A New Approach to Differential Extraction

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Abstract

Significant progress has been made in the high throughput processing of casework samples over the last few years. One remaining bottleneck has been the inability to separate sperm and epithelial cells from sexual assault cases in an efficient manner. We have developed a new method of differential extraction that employs a combination of phase separation and differential centrifugation. After performing a selective lysis of epithelial cells, the sample is efficiently centrifuged out of the solid support and into a tube containing a nonaqueous Separation Solution that is more dense than water but less dense than sperm. The aqueous solution that contains the epithelial DNA remains on top of the Separation Solution. The sperm cells migrate through the Separation Solution and form a tight pellet at the bottom of the tube. After removing the epithelial DNA-containing buffer, the tube is rinsed twice with water to dilute and remove the remaining aqueous DNA containing buffer. No centrifugation is required for this step. DNA in the resulting fractions can then be purified with DNA IQ™. The process of separation and DNA purification takes abut 2 hours and is very easy to learn. Plasticware is currently being developed to allow processing of the samples in a 96-well format.

Introduction

The preparation and analysis of casework samples is a time-consuming process with several bottlenecks. While automation was applied to the processing of database samples several years ago, the diverse nature of casework samples impeded the development of high throughput methods for these sample types. Three years ago, Promega developed an automated method to purify DNA from samples following a manual preprocessing step. This method used DNA IQ™ chemistry on a Beckman Coulter Biomek® 2000 Workstation. Two years ago, Promega developed an automated human-specific quantitation method integrating AluQuant® chemistry on the same workstation. Last year, Promega and Beckman Coulter jointly developed a versatile Normalization Wizard and PCR setup method. These systems have been shown to dramatically increase the efficiency of both large (1) and small laboratories (2). As several organizations are developing and beginning to use expert systems that intelligently perform fragment analysis of STR products, the remaining bottleneck to a completely automated DNA laboratory is the upfront processing of samples.

Sexual assault samples make up a large percentage of casework samples. These samples are typically bound to solid matrices and require separation of different cell types. In 1985, Gill *et al.* (3) developed a method to selectively enrich for sperm cells in the presence of an excess of epithelial cells. After controlled Proteinase K lysis of epithelial cells in the absence of DTT, the sample is centrifuged in a spin basket to separate the solid matrix from intact sperm and the buffer containing DNA from lysed epithelial cells.

One limitation of this traditional method is the difficulty in removing soluble DNA from the

sperm pellet. The many washes and centrifugations that the traditional method uses to accomplish this process are laborious and time-consuming and results in a delicate balance between clean separation and loss of sperm.

Prior attempts to improve on this method and to develop a high throughput process have met with varied degrees of success. Sperm-specific antibodies attached to solid supports have been used to capture sperm but this approach results in low yields due to mechanical shearing of these complexes and variable epitope accessibility.

Selective filtration through membranes has also been used to separate the smaller sperm cells from the larger epithelial cells. This method is inefficient due to the tendency of sperm to clump and the clogging of the membrane with excess epithelial cells, or the lysis of epithelial cells. The end result is considerable carryover of one cell type into the other and low yields. Selective lysis of epithelial cells prior to separation improves this process but this method as well as the antibody approach still must address an efficient way to remove the sample from the solid support.

The Differex™ System

In the process of designing a simple, high throughput method to separate sperm from epithelial cells two features were emphasized. First, the extraction process must efficiently extract sperm from a solid matrix and retain that sperm during the separation process (high yield). Second, each fraction must have minimal carryover of the other cell type (high purity).

The standard selective Proteinase K digestion of epithelial cells in the absence of DTT, followed by centrifugation, is one of the best methods to obtain a high sperm yield and purity. Proteinase K digestion loosens the attachment of cells to solid supports and reduces the degree of clumping, increasing yields and allowing for better separation. This selective digestion also converts the typically large excess of epithelial cells into soluble DNA, which is more easily separated from intact sperm. Centrifugation is essential to efficiently remove both the sperm cells and epithelial DNA-containing buffer from the matrix. For these reasons, a selective Proteinase K digestion and centrifugal extraction has been incorporated into the Differex™ System protocol.

