (data not shown). While the data supports these theories, microscopic analysis of resulting fractions after non-sperm lysis with *prep*GEM™ could also determine whether this treatment impacts the stability of the cell pellet, which could lead to leaking of sperm cells into the downstream non-sperm chamber – negatively impacting the M:F ratios. Additionally, previous centrifugal microdevice studies have suggested the possibility of pelleted cells to diffuse back into solution once centrifugation ceases [42]; this further highlights the necessity of higher spin speeds for generating more stable cell pellets, as well as automated laser valving and spinning to reduce time between pelleting and supernatant removal.

Resulting STR profiles from all microdevice samples also exhibited worsened interlocus balance for unshared alleles compared to those from samples processed using the in-tube assay. When performed in-tube, resulting CV of LPH:TPH was 0.60 ± 0.141 and 0.50 ± 0.167 in non-sperm and sperm fractions, respectively (data not shown). These values increased to 0.69 ± 0.250 and 0.68 ± 0.217 for non-sperm and sperm fractions, respectively, for the microdevice assay – signifying more variation in peak heights and thus worse interlocus balance (data not shown). Interestingly, two non-sperm fraction STR profiles from microdevice samples experienced allelic dropout at larger loci for the female contributor despite exhibiting major female profiles; this was not observed for in-tube samples (data not shown). Although the same lysis methods were incorporated for the in-tube and microdevice assays, there were two differences in the protocol that could potentially explain this observation. Firstly, total non-sperm lysis volume for the in-tube

assay was 50 µL while the total volume for the microdevice assay was reduced to 30 µL in an effort to decrease reagent volumes and associated costs. Further, the 95ºC step was shortened to 30 seconds on the microdevice (versus two minutes in-tube) to avoid fluid loss due to evaporation; this could have exacerbated the inhibitory effect of the *prep*GEM™ technique on resulting STR amplification. Although not reported in the literature, and despite internal PCR control (IPC) C_q values within the normal range, studies within our laboratory have also indicated the potential for *prep*GEM™ lysates to exhibit inhibited STR profiles when their DNA concentrations are lower (i.e., when the dilution factor is less prior to STR amplification and thus more neat lysate is processed) (data not shown). These reasons, rather than the microdevice materials themselves, are believed to have caused ski slope effects in resulting STR profiles, especially given the fact that sperm fractions from the microdevice were not as impacted as non-sperm fractions.

CONCLUSIONS

The traditional processing of sexual assault samples remains a very manual, tedious, and inefficient technique that often culminates in complex DNA profile interpretation, fewer case resolutions, and contributes to persistent SAECK backlogs. While many studies have attempted to improve this technique, most forensic DNA labs still employ the traditional differential lysis method that was developed in the 1980s – in part because it is already well accepted and validated, but also because newer methods often fail to consistently demonstrate improvements in STR profiling results.

Given that two of the primary limitations of the traditional differential method are lysis inefficiency and long incubation times, this study aimed to develop a modified differential cell lysis that could quickly and effectively accomplish both fractional separation and lysis. Further,

reduced volumes were implemented to minimize costs and aid in analysis of lower sperm inputs. Previous studies have demonstrated the ability of enzymatic [14–20] and alkaline [12,43–45] techniques to effectively lyse cells and obviate the need for DNA purification; thus, we proposed a combined enzymatic and alkaline differential cell lysis assay for processing sexual assault samples. This method can be accomplished in 50 – 60 µL and generate fractional lysates from swab cuttings in approximately 25 minutes. Not only was this method developed for a traditional, in-tube format, but it was also explored within a centrifugal microdevice to enable automation.

Overall, the in-tube *prep*GEM™/alkaline technique described herein could retain 72.0 ± 18.3%, 15.8 ± 14.2%, and 46.4 ± 29.6% of total DNA in sperm fractions for semen, vaginal, and mixture eluates, respectively. Resulting sperm fraction STR profiles for mixtures processed with this technique exhibited mean M:F ratios of 1.74:1 and a M:F fold-improvement of 3.01 ± 2.3 compared to unseparated controls. Further, 5.90 ± 7.8 unshared male contributor alleles were recovered in sperm fractions, while MF ratios and peak heights within resulting non-sperm fractions demonstrated enrichment of the female contributor.

Preliminary transition of this assay onto a centrifugally driven microdevice platform revealed that pelleting and trapping of intact cells was possible with the proposed architecture. Microscopy for semen eluates demonstrated that cell pellets could be retained after transfer of the supernatant into an adjacent chamber. Ultimately, however, resulting STR profiles from processed mixture swabs revealed only minor sperm enrichment for sperm cell fractions. Although this assay failed to exhibit the same level of fractional separation as the in-tube method, it still recovered a small number of unshared male alleles in resulting sperm fractions compared to unseparated controls

and generated non-sperm fractions trending toward major female DNA profiles. Although the microdevice assay appears to retain fewer sperm cells and/or lyse fewer non-sperm cells than the in-tube assay, STR profiling results were complicated by potential inhibition (i.e., ski slope); thus, additional studies are needed to investigate the potential of and improve upon this technique. Architectural adjustments should be investigated to overcome evaporation during the 95ºC step after *prep*GEM™ lysis, which would allow extension of this step and possibly reduce the inhibitory effects of *prep*GEM™. In addition, microscopic assessment of cell pellets after non-sperm lysis, upgrading the motor to accomplish higher spin speeds and thus stabilize the pellet and reduce time, and automation of laser valving to decrease potential of cell resuspension/diffusion are all avenues that should be explored.

