Application of Leica™ Laser Microdissection Microsystem to Expedite Forensic Sexual Assault Casework

Langley, K.B., Wojtkiewicz, P.W., North Louisiana Criminalistics Laboratory, 1115 Brooks St, Shreveport, LA 71101

INTRODUCTION

In 1996, the medical field first used Laser Microdissection (LMD) as a diagnostic tool for cancer research (1). Since then, neurobiology laboratories found this instrument particularly useful for isolating and studying the transcriptional and translational processes of individual neurological cells. In 2003, the Forensic Science Services investigated LMD as a tool for forensic DNA analysis for identifying and isolating sperm and epithelial cells in sexual assault samples (2). Contemporaneously, in the United States, researchers were actively investigating LMD applications on simulated sexual assault samples, and the findings were presented at the 2004 national AAFS meeting in Dallas, TX, USA (3).

The North Louisiana Criminalistics Laboratory (NLCL) identified the current differential extraction process as a bottleneck in examining sexual assault evidence. LMD was proposed to expedite DNA analysis of sexual assault forensic evidence in the laboratory by offering the following advantages: 1) complete separation of the cellular components, 2) complete elimination of the traditional differential extraction technique while maintaining the ability to get full DNA profiles, 3) greater sensitivity by counting and collecting small amounts of sperm DNA (< 450 pg) from large epithelial cell populations, and lastly, 4) possible elimination of traditional quantitation of these samples.

It was speculated that the effect of traditional PCR challenges would be minimized by the ability of LMD to excise the cell nuclei from extraneous components of the sample. The assumption was that clean DNA profiles should be obtained, thereby simplifying their interpretation. Consequently, difficult statistical interpretations would generally be eliminated. Other possibilities were that there might be less potential for capturing contaminants/inhibitors in these DNA samples, and therefore, it may be possible to increase PCR cycles to enhance the sensitivity of LCN sperm analysis.

The NLCL currently employs traditional organic extraction for purifying DNA in evidentiary samples. Since sexual assault cases comprise approximately 45% of all DNA casework, it is apparent this type of evidence makes up the bulk of DNA extractions. The organic extraction process becomes a cumbersome and lengthy process when coupled with screening and subsequent preferential lysis of separate cellular components comprising sexual assault samples. This process takes nearly two days in the lab. Additionally, current traditional differential extractions do not assure complete separation of epithelial and sperm DNA.

The major problem encountered in traditional differential extraction is the incomplete separation of the perpetrator's DNA from the victim's DNA, which results in a mixture of DNA profiles. This produces more difficult interpretations and statistical calculations of the DNA profile; and ultimately, explanations of scientific results to a jury. Lastly, traditional differential extraction often is unsuccessful in isolating sperm from sexual assault samples which contain a ratio of epithelial cells that greatly exceeds the spermatozoa counterpart. Sexual assault samples that contain few sperm provide an even greater challenge for isolation when analyzing sexual assault samples using the traditional differential extraction process.

Laser microdissection offers a promising alternative to traditional differential extractions of sexual assault samples. The NLCL purchased the Leica™ AS Laser Microdissection Microsystem with the hope that it would expedite the separation of sperm and epithelial DNA by identifying and simultaneously excising individual sperm and epithelial nuclei. Thus, laser microdissection offers a considerable opportunity for decreased analysis times and decreased analyst time, and ultimately, decreased sexual assault DNA casework turnaround times.

MATERIALS AND METHODS

Laser Microdissection

Laser Microdissection with the Leica™ Laser Microdissection Microsystem integrates microscope and laser functions to allow the user to simultaneously identify and cut desired cellular components from a sample for further analysis. Images are captured by a camera, which is combined with image analysis software, to identify cells and target the cutting region. The user has the ability to cut and collect cells (e.g., epithelial cells and spermatozoa) into separate tubes.

The microscope utilizes a motorized stage with xyz motion capabilities. Slides are mounted inverted onto the microscope stage, where the desired cellular components are excised from the slide by the laser passing through the glass to cut the foil membrane. Gravity facilitates the capture of the dissected cellular components directly into PCR tubes located in a substage tube holder. The microscope also has fluorescent capabilities; this optional component provides the user more versatility for sample visualization.

Slide Preparation

LMD requires the use of special polyethylene-naphthalene (PEN) slides. The foil is glued on the glass slide at its perimeter. The sample is placed on the foil by smearing or spotting sample, where it can be stained or fluorescent dyes added.

