J Forensic Sci, July 2006, Vol. 51, No. 4
doi:10.1111/j.1556-4029.2006.00180.x
Available online at: www.blackwell-synergy.com

Christine T. Sanders,[1,2] *M.S.; Nick Sanchez,*[3] *B.A.; Jack Ballantyne,*[2] *Ph.D.; and Daniel A. Peterson,*[1] *Ph.D.*

Laser Microdissection Separation of Pure Spermatozoa from Epithelial Cells for Short Tandem Repeat Analysis*

ABSTRACT: Short tandem repeat (STR) analysis is a valuable tool in identifying the source of biological stains, particularly from the investigation of sexual assault crimes. Difficulties in analysis arise primarily in the interpretation of mixed genotypes when cell separation of the sexual assailant's sperm from the victim's cells is incomplete. The forensic community continues to seek improvements in cell separation methods from mixtures for DNA typing. The feasibility of applying laser microdissection (LMD) technology to precisely separate sexual assault cell mixtures by visual inspection coupled with laser dissection was assessed through three experiments. First, various histological stains were evaluated for use with LMD and DNA analysis. Second, different DNA isolation methods were evaluated on LMD-collected cells. Finally, STR analysis was performed on LMD-separated sperm cells from mixtures of semen and female buccal epithelial cells. The results indicated (a) hematoxylin/eosin staining performed best in its ability to differentiate sperm and epithelial cells while exhibiting the least negative effect on further downstream analysis; (b) both QIAamp® and Lyse-N-Go™ methods were useful for recovery of DNA from LMD-collected sperm cells; and (c) LMD separation provided clear STR profiles of the male donor with the absence of any additional alleles from the female donor. This report describes an efficient, low-manipulation LMD method for the efficient separation of spermatozoa from two-donor sperm/epithelial cell mixtures.

KEYWORDS: forensic science, DNA typing, DNA mixtures, cell separation, histology, DNA isolation

Forensic evidence comprised of biological mixtures is a common occurrence, particularly in sexual assault crimes. Typically, the sperm cells are the component of interest, while the victim's cells from either a vaginal, rectal, oral, or other body swab complicate the genotyping of the assailant. The preferential lysis method (1) has been the forensic standard for separating sperm cells from epithelial cells. This method utilizes cell-specific differences in membrane chemical composition by first lysing the nonsperm cells without disrupting the sperm cells, and then washing away any residual exogenous DNA from the intact sperm cells. Although this method can generally provide two cellular fractions, one comprising of sperm cell DNA and the other of nonsperm DNA, the separation is not always complete. There may be carryover from one cell fraction to another, making eventual genotype interpretation and further statistical analysis challenging. Additional limitations to this technique are the premature lysis and loss of sperm cells in the first digestion and the multiple liquid transfers and washing steps that reduce cell recovery. The development of a method capable of fully separating pure populations of spermatozoa from epithelial cells while conserving sample would enable analysts to interpret DNA typing patterns with less difficulty.

An alternative to a chemical separation, such as the preferential lysis method, is separation by direct physical selection of the target cells from a mixture. Various physical separation methods have been explored (2–5); however, the high degree of precision required for a pure separation has not been available previously. Recent advances in microscopic instrumentation now permit direct visualization, dissection, and recovery of specific cells and tissue from microscope slides using laser illumination; this approach is called laser microdissection (LMD). LMD technology has been increasingly used in biomedical research applications to harvest selected cells from histological sections of complex tissues (6–9). In this study, LMD was evaluated and a method was developed to separate pure populations of sperm cells from semen/epithelial cell mixtures compatible with downstream short tandem repeat (STR) analysis. A series of three experiments were performed to assess LMD technology. First, the effect of various histological stains on downstream analysis from stained LMD cells was examined. Second, DNA isolation methods were compared when applied to LMD-collected cells. Finally, the separation capability of LMD to recover sperm cells from epithelial cells in a stained mixed specimen was determined.

Materials and Methods

Sample Collection

Liquid semen samples were obtained from stock internal laboratory standards stored at −20°C. Working solutions of the semen samples were prepared to a 1:10 dilution in sterile water for all samples. Buccal swabs were obtained from female subjects by sterile cotton swabs, dried, and then stored at −20°C. Each swab

[1]Department of Neuroscience, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064.
[2]Department of Chemistry, University of Central Florida, Orlando, FL 32816.
[3]Scientific Investigation Division, Los Angeles Police Department, Los Angeles, CA 90012.

*Part of this research was presented in oral form at the American Academy of Forensic Sciences meeting in Dallas, TX, on February 19, 2004; the California Association of Criminalists meeting in Ventura, CA, on October 27, 2004; the Louisiana Association of Forensic Scientists meeting in Baton Rouge, LA, on December 2, 2004; the Mid-Atlantic Association of Forensic Scientists meeting in Pittsburgh, PA, on May 19, 2005; and the International Association of Forensic Sciences meeting in Hong Kong on August 25, 2005.

Received 13 Nov. 2005; and in revised form 25 March 2006; accepted 26 March 2006; published 23 June 2006.