Overall, this study demonstrated the ability of a combined enzymatic and alkaline lysis technique to differentially lyse non-sperm and sperm cells within forensically relevant samples. Sperm fractions exhibited enrichment of male contributions and even recovered unshared male contributor alleles – results which could inherently simplify mixture profile deconvolution and lead to additional points of inclusion for statistical analysis. The ability of this method to accomplish cell elution and fractional lysis within 25 minutes (in comparison to approximately 170 minutes when using the QIAamp® DNA Investigator kit [46]) also enables much quicker and more efficient processing of samples, which could help reduce SAECK backlogs. Further, the reduced volumes and the ability to go directly into STR amplification without further DNA purification make it much more time and cost efficient compared to the traditional differential lysis. Although transition of this technique onto a microdevice platform was not as successful as the in-tube assay, it still demonstrated the ability to pellet intact cells on a microdevice (both in

general and following non-sperm lysis), remove the supernatant to a separate chamber, and control fluidic movement in such a way that would reduce contamination potential, analyst intervention, and variability from sample-to-sample (or analyst-to-analyst). Not only is this significant for forensic DNA analysis, but other fields that isolate and analyze various cell types could benefit from such an automated device. Since this microdevice only incorporates elution, pelleting/lysis, and separation modules, the applications are endless and could be customized for the end user. This microdevice also obviates the need for beads and antibodies to accomplish cell separation – which are potentially cost prohibitive. Future optimization studies that evaluate higher spin speeds and cell trap design modifications should be performed to enhance the cell pelleting and retainment capabilities of this microdevice. Additionally, because there is evidence of intact non-sperm cells after treatment with *prep*GEM™, alternative non-sperm lysis techniques should be explored to further optimize this assay – with the goals of maximizing non-sperm lysis, minimizing premature sperm lysis, and limiting inhibition.

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SUPPLEMENTAL TABLES & FIGURES

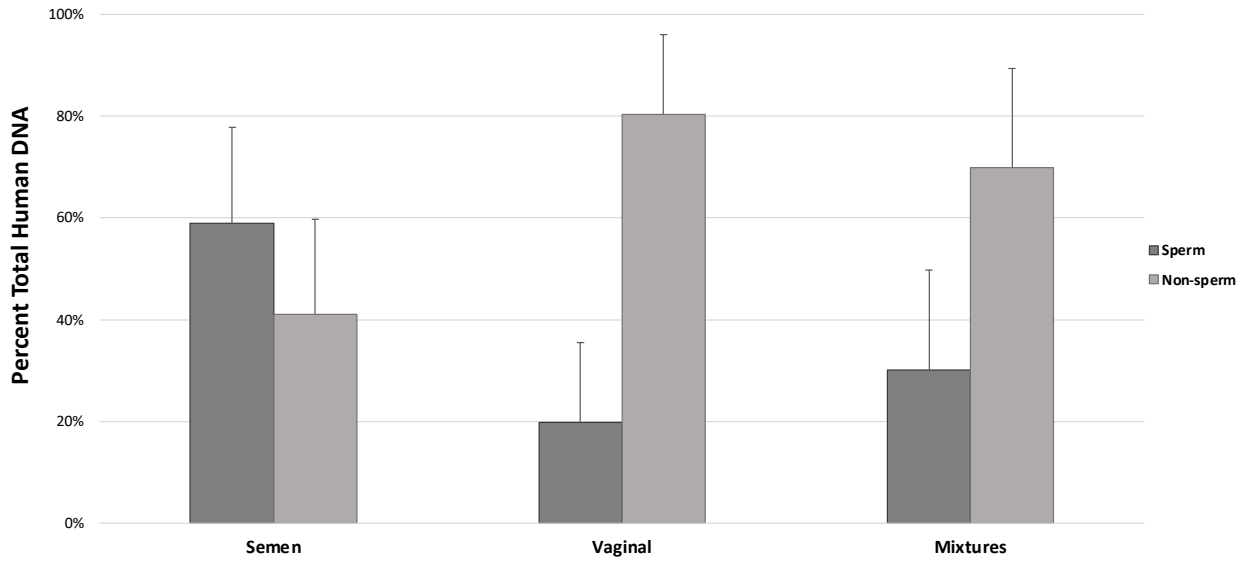

FIGURE S1 – Percentage of total DNA retained in sperm and non-sperm fractions after treatment of semen, vaginal, and mixture eluates (n = 10) with the combined enzymatic and alkaline lysis in-tube approach using 2X *prep*GEM™ enzyme. This technique was able to retain 59.0 ± 18.7% of total DNA in sperm fractions for semen, while leaving behind only 19.7 ± 15.7% of total DNA in sperm fractions for vaginal eluates. 30.2 ± 19.5% of total DNA was retained in sperm fractions for mixtures.