Following the selective Proteinase K digestion, the key to obtaining pure sperm and epithelial fractions is an efficient separation strategy. This has been accomplished with the Differex™ System through a combination of phase separation and differential centrifugation. The digested sample in buffer is placed into a spin basket seated in a tube containing a nonaqueous solution. The Differex™ System Separation Solution is not miscible with water and is more dense than water but less dense than sperm. Additionally, it is nontoxic and biodegradable, so it can be used in an open environment and disposed of easily. During centrifugation, the sperm are pulled from the solid matrix and rapidly move through the Separation Solution to form a tight pellet at the bottom of the tube. The soluble DNA remains in the aqueous buffer, which forms a layer on top of the denser Separation Solution (Figure 1).

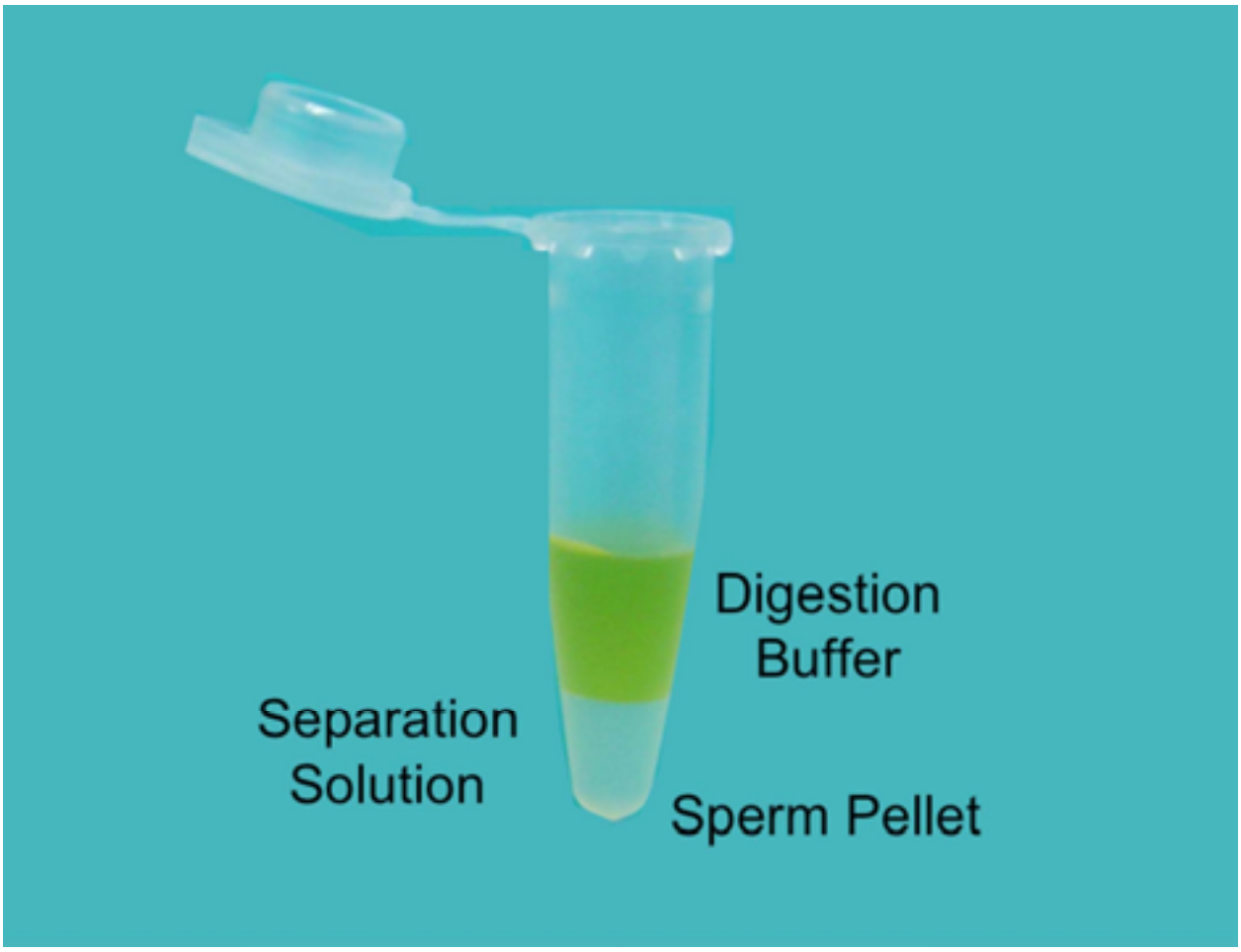

Figure 1. The Differex™ System. The Differex™ System is based on a concept of phase separation and differential centrifugation. After a differential lysis of epithelial cells in a Proteinase K containing Digestion Buffer the sample is centrifuged out of the solid support with the help of a spin basket placed above a nonaqueous Separation Solution. The epithelial DNA in the yellow aqueous Digestion Solution remains on top of the clear dense Separation Solution. The dense sperm migrate through the Separation Solution and form a tight pellet on the bottom of the tube. The Separation Solution forms a physical barrier between the sperm pellet and the aqueous DNA containing Digestion Buffer.