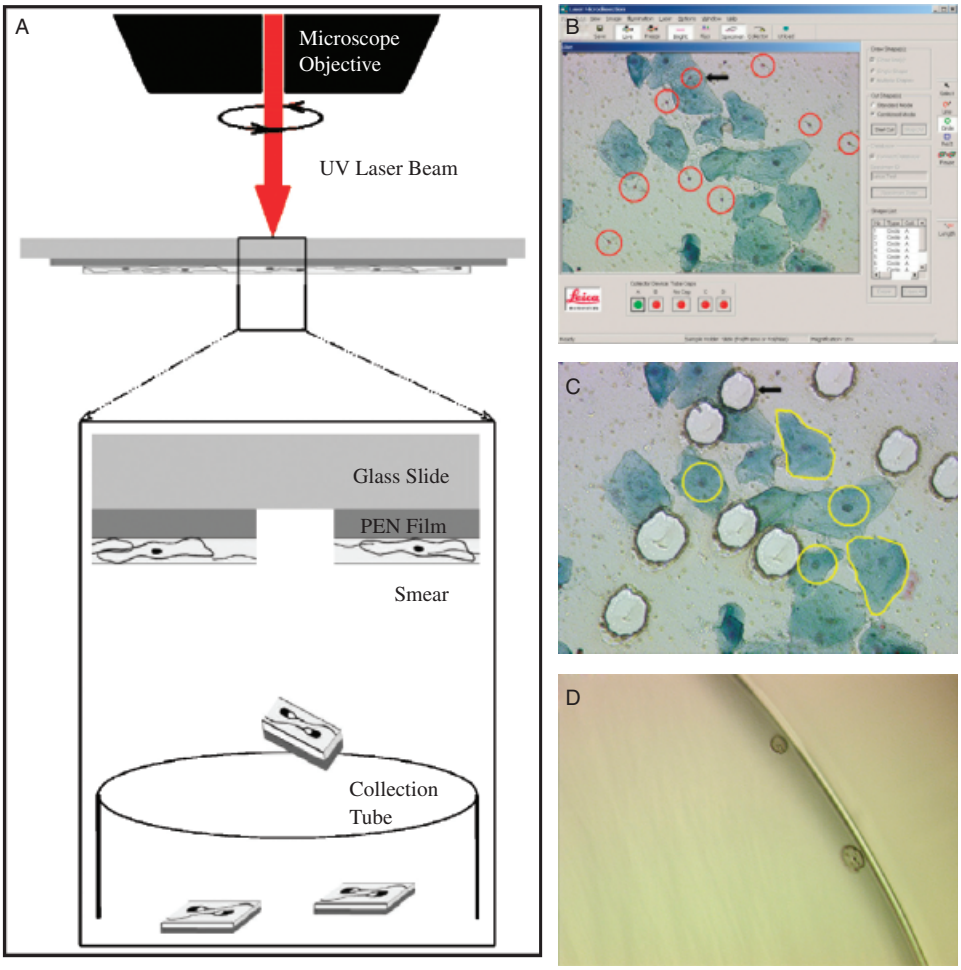

FIG. 1—*Laser microdissection (LMD) on the Leica AS LMD. (A) The plastic-covered glass slide rests inverted on the microscope stage with the cell smear facing down. The laser is focused through the objective to dissect around sperm cells, cutting the PEN film. The magnified cross section illustrates sperm cells adhered to PEN film dropping into the collection tube cap directly below stage. (B) Leica AS LMD collection software window (version 4.1.3) with operator-drawn circles (in red) around sperm cells. (C) After laser microdissection of sperm cells, cuts can be verified (example indicated by black arrow) and epithelial cells are targeted by operator for dissection (in yellow). (D) Postcollection feature of instrument allows inspection of the collection tube to confirm recovery of sample.*

was agitated in sterile water and the epithelial cell pellet was recovered in a 50 µL working solution. All procedures involving human subjects were in accordance with the Rosalind Franklin University Institutional Review Board.

Samples/LMD Slide Preparation

Mixtures were prepared by combining 25 µL of the epithelial cell pellet working solution with 10 µL of the 1:10 semen working solution. Two microliters of both the mixtures and single source working solutions were smeared over a 7 mm diameter circle on a PEN slide (Leica Microsystems, Brannockburn, IL) and dried at room temperature. The PEN slide is a glass microscope slide covered with a 2-µm-thick polyethylene naphthalate (PEN) plastic membrane, which is adhered close to the edges of the slide.

Histological Staining

Sterile filtered solutions of hematoxylin/eosin (H&E), nuclear fast red/picroindigocarmine (CTS, also known as "Christmas tree stain"), methyl green (MG), Wright's stain (WRT), and acridine orange (AO) were used for staining of cells. The durations for which slide smears were exposed to chemical stains in the histology comparison study were as follows: H&E—Mayer's hematoxylin for 5 min and then eosin for 5 min; CTS—nuclear fast red for 5 min and then picroindigocarmine for 30 sec; WRT—Wright's stain for 5 min; AO—acridine orange for 4 min; and MG—methyl green for 5 min. Unstained control smears were rinsed with 95% ethanol for 5–10 sec. Slides were vacuum-desiccated and stored at -20°C. Desiccation was repeated at room temperature immediately before LMD. A modified protocol of H&E staining (H&E Modified) was performed on the smears used in the isolation comparison and mixture separation studies in which exposure times to chemical stains were reduced to the following: Mayer's hematoxylin for 1 min and then eosin for 10 sec.

LMD

The Leica AS LMD instrument (Leica Microsystems) is a computer-controlled, motorized, upright laboratory microscope integrated with a 337 nm UV laser. The cells of interest are visualized and marked through the computer software. Then, the pulsed laser beam is directed through the objective lens passing through the inverted glass microscope slide to the plastic PEN film on which the sample resides. Laser ablation occurs around the cell(s) of interest and the material is collected by gravity into the

cap of a PCR tube below the stage. The technique is illustrated in Fig. 1. Groups of 300, 150, or 75 sperm and epithelial cells were dissected by LMD from the prepared smears under the $\times 40$ objective. Upon laser dissection, cells were automatically deposited into the caps of 0.2 mL thin-walled PCR tubes containing 20 μL of the appropriate collection buffer for each DNA isolation method described below.

DNA Isolation

Qiagen QIAamp®—LMD cells collected in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8) were extracted using the QIAamp® DNA Micro Kit (Qiagen, Valencia, CA). The DNA isolation was performed according to the manufacturer's recommendations for microdissected samples with the addition of dithiothreitol (DTT) to a final concentration of 30 mM in the lysis step. The final elution volume ranged from 20 to 25 μL.

MicroLYSIS®—LMD cells collected in MicroLYSIS® reagent (Microzone Ltd., West Sussex, U.K.) were extracted using 20 μL of reagent with the addition of DTT (30 mM) and incubated in the thermal cycler according to the manufacturer's recommendations as follows: 65°C for 5 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min, 96°C for 30 sec, and 20°C hold. LMD sample collection, lysis, and PCR were all performed in the same 0.2 mL thin-walled tube.

Lyse-N-Go™—LMD cells collected in Lyse-N-Go™ reagent (Pierce Chemical Co., Rockford, IL) were extracted using 20 μL of reagent with the addition of DTT (30 mM) and incubated in the thermal cycler according to the manufacturer's recommendations as follows: 65°C for 30 sec, 8°C for 30 sec, 65°C for 90 sec, 97°C for 180 sec, 8°C for 60 sec, 65°C for 180 sec, 97°C for 60 sec, 65°C for 60 sec, and 80°C for 5 min. LMD sample collection, lysis, and PCR were all performed in the same 0.2 mL thin-walled tube.

PCR Conditions

DNA Amplification was performed using the AmpF*l*STR® Profiler Plus Kit™ (Applied Biosystems, Foster City, CA) for nine STR loci (D351358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) plus amelogenin using a Bio-Rad iCycler to carry out the PCR. Standard PCR was performed according to the manufacturer' recommendations as follows: 21 μL AmpF*l*STR® PCR Reaction Mix (Applied Biosystems, Foster City, CA), 1 μL AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 11 μL AmpF*l*STR® Profiler Plus Primer Set (Applied Biosystems), and 20 μL sample DNA; thermal cycling conditions—incubate at 95°C for 11 min (polymerase activation); 94°C for 1 min (denaturation), 59°C for 1 min (annealing), 72°C for 1 min (extension) for 28 cycles; and then 60°C for 45 min (final extension). In addition, extended cycles were used with PCR conditions as follows: 25 μL of PCR product amplified under the standard conditions were removed and added to a new tube with 0.25 μL of AmpliTaq Gold® DNA Polymerase, and then PCR was performed for six additional cycles as above.

Electrophoresis Conditions

One-and-a-half microliters of each PCR product was denatured in 24 μL of HI-DI formamide with 1 μL of ROX 500 size standard (Applied Biosystems). Electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using a 5 sec injection time for the histology study and an ABI Prism 3100 Genetic Analyzer using an 11 sec injection time for the DNA isolation and mixture studies.