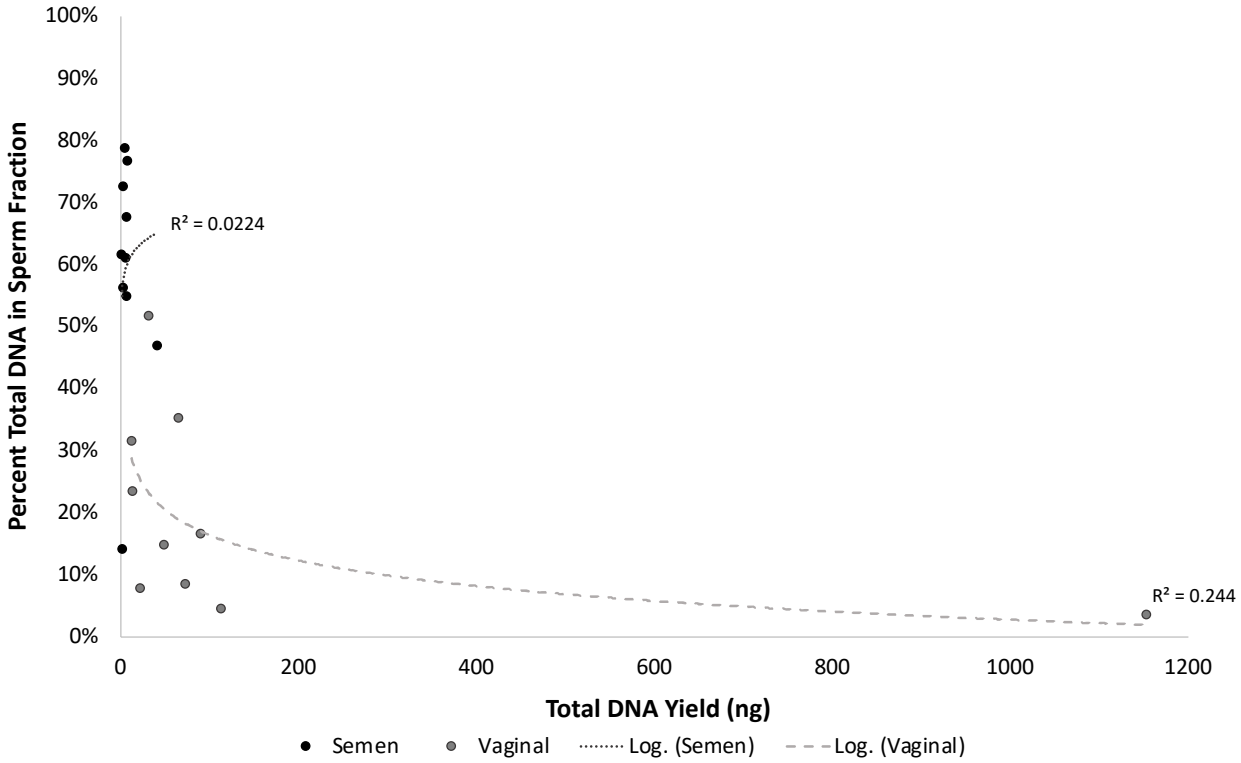

FIGURE S2 – Percentage of total DNA retained in sperm fractions for semen and vaginal eluates in-tube versus the total DNA yield when 2X *prep*GEM™ enzyme was implemented for non-sperm lysis. Logarithmic trendlines ("Log.") indicated no correlation between cellular input (i.e., total DNA yield) and the percentage of DNA retained in sperm fractions. However, reduced percentages of seminal DNA were retained in sperm fractions compared to the original method.

TABLE S1. Male-to-female (M:F) ratios after DNA quantification and STR profiling for unseparated controls, sperm fractions, and non-sperm fractions from mixture samples processed with 2X *prep*GEM™ enzyme.

Sample	DNA Quantification			STR Profile Analysis			
	M:F Ratio			M:F Ratio			M:F Fold-Improvement (S/Unseparated)
	Unseparated	Sperm Fraction	Non-sperm Fraction	Unseparated	Sperm Fraction	Non-sperm Fraction	
Mixture 1	1 : 32.3	1 : 10.7	1 : 47.1	1 : 2.32	1 : 1.33	1 : 4.37	1.74
Mixture 2	1 : 37.8	1 : 26.3	1 : 47.2	1 : 13.43	1 : 8.41	1 : 15.07	1.60
Mixture 3	1 : 18.2	1 : 1.8	1 : 31.9	1 : 4.21	1.71 : 1	1 : 9.87	7.20
Mixture 4	1 : 157.5	1 : 19.1	1 : 79.6	1 : 15.74	1 : 3.75	1 : 15.44	4.20
Mixture 5	1 : 2.8	1.4 : 1	1 : 4.6	1.69 : 1	9.73 : 1	1 : 1.80	5.75
Mixture 6	1 : 15.3	1 : 4.8	1 : 29.2	1 : 3.78	1 : 1.82	1 : 9.49	2.08
Mixture 7	1 : 3.7	1 : 1.4	1 : 3.1	2.06 : 1	3.19 : 1	1.49 : 1	1.55
Mixture 8	1 : 56.0	1 : 29.4	1 : 103.6	1 : 13.42	1 : 5.91	1 : 17.01	2.27
Mixture 9	1 : 12.4	1 : 6.0	1 : 17.4	1 : 5.34	1 : 3.42	1 : 5.06	1.56
Mixture 10	1 : 14.5	1 : 9.0	1 : 24.6	1 : 6.92	1 : 4.36	1 : 8.70	1.58
AVERAGE	**1 : 10.2**	**1 : 3.01**	**1 : 13.06**	**1 : 1.91**	**3.35 : 1**	**1.70 : 1**	**2.95 ± 2.1**

