Micro-Manipulation and Isolation Techniques for the Collection of Spermatozoa from Smear Slides and Subsequent Analysis of DNA

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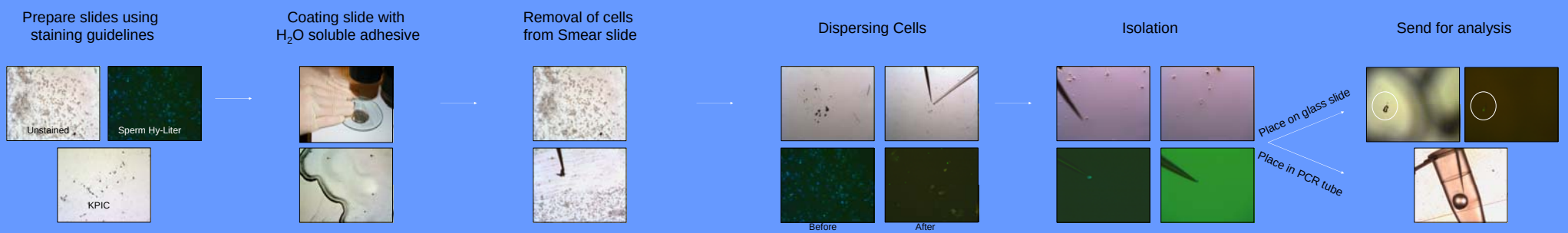
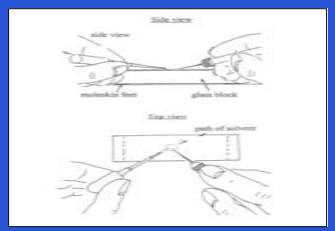
Preparation of Cells for Isolation

The smear slide is coated with a thin layer 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-LiterTM stained were transferred to 3 separate tubes; 40 unstained, 40 KPIC stained and 40 Sperm Hy-LiterTM 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.
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 TM and 3 tubes with 40 picks from the same sources, making a total of 18 tubes plus controls, were prepared.
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.



Isolation Materials

Isolation Technique



DNA Analysis

Pyrosequencing

Extraction and Amplification -- A mixture of Lyse-N-Go reagent and DTT were added to each sperm cell sample and thermally cycled according to protocol. Volumes of each sample were then brought up to 25 ul with molecular grade water. Two regions (136 bp and 137 bp) of the mitochondrial genome were amplified by adding 8 ul of these extracts to standard PCR reactions. A second amplification was performed using 1 ul of the initial amplicon and the same reaction conditions. Both PCR reactions were cycled under the same parameters (94°C for 2 minutes, 35 cycles: 94°C for 30s, 55°C for 30s, 68°C for 45s, 4°C hold) using a Gene Amp PCR System 9700. Following each 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.

Pyrosequencing -- Two mtDNA SNPs were examined in this study using Pyrosequencing. All reagents were mixed and used at room temperature. 10µL of PCR product was placed into a 96 well PCR plate. 70µL of Streptavidin Sepharose beads/Binding buffer was added to the PCR product. The 96 well filter plate was then mixed on a plate vortex at 1500 rpm for 10 minutes. During this time, a total of 15pmole of extension primers were added to the appropriate wells of the well plate. After mixing, the PCR plate was transferred to the vacuum prep station, where the PCR bound Streptavidin beads were captured and subsequently rinsed with 200 µL of 70% Ethanol, denatured in 200 µL of Denaturation Solution (0.2M NaOH) and washed in 200 µL of Washing Buffer (10mM Tris-Acetate, pH 7.6). The single-stranded PCR product bound beads were then deposited into the appropriate well with sequencing primers. The well plate was then heated at 90°C for 2 minutes on a PSQ 96 Sample Prep Thermoplate Low. The low plate is then allowed to cool to room temperature and then placed in the PSQ 96MA instrument. The PSQ 96 SNP Reagent Cartridge was filled with the appropriate amount of each nucleotide Enzyme and Substrate. The 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.

Mitochondrial

Method
Unstained sperm, Christmas tree-stained sperm, and sperm labeled with Hy-liter were picked by manual microdissection and sorted into tubes such that 20 or 40 sperm per tube were available for DNA analysis. The sperm were immobilized in water-soluble gum for easier manipulation and to avoid loss on the inner surface of labware such as tubes and pipettes.

DNA extraction using DTT, Proteinase K, PCIA, and PCR amplification were carried out to determine if mitochondrial DNA analysis could be performed on samples treated in the above manner. The amplification target was a 281 base-pair fragment from the mitochondrial DNA hypervariable region 1. Success of amplification was judged by a 1% agarose yield gel, and DNA sequencing was carried out to determine the mitochondrial DNA 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 consisting of water soluble gum blanks with no sperm were extracted and amplified in parallel with the sperm to investigate whether the system for collecting sperm was free of contamination with exogenous DNA.