The aqueous buffer, which contains the epithelial DNA, is removed and reserved for DNA purification, as an epithelial fraction. A yellow dye has been added to the Digestion Buffer, which aids in the removal of both the Digestion Buffer and the wash solution and gives an indication of the wash efficiency. This dye partitions in the aqueous solution, is effectively removed during DNA purification and does not interfere with amplification. The distinct separation of phases and the yellow color of the aqueous phase remove the guesswork from this process and all but eliminate the learning curve of this new method and variation between examiners.

Because the sperm DNA is purified in the same tube, all the epithelial DNA-containing Digestion Buffer must be removed. Water is used to rinse the sides of the tube and dilute the

yellow Digestion Buffer which remains as a thin film or small bubble following removal of the bulk of the Digestion Buffer. The water does not mix with the lower layer and is removed after 30 seconds without centrifugation. A second water wash ensures that all the Digestion Buffer is removed. As this water wash is removed, up to half of the Separation Solution can be removed to collect any cell debris that is present at the interface between the two solutions. The overall process is shown in Figure 2.

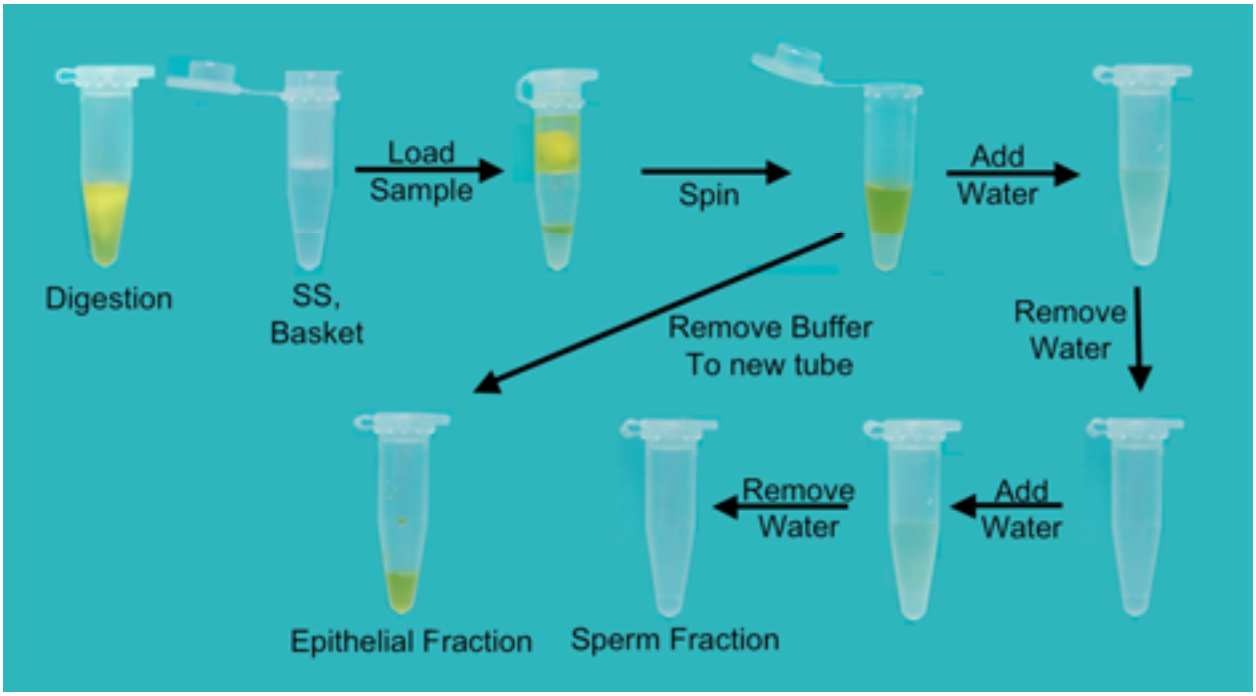

