Promega

Technical Bulletin

Differex™ System

INSTRUCTIONS FOR USE OF PRODUCTS DC6800 AND DC6801.

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Differex™ System

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I. Description

In 1985, Gill *et al.* (1) developed a method to enrich for sperm cells in the presence of an excess of epithelial cells. After a controlled lysis of the epithelial cells in the absence of a reducing agent, the sample is centrifuged in a spin basket to remove intact sperm and buffer containing the DNA from lysed epithelial cells from the solid matrix. The centrifugation pellets the sperm and cell debris. The solution containing the epithelial DNA is removed, but because of the loose nature of the pellet, some solution containing epithelial DNA remains. To obtain sperm free of epithelial DNA, several washings and recentrifugations are required. Although time consuming and labor intensive, the process has remained the method of choice without any major changes for almost two decades. This process strikes a balance between epithelial DNA removal and loss of sperm during the washings. Due to the competing nature of removing epithelial DNA and losing sperm, considerable time is spent by examiners to determine how much sample to use and how many times to wash, and so the examiner's level of experience plays an important role in determining the success of the separation process.

The Differex™ System[(a)] uses the standard proteinase K digestion and a combination of phase separation and differential centrifugation for the separation of sperm and epithelial DNA. After a standard proteinase K digestion, the sample and buffer are placed in a spin basket seated in a tube containing a nonaqueous, nontoxic, biodegradable Separation Solution. This solution is denser than, and nonmiscible with, the aqueous buffer but is less dense than sperm. During centrifugation, the sperm pellets to the bottom of the

tube. The aqueous solution containing the epithelial DNA remains on top of the nonaqueous solution. Removal of the aqueous solution is aided by the yellow color of the buffer. Only a thin film of the aqueous solution usually remains. This contaminating solution is easily removed by adding water to dilute the remaining DNA solution. The water is not miscible with the bottom solution and can be easily removed without additional centrifugation. The use of a dense nonaqueous fluid instead of a membrane eliminates clogging issues, and the nonaqueous nature of the solution helps to form a tight sperm pellet and keep cell debris away from the sperm pellet.

Following the efficient removal of the solution containing the epithelial DNA, the sperm DNA is isolated using the DNA IQ™ System[b] (Cat.# DC6701). DNA IQ™ Lysis Buffer containing DTT is added to the pellet to dissolve the nonaqueous solution and lyse the sperm. The DNA IQ™ Resin is then added, and the sperm DNA is purified following the DNA IQ™ purification protocol. The epithelial DNA is purified in a similar manner by adding two volumes of DNA IQ™ Lysis Buffer with DTT and the DNA IQ™ Resin. Figure 1 shows the amplification results of DNA isolated from a 4-year-old swab.

The total time for separating sperm from epithelial cells after addition of the sample to the proteinase K-containing Digestion Solution is approximately 90 minutes, which includes a 60-minute proteinase K digestion. DNA purification requires 30 minutes, so separation and purification can be accomplished in as little as 2 hours. Although more time is required, DNA can also be purified from the separated epithelial and sperm fractions using phenol:chloroform-based methods.

The Differex™ System provides an easy and efficient method of removing the solution containing the epithelial DNA. Variability between examiners should be reduced. In addition, since only one centrifugation is required, fewer sperm should be lost during the process. Finally, the familiar proteinase K digestion from the standard methodology is retained for the upfront processing of the solid support.

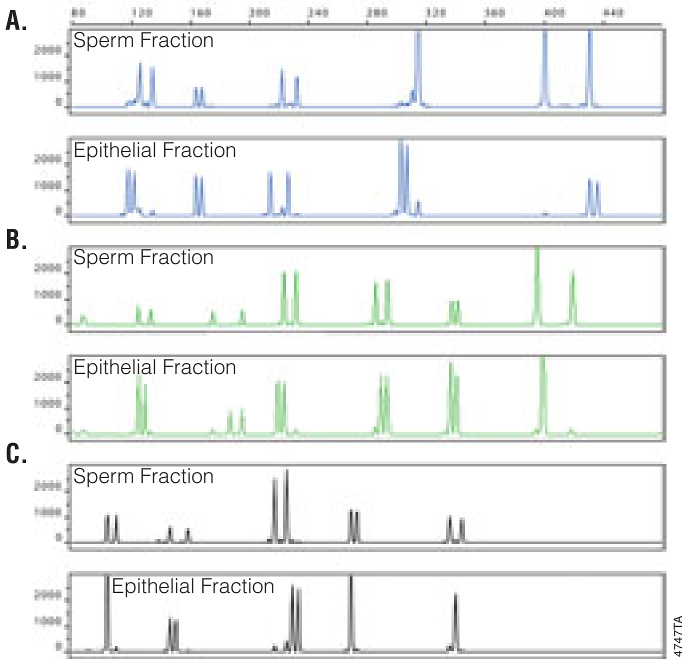

Figure 1. Analysis of DNA purified from a 4-year-old vaginal swab. Sperm and epithelial DNA were separated from a vaginal swab stored at room temperature for 4 years using the Differex™ System. DNA was isolated from each fraction using the DNA IQ™ System. The DNA (1/400th of the epithelial fraction and 1/80th of the sperm fraction) was amplified with 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.

