DIFFEREX™ SYSTEM

A New, Rapid Method to Separate Sperm and Epithelial Cells

By Allan Tereba, Laura Flanagan, Paraj Mandrekar and Ryan Olson
Promega Corporation

The key to obtaining pure sperm and epithelial fractions is an efficient separation strategy. The Differex™ System accomplishes this through a combination of phase separation and differential centrifugation.

SUMMARY

In a revolutionary advance to an age-old process, we developed the new Differex™ System[(a)] to separate sperm and epithelial cells from sexual assault samples in as little as 2 hours, including DNA purification. This method involves a Proteinase K-selective digestion of epithelial cells, followed by differential centrifugation and phase separation, and is amenable to a high-throughput format. The Differex™ System leads seamlessly into the DNA IQ™ System[(b)], which has been automated, but is also compatible with DNA isolation using phenol/chloroform methods.

BACKGROUND

The use of automation to process casework samples has increased the efficiency of both large (1) and small laboratories (2). Unfortunately, the nature of most evidentiary samples prevents complete automation. This is especially true with sexual assault samples that are 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 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 inability to efficiently separate soluble DNA from the cell pellet. The high number of washes and centrifugations is laborious and time-consuming and results in a delicate balance between clean separation and loss of sperm. Other differential extraction protocols that seek to remedy these drawbacks have been examined. In spite of this, the 19-year-old method has remained the preferred method for forensic laboratories. However, the amount of time required to process sexual assault samples using this method has resulted in a steady increase in backlogs, which effectively limits the usefulness of felon databases.

SEPARATION: A MIX OF OLD AND NEW TECHNOLOGIES

A successful differential extraction process must efficiently extract sperm from a solid matrix and retain that sperm during the separation process (high yield). Also, each fraction must have only minimal carryover of other cell types (high purity). Standard proteinase K digestion in the absence of DTT, followed by centrifugation, is one of the best methods to obtain high sperm yield and purity. Semen sticks to material, especially cotton, and sperm cells clump and attach to epithelial cells and cell debris. Proteinase K digestion loosens the attachment to solid supports and reduces the degree of clumping, increasing yields and allowing better separation. This selective digestion also converts the large excess of epithelial cells into soluble DNA, which is more easily separated from intact sperm. Centrifugation is essential to efficiently pull the sperm out of the fibers and to extract the entire volume of epithelial DNA-containing buffer from the matrix. For these reasons, we have incorporated a proteinase K-selective digestion and centrifugal extraction into

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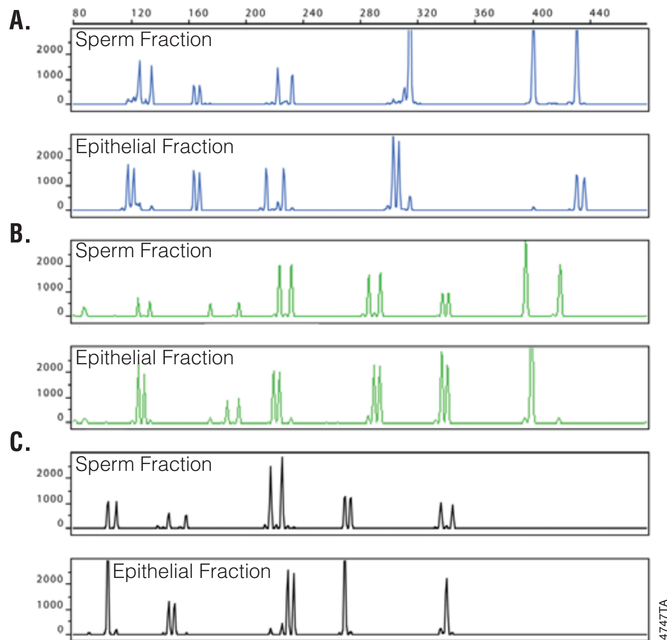