Figure 2. Differex™ System flow diagram. A sample containing sperm and epithelial cells on a solid support is digested with a Proteinase K Digestion Buffer and then placed in a spin basket above a nonaqueous Separation Solution. After centrifugation, the upper Digestion Buffer layer is removed for purification of epithelial DNA. The walls of the tube are rinsed twice with water to dilute the remaining Digestion Buffer. The water is removed without centrifugation. The sperm pellet and remaining Separation Solution are ready for purifying sperm DNA.

After washing, the tube contains a sperm pellet beneath the remaining Separation Solution. Two or more volumes of DNA IQ™ Lysis Buffer containing DTT are added to lyse the sperm and to solubilize the Separation Solution. DNA IQ™ Resin is then added, and the sperm DNA is purified (Figure 3). At the same time, 2 volumes of DNA IQ™ Lysis Buffer with DTT and DNA IQ™ Resin are added to the epithelial fraction collected earlier to allow epithelial DNA purification. Processing 100µl of the epithelial fraction is typically sufficient to obtain enough DNA for genotype analysis. The total time from the start of the Proteinase K digestion to purified DNA is as little as 2 hours for most samples.

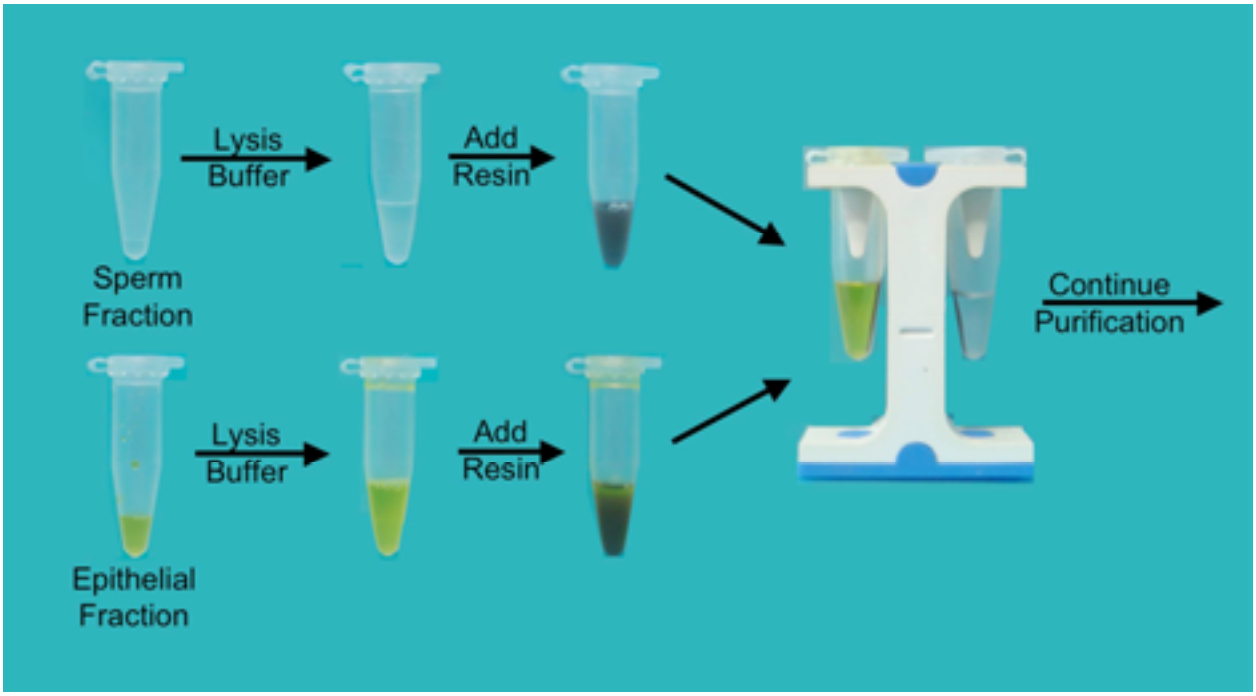

Figure 3. Purification of DNA from the separated fractions. At least 2 volumes of DNA IQ™ Lysis Buffer containing DTT is added to the sperm and epithelial fractions. The Lysis Buffer lyses the sperm and solubilizes the Separation Solution. DNA IQ™ Resin is added to bind the DNA and then the samples are placed on a magnet. The solution is removed and the Resin is washed several times. Elution buffer is added and the DNA is eluted with heat. The purification of DNA can also be performed using a robotic workstation.