II. Product Components

Product	Size	Cat. #
Differex™ System	50 samples	DC6801

Not for Medical Diagnostic Use. Includes enough reagents for 50 samples:

- 25ml Digestion Buffer
- 5ml Separation Solution*
- 2 × 25ml Nuclease-Free Water
- 1 Protocol

Product	Size	Cat. #
Differex™ System	200 samples	DC6800

Not for Medical Diagnostic Use. Includes enough reagents for 50 samples:

- 100ml Digestion Buffer
- 20ml Separation Solution*
- 2 × 150ml Nuclease-Free Water
- 1 Protocol

Storage Conditions: Store all components at room temperature.

*The Separation Solution is nontoxic and biodegradable and can be used outside of a hood in a room with good airflow.

III. Separation of Sperm from Epithelial Cells

Follow current guidelines when determining the size of sample to process. Carryover of epithelial DNA into the sperm fraction can be minimized by using only enough sample to obtain sufficient amounts of sperm for analysis.

Materials to Be Supplied by the User for Differential Extraction

- Proteinase K (10–20mg/ml) (Cat.# V3021)
- barrier tips
- microcentrifuge tubes (Promega Microtubes, 1.5ml, Cat.# V1231)
- DNA IQ™ Spin Baskets (Cat.# V1221)
- microcentrifuge
- heat block or oven at 56°C or 37°C

Materials to Be Supplied by the User for DNA Purification Using the DNA IQ™ System

- DNA IQ™ System (Cat.# DC6701 or DC6700)
- ethanol
- isopropyl alcohol
- DTT (Cat.# V3151)
- heat block or oven at 65°C

A. Preparation of Digestion Solution

1. Add proteinase K to the Digestion Buffer to a final concentration of 270µg/ml to prepare the Digestion Solution. Each sample will require 0.4ml of Digestion Solution.

   Do not store and reuse the Digestion Solution once the proteinase K has been added. The concentration of proteinase K can be adjusted for the number of epithelial cells and the quality of the sample based on previous experience with differential lysis.

2. Mix and use immediately.

B. Differential Extraction Protocol

1. Place the solid support containing sperm in a microcentrifuge tube.

2. Add 0.4ml of yellow Digestion Solution prepared in Section III.A to the sample.

   Note: You may use 0.5ml of Digestion Solution. However, droplets of buffer will remain on the side of the tube after centrifugation. This may result in carryover of epithelial DNA in the sperm fraction unless a third water wash is performed (repeat Steps 9 and 10 a third time).

3. Close the tube cap and vortex at high speed for 30 seconds. Be sure to keep the tube vertical while vortexing. Place the tube at 56°C for 1 hour. Alternatively, the sample can be placed at 37°C for 2 hours.

   Note: The proteinase K concentration and digestion time may be adjusted.

4. Place 100µl of Separation Solution in a clean microcentrifuge tube and place a DNA IQ™ Spin Basket on top of the tube.

5. After the proteinase K digestion (Step 3), remove the solid support from the Digestion Solution and place it in the spin basket prepared in Step 4. Slowly pipet the remaining Digestion Solution into the spin basket. Some of the solution may flow through the spin basket.

6. Close the cap on the spin basket and centrifuge for 10 minutes at maximum speed (14,000rpm) in a microcentrifuge at room temperature. After centrifugation, the tube will contain a small, slightly yellow or white pellet of sperm, a clear, lower layer of Separation Solution and an upper, yellow layer containing epithelial DNA in Digestion Solution.

7. Remove and discard the spin basket. Remove any yellow Digestion Solution from the tube cap with a Kimwipes® tissue or pipette. Alternatively if any Digestion Solution remains in the tube cap, perform a brief centrifugation to force the contents to the bottom of the tube. To avoid carryover of epithelial DNA into the sperm fraction, be sure that no liquid remains in the cap after the centrifugation.

8. Remove and reserve as much of the yellow solution as possible for purification of the epithelial DNA in Step 13. Some Separation Solution may be removed along with the yellow buffer.

   Note: It is not necessary to remove all droplets from the side of the tube. The droplets will be removed during the wash step.

9. Pipet 500µl of water on top of the Separation Solution, washing the sides of the tube to remove any droplets of yellow buffer. Some mixing of the water and Separation Solution will not affect the results, but be careful not to disturb the pellet.

10. Wait 30 seconds or more, then remove and discard the upper water layer, up to a third of the clear Separation Solution and any cell debris near the boundary of the two solutions. This wash step dilutes and removes buffer containing the epithelial DNA at the interface and on the sides of the tube. Do not disturb the pellet.