Data Analysis

GeneScan 3.1.2 and Genotyper 2.5.2 (Applied Biosystems) software was used to analyze the electrophoresis data. Baseline correction, matrix correction, and light smoothing were applied to all samples. The PCR amplification of human DNA using the Profiler Plus™ kit is such that a fluorescent dye is incorporated into each amplicon through a 5′-end-labeled oligonucleotide primer; therefore, the fluorescent signal detected is a measure of quantity of the amplified target. Sample peak heights, in relative fluorescent units (RFU), of all true alleles were used for quantitative analysis and heterozygous peak ratio calculations. The minimum peak height threshold was set at 50 RFU to allow for detection of all peaks clearly above background. Data compilations were performed using Microsoft Excel 2001 and GraphPad Prism 4.0 including mean, standard error, unpaired *t*-test, and ANOVA analysis with Bonferroni post-hoc test.

Studies

LMD Histology Comparison—Samples from six donors (three semen and three oral swabs) were examined. Six slides were prepared for each single source donor specimen and stained with H&E, CTS, MG, WRT, and AO including an unstained control as described in the histology methods. Cell identification was performed at a magnification under the $\times 40$ and $\times 63$ objectives using brightfield and fluorescence microscopy on a Leica AS LMD microscope. Scores were assigned to describe the stain's ability to facilitate cell identification as follows: double minus ($--$) = cannot identify or highly challenging; minus ($-$) = poor; plus/minus ($+/-$) = satisfactory; plus ($+$) = good; and double plus ($++$) = excellent. Collections of 300 sperm cells and 150 epithelial cells were recovered by LMD representing equivalent amounts of starting DNA material from the haploid sperm and diploid epithelial cells. Cells were isolated using the Qiagen QIAamp® DNA isolation method, followed by STR analysis using standard PCR conditions with 20 μL of undiluted DNA extract from all samples. RFU values were tabulated for each sample at all loci and compared with values of the unstained specimen to determine the relative PCR product yields.

LMD DNA Isolation Comparison—Samples from 10 donors (five semen and five oral swabs) were examined. Collections of 300 sperm cells and 150 epithelial cells stained with H&E Modified were collected by LMD in triplicate to compare Qiagen

TABLE 1—*Microscopic identification scores of sperm and epithelial cells for each histology stain.*

		Histological Stain					
	Sample	UNSTN	H&E	CTS	MG	WRT	AO
Spermatozoa	1	+/−	+	+	−−	−	+
	2	+/−	+	++	−	−−	+
	3	+/−	+	++	−−	−	+
Buccal Cells	1	+/−	+	++	−	−−	+/−
	2	−	+	++	−	−−	−
	3	+/−	+	++	−−	−−	+

UNSTN, not stained; H&E, hematoxylin/eosin; CTS, nuclear fast red/picroindigocarmine; MG, methyl green; WRT, Wright's stain; AO, acridine orange; −−, cannot identify or highly challenging; −, poor; +/−, satisfactory; +, good; ++, excellent.

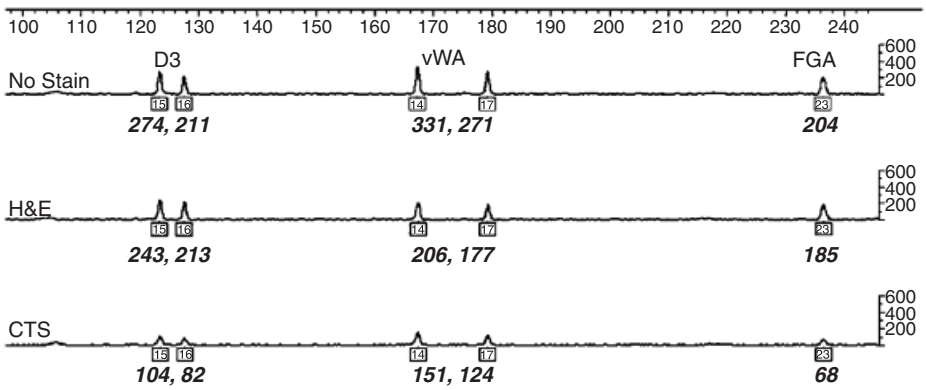

FIG. 2—*Peak height comparison of histologically stained cells. Examples of short tandem repeat plots at the D351358, vWA, and FGA loci from 150 oral epithelial cells with no stain, hematoxylin/eosin, and Christmas tree stain. The italicized numbers below the plot are the relative fluorescent units values of each peak.*

QIAamp®, MicroLYSIS®, and Lyse-N-Go™ DNA isolation methods. All samples were processed under standard PCR conditions. RFU values were tabulated for each sample at all loci to determine the relative PCR product yields.

LMD Mixture Study—Five mixed cell samples from 10 donors (five semen and five oral swabs) were examined. Collections of 300, 150, and 75 sperm cells stained with H&E Modified were separated by LMD from the mixtures. A serial dilution of a human DNA standard was included in the analysis, amplifying 2, 1, 0.5, 0.25, and 0.125 ng of DNA to compare with LMD-collected cell samples. All samples were processed using Lyse-N-Go™ DNA isolation applying both standard and extended cycles PCR.

Results

Histology Comparison

To ascertain an appropriate histological stain for LMD recovery, several common stains were tested for their utility in sperm and epithelial cell identification and their effect on downstream DNA analysis. Identification scores were assigned to cells microscopically examined from PEN slides without a coverslip (Table 1). Unstained specimens could be identified under brightfield conditions although the process at times was slow and laborious when sperm tails were detached. Both H&E and CTS readily provided morphological discrimination of spermatozoa and epithelial cells. Both WRT stain and MG staining resulted in poor visualization of sperm cells, making identification difficult. The penetration of MG was difficult to control and the WRT staining method appeared to cause some deformation either in the epithelial cells or the PEN membrane, hindering identification. AO performed well for identification of sperm, although it appeared that differentiation among a concentrated field of epithelial cells might be challenging as the larger epithelial cells brightly fluoresced, potentially masking hidden sperm cells. Because measures were based upon a single evaluator, statistical analysis was not applied.

STR data from H&E, CTS, AO, and unstained cells were compared. Figure 2 illustrates capillary electrophoresis data in

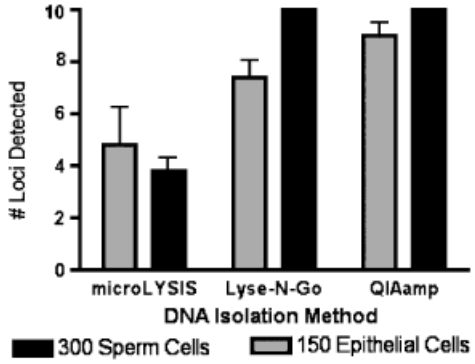

FIG. 3—*Detection of short tandem repeat loci using different isolation methods. Samples comprising 300 sperm and 150 oral epithelial cells were subjected to three DNA isolation methods: MicroLYSIS®, Lyse-N-Go™, and QIAamp®. The mean number of loci (n = 5) detected out of 10 possible Profiler Plus markers is shown from each group.*