Although more time consuming, the DNA from each fraction can also be purified using phenol:chloroform extraction. The Separation Solution is removed from the sperm pellet and an aqueous solution containing a detergent and DTT is added to lyse the sperm. Phenol:chloroform is then added. A Microcon® unit must be used to concentrate and remove inhibitors from the DNA solution. Phenol:chloroform can be added directly to the epithelial fraction. A Microcon® unit must be used to concentrate and remove inhibitors from this fraction also. Some yellow dye will typically remain but will not interfere with amplification.

Results using the Differex™ System

The effectiveness of the Differex™ System was demonstrated by processing half of a vaginal swab containing added semen and then purifying the DNA using the DNA IQ™ System. DNA from each fraction was amplified with PowerPlex® 16, and analyzed on an ABI Prism™ 3100 Genetic Analyzer. The genetic profile of each dye with the sperm fraction displayed above the epithelial fraction is shown in Figure 4. Effective separation was obtained with only very minor carryover of one fraction into the other.

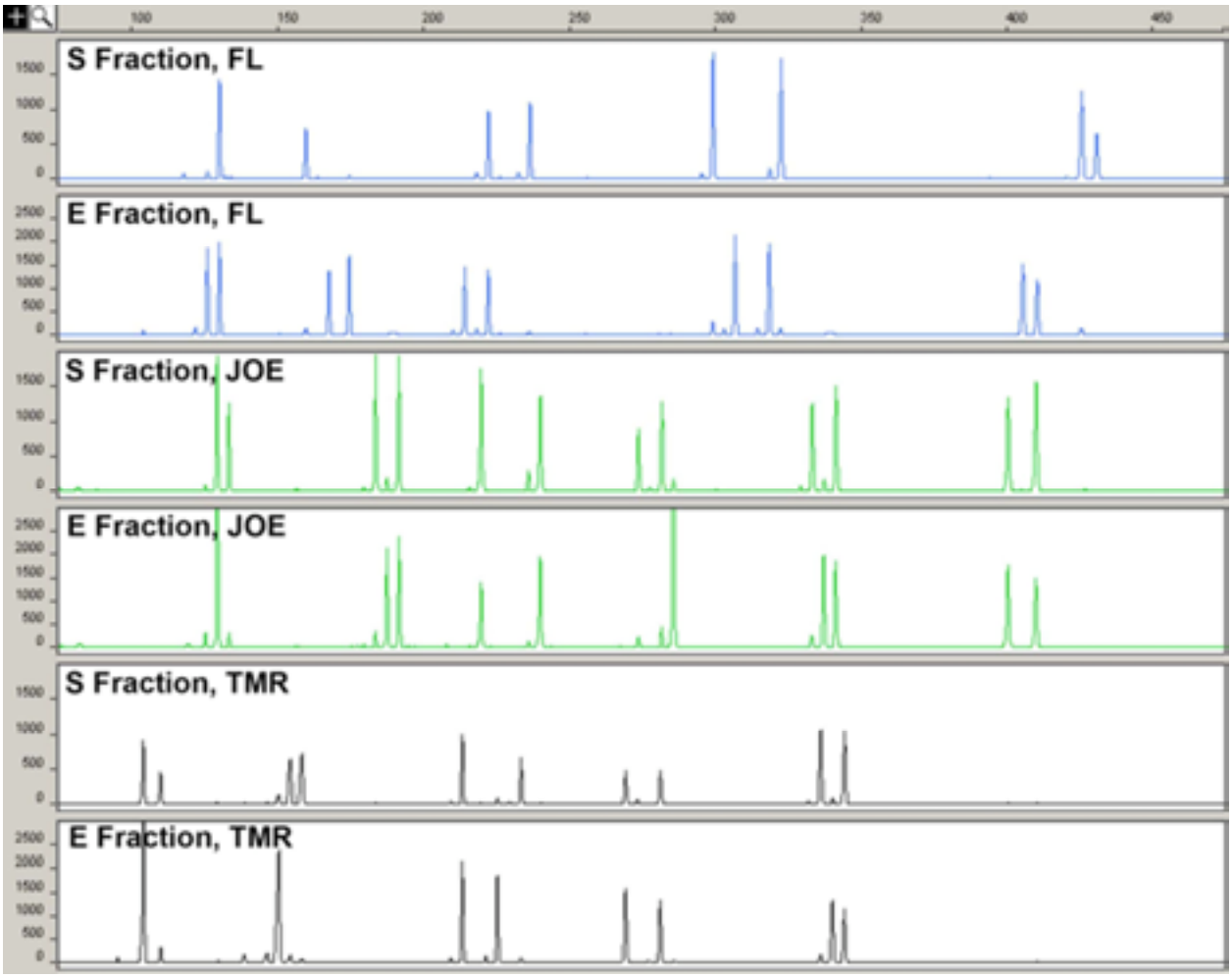

Figure 4. Analysis of fresh vaginal swabs with added semen. Half vaginal swabs with added semen were processed with the Differex™ System and DNA from the two fractions was purified using DNA IQ™ System. The resulting purified DNA was amplified with PowerPlex® 16 and analyzed on an ABI Prism™ 3100 Genetic Analyzer. The patterns are displayed by dye color with the sperm fraction above the epithelial fraction.

To determine whether the Differex™ System would work on old samples, a vaginal swab containing added semen and stored for approximately 4 years at room temperature was split in half and each sample processed using the Differex™ System. Following DNA purification with DNA IQ™ the fractions were amplified with PowerPlex® 16 and analyzed on an ABI Prism 310 Genetic Analyzer. Figure 5 shows the genetic profile of the loci labeled with TMR for each fraction of the 2 samples. Only a minor amount of epithelial DNA was observed in the sperm fraction.