Two methods of sample preparation onto the PEN slide were considered. Initially, smears were prepared from 25µL of sample pellet using a method similar to that done in a clinic for RBC counts. This method was acceptable if a large number of cells were present in the sample; however, for the type of samples being studied in this research, smearing was inefficient. The smear resulted in a sparsely populated slide, causing the user to spend excessive time locating desired cells. For forensic-like samples, slides were prepared by a simple spotting technique. Approximately 25µL of the sample pellet was transferred to the foil membrane and allowed to dry at 56°C for 15-30 minutes. The slides were stained using nuclear fast red (NFR) for 2-3 minutes. Previous studies have shown that NFR staining can be used to identify nuclei and is reliably used with PCR (4).

Spermatozoa from a three year old frozen sample were used for initial studies. The sperm cells were diluted to an appropriate concentrate and then dried onto cotton swabs. The swabs were soaked in 1x PBS for 1 hr. with continuous shaking. The extracted cells were then pelleted by centrifugation and the pellet spotted onto a slide. Later studies were done with a known mixture of sperm and epithelial cells.

Sperm Lysis Technique

Since cellular components were being cut into PCR tubes, direct amplification was first considered. For direct amplification, cells were dissected from PEN slides containing the specimen into 25µL DEPC-treated water. The cells were dried in the tubes, PCR master mix was then added directly to the dried, concentrated cells, and PCR was performed using standard cycling conditions. PCR cycles were varied experimentally, and the resulting amplified products were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

Alternately, pre-amplification cell lysis procedures were tested. The first method tested was Lyse-N-Go™ PCR Reagent (LNG, Pierce, Rockford, IL). This solution was added either to concentrated cells in the PCR tube or cells were excised directly into the Lyse-N-Go™. The cells were then lysed using a thermal cycler program specified by the manufacturer. PCR master mix was added directly to this LNG/digested cell solution and PCR was performed using standard cycling conditions. PCR cycles were varied experimentally and the resulting amplified products were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

A second pre-amplification lysis method was examined using recombinant ProteinaseK (recProK, Roche Applied Science, Indianapolis, IN) in combination with heating to lyse cells and digest cellular components (5). For this method, cells were dissected and concentrated as described for the direct amplification procedure. AmpFℓSTR® PCR reaction mix and an optimized concentration of the recProK was added to the concentrated cells. The resultant lysis solution was incubated in a thermal cycler using the following parameters: 3 hours at 56°C, 10 minute hold at 94°C, and a final hold at 8°C (5). Immediately following thermal cycler incubation, a solution of AmpFℓSTR®

Primer/Taq Gold was added to the lysed cell solution. PCR was performed using standard cycling conditions. PCR cycles were varied experimentally, and the resulting amplified products were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

In some of the experiments, dithiothreitol (DTT, 40mM) was used to enhance lysis with LNG or recProK. Also, as a substitute to increasing PCR cycles, reduced volume PCR (RV-PCR) in combination with the recProK/DTT pre-amplification lysis was explored to detect DNA profiles for samples containing less than 450pg (150 spermatozoa) (6).

RESULTS

At the NLCL, three techniques to obtain DNA profiles from cells collected via LMD were explored and compared: 1) direct amplification of collected cells via addition of AmpFℓSTR® reaction components, 2) lysis of collected cells by Lyse-N-Go™ or by 3) ProK or ProK/DTT addition before amplification (5). All were investigated using increased PCR cycles in an effort to get full DNA profiles for various quantities of cells.

Experiments using direct amplification showed only partial DNA profiles with nearly 1ng DNA input (150 epithelial cells) and 450pg DNA (150 spermatozoa). Increasing PCR cycles to improve sensitivity gave only limited success. Collected epithelial cells containing as little as 300pg DNA (50 cells) gave better DNA profiles over the same quantity amplified with fewer PCR cycles. Collected spermatozoa containing 450pg DNA (150 cells) gave a nearly full profile. However, when direct amplification was coupled with increased PCR cycles to obtain DNA profiles, artifacts masked as true alleles were observed in most instances. Additionally, direct amplification of even optimal quantities with increased PCR cycles did not guarantee full DNA profiles.

Pre-amplification lysis using LNG with 50µL and 25µL volumes was attempted and gave partial DNA profiles similar to direct amplification. This technique showed little improvement over the direct amplification approach (Figure 1.).