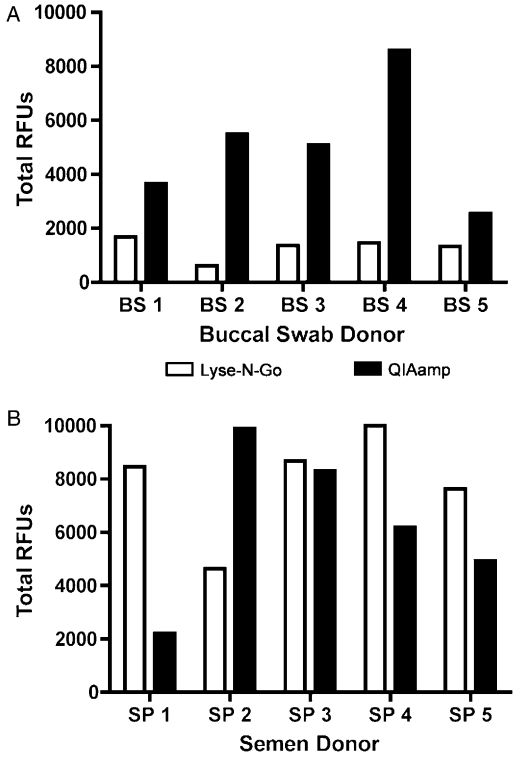

FIG. 4—*Total PCR product detected from laser microdissection-collected cells using Lyse-N-Go™ and QIAamp® DNA isolation methods: (A) 150 epithelial cells each from five buccal swab donors, (B) 300 sperm cells each from five semen donors.*

the blue spectra, showing a decrease of RFU peak height exhibited by stained epithelial cells. Total RFU values of all Profiler Plus loci from the stained sperm and epithelial cells were compared with that of the unstained control to determine the relative percent PCR yield. Combined data from sperm and epithelial cells were analyzed by ANOVA, followed by Bonferroni post-hoc test, excluding one semen donor sample due to the amplification failure of five loci from an unstained control. Stained specimens showed a significant decrease in total RFU values compared with unstained specimens. H&E samples exhibited RFU values $62.4 \pm 6.6\%$ of that observed by the unstained control ($p < 0.01$). CTS samples exhibited RFU values $42.6 \pm 5.5\%$ of that observed by the unstained control ($p < 0.001$). Cells stained with AO produced no amplified product in all samples tested.

DNA Isolation Comparison

Three isolation methods were compared to evaluate their effectiveness in DNA extraction from stained cells and to develop a method enabling successful STR analysis of LMD samples. Figure 3 shows the mean values of the number of loci detected from samples processed with three different isolation methods.

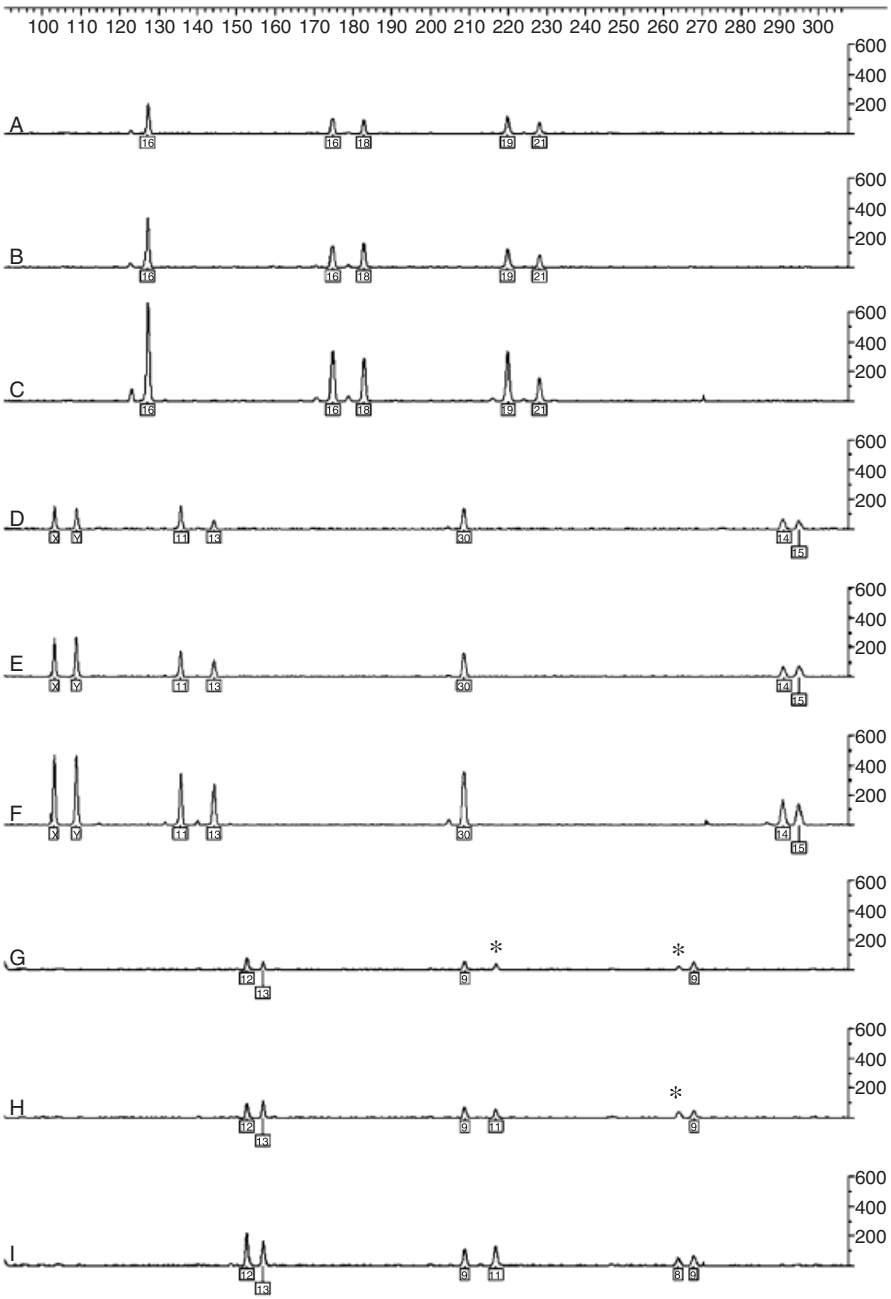

FIG. 5—*Profiler Plus plots of sperm separated by laser microdissection from a mixture. Blue loci of (A) 75 sperm, (B) 150 sperm, and (C) 300 sperm. Green loci of (D) 75 sperm, (E) 150 sperm, and (F) 300 sperm. Yellow loci of (G) 75 sperm, (H) 150 sperm, and (I) 300 sperm (y-scale at 600 relative fluorescent units). Allelic dropout observed in yellow loci for 75 and 150 cells indicated with an asterisk.*

TABLE 2—*Number of male donor alleles detected from LMD sperm fraction.*

Sample	# Expected Alleles	# Alleles Detected ≥ 50 RFU		
		"75 Sperm"	"150 Sperm"	"300 Sperm"
1	18	16	17	18
2	19	18	19	19
3	19	13	19	19
4	17	5	17	17
5	20	16	17	20

LMD, laser microdissection; RFU, relative fluorescent units.