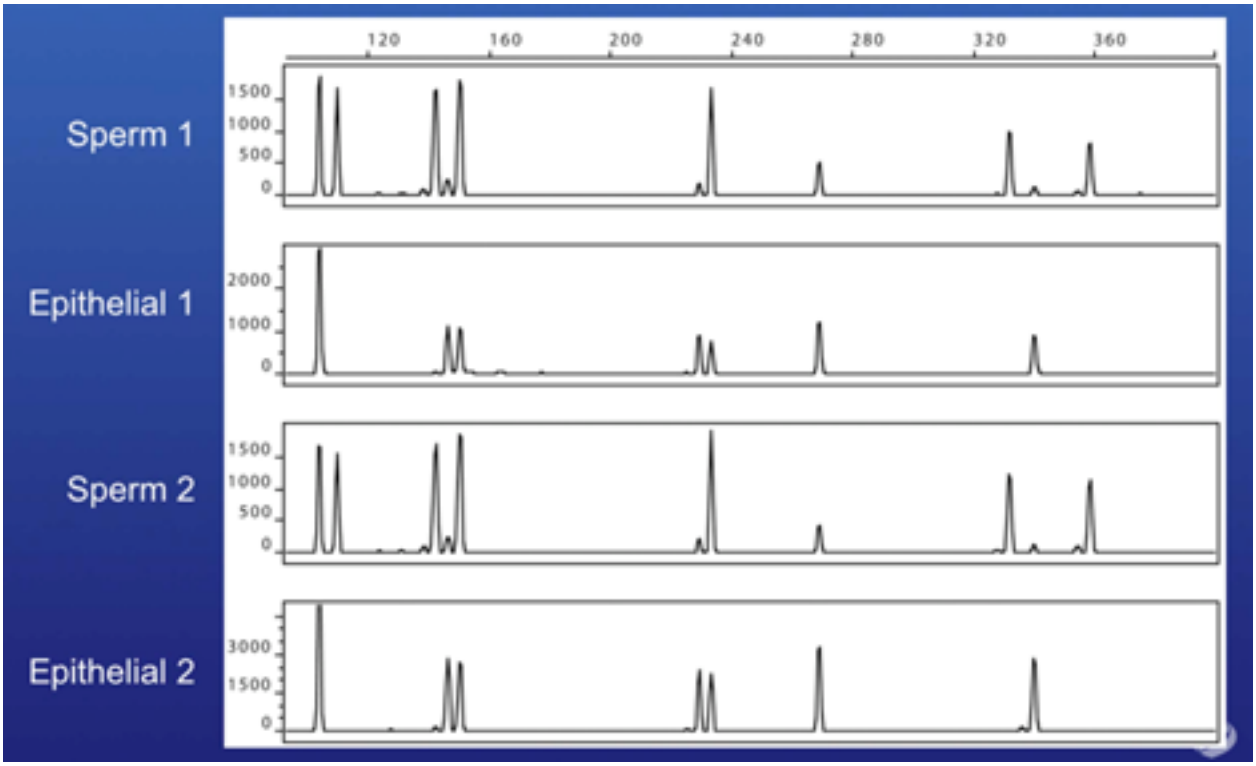

Figure 5. Analysis of old vaginal swabs with added semen. Half vaginal swabs with added semen that were prepared and stored at room temperature for approximately 4 years were processed with the Differex™ System and DNA from the two fractions was purified using the DNA IQ™ System. The resulting purified DNA was amplified with PowerPlex® 16 and analyzed on an ABI Prism™ 310 Genetic Analyzer. The patterns in the TMR channel are displayed for the two samples with the sperm fraction above the epithelial fraction.

The sensitivity of the Differex™ System was determined by placing decreasing amounts of semen on vaginal swabs, processing the samples, amplifying the purified DNA with PowerPlex® 16 and then analyzing the resulting peaks generated on an ABI Prism™ 3100 Genetic Analyzer. Figure 6 shows the TMR channel of samples containing 1.0, 0.25, 0.1, 0.05 and 0.01µl of semen per half swab. The bottom panel contains the epithelial fraction, which had no observable male-specific peaks in any replicate samples. At 1.0 and 0.25µl of semen there was only a low level of epithelial carryover into the sperm fraction. At 0.1µl of semen there was about a 10% carryover of epithelial DNA, which varied from locus to locus depending on how robust the primer set was. At 0.05µl of semen the sperm and epithelial specific peaks were about equal and at 0.01µl of semen only a few sperm-specific peaks were observed. Thus, the sensitivity of the Differex™ System is somewhat below 0.05µl of semen.