In addition to testing collected cells with recProK only, samples were also tested using recProK with an addition of DTT. The addition of DTT clearly showed enhanced peak heights for the DNA profile. RecProK and recProK with DTT for pre-amplification lysis and digestion gave the best results when compared to direct amplification and Lyse-N-Go™ methods. Nearly full DNA profiles were observed when using recProK or recProK/DTT for pre-amplification lysis (Figure 1.).

Using 30 PCR cycles with the 25µL PCR reaction volume was reliable with recProK/DTT pre-amplification lysis of collected cells. Alleles detected in all samples tested gave the correct DNA profile. 17 of 18 expected alleles were observed in a sample containing approximately 450pg DNA (150 spermatozoa). PCR cycles >30 with recProK/DTT pre-amplification lysis improved sensitivity of the 25µL PCR reaction volume, but sample DNA profiles were compromised. Since the addition of RV-PCR was an improvement over the 25µL reaction and was reproducible, the recProK/DTT pre-amplification lysis procedure was developed to include RV-PCR (Figure 2.).

Additionally, the ability of the recProK/DTT pre-amplification lysis method to generate DNA profiles from decreasing numbers of spermatozoa and epithelial cells was tested. Full DNA profiles were obtained from samples with as little as ~ 300pg DNA (100 spermatozoa) and 17 of eighteen 18 expected alleles were observed in one sample containing ~ 150pg DNA (50 spermatozoa) (Figure 3.).

CONCLUSIONS

Since epithelial cells and spermatozoa were collected directly into PCR tubes using LMD, direct amplification of collected cells was considered. Direct amplification had the advantage of contributing the least amount of volume and sample manipulations since the sample was dried and the PCR master mix (with the appropriate amount of water added to substitute for a DNA extract) was added directly to the tube. However, direct amplification with increased PCR cycles was determined to be an unreliable method.

A method which could lyse and digest the cells was considered to be a better alternative. Successful PCR requires cellular lysis and digestion to expose the cellular DNA. Spermatozoa, by their nature, already are difficult

to lyse, which is the basis of the differential extraction procedure. In addition, if a lysis method was amenable to PCR, this would significantly reduce the procedure time. Therefore, pre-amplification lysis procedures were considered to enhance exposure of the cell DNA. A pre-amplification procedure had two key constraints: limit on input volume and it must not adversely affect PCR. The lysis reaction volume was particularly important, especially if reduced volume techniques (vs. increased PCR cycles) were to be used to increase overall sensitivity (6). Currently, novel pre-amplification lysis techniques to the forensic biology field are being explored (3, 4).

Lyse-N-Go™ was a likely candidate as a pre-amplification agent since it was designed so that PCR reaction components could be added directly to the tube containing the lysis buffer and cells. This technique had the advantage of requiring no additional manipulations to the lysed cells, while at the same time allowing for addition of PCR components directly to the solution. Also, lysis time was very short (20 min.). One disadvantage of LNG was that if the dissected cells were cut directly into the solution, evaporation occurred during microscopic examination and laser microdissection, resulting in an unknown loss of volume. Another negative aspect was that the reaction was designed for a 50µL PCR reaction, whereas the NLCL currently employs a 25µL reaction volume for casework.

Another possible candidate to assist pre-amplification lysis was the addition of recProK. DTT was added to some reactions to investigate whether sperm lysis would be enhanced as it does for traditional differential extractions. The solution containing the recProK/DTT did not contain additional components to adversely affect PCR or drying of the cells. The PCR reaction components could be added directly to the collection tube after lysis. This technique had the added advantage of requiring no additional manipulations to the lysed cells. Two disadvantages to this approach were that the suggested lysis time in the thermal cycler (3.5 hrs.) used for this research was longer than that for the LNG, and a high concentration of recProK inhibited PCR (5).

Comparison of different approaches to achieve cellular lysis and facilitate successful PCR required a method that would reliably lyse and digest the cellular components in the PCR collection tube. The recProK/DTT pre-amplification lysis technique gave the best overall results for spermatozoa amplification. Implementation of LMD in combination with pre-amplification lysis offers a promising alternative to traditional differential extractions of sexual assault samples.

An additional benefit of using LMD to process sexual assault samples is that it allowed for simultaneous screening and cutting of the target cells. Counting of the cells as dissection occurs was a simple way to asses the DNA quantity of collected cells. This investigation showed that counting cells is in fact a good assessment of the quantity of DNA present in these samples. Therefore, it is proposed that traditional quantitation of LMD samples from sexual assault evidence processing can be eliminated.