Figure 1. Analysis of DNA purified from a 4-year-old vaginal swab containing added semen. Sperm and epithelial fractions were separated from a vaginal swab stored at room temperature for 4 years using the Differex™ System. DNA was isolated from each fraction using the DNA IQ™ System. The DNA (1/400th of the epithelial fraction and 1/80th of the sperm fraction) was amplified using the PowerPlex® 16 System and analyzed on an ABI PRISM® 310 Genetic Analyzer. **Panel A.** Results in the fluorescein channel. **Panel B.** Results in the JOE channel. **Panel C.** Results in the TMR channel.

the Differex™ System protocol. This protocol eliminates the inefficient wash steps that are integral to the traditional method.

Following an efficient proteinase K digestion, the key to obtaining pure sperm and epithelial fractions is an efficient separation strategy. We have accomplished this with the Differex™ System through a combination of phase separation and differential centrifugation. The digested sample and buffer are placed in a spin basket seated in a tube containing a nonaqueous Separation Solution. The Separation Solution is not miscible with water and is more dense than water but less dense than sperm. In addition, 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 more dense Separation Solution.

The aqueous buffer containing the epithelial DNA is removed and reserved for DNA purification. To aid in removing this solution and to gauge washing efficiency, we have added a yellow dye that partitions into the aqueous phase. This dye 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 removes the guesswork from the process and all but eliminates the learning curve of this new method and variation between examiners.

The Separation Solution is washed to eliminate the thin film of DNA-containing buffer at the interface between the two layers and any droplets on the side of the tube following removal of the aqueous layer. Water is placed on top of the Separation Solution to dilute this buffer. The water does not mix with the lower layer and is removed after 30 seconds without centrifugation. 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. This debris consists mainly of mucus and cell membranes.

After washing, the tube contains a pellet of sperm and the remaining Separation Solution. Two or more volumes of DNA IQ™ Lysis Buffer containing DTT are added to lyse the sperm and solubilize the Separation Solution. The DNA IQ™ Resin is then added, and the sperm DNA is purified. At the same time, 2 volumes of DNA IQ™ Lysis Buffer with DTT and the DNA IQ™ Resin, are added to the epithelial fraction collected earlier to allow epithelial DNA purification. The total time from the start of the proteinase K digestion to purified DNA is as little as 2 hours for most samples.

FACTORS THAT AFFECT THIS METHOD

The success of this method depends on sample quality and the proteinase K digestion. Samples that contain degraded sperm will have some sperm DNA in the epithelial fraction. This carryover will be no different than that observed using the current standard method. Incomplete proteinase K digestion will result in epithelial cells pelleting with the sperm and thus epithelial DNA contamination in the sperm fraction. Again this type of contamination is also observed using the current standard method. In every other aspect, the new method will be superior to the standard method. Using 1 centrifugation instead of 4 or more, fewer sperm cells are lost, resulting in higher yields. Since the pellet is effectively separated from the aqueous, DNA-containing buffer, there should be less contamination of epithelial DNA into the sperm fraction due to soluble DNA associating with the pellet.

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RESULTS

The effectiveness and limitation of the Differex™ System is demonstrated in Figures 1 and 2. One half of a vaginal swab containing added sperm and stored for approximately 4 years at room temperature was processed as described above. DNA was purified from 100µl (1/5th) of the epithelial fraction using the DNA IQ™ System. A total of 0.5µl of DNA (1/400th of the epithelial fraction) from this mock sample was amplified using the PowerPlex® 16 System[c,e,f], and the amplification was analyzed on an ABI PRISM® 310 Genetic Analyzer. The DNA IQ™ System was used to purify DNA from the sperm pellet by adding 2 volumes of DNA IQ™ Lysis Buffer with DTT to the Separation Solution. A total of 0.5µl of the sperm DNA fraction (1/80th) was amplified and analyzed as described above.