CHAPTER SIX:

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The ever-increasing sexual assault evidence collection kit backlog within the United States has prompted the search (and desperate need) for a more efficient, cost-effective, and rapid processing technique that can separate male and female contributions from evidentiary samples. Antibodies have shown great promise for targeting and isolating sperm cells within these samples; however, optimization studies are needed to address reported sensitivity and reproducibility issues. Even further, the transition of any optimized technique into a microdevice environment would further promote its adoption within the forensic science community – given the well-established benefits of automation, including reduced sample and reagent consumption. Ultimately, development of a reproducible sperm isolation technique combined with quick and efficient lysis of resulting fractions is sorely needed to reduce time and cost, and automation of such a technique within a microfluidic platform could greatly enhance sexual assault sample processing efficiency.

This research initially evaluated several direct-to-amplification lysis techniques for processing of sexual assault samples in an effort to reduce time and cost. Although dithiothreitol (DTT) has been the primary reagent employed for the lysis of spermatozoa for many years, the work herein revealed that residual DTT in samples that move directly into a PCR-based method can artificially increase DNA quantities of various qPCR targets (e.g., small autosomal, large autosomal, and male) *and* impact predicted male-to-female DNA ratios. Not only does this affect downstream STR amplification, but it would also potentially alter decision making for samples at the quantification step. Thus, several promising, alternative direct-to-amplification sperm lysis techniques were identified and tested. Results indicated that Promega's Casework Direct kit, NP-40 cell lysis buffer, HGH (i.e., HTF media + glutathione + heparin), and alkaline solution (i.e., 4

μL 1M NaOH) could generate sufficient DNA quantities and STR profiles with adequate peak heights, peak height ratios, and interlocus balance. While all techniques were quick and efficient, lysis using the proposed alkaline solution was identified as the optimal method – providing amplification-ready lysates within five minutes.

Upon identification of novel direct-to-amplification lysis techniques, this research set out to develop a PH-20 antibody-mediated cell isolation assay which could isolate sperm cells within sexual assault samples. Ultimately, a 35-minute assay using biotin-conjugated polyclonal PH-20 antibodies and streptavidin-coated Microbubbles was developed. This method could retain 58.0 ± 15% of seminal DNA within semen, as well as improve M:F ratios 2.76 ± 0.92-fold ($p = 0.041$) within STR profiles of processed mixture samples. While this was both a statistically *and* practically significant development, further evaluation identified the prominence of female contributor DNA within the residual supernatant/"subnatant" of samples, highlighting the need to remove as much "subnatant" as possible during fractional separation, perform a singular wash step, *and/or* implement DNase treatment. Additional studies which evaluate other antibodies – or even aptamers – are necessary to determine whether PH-20 is the best target antigen. Further, antibodies which target other forensically relevant cells could be important for situations in which sperm are not expected to be present.

This assay was then transitioned onto a centrifugal microdevice – with the goal of providing automation and reduced variability. Proof of concept studies demonstrated the feasibility of employing Microbubbles and buoyancy activated cell sorting (BACS) on a centrifugal microdevice platform. With change in center of rotation of the device and spin speed adjustments,

Microbubbles were sufficiently homogenized/mixed and aggregated. Precise control of the position of Microbubbles within the microdevice chamber was achieved, while valve opening and "subnatant" transfer could be performed without significant loss of Microbubbles. Subsequently, the aforementioned PH-20 antibody-mediated assay was preliminarily evaluated on the microdevice. Although M:F ratios in resulting antibody-bound fractions were similar to unseparated controls, and thus not ideal, this assay still demonstrated the potential to enrich for target cells and accomplish BACS in an automated fashion. Future studies should investigate microdevice architecture (e.g., increased depth of chambers and reduced evaporation), hardware (e.g., attachments for automated mixing), and antibody-binding protocol modifications to improve upon this technique.