The procedure developed to date for the laser microdissection should help significantly reduce the analysis time for sexual assault evidence samples, a large portion of DNA case samples at the NLCL. This will lead to decreased sexual assault DNA casework turnaround times, a fundamental goal in forensic DNA laboratories. This procedure also lends itself to decreasing analyst time, freeing up the analyst for other tasks required during a normal workday. The NLCL envisions implementing a new way of processing and storing sexual assault samples. Ultimately, the use of LMD to process sexual assault casework at the NLCL will improve and streamline sexual assault evidence analysis.

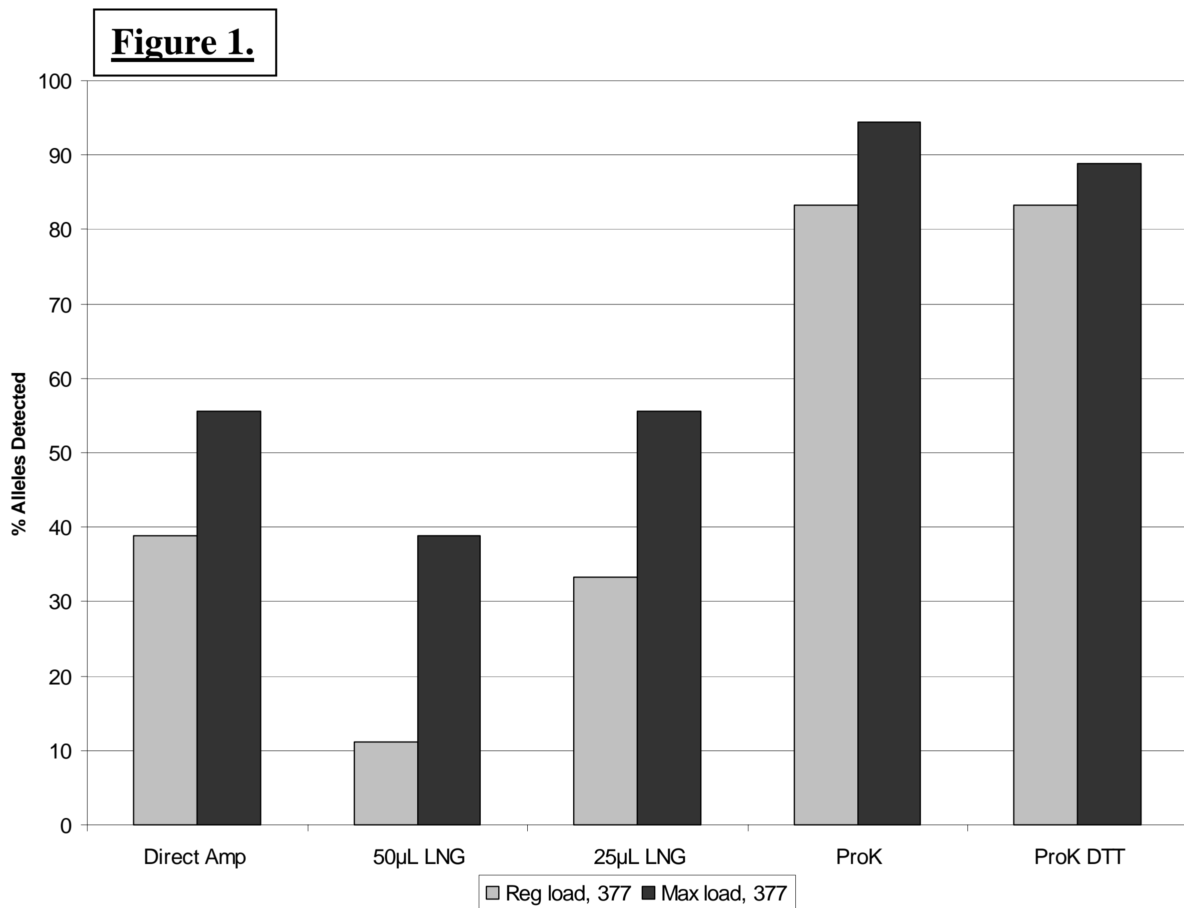

Figure 1. Comparison of techniques used to obtain DNA profiles from 150 spermatozoa collected using LMD. All samples were amplified using AmpFℓSTR® Profiler Plus and 30 PCR cycles. Samples were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

