MICRO-MANIPULATION AND ISOLATION TECHNIQUES FOR THE COLLECTION OF SPERMATOZOA FROM SMEAR SLIDES AND SUBSEQUENT ANALYSIS OF DNA

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The separation of spermatozoa from a mixture of assailant's spermatozoa and victim's epithelia should allow for a more certain identification of the assailant's DNA type. Although separation has been accomplished by a variety of other methods such as differential extraction[1] and by the use of microfabricated devices[2], the direct isolation and pooling of individual spermatozoon has received little attention because of difficulty validating appropriate techniques and the cost of instrumentation such as that required for Laser Capture Micro-dissection. Furthermore, the other techniques that are used for separating spermatozoa and epithelial cells often reduce the number of spermatozoa recovered and thereby limit the amount of DNA available for typing.[3]

It has been estimated that 500pg (~ 150 spermatozoa) of DNA is required for reliable real-time PCR quantification.[4] On the other hand, fewer spermatozoa may be needed and more sensitive techniques devised if the spermatozoa can be isolated and cleaned of the contaminating epithelia. Mitochondrial or pyrosequencing techniques were shown in this study to be more sensitive methods for characterizing and comparing the donor's DNA. Applying particle manipulating techniques used in McCrone Associates' laboratory for decades[5], individual sperm were isolated from stained smears and transferred to sterile tubes for DNA analysis. Spermatozoa were isolated from both Kernechtrot-Picroindigocarmine (KPIC, Christmas Tree) stained smears and smears stained with Independent Forensics' Sperm Hy-Liter™ while employing fluorescence microscopy.

The smear is coated with a thin coat of water soluble adhesive [3M; Water Soluble Tape] and each spermatozoon can be individually selected. While being observed with the microscope, the spermatozoon is picked from the slide surface with a finely pointed tungsten needle. The isolated spermatozoa are held intact by the adhesive and are transported to sterile tubes. Each pick requires approximately 10 seconds. The tubes are then processed for DNA. These isolation and manipulation techniques can easily be incorporated into an everyday screening process, and the amount of training that personnel would need in order to achieve desirable results is minimal.

Samples of spermatozoa were prepared for mitochondrial analysis as follows: 20 unstained, 20 KPIC stained and 20 Sperm Hy-Liter™ stained were transferred to 3 separate tubes; 40 unstained, 40 KPIC stained and 40 Sperm Hy-Liter™ stained were likewise transferred to 3 tubes. A negative control with the tube containing only soluble gum was processed with each batch along with six blank tubes, right out of the package. A saliva swab from the donor of the sperm was used as a reference sample.

DNA extraction using DTT, Proteinase K, PCIA, and PCR amplification were carried out to determine if mitochondrial DNA (mDNA) analysis could be performed on samples. The amplification target was a 281 base-pair fragment from the mDNA hypervariable region 1. Success of amplification was judged by a 1% agarose yield gel, and DNA sequencing was carried out to determine the mDNA profile of the sperm donor. A known buccal swab sample of the sperm donor was used to confirm that the correct profile was obtained. Negative extraction controls were amplified in parallel with the sperm to investigate whether the system for collecting sperm was free of contamination with exogenous DNA.

A similar set of samples were analyzed by pyrosequencing except that two additional duplicates were included. In other words, 3 tubes containing 20 picks from unstained, KPIC stained and Sperm Hy-Liter™ and 3 tubes with 40 picks from the same sources, making a total of 18 tubes plus controls, were prepared. Lyse-N-Go reagent with DTT was used to extract samples followed by standard PCR amplification. Amplicons were visualized on a 4% agarose E-gel using a 50bp DNA ladder as reference. Gel documentation was performed with a Gene Genius Bio Imaging System (SynGene). Streptavidin Sepharose beads/Binding buffer was added to 10µL of PCR product in a 96 well PCR plate followed by extension primers. PCR bound Streptavidin beads were captured and subsequently rinsed. PCR product bound beads were then deposited into the appropriate well of the flat-bottom well plate with sequencing primers. The PSQ 96 SNP Reagent Cartridge was then added to the PSQ 96 instrument and the Pyrosequencing software was run using the "instrument parameters code 0002." The results were then analyzed with the Pyrosequencing 96 MA SNP Analysis Software.

With hand micro-manipulation, spermatozoa can be isolated from the epithelial cells and collected from smear slides. Approximately 20 spermatozoa can be analyzed successfully for DNA. The need for expensive and time consuming digestion processes is eliminated, and one can achieve desirable DNA results with fewer spermatozoa on the smear.

References

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