Given the possible cost prohibitive nature of BACS, this research also evaluated the ability to achieve fractional separation with a differential cell lysis assay involving a combined enzymatic and alkaline approach, as well as simple centrifugation. With this assay, 72.0 ± 18.3% and 15.8 ± 14.2% of total DNA was retained in sperm fractions for semen and vaginal samples, respectively. Further, STR profiles of processed mixture samples exhibited 3.01 ± 2.3-fold improvement in M:F ratios and revealed 5.90 ± 7.8 unshared male contributor alleles in sperm fractions that were otherwise undetected in unseparated controls. Transition of this assay onto a centrifugal microdevice with a modified cell trap module demonstrated only minor enrichment of male DNA contributions and was thus not as successful as the in-tube assay. However, this work did demonstrate proof of concept for cell trapping and fractional separation using centrifugal microfluidics, which is an important step. Overall, the data indicated that using *prep*GEM™ enzyme and alkaline solution for lysis of non-sperm and sperm cells, respectively, could enrich for

male DNA within sperm fractions and required only 25 minutes of overall processing time. In future studies, alternative, direct-to-amplification *non-sperm* cell lysis methods should be explored to replace the use of *prep*GEM™. Investigation and development of such methods should focus on more efficient and comprehensive lysis of non-sperm cells while ensuring that downstream STR amplification is not inhibited. Further, implementing higher spin speeds and automated laser valving within the cell trap microdevice should be explored to improve intact cell retainment.

IMPACT

The data and results from this research will contribute to local *and* global scientific communities. Several alternative direct-to-amplification lysis methods – Casework Direct, NP-40 buffer, HGH, and alkaline solution – were developed herein. These methods provide the field with multiple options for obtaining DNA from sperm cells within minutes at decreased volume and time, ultimately reducing costs associated with the forensic DNA workflow. They are also compatible with microdevice platforms, providing an opportunity for automation.

Further, the enrichment of sperm cells within sexual assault samples is critically important for forensic casework, as it would prevent or reduce the occurrence of DNA mixture samples and therefore simplify data analysis on the back end of the forensic DNA workflow. The PH-20 antibody assay developed by this research provides the forensic science community with a means of enriching for male DNA contributions with reduced time; it also serves as a stepping-stone for further optimization and automation studies by others in the community. As noted, the currently implemented and researched techniques for handling sexual assault samples are fraught with laborious, time-consuming, and inefficient processes that accumulate substantial costs when it

comes to instrumentation, reagent consumption, analyst labor, and justice system expenses. This project developed two specific, sensitive, and efficient in-tube cell isolation assays for sperm cell enrichment – one that utilizes a sperm-specific PH-20 antibody, and one that implements a combinatorial enzymatic and alkaline differential cell lysis approach with simple centrifugation. These assays require less than 45 minutes to go from swab cutting to lysate, are relatively cheap, and involve fewer manual interventions in comparison to currently employed methods.

Proof of concept studies herein also demonstrated that these differential cell lysis and BACS assays could be transitioned to an automatable microdevice platform, leading to substantial time and cost savings, reduced sample and reagent consumption, and completion of several steps in the DNA workflow in a closed environment. This technique, if further optimized, commercialized, and introduced into forensic labs, could be used to help tackle the SAECK backlogs reported throughout the US and the world. With a system developed from this approach, more samples could be processed in a shorter timeframe while reducing the occurrence of DNA mixtures, leading to more certainty in STR profiling results and thus better scientific evidence in the courtroom. These techniques also provide impact and applications outside of the forensic DNA community. This research offered the first characterization of buoyant Microbubbles within a centrifugal microfluidic environment, serving as evidence of the ability to automate any antibody or aptamer-mediated BACS assay which utilizes this solid support. Further, the development of a centrifugal microdevice containing a cell trap module within this research has implications for the isolation and analysis of various cell types throughout the biological and biomedical communities.

VITA

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ResearchGate: https://www.researchgate.net/profile/Brittany-Hudson

EDUCATION

Virginia Commonwealth University **August 2018 – December 2022**
Overall GPA: 4.0

Doctor of Philosophy in Integrative Life Sciences

Dissertation: Development of novel, miniaturized sperm cell isolation techniques for sexual assault samples

Marshall University **August 2016 – May 2018**
Overall GPA: 4.0 (summa cum laude)

Master of Science in Forensic Science with concentrations in DNA Analysis, Crime Scene Investigation, and Digital Forensics

Virginia Commonwealth University **August 2012 – May 2016**
Overall GPA: 4.0 (summa cum laude)

Bachelor of Science in Forensic Science with a concentration in Forensic Biology

Bachelor of Science in Criminal Justice with a concentration in Forensic Crime Scene Investigation

Minor in Chemistry

University of North Dakota **December 2014 – December 2015**

Certificates of completion obtained from noncredit online courses offered through the University of North Dakota's School of Medicine as part of the "Death Investigation Training" program

PUBLICATIONS

Alternative direct-to-amplification sperm cell lysis techniques for sexual assault sample processing **March 2022**

Sarah K. Schellhammer, Brittany C. Hudson, Jordan O. Cox, Tracey Dawson Green

Comparison of DNA typing success in compromised blood and touch samples based on sampling swab composition **July 2021**