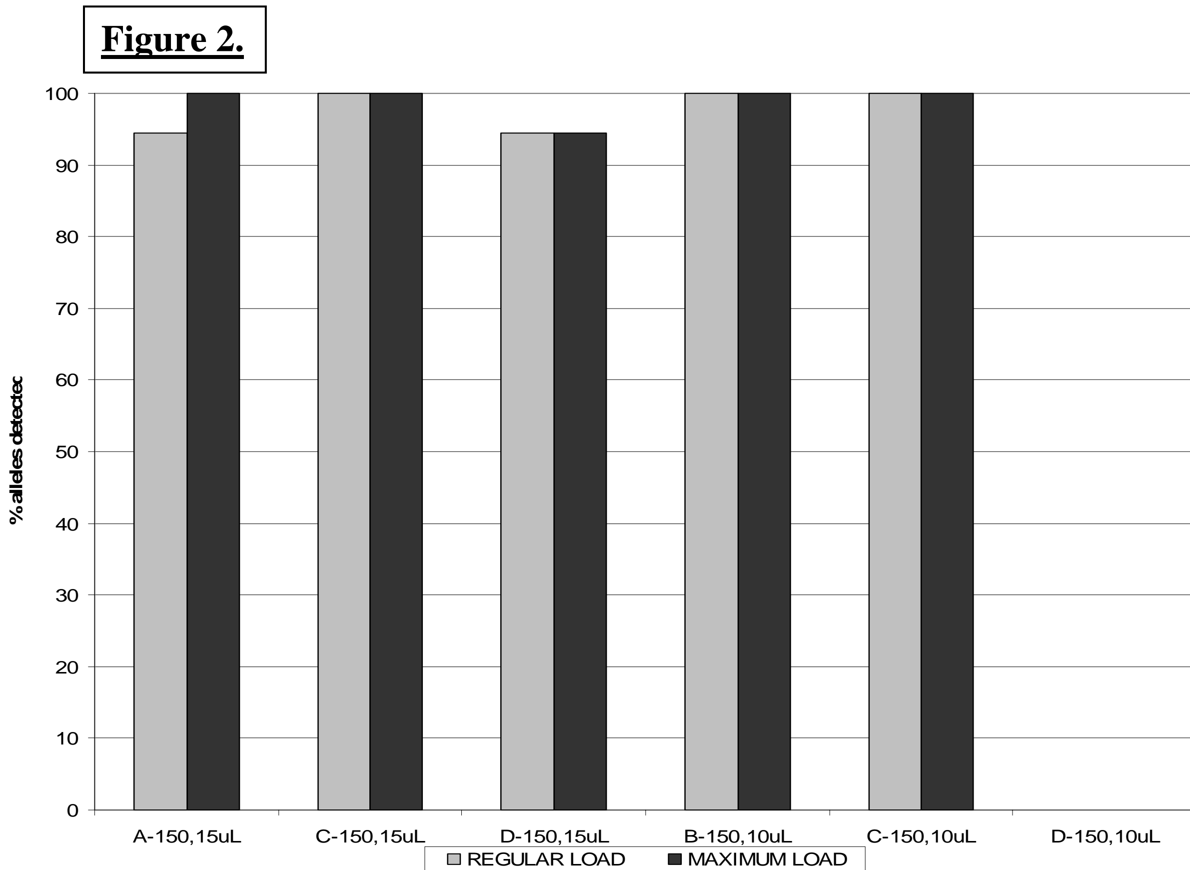

Figure 2. Reproducibility of the DNA profile from 150 spermatozoa with recProK/DTT pre-amplification lysis in combination with RV-PCR. All samples were amplified using AmpFℓSTR® Profiler Plus and 30 PCR cycles. Samples were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