MicroLYSIS® performed poorly for both sperm and epithelial cell samples with a high degree of allelic drop-out. Both Lyse-N-Go™ and QIAamp® methods successfully isolated sperm DNA ("300 cell" count) such that all loci were detected in 100% of the samples; however, results from epithelial cell extractions ("150 cell" count) varied. On average, 74 ± 6.8% of the female donors' loci were detected using Lyse-N-Go™ on epithelial cells while 90 ± 5.4% of loci were detected using the QIAamp® method; however, within this study population, the difference could not be deemed statistically significant ($p > 0.05$, ANOVA Bonferroni post-hoc test).

RFU (signal intensity) is a measure of PCR product quantity. Therefore, RFU values from Lyse-N-Go™ and QIAamp® samples were compared to evaluate the amount of PCR product produced. Figure 4 summarizes the total fluorescence signal detected in total RFUs (the sum of the peak heights at all loci) for each LMD sample comparing Lyse-N-Go™ and QIAamp® methods. QIAamp® extractions produced RFU values approximately 75% higher than the Lyse-N-Go™ method, a significant increase ($p < 0.05$, paired *t*-test), when used to extract epithelial cells. When applied to sperm cells, the Lyse-N-Go™ method resulted in higher observed RFU values in five out of the six samples compared with the QIAamp® method. However, average RFU values did not exhibit a statistically significant difference within this sperm sample population ($p > 0.05$, paired *t*-test).

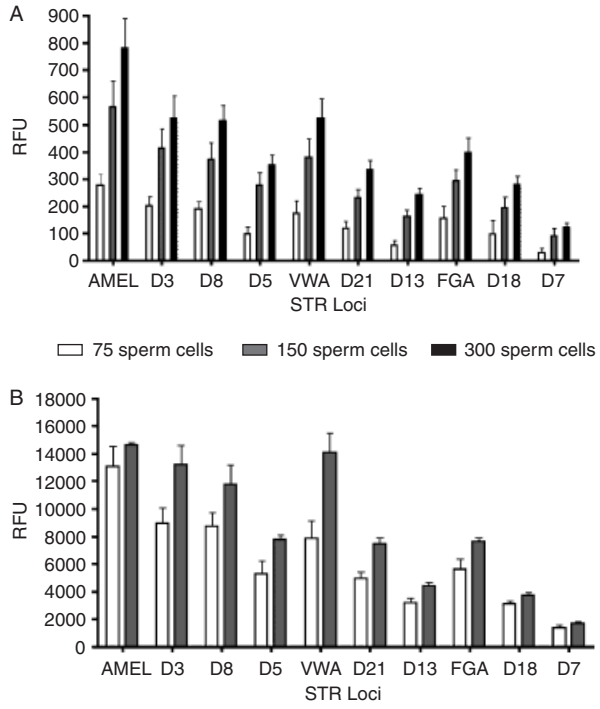

FIG. 7—*Fluorescent signal at each locus for standard and extended cycles PCR. Total relative fluorescent units peak values at each locus were averaged for (A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and (B) 75 and 150 sperm cell specimens using extended cycles PCR.*

Mixture Separation Study

To establish the separation capability of LMD, sperm cells from semen and female oral epithelial cell mixtures were recovered, followed by DNA analysis. STR plots of sperm cells recovered from semen/buccal mixtures are illustrated in Fig. 5. In all samples tested, the semen donors' genotypes were detected with

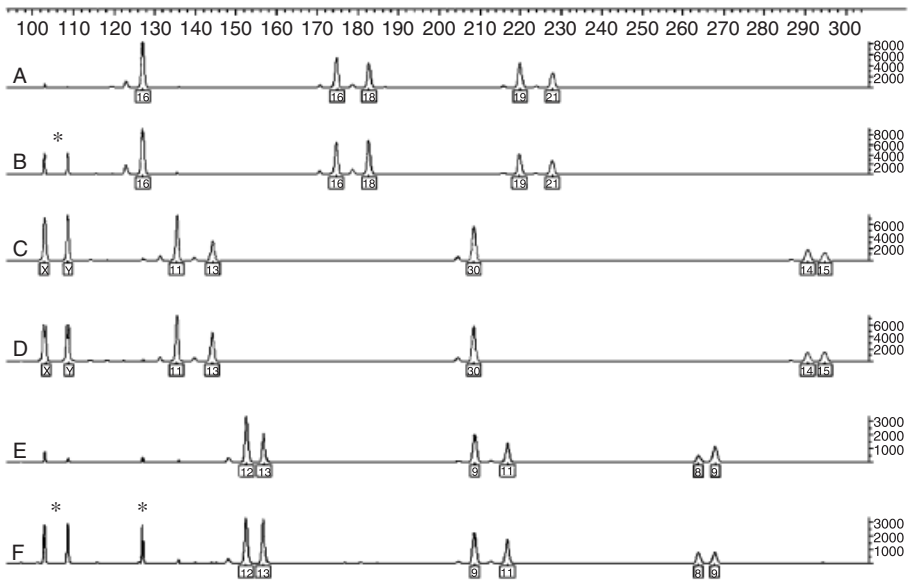

FIG. 6—*Profiler Plus plots of laser microdissection collected sperm cells from a sperm/epithelial cell mixture using extended cycles PCR. Blue loci of (A) 75 sperm, and (B) 150 sperm. Green loci of (C) 75 sperm and (D) 150 sperm. Yellow loci of (E) 75 sperm and (F) 150 sperm. All alleles detected from male donor without female carryover. Notable spectral pull-up observed from "150 sperm" samples indicated with an asterisk.*

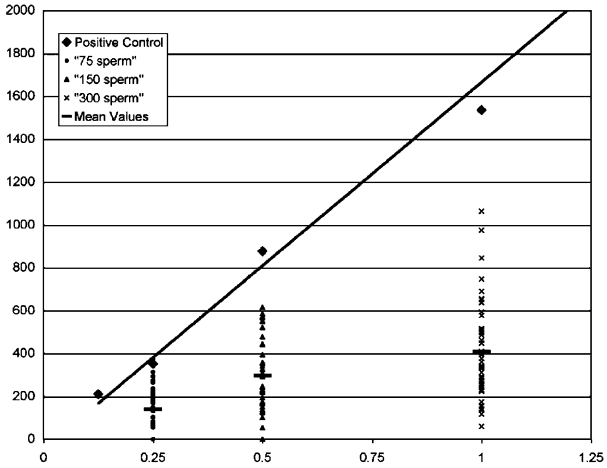

FIG. 8—*PCR product level of 75, 150, and 300 cells under standard PCR conditions. Five positive control dilutions of 0.125, 0.25, 0.5, 1.0, and 2.0 ng of Human DNA (2.0 ng not shown) plotted against observed total relative fluorescent units (RFU) values. Total RFU values for 75, 150, and 300 sperm samples were plotted on the x-axis at the corresponding maximum theoretical DNA quantity.*

the absence of any alleles known to originate from the female buccal cell donors. Under standard PCR conditions (28 cycles), all samples containing 300 LMD sperm displayed all 10 loci of the sperm donors. Samples containing 150 sperm exhibited on average 96 ± 3% of the male donors' alleles and samples containing 75 sperm cells displayed on average 72 ± 12% of the male donors' alleles. The number of alleles detected above threshold (≥ 50 RFU) is tabulated for each specimen in Table 2.