Chastyn Smith, Jordan O. Cox, Ciara Rhodes, Carolyn Lewis, Memunatu Koroma, Brittany C. Hudson, Tracey Dawson Cruz, Sarah J. Seashols-Williams

The effects of dithiothreitol (DTT) on fluorescent qPCR dyes **March 2021**

Brittany C. Hudson, Jordan O. Cox, Sarah J. Seashols-Williams, Tracey Dawson Cruz

Comparison of Cyanoacrylate Fuming Techniques of Bloody and Latent Fingerprints and the Examination of Subsequent DNA Success **February 2020**

Jessica Joy, Jordan O. Cox, Brittany C. Hudson, Julissa Armstrong, Marilyn T. Miller, Tracey Dawson Cruz

ORAL PRESENTATIONS

A Novel Sperm Isolation Technique Using Buoyancy Activated Cell Sorting (BACS) and PH-20 Antibody **June 19, 2022**

Brittany C. Hudson, M.S.; Tracey Dawson Green, Ph.D.*

- Proceedings of the 2022 Gordon Research Seminar (GRS) on Forensic Analysis of Human DNA in West Dover, Vermont

Alternative Sperm Cell Lysis Methods for Sexual Assault Samples **February 24, 2022**

Sarah K. Schellhammer, M.S.; Brittany C. Hudson, M.S.; Jordan O. Cox, M.S.; Tracey Dawson Green, Ph.D.*

- Proceedings of the 2022 American Academy of Forensic Sciences annual meeting in Seattle, Washington

The effects of dithiothreitol (DTT) on fluorescent qPCR dyes **September 23, 2021**

Brittany C. Hudson, M.S.; Jordan O. Cox, M.S; Sarah J. Seashols-Williams, Ph.D., Tracey Dawson Cruz, Ph.D.*

- Proceedings of the 2021 Mid-Atlantic Association of Forensic Scientists annual meeting

The Development of RED-BLEU: A UV/Vis Assay Following Colorimetric Detection of EDTA **February 17, 2021**

Sarah K. Schellhammer, B.S.; Brittany C. Hudson, M.S.; Alexandra M. Wright, B.S.; Catherine Cupples Connon, Ph.D*

- Pre-recorded oral presentation for the 2021 American Academy of Forensic Sciences annual meeting (virtual)

Optimization of an in-tube bead-mediated cell separation assay using sperm-specific PH-20 antibody **September 4, 2020**

Brittany C. Hudson, M.S.; Yolanda Correia, M.S.; Chelsie Testerman, M.S.; Jordan O. Cox, M.S; James P. Landers, Ph.D; Tracey Dawson Cruz, Ph.D.*

- Pre-recorded oral presentation for the 2020 Mid-Atlantic Association of Forensic Scientists annual meeting

An Exploration of EDTA Detection Within Forensically Relevant Blood Samples **February 21, 2020**

Brittany C. Hudson, M.S.; Matthew C. Rodriguez, B.S.; Catherine Cupples Connon, Ph.D.*

- Proceedings of the 2020 American Academy of Forensic Sciences annual meeting in Anaheim, California

Cell Isolation Techniques: Antibodies and Aptamers **November 12, 2019**

*Brittany Hudson, M.S.**

- Lecture given to the Fall 2019 FRSC 686 (Emerging Molecular Application) class at Virginia Commonwealth University

Internal Validation of Dubai Police Headquarters' Science Research Laboratory **October 27, 2017**

Brittany Hudson, B.S.; Taylor Beatty, B.S.**

- Presentation/defense of internship and directed research given to the Fall 2017 Seminar class at Marshall University

Is Less Actually More? An Evaluation of Reduced Volume Reactions for Forensic Applications **April 17, 2017**

*Brittany Hudson, B.S.**

- Lecture given to the Spring 2017 Seminar class at Marshall University

POSTER PRESENTATIONS

A Novel Sperm Isolation Technique Using Buoyancy Activated Cell Sorting (BACS) and PH-20 Antibody **June 19-22, 2022**

Brittany C. Hudson, M.S.; Tracey Dawson Green, Ph.D.*

- 2022 Gordon Research Conference (GRC) on Forensic Analysis of Human DNA in West Dover, Vermont

A Novel Differential Protocol for Sexual Assault Samples Using a Target-Specific Antibody-Bead Complex **February 27, 2020**

Chelsie Testerman, M.S.; Jordan Cox, M.S.; Molly Woodson, M.S.; Yolanda Correia, M.S.; Brittany Hudson, M.S.; Kemper Gibson, M.S.; James Landers, Ph.D.; Tracey Dawson Cruz, Ph.D.*

- 2020 Annual Integrative Life Sciences Program Research Showcase at Virginia Commonwealth University

An Evaluation of Capillary Array Variance for the Validation of STR Amplification Kits on the 3500 Genetic Analyzer **April 20, 2018**

Brittany Hudson, B.S.; Taylor Beatty, B.S.; Haifa Al Bastaki, B.S.; Kelly Beatty, M.S.F.S.; Laura Kuyper, M.S.F.S.; Rashed Al Shamsi, B.S.*