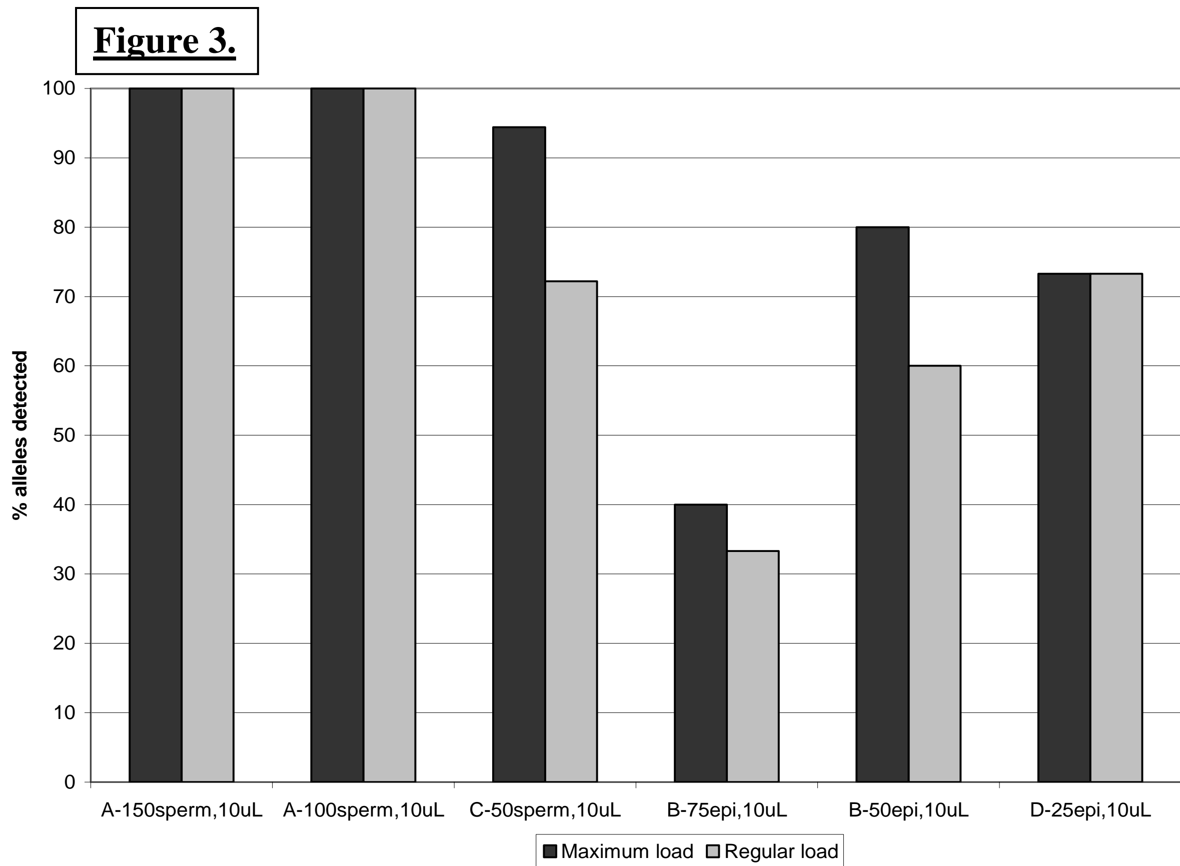

Figure 3. Sensitivity of pre-amplification lysis treatment in combination with RV-PCR (10µL volume) was tested by decreasing the number of input cells. All samples were amplified using AmpFℓSTR® Profiler Plus and 30 PCR cycles. Samples were analyzed by gel electrophoresis using the ABI PRISM 377 Genetic Analyzer. DNA profiles were typed using GeneMapper™ Version 3.1 software for data analysis.

REFERENCES

1 – Emmert-Buck MR, Bonner RF, Smith PD, *et al*. Laser capture microdissection. Science 1996;274:998-1001.

2 – Elliott K, Hill DS, Lambert C, *et al*. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Science International 2003;137:28-36.

3 – Sanders CT, Peterson DA. Separation of spermatozoa and epithelial cell mixtures by laser microdissection for forensic DNA analysis. In: Proceedings of the 56th Annual Meeting of American Academy of Forensic Sciences; 2004 Feb. 16-21; Dallas (TX): Colorado Springs, Co: American Academy of Forensic Sciences, 2004; 36

4 – Sanders CT, Peterson DA. STR analysis of laser microdissection cells for mixed stain samples. In: Louisiana's First Annual DNA Scientific Meeting; 2004 Dec. 2-3; Baton Rouge (LA)

5 – Discovery and analysis of differentially expressed genes in single cells and cell populations. Current Protocols in Molecular Biology, Spring 2005, Ch. 25, Unit 25A.2, Preparation of single cells from solid tissues for analysis by PCR, Basic Protocol 2: Analysis of single cells by PCR.

6 – Gaines, ML, Wojkiewicz, PW, Valentine JA, Brown, CL. Reduced-volume PCR amplification reactions using the AmpFℓSTR™ Profiler Plus kit. J Forensic Sci 2002;47(6):1224-37.

7 – Curran S, McKay JA, McLeod, HL, Murray GI. Laser capture microscopy. J Clin Pathol: Mol Pathol 2000;53:64-68.