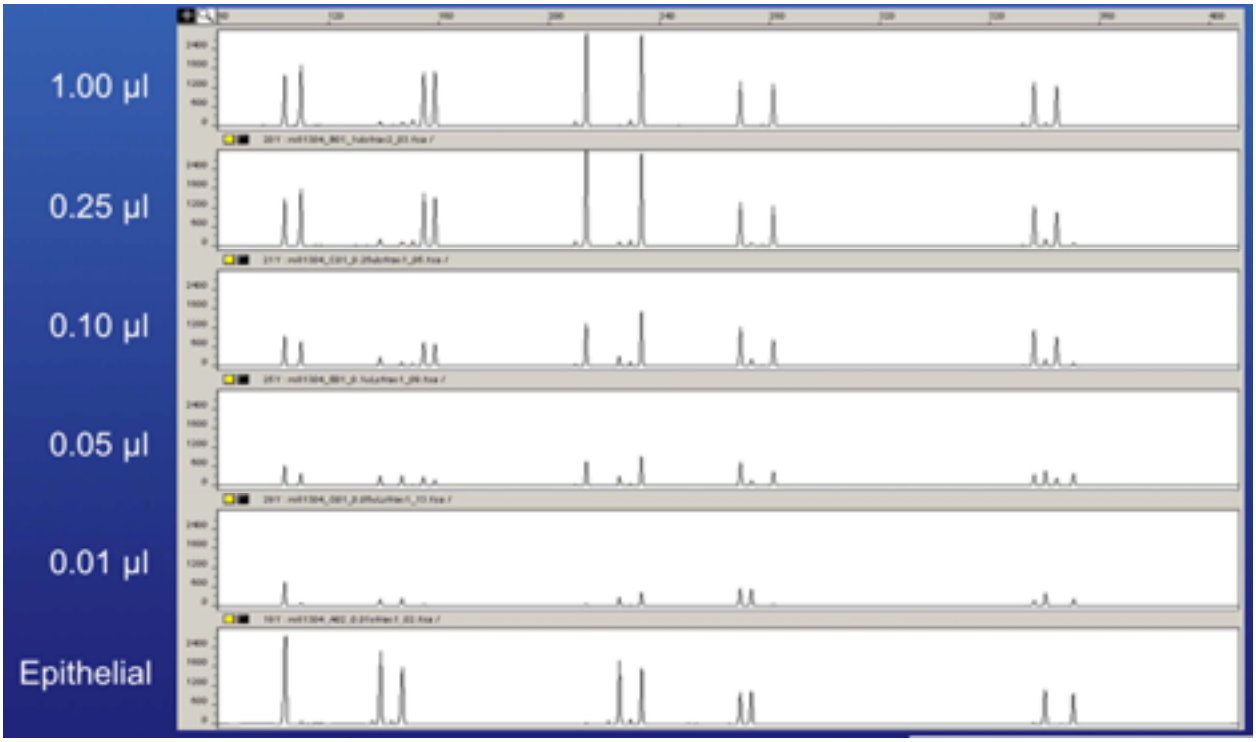

Figure 6. Sensitivity of the Differex™ System. Half vaginal swabs with various amounts of added semen were processed with the Differex™ System and DNA was purified using the DNA IQ™ System. The resulting purified DNA was amplified with PowerPlex® 16 and analyzed on an ABI Prism™ 3100 Genetic Analyzer. The patterns in the TMR channel are displayed for the sperm fractions containing 1.0, 0.25, 0.1, 0.05 and 0.01µl of semen while the bottom panel displays an epithelial fraction.

Factors Affecting Differential Extraction

There are several factors that can affect the efficiency of separating sperm and epithelial cells. Most of these factors affect any differential extraction method. The quality of the sample will determine whether effective separation can occur. Samples that contain very little sperm such as vaginal swabs that are over 72 hours post coital will frequently have significant epithelial carryover in the sperm fraction. Old samples, especially those stored in a humid environment, will contain lysed sperm, which will result in sperm DNA in the epithelial fraction.

The efficiency of the selective Proteinase K digestion will also affect the purity of the two fractions. Over digestion may result in sperm lysis and the presence of some sperm DNA in the epithelial fraction. Under digestion may not lyse all the epithelial cells, which will pellet with the sperm cells. This will result in the presence of epithelial DNA in the sperm fraction. The presence of a large amount of mucus may also inhibit efficient Proteinase K digestion and result in epithelial DNA being present in the sperm fraction.

In addition to issues that affect almost all differential extraction methods, there is one issue that specifically affects the efficiency of the Differex™ System. Because the purification of sperm DNA is performed in the same tube as the separation, it is important to remove all traces of the

epithelial DNA-containing digestion buffer. Droplets on the sides of the tube and on the tube cap must be removed. The droplets on the sides of the tube and any remaining buffer at the interface of the two layers are effectively removed by rinsing the sides of the tube with two water washes. The color of the water wash is a good indication as to the effectiveness of the wash.

