Differential extraction using Alkaline Lysis and Pressure cycling

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Issues in DNA extraction

1. Removal of cells from a substrate – release from swabs, clothing

2. Removal of DNA from the cells using detergents, enzymes, etc.– time-consuming and cumbersome

3. Differential extraction of male vs. female cells

**Vuichard et al.- nine different laboratories used different protocols for differential digestion and reported high levels of male DNA loss and user based variations.**

**Vuichard S., Borer U., Bottinelli M., Cossu C., Malik N., Meier V., Gehrig C., Sulzer A, Morerod M., Castella V. Investigative genetics 2011, 2 (1), 11.**
Mathematically the problem is demonstrated by 2 equations

**Equilibrium**
- Cells on surface vs swab
- Cells on swab vs in vial
- DNA recovered vs cell debris

\[ K_{eq} = \frac{[\text{DNA}_{\text{isolate}}]}{[\text{DNA}_{\text{cellular debris}}]} \]

**Recovery**
- Cells from surface
- Cells from swab
- DNA from cell debris

\[ \%\text{Recovery} = 100 \times \frac{\text{pg purified DNA}}{\text{pg total input DNA}} \]
Differential Extraction


1) DNA from swab is added to detergent and proteinase to lysis the epithelial cells without lysing the sperm cells.

2) The sample is centrifuged to pellet intact sperm cells and supernatant removed.

3) The pellet is subjected to additional washes to remove residual female DNA.

4) Sperm cells are treated with DTT to reduce protein disulfide bonds, lyse cells and release male DNA.
Protamines replace histones in sperm cells.

They permit tight compacting of the genome and protect it from environmental insults and mutations.

The proteins have more + charged arginine and other basic proteins to permit tight packing of − charged DNA.

Furthermore numerous cysteine residues occur which permit tight crosslinking of the DNA.

Certain portions of sperm DNA have histones, these are assumed to involve early embryonic development and may also exert epigenetic control of parental DNA.
Protamines replace histones in sperm cells.

- They permit tight compacting of the genome and protect it from environmental insults and mutations.
- The proteins have more arginine and other basic proteins to permit tight packing of negatively charged DNA.
- Furthermore, numerous cysteine residues occur which permit tight crosslinking of the DNA.
- Certain portions of sperm DNA have histones; these are assumed to involve early embryonic development and may also exert epigenetic control of parental DNA.
Differential extraction

Step 1: removal of cells from swab
Step 2: Lysis and separation of female epithelial DNA
Step 2: Lysis and purification of male sperm DNA

ISSUES
Recovery of DNA from evidence
Removal of DNA from swab
Sperm cells may lyse during initial digestion affecting female epi fraction
Female cells may not completely digest affecting male sperm fraction
Differential Extraction (cutting from comforter)

Sample was mostly female, differential extract recovered male alleles as mixture.

Female Fraction

| 103.56 |
| 144.84 |
| 149.38 |
| 153.76 |
| 158.21 |

Comforter stain (male fraction)

| 103.54 |
| 144.84 |
| 153.76 |
| 158.21 |

Victim (13,15), (30,32.2), (15,16)
The absence of sperm does not mean rape didn’t occur

- Clinical evidence of sperm is absent in approximately 37% of rapes

- 1/3 of rapists exhibit some form of sexual dysfunction during their assaults, a finding consistent with the above

- Laws requiring the presence of sperm to confirm an act of rape fail to recognize that the assault is a pseudosexual act motivated more by feelings of hostility and power than sexual desire.

Alternatives to differential extraction

- Y STR typing
- Laser microdissection
- Cell sorting via flow cytometry
- Pressure cycling
Pressure Cycling Technology (PCT)

Barocycler® NEP2320 (Pressure BioSciences Inc.)
User controlled variables:

a) Pressure (5-45k psi)
b) Holding time at ambient pressure (T1)
c) Holding time at target pressure (T2)
d) No. of cycles (1-99)
e) Temperature

Micro Tubes
- PULSE™ tubes (50-150 µl)
- (up to 300 µl)
Mechanism of pressure-based lysis
lipids are highly compressible

A

Lipid bilayer

Membrane protein

Hydrophobic tails

Hydrophilic heads

Ambient pressure

B

Application of high pressure

C

High pressure released
Hypothesis

- Sperm and epithelial cells should respond differently to pressure cycling based on their different composition

  - Epithelial cells are larger, with more diffuse structures. They should be more distorted by pressure, and thus more sensitive to its effects.

  - Sperm DNA is associated with protamines, proteins with a high cysteine content, crosslinked with disulfide bridges—dense packing of DNA (12-18% cysteine)

  - Epithelial cell nuclei are surrounded by histone proteins. These are not cross linked and have lower overall + charge. (0.2% cysteine)
Initial work with pressure cycling
Microscopic studies

Cell Visualization in PBS

- Cells stained with 0.4% Trypan blue (dye exclusion method) following Pressure treatment
- Color indicates PCT treatment is causing take-up of dye and partial disruption of cell walls

Vaginal epithelial cells

Sperm cells
Initial response to pressure treatment

Suspended cells in 1X PBS buffer subjected to pressure treatment vs. standard Proteinase K as measured by real-time PCR

Low recovery

* Mean (n=3) ± SE
Improving DNA recovery

Reducing agent (DTT)- Sperm DNA recovery
Detergent, temperature- Epithelial DNA recovery

* Mean (n=3) ± SE
Effect of higher concentrations of DTT

1) Increase in recovery
2) Loss of selectivity

* Mean (n=3) ± SE
Alternate reducing agent - TCEP

Goal: improve reduction of dithiol linkages for sperm protamines

Advantages to TCEP

- Water solubility
- Odorless
- Wide pH range
- Resistant to air oxidation

Disadvantage

TCEP is not particularly stable in phosphate buffers

A comparison: DTT vs. TCEP

Switching to **TCEP** caused an increase in selectivity between sperm cell and epithelial cell lysis.

**DTHiothreitol (DTT)**

**TCEP**

Recovery- **73%±4**  
Selectivity- **1.5/1**

Recovery- **58%±4**  
Selectivity- **9/1**
DNA recovery from swabs

1. The next step was to access real samples and ideally combine cell removal and lysis using high pressure pulsing

2. To our great dismay, the recovery was very poor from cotton swabs. Other materials fared no better [flocked swabs, polyester swabs, etc.]

3. Many studies in the literature support this finding)
Effect of PCT on DNA recovery from cotton swabs

Poor DNA recovery from cotton swabs

- Mean (n=3) ± SE

- 45000 psi, 60 cycles (in the presence of 20 mM TCEP)

* Mean (n=3) ± SE
Improving DNA recovery from cotton swabs using alkaline lysis

Key issues were the speed of the procedure - 2 hrs
Alkali can simultaneously release cells from swabs and lyse them
Strong detergents and chaotropic salts which can create inhibition are removed.

But we wanted to avoid DNAse treatment and improve selectivity using pressure cycling
Effect of Alkaline lysis on DNA recovery good improved recovery/ poor selectivity

* Mean (n=3) ± SE
Optimization of selectivity using pressure cycling

0.4 N NaOH (at room temp)

- High recovery of female DNA
- Excellent selectivity for male DNA

* Mean (n=3) ± SE
Development of differential extraction protocol using PCT treatment and Alkaline lysis

Transfer swab to PULSE™ tube and add 0.4 N NaOH

PCT
20,000 psi for 10 cycles

Transfer swab to another tube and add 0.4 N NaOH

Incubate at 95°C for 5 min

Discard the swab and purify the eluate via PCIA