- 2018 Annual College of Science Research Expo at Marshall University

WORK EXPERIENCE

Teaching Assistantship – Molecular Capstone Laboratory (BIOZ 476) **January 2022 – December 2022**

Paid teaching assistantship at Virginia Commonwealth University

- Direct and train senior-level undergraduates on ELISA, DNA extraction, PCR, agarose gel electrophoresis, PAGE, and sterile mammalian cell culture techniques

Graduate Research Assistantship **August 2018 – December 2022**

Paid graduate assistantship at Virginia Commonwealth University

- Develop and review SOPs/lab protocols, maintain instruments, and perform research
- Supervise and train other lab members on DNA extraction, quantification, STR amplification, and capillary electrophoresis

Teaching Assistantship – Biological Concepts Laboratory (BIOZ 101) **August 2021 – December 2021**

Paid teaching assistantship at Virginia Commonwealth University

- Instructed students on the scientific method, water, enzymes, macromolecules, fermentation, immunology, and citizen science

Sales Associate at Pleasants Hardware Co. **April 2015 – March 2016; May 2018 – December 2018**

Part-time job as a sales associate/cashier in Richmond, VA

- Stocked shelves, assisted customers, answered phones, assembled store merchandise, and maintained store

Graduate Research Assistantship **August 2016 – May 2018**

Paid graduate assistantship at Marshall University (Crime Scene House)

- Maintained the Crime Scene House, involving inventory of chemicals and materials, cleaning, and security
- Held tours, demonstrated fingerprinting and bloodstain pattern analysis, and created mock crime scenes
- Evaluated different methods for performing cross-sections on fibers

Organic Chemistry Teaching Assistant – VCU **August 2015 – May 2016**

Facilitated the learning of organic chemistry through class questions, weekly help sessions, and catalysis of group interactions

- Implemented and promoted teamwork, leadership, critical thinking, and problem-solving skills as it pertains to learning and understanding organic chemistry
- Spearheaded weekly review sessions to tutor and prepare students for quizzes and exams

RESEARCH EXPERIENCE

Graduate Research Assistantship – Sperm Cell Isolation in Sexual Assault Samples **August 2018 – December 2022**

Research guided by Dr. Dawson Green at Virginia Commonwealth University

- Development, evaluation, and optimization of alternative cell lysis techniques which obviate purification.
- Development and optimization of a method to separate male and female cells using sperm-specific antibodies.
- Development and optimization of an automated microdevice platform capable of performing sperm and non-sperm cell separation, DNA extraction, and PCR amplification from sexual assault samples.

Collaborative Research – EDTA Detection in Blood **August 2018 – December 2022**

Research guided by Dr. Connon at Virginia Commonwealth University

- Development, optimization, and validation of a colorimetric method for the detection of EDTA within blood samples.
- Funded by the 2018 FSF Lucas Research Grant.
- Consulted with an Innocence Project case from New York.

Graduate Research Assistantship – EDTA Detection in Blood **January 2017 – May 2018**

Research guided by Dr. Rushton and Dr. Waugh at Marshall University

- Evaluated and developed a method for the detection of EDTA within control samples using a colorimetric indicator.

Internship – Dubai Police General Headquarters' Science Research Laboratory **May 2017 – August 2017**

Paid internship for an internal validation project for the Biology and DNA section of the laboratory that was conducted as part of a graduation requirement for the Marshall University Forensic Science Graduate Program

- Validation studies conducted in accordance with ISO/IEC 17025:2005 guidelines, as well as FBI Quality Assurance Standards and SWGDAM recommendations (when possible).
- Validation/verification of the PrepFiler Express™ and PrepFiler Express BTA™ Forensic DNA Extraction Kits on the Applied Biosystems AutoMate Express™ DNA Extraction System was performed using various reference and mock casework samples.
- Validation of the Quantifiler® Trio DNA Quantification Kit on the Applied Biosystems® 7500 Real-Time PCR System was performed. Included repeatability, reproducibility, limit of detection, shelf life of the standards, and mixture studies.
- Evaluated variance of the capillary array on the Applied Biosystems™ 3500 Genetic Analyzer using allelic ladder samples, where repeatability and reproducibility of the precision in sizing and relative quantity were examined.
- Validations of the GlobalFiler™, GlobalFiler™ Express, and Yfiler™ Plus PCR Amplification Kits were performed using DNA control 007, buccal samples, and blood samples on FTA cards. An analytical threshold study and sensitivity study were performed for each amplification kit. Additionally, mixture studies were performed for the GlobalFiler™ and Yfiler™ Plus kits, and a stochastic threshold study was performed for the GlobalFiler™ and GlobalFiler™ Express kits. Thermal cycling and injection parameters were optimized for each kit.
- Validation reports and SOPs were generated for each study performed and each kit/method validated.
- Culminated in an oral presentation and thesis paper, both of which can be found on the Marshall Forensic Science Program website or, alternatively, made available upon request.

Independent Study – Molecular Biology/DNA Analysis **January 2016 – July 2016**

Research guided by Dr. Connon, Dr. Dawson Cruz, and Dr. Seashols-Williams at Virginia Commonwealth University

- Validation and optimization of 6.25 µl and 12.5 µl PCR amplification reaction volumes for the AmpFlSTR® Identifiler® PCR Amplification kit on the ProFlex™ PCR System Dual 96-well thermal cycler.
- Validation and optimization of the Quantifiler® Trio DNA Quantification Kit on the Applied Biosystems® 7500 Real-Time PCR System, with studies covering repeatability, reproducibility, life of standards, and half volume reactions.

Independent Study – Bloodstain Pattern Analysis **January 2016 – May 2016**

Research guided by Dr. Marilyn Miller at Virginia Commonwealth University

- Assessment of the volume of blood needed to drip from a porous surface (e.g., cotton sock) compared to a nonporous surface (e.g., knife).
- Conducted to aid in crime scene reconstruction for a criminal case.

HONORS AND CERTIFICATIONS

Akadeum Life Sciences' Rising Innovator Research Award **June 2022**

MAAFS Annual Student Scholarship Award **August 2020**

- Awarded to three students each year during the MAAFS annual meeting

MUFSC Recognition of Academic Excellence **May 2018**

- Awarded to students graduating with an overall GPA of 3.9 or higher

Cellebrite Certified Operator (CCO) Certification **February 2018 – December 2020**

American Society of Crime Laboratory Directors Scholarship **August 2016**

Outstanding Undergraduate Award **May 2016**

- VCU Department of Forensic Science

Dean's Scholar Award **April 2015 and April 2016**

- Awarded to students classified as seniors by credit hours with a 4.0 GPA

Dean's List **December 2012 – May 2016**

- Awarded to students with an overall GPA of 3.5 or higher for each semester

MEMBERSHIPS & ACTIVITIES

American Academy of Forensic Sciences – Student Member **February 2022 – Present**

Delta Delta Epsilon – Zeta Chapter **January 2017 – Present**

Omicron Delta Kappa Honor Society **April 2015 – Present**

National Society of Collegiate Scholars **January 2013 – Present**

Crime Scene Shadowing **September 2017 – May 2018**

- Shadowed the Huntington Police Department's Forensic Investigations Unit to help process crime scenes and get hands-on experience with documentation, collection, and bloodstain pattern analysis

American Association for the Advancement of Science **September 2016 – May 2018**

Marshall University Forensic Identification Association **August 2016 – May 2018**

- Community outreach experience for junior high and high school students via mock crime scene and trial

Student United Way at VCU **January 2015 – May 2016**

Organic Chemistry Teaching Assistant at VCU **August 2014 – May 2016**

- Served as an organic chemistry teaching assistant for volunteer and credit-based experiences prior to employment

VCU Forensic Science Student Club **August 2012 – May 2016**

Women's Club Soccer at VCU – Co-Captain and Treasurer **August 2012 – May 2016**

- Planned and led practices, motivated team during games, recruited new players, and handled all finances

CONTINUING EDUCATION

29th Congress of the International Society for Forensic Genetics (Washington, DC) **August 2022**

- Attendee

Gordon Research Conference on Forensic Analysis of Human DNA (West Dover, VT) **June 2022**

- Attendee, speaker

Gordon Research Seminar on Forensic Analysis of Human DNA (West Dover, VT) **June 2022**

- Attendee, speaker

Mid-Atlantic Association of Forensic Scientists – Annual Meeting (Newport News, VA) **May 2022**

- Attendee, volunteer

American Academy of Forensic Sciences – 74th Annual Meeting (Seattle, WA) **February 2022**

- Attendee, speaker

Applied Biosystems™ 3500 Genetic Analyzer Install and Training **July 2021**

- Four hours of instruction and training at Virginia Commonwealth University

Applied Biosystems™ RapidHIT ID System Install and Training **July 2021**

- Five hours of instruction and training at Virginia Commonwealth University

Mid-Atlantic Association of Forensic Scientists – Annual Meeting (Pocono Manor, PA) **September 2021**

- Attendee, speaker

American Academy of Forensic Sciences – 73rd Annual Meeting (virtual) **February 2021**

American Academy of Forensic Sciences – 72nd Annual Meeting (Anaheim, CA) **February 2020**

- Attendee, speaker

HID Future Trends in Forensic DNA Technology – ThermoFisher seminar series (Arlington, VA) **November 2019**

- Attendee

Mid-Atlantic Association of Forensic Scientists – Annual Meeting (Morgantown, WV) **May 2019**

- Attendee, volunteer

American Academy of Forensic Sciences – 71st Annual Meeting (Baltimore, MD) **February 2019**

- Attendee, volunteer

American Academy of Forensic Sciences – 70th Annual Meeting (Seattle, WA) **February 2018**

- Attendee, volunteer

American Academy of Forensic Sciences – 69th Annual Meeting (New Orleans, LA) **February 2017**

- Attendee, volunteer

Mid-Atlantic Association of Forensic Scientists – Annual Meeting (Richmond, VA) **May 2016**

- Attendee, volunteer