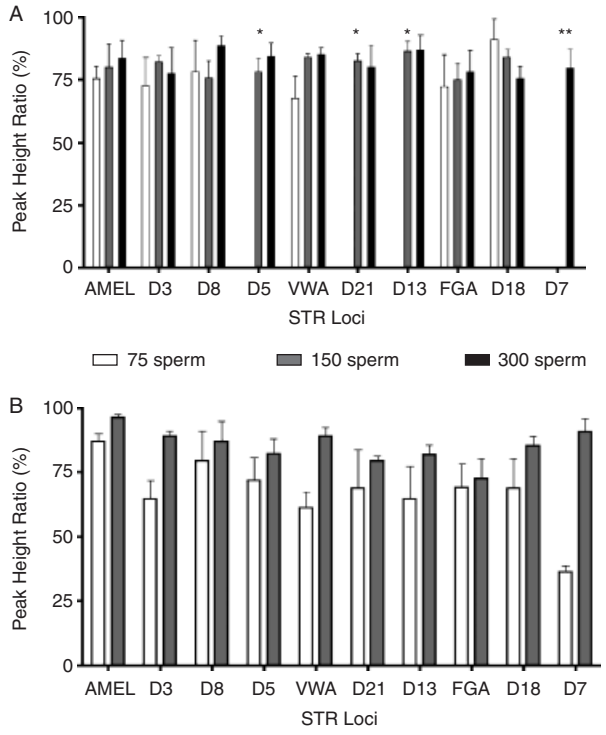

FIG. 9—*Peak height ratios at each locus for standard and extended cycles PCR. Peak height ratios of heterozygous loci were averaged for (A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and (B) 75 and 150 sperm cell specimens using extended cycles PCR. *Insufficient heterozygous data for analysis.*

Using extended cycles (six additional cycles) PCR, 100% of samples containing 75 and 150 sperm cells exhibited all of the sperm donor alleles. Nonoverlapping female alleles were not detected in any samples using a total of 34 PCR cycles demonstrating the collection of a pure population of sperm cells without female DNA contamination as illustrated in Fig. 6. Data from extended cycles PCR containing 300 sperm were not included in this report owing to the preponderance of peak heights above the linear range of the instrument and an abundance of PCR artifacts typical of increased PCR cycles such as increased stutter and minus-A nucleotide products.

The relative quantities of PCR product were examined from the three LMD collection amounts of sperm cells. Figure 7 summarizes the total fluorescence signal detected at each locus for the three collection amounts using standard and extended cycles PCR. In general the total RFUs detected increased as a function of the number of LMD-collected sperm cells. Assuming one human haploid cell contains 3.3 pg of genomic DNA, the examination of 75, 150, and 300 sperm cell amounts contains approximately 0.25, 0.5, and 1.0 ng of DNA, respectively, prior to DNA isolation. Signal intensity was compared from the experimental samples with a dilution series of the AmpF*l*STR® DNA-positive control under standard PCR conditions. Figure 8 shows a plot with regression line of the positive DNA control analyzed from 0.125 to 2 ng. As expected, the positive control showed a linear relationship between the RFU value and the quantity of DNA ($r^2 = 0.9961$). The mean values of the LMD samples plotted on the same graph maintained a linear relationship ($r^2 = 0.9179$) with RFU values 2.5–3.7 times less than the positive control values. This reduction likely reflects DNA isolation inefficiency, but it is not possible to measure this precisely due to inherent inaccuracies with the quantitation of the positive control by the manufacturer.

Peak height ratio, which is defined as the height of the lower peak divided by the height of the higher peak—expressed as a percentage, was calculated at heterozygote loci. Samples that displayed only one allele at a locus where the donor was heterozygous were excluded from the calculations. Peak height ratios are displayed in Fig. 9. The mean peak height ratios over all loci under standard conditions were 76.3 ± 3.3% for "75 sperm," 81.1 ± 1.3% for "150 sperm," and 82.0 ± 1.4% for "300 sperm" samples. The mean peak height ratios for extended cycles analysis were 67.0 ± 4.2% for "75 sperm" and 85.2 ± 2.1% for "150 sperm" samples.

Discussion

The results of this study demonstrate through STR genotyping that LMD of mixed cell populations achieves pure separation of sperm with no DNA contamination from exogenous buccal epithelial cells. The considerations for using LMD are discussed below.

Histology Study

The separation and recovery of sperm cells by LMD for DNA analysis differs from the preferential lysis method in that LMD is best performed when the material is stained for a more accurate and efficient microscopic identification of the cells of interest. It is important that the histological dyes chosen do not interfere with downstream analysis of the sample DNA material. PCR inhibition of genomic DNA by dyes and fixatives has been observed with gross, stained tissue samples (10,11). However, the negative effects on DNA analysis from histological dyes can be reduced

when the tissue is recovered using LMD instead of manually dissecting tissue (12). This contrast is most likely attributable to the amount of tissue sampled as the cellular material collected by LMD is microscopically small and the instrument's ability to excise precisely the area of interest results in a low contamination of dye substances into further downstream analysis.

The objective of this part of the study was to identify dye chemistry with the least risk of degradative or inhibitory properties while still achieving good visual identification of the target cells. Five common stains were chosen to investigate their effect on downstream analysis of LMD-collected material. Nuclear fast red/picroindicocarmine, also known as the CTS, is universally used to differentiate sperm cells from epithelial cells in stain identification of sexual assault evidence (13). H&E is conventionally used as a nuclear stain in pathology laboratories and has been successfully used to recover LMD tissue for nucleic acid analysis (12). H&E is also a popular choice for differentiation of sperm cells from epithelial cells in European forensic laboratories. MG is a one-component nuclear dye believed to bind to the negatively charged DNA in nuclei showing no adverse effect on laser microdissected tissue by producing consistent amplification from manually dissected tissue (10). WRT stain (azure blue/eosin) is a commonly used stain for blood smears (14). AO is a fluorescent stain used to visualize sperm from vaginal swabs particularly from samples with dense epithelial cell populations (15).

The overall performance of each stain was determined by considering both cell discrimination ability and genotyping results. Of the histological stains evaluated in this study, H&E performed the best. It readily provided morphological discrimination of spermatozoa and epithelial cells, which is consistent with the findings reported by Allery et al. (16). The use of H&E, however, resulted in lower RFU values compared with unstained specimens. This supports reports indicating that hematoxylin produces less PCR product than unstained controls in laser-microdissected tissue sections (10,12). Although the mechanism responsible for the reduced yield is not completely understood, hematoxylin-bound DNA seems resistant to complete digestion, which may make the DNA less available for enzymatic replication (17). In addition, while Eosin Y has shown no effect on PCR yield in laser micro-dissected tissues (10), it is an acidic dye that could be responsible for DNA damage. Despite the observed reduction in PCR product, the use of H&E did not prevent the acquisition of sufficient PCR product for successful STR genotyping. Shortened exposure times of H&E staining were used as a simple tactic to reduce the uptake of dyes by the cells and lessen the negative effect of these chemicals in subsequent studies.