High Throughput Differential Extraction

One design consideration central to the development of the Differex™ System was the necessity that it could be used in a high throughput manner. The use of liquid phase separation and the incorporation of the extraction and separation steps into a single centrifugation have resulted in a rapid method that can be easily automated following the centrifugation step. To increase the efficiency of the digestion and centrifugation steps, Promega is developing a device that will allow these two steps to be performed in the same unit without the need to transfer samples. Following centrifugation the layered phases and pelleted sperm will be in a 96-well format ready to be placed on a robotic workstation. The method being developed will perform the separation and progress directly into DNA purification using DNA IQ™ chemistry.

Conclusion

A new method for separating sperm and epithelial cells has been developed. The Differex™ System uses a combination of phase separation and differential centrifugation to separate epithelial DNA from sperm. Following a selective Proteinase K digestion of epithelial cells the sample is centrifuged out of the solid support and onto a nonaqueous Separation Solution. The epithelial DNA remains on top in the upper layer and the sperm migrate through the Separation Solution to form a tight pellet. This system is rapid, taking 2 hours including DNA purification, and is easy to learn. The Differex™ System works on both fresh samples and samples stored for several years. The system has been designed to flow seamlessly into the DNA IQ™ System purification and can be processed in a high throughput format.

References

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2. Crouse, C. (2003) Successful validation and implementation of semi-automated DNA protocols in a small forensic caseworking laboratory. *The Fourteenth International Symposium on Human Identification*, Promega Corporation.
3. Gill, P., Jeffreys, A.J. and Werrett, D.J. (1985) Forensic application of DNA ‘fingerprints’. *Nature* 318, 577–9.