Figure 1 shows the amplification results with DNA isolated from the sperm and epithelial fractions. Importantly, the amount of sperm recovered, based on peak height and the amount of sample amplified, shows that the Differex™ System can isolate sufficient quantities of sperm from 4-year-old samples for amplification. Also, there is only minor DNA carryover between the 2 fractions. The sperm fraction contained only minor amounts of epithelial DNA, allowing us to obtain a clean genotype. In contrast, the epithelial fraction contained noticeable, but relatively low, levels of sperm DNA. This carryover is due to sperm lysis, which could have occurred during storage of the swab or during the proteinase K digestion.

Figure 2 shows the amplification results with DNA isolated from one half of a vaginal swab taken 11 hours postcoital and stored for about 4 years at room temperature. The processing was identical to the mock sample. These results demonstrate that the Differex™ System can efficiently process samples typical of those stored from old cases. The peak heights were similar to those obtained with the mock sample, showing good recovery of sperm after 4 years. The male DNA was easily genotyped and female peaks clearly identified. In this case, the epithelial fraction showed no significant sperm-DNA peaks, but the sperm fractions had approximately 10 to 15% contaminating epithelial DNA. This carryover is likely due to incomplete digestion of the epithelial cells, which can also occur with the current differential extraction method.

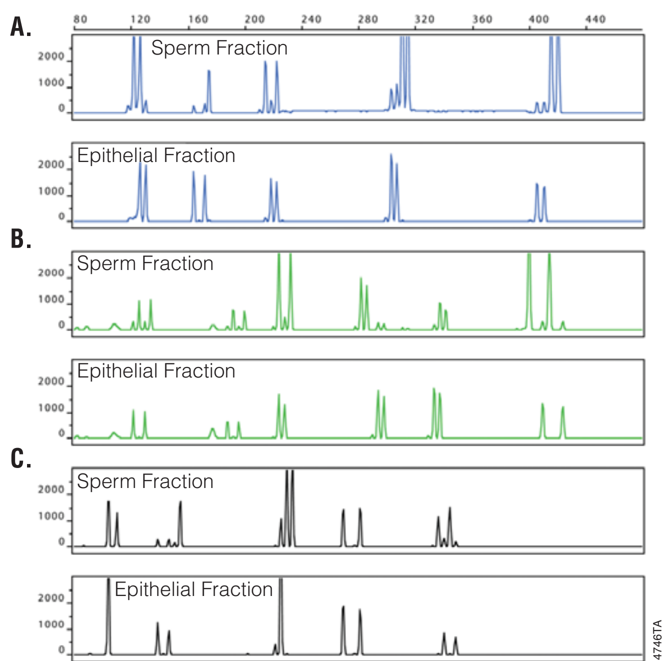

Figure 2. Analysis of a 4-year-old vaginal swab taken 11 hours postcoital. Sperm and epithelial DNA were separated from a vaginal swab stored at room temperature for 4 years using the Differex™ System. DNA was isolated from each fraction using the DNA IQ™ System. The DNA samples (1/400th of the epithelial fraction and 1/80th of the sperm fraction) were amplified using the PowerPlex® 16 System and analyzed on an ABI PRISM® 310 Genetic Analyzer. **Panel A.** Results in the fluorescein channel. **Panel B.** Results in the JOE channel. **Panel C.** Results in the TMR channel.

HIGH THROUGHPUT AND AUTOMATION

The Differex™ System is a new method for differential extraction that uses single tubes for digestion and requires transfer of the sample to a spin basket, not unlike the current method. Following centrifugation, the sperm and epithelial fractions can be manually placed in a 96-well, deep-well plate for automated DNA purification, or the tubes themselves can be placed on a workstation for processing.

We are currently developing a 96-well unit that will allow both digestion and centrifugation in the same unit without the need to transfer samples. This 96-well unit can then be placed on a workstation for automated DNA purification. The Differex™ System will also allow the processing of reference buccal and blood card samples in the same 96-well unit. This unit should be available in early 2005.

REFERENCES

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