Although the CTS provided excellent morphological discrimination of spermatozoa and epithelial cells, its use produced significantly lower RFU values than H&E specimens ($p < 0.05$, paired *t*-test). This loss may be owing to the picric acid component as highly acidic solutions will depurinate nucleic acids (18) damaging DNA. In addition, indigo carmine, used in the textile industry for dyeing denim, is a known inhibitor of PCR (19), further causing low yields. The use of nuclear fast red stained paraffin-embedded tissues before LMD has produced a superior yield over other histological stains (17) and, used alone, may be an approach to increasing yield if found sufficient for morphological identification.

AO, the only fluorescent stain used in this study, may have provided good visual identification of sperm but differentiation from epithelial cells became more difficult among high concentrations of epithelial cells and/or sperm without tails. It, however, proved not to be compatible with downstream analysis. AO intercalates with double stranded DNA and binds electrostatically to the phosphate backbone (20), which may hinder primer access to the template.

DNA Isolation Comparison

Unique challenges were faced when developing and determining which DNA isolation method is appropriate for LMD cells. When confronted with molecular DNA analysis of histologically stained cells, a method that could remove Taq inhibitors from the sample would be advantageous. At the same time, conservation of DNA from the recovered cells is crucial. The time required to collect sperm cells is approximately 15–20 min per 100 sperm cells using the Leica system software version 4.1.3.; therefore, minimizing the necessary number of cells required reduces overall analysis time. In addition, a DNA isolation method that conserves the DNA and provides a concentrated extract such that the entire quantity can be used for PCR is important for the recovery of very minute evidence samples for subsequent low copy number (LCN) analysis.

Samples collected by LMD are held in a collection cap that has a working volume of 20–30 μL. The goal of this study was to incorporate and develop a DNA isolation method that can work in this small-volume format and preferably in a single-tube format amenable to automation. Although Chelex-100 (Bio-Rad Laboratories, Hercules, CA) is widely used in forensic casework (21), preliminary studies of this project demonstrated that the use of Chelex resin was a poor method for the extraction of DNA from LMD cells, as it was difficult to use in a low-volume format, challenging to remove all the liquid from the resin beads, and resulted in little or no interpretable STR results (data not shown).

QIAamp® spin columns have been successfully used to isolate DNA from forensic casework (22) and laser capture microdissection samples (23). This method uses a column containing a silica-based membrane that binds nucleic acids. Through a series of washes and elution steps, proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are removed. This method provides a relatively pure DNA extract but requires sample transfers, washes, and elution steps that may increase the chances of sample loss and potential cross contamination.

An alternative approach to DNA extraction is the use of one-step commercial buffers such as MicroLYSIS® reagent and Lyse-N-Go™ reagent, which are designed to lyse cells ready for PCR in one tube. These solutions allow the release of DNA through a series of heating and cooling, causing the cells and their organelles to lyse open in addition to promoting inactivation of endogenous nucleases. LMD collection, lysis, and PCR can all occur in a single tube requiring little manipulation resulting in conservation of the sample and prevention of sample-to-sample contamination.

The results of this study showed that the QIAamp® method performed the best for the DNA analysis of stained LMD-recovered epithelial cells by clearly producing higher RFU values than the Lyse-N-Go™ method. However, this was not the case when the cells isolated were sperm. The average RFU values did not differ between the Lyse-N-Go™ and QIAamp® methods when used for DNA isolation of sperm cells. This contradiction may be explained by differences in the amount of cellular material collected by LMD from the two different cell types.

The nucleus of a human cheek cell is approximately 5 μm in diameter, whereas a sperm cell head is approximately 5 μm by 3 μm. In this experiment, the nuclear material of the buccal cell was collected by recovering the whole epithelial cell body, which

is several times larger than the sperm cell. The amount of biological material including bound histological chemicals is therefore expected to be greater from the epithelial cells than the much smaller collected sperm heads. The QIAamp® kit is designed to remove proteins and possibly other contaminants that can inhibit PCR, improving DNA yield from the epithelial cell samples. No such purification is performed using the Lyse-N-Go™ method, leaving effective contaminants in the PCR reaction. In contrast, the sperm cell samples most likely contributed a smaller concentration of inhibitory histological dyes into the PCR reaction than the LMD-collected epithelial cells. This may have allowed the sperm cell samples to benefit from the Lyse-N-Go™ method's ability to conserve sample.

The technique of diluting a DNA extract to reduce inhibitors and facilitate amplification, although with reduced sensitivity, is well documented (24). A similar approach of dissecting only the nuclei of the larger epithelial cells may reduce the contribution of inhibitory or degradative dyes into downstream analysis while maintaining the same concentration of DNA. In addition, it can be anticipated that as fewer amounts of cells are collected by LMD the concentration of inhibitors would decrease, making collection of minute numbers of cells for LCN analysis more amenable to nonpurification, one-step lysis buffers such as Lyse-N-Go™. Therefore, consideration of cell type and number should be a factor in the choice of DNA extraction method to address the presence of potentially inhibitory histological dyes when using LMD.

Mixture Separation Study

The primary goal of this research was to develop a method for the pure cell separation from a sperm/epithelial mixture amenable to forensic STR analysis. Gill developed a preferential lysis method (1), which was subsequently modified (25), becoming the "gold standard" for separation of sperm from victim cells in sexual assault cases. However, this method is often beset by incomplete separation, resulting in female DNA contaminating the sperm fraction, and the recovery of sperm may not be efficient enough with minute numbers of sperm. The forensic research community has continued to investigate alternative improved methods for cell separation including flow cytometry by fluorescence-activated cell sorting (5), microchip-based sperm and epithelial cell separation (4), and membrane filtration (2).

LMD has several potential advantages over previous separation methods. It requires only minimal manipulation of the sample and works by direct microscopic visualization, making it suitable for minute quantities of sperm. The first generation of this technology was termed laser capture microdissection (LCM) (Arcturus Bioscience, Mountain View, CA). LCM technology involves the use of a laser to microscopically melt a thermoplastic film onto a target cell embedding and lifting the cell from the slide. This technology has been used to recover sperm from microscope slides (3). While LCM allows the recovery of an enriched sperm fraction, female carryover can be relatively common from cell mixtures (3). Carryover can be due to female DNA from lysed cells adhering to the sperm (26). Alternatively, it could be due to the nonspecific attachment of surrounding cells to the plastic membrane. Despite transfer of female DNA in the male fraction, LCM performs significantly better than the preferential lysis method in its ability of separate sperm from vaginal epithelial cells (3).

More recent advances in LMD methodology as used in this study allow a more precise dissection of cells (Fig. 1). In addition, sample recovery can be verified visually in a postcollection mode, allowing the user to inspect the cells collected microscopically. Thus, LMD can clarify the cell source attribution of any genetic profile obtained.