    Do not add the Separation Solution and cell debris to the fraction that will be used to purify epithelial DNA. Be careful not to disturb the small pellet at the bottom of the tube.

11. Perform a second water wash by repeating Steps 9 and 10.

    Note: The color of the first water wash in Step 9 is an indication of the success in removing the yellow layer containing the epithelial DNA. If the first water wash is clear and the sample contains a low number of epithelial cells, a second water wash may not be necessary.

12. Separation is complete. To extract DNA from the sperm fraction, add 200µl or at least two volumes of DNA IQ™ Lysis Buffer containing DTT. Vortex briefly. The Separation Solution will completely dissolve in the Lysis Buffer, and the sperm will lyse. Add 7µl of DNA IQ™ Resin, mix and proceed with DNA purification as described in the *DNA IQ™ System – Small Sample Casework Technical Bulletin* #TB296 (Section IV.C, Step 4). See Note 3.

13. Epithelial DNA is purified from the yellow solution reserved in Step 8. Add 2 volumes of DNA IQ™ Lysis Buffer with DTT to the yellow solution and mix. Add 7µl of DNA IQ™ Resin, mix and proceed with DNA purification as described in the *DNA IQ™ System – Small Sample Casework Technical Bulletin* #TB296 (Section IV.C, Step 4). See Note 3.

Notes:

1. For most samples, 100µl of the yellow solution reserved in Step 8 is sufficient to obtain enough epithelial DNA for genotype analysis. DNA from the entire epithelial fraction can be purified if desired. However, because the DNA IQ™ Resin has a limited binding capacity of 100–300ng, any excess DNA will be lost.

2. The yellow dye will not interfere with DNA amplification and will be removed during the purification process.

3. Alternatively, DNA can be purified using a phenol:chloroform-based protocol. The Separation Solution is removed from the sperm pellet before adding an aqueous solution containing detergent and DTT. When performing phenol:chloroform extraction, the DNA must be concentrated and washed using a Microcon® centrifugal filter unit.

VI. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Large tight cell pellet (epithelial cells in sperm fraction)	Incomplete lysis of epithelial cells. The proteinase K digestion was incomplete. Use a new vial of proteinase K, and digest the samples for up to 2 hours. Perform the digestion at 56°C.
Large diffuse cell pellet	Large amount of mucus in the sample. Use less sample or digest the sample with proteinase K for a longer time at 56°C. Diffuse pellets primarily contain cell debris with little DNA and can be removed without disturbing the tight sperm pellet.
A bubble of yellow buffer forms within the Separation Solution	Yellow buffer trapped by large amount of cell: debris: • Do not confuse the normal, slightly large amount of cell yellow color of the tight sperm pellet with a bubble of yellow buffer. Carefully remove any bubbles of yellow solution without disturbing the solid sperm pellet. • Perform a longer proteinase K digestion.
Sperm DNA in epithelial fraction or low number of sperm	Degraded sample. Samples containing a large number of lysed sperm due to poor storage conditions will contain some sperm DNA in the epithelial fraction.
	Overdigestion of sample. Proteinase K digestion of some samples containing few epithelial cells may result in lysis of some sperm cells. Use less proteinase K, a lower temperature for digestion or a shorter incubation time.
	Too little sample used: • Use more sample. Samples taken more than 72 hours postcoital have very few sperm. • Consider using the PowerPlex® Y System to amplify DNA purified from the entire sample.

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V. Reference

1. Gill, P., Jeffreys, A.J. and Werrett, D.J. (1985) Forensic application of DNA 'fingerprints'. *Nature* **318**, 577–9.

VI. Related Products

Product	Size	Cat#
DNA IQ™ System	100 reactions	DC6701
	400 reactions	DC6700
Tissue and Hair Extraction Kit (for use with DNA IQ™)	100 reactions	DC6740

For Laboratory Use

Accessory Components

Product	Size	Cat#
Proteinase K (Dry Powder)*	100mg	V3021
DTT, Molecular Grade (Dry Powder)	5g	V3151
DNA IQ™ Spin Baskets**	1,000/bag	V1221
Microtubes, 1.5ml	1,000/bag	V1231
MagneSphere® Technology Magnetic Separation Stand (two-position)	1.5ml	Z5332
MagneSphere® Technology Magnetic Separation Stand (twelve-position)	1.5ml	Z5342
MagnaBot® 96 Magnetic Separation Device	1 each	V8151

*For Laboratory Use.
**Not for Medical Diagnostic Use.

[a]Patent Pending.

[b]U.S. Pat. Nos. 6,027,945, 6,368,800 and 6,673,631, Australian Pat. No. 732756 and European Pat. Nos. 0 895 546 and 1 204 741 have been issued to Promega Corporation for methods of isolating biological target materials using silica magnetic particles and simultaneous isolation and quantitation of DNA. Other patents are pending.

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