STR results from this study demonstrate that pure populations of sperm are recovered from semen/epithelial cell mixtures, and amplifications at higher cycle numbers further show the absence of any female DNA in the sperm fraction. In this study, the number of sperm cells tested ranged from 75 to 300, and therefore addresses DNA analysis below the 1ng limit recommended by the AmpF*l*STR® kit, a practice routinely performed in forensic casework. The data did show a loss in yield associated using the LMD method. Whether this is due to the viability of nuclear material, extraction effectiveness, or PCR efficiency are areas of future study. Nevertheless, using standard PCR conditions, genotyping can be obtained from 75 to 300 sperm cells, with most heterozygous peak ratios above 70%, an acceptable industry standard (27). Samples exhibiting peak height ratios below 70% were most likely a result of low amounts of haploid cells and the presence of PCR inhibitors.

The technique of performing PCR for six additional cycles on the PCR product amplified first for 28 cycles was used in this study primarily to detect any potential female contamination in the sperm fraction. However, it also increased signal intensity, providing full Profiler Plus genotypes from the "75" and "150" sperm cell samples when allelic-dropout or partial profiles were observed under standard PCR from the same samples. Increasing PCR cycles above the optimized range can cause preferential amplification of one allele (28), which was evidenced in the lower overall peak height ratios observed in the "75 sperm" samples. Nevertheless, the preferential amplification did not affect the accurate and complete genotyping of the samples tested. This suggests the potential of LMD in recovering sperm fewer than 75 cells for LCN analysis.

Conclusions

The results of this study demonstrate that LMD is an effective technique for recovering spermatozoa from a sperm/epithelial cell mixture. LMD collects pure populations of sperm with no apparent cross contamination from buccal epithelial cells. H&E staining can be effectively used for sperm identification in conjunction with LMD separation for STR genotyping. Used in combination with the Lyse-N-Go™ extraction procedure, the LMD method is a simple, low-manipulation method for the analysis of sperm cells. This has the potential of facilitating analysis of low numbers of cells. An additional benefit of using the LMD method, whether it is for mixture separation or single source cell recovery, is that laborious intermediary DNA quantification analysis may be eliminated. Instead, cells can easily be counted during LMD collection and extrapolated into an estimate of DNA quantity added to the PCR reaction.

This work will continue by testing mixtures with high concentrations of epithelial cells, and its application to recover minute numbers of sperm cells for LCN analysis. Further studies are warranted to establish whether testing of genuine casework specimens responds to LMD separation in a manner similar to the mock mixed-cell specimens of this study.

Acknowledgments

This project was supported by grant #2004-DN-BX-K215 awarded by the National Institute of Justice, Office of Justice

Programs, U.S. Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. The products and manufacturers discussed in this document are presented for informational purposes only and do not constitute product approval or endorsement by the U.S. Department of Justice.

References

1. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA 'fingerprints'. Nature 1985;318(6046):577–9.
2. Chen J, Kobilinsky L, Wolosin D, Shaler R, Baum H. A physical method for separating spermatozoa from epithelial cells in sexual assault evidence. J Forensic Sci 1998;43(1):114–8.
3. Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Sci Int 2003;137:28–36.
4. Horseman KM, Barker SLR, Ferrance JP, Forrest KA, Koen KA, Landers JP. Separation of sperm and epithelial cells in a microfabricated device: potential application to forensic analysis of sexual assault evidence. Anal Chem 2005;77(3):742–9.
5. Schoell WM, Klintschar M, Mirhashemi R, Pertl B. Separation of sperm and vaginal cells with flow cytometry for DNA typing after sexual assault. Obstet Gynecol 1999;94(4):623–7.
6. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser capture microdissection. Science 1996;274 (5289):998–1001. Comment in: Science 1996 Nov 8;274(5289):921–2.
7. Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends Genet 1998 Jul;14(7):272–6.
8. Luzzi V, Holtschlag V, Watson MA. Expression profiling of ductal carcinoma in situ by laser capture microdissection and high-density oligonucleotide arrays. Am J Pathol 2001;158(6):2005–10.
9. Rook MS, Delach SM, Deyneko G, Worlock A, Wolfe JL. Whole genome amplification of DNA from laser capture-microdissected tissue for high-throughput single nucleotide polymorphism and short tandem repeat genotyping. Am J Pathol 2004;164(1):23–33.
10. Murase T, Inagaki H, Eimoto T. Influence of histochemical and immunohistochemical stains on polymerase chain reaction. Mod Pathol 2000;13(2):147–51.
11. Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R, Jonas U. Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. Am J Pathol 2000;156(4):1189–96.
12. Ehrig T, Abdulkadir SA, Dintzis SM, Milbrandt J, Watson MA. Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. J Mol Diagn 2001;3(1):22–5.
13. Oppitz E. Eine neue Farbemethode zum Nachweis der Spermien bei Sittlichkeitsdelikten (A new colouring method for the determination of sperm cells in sexual assault cases). Arch Kriminol 1969;144:145–8.
14. Walker HK, Hall WD, Hurst JW, editors. Clinical methods: the history, physical, and laboratory examinations. 3rd ed. Stoneham: Butterworth-Heinemann, 1990.
15. Mercurio M, Silenieks T, Royds D. An evaluation of the acridine orange and Christmas tree stains for the identification of spermatozoa. Forensic Bull 1997.
16. Allery JP, Telmon N, Mieusset R, Blanc A, Rouge D. Cytological detection of spermatozoa: comparison of three staining methods. J Forensic Sci 2001;46(2):349–51.
17. Burton MP, Schneider BG, Brown R, Escamilla-Ponce N, Gully ML. Comparision of histologic stains for use in PCR analysis of microdissected, paraffin-embedded tissues. Biotechniques 1998 Jan;24(1): 86–92.
18. Moore J, Aros M, Steudel K, Cheng K. Fixation and decalcification of adult zebrafish for histological, immunocytochemical, and genotypic analysis. Biotechniques 2002;32:296–8.
19. Larkin A, Harbison S. An improved method for STR analysis of blood-stained denim. Int J Legal Med 1999;112(6):388–90.
20. Lerman LS. Structural considerations in the interaction of DNA and acridines. J Mol Biol 1961;3:18–30.
21. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 1991;10(4):506–13.
22. Greenspoon SA, Scarpetta MA, Drayton ML, Turek SA. QIAamp spin columns as a method of DNA isolation for forensic casework. J Forensic Sci 1998;43(5):1024–30.
23. Martino D, Giuffre G, Staiti N, Simone A, Le Donne M, Saravo L. Single sperm cell isolation by laser microdissection. Forensic Sci Int 2004; 146(Suppl):S151–3.
24. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997;63(10):3741–51.
25. Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sato H, et al. The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. Forensic Sci Int 1995;72(1):25–33.
26. Spadafora C. Sperm cells and foreign DNA: a controversial relation. Bioessays 1998;20(11):955–64.
27. Applied Biosystems. AmpFlSTR Proliler Plus user's manual. Foster City, CA: Applied Biosystems, 1998.
28. Gill P. Application of low copy number DNA profiling. Croat Med J 2001;42(3):229–32.

Additional information and reprint requests:
Christine T. Sanders, M.S.
Department of Neuroscience
Rosalind Franklin University of Medicine and Science
3333 Green Bay Road
North Chicago, IL 60064
E-mail: geneskr@alumni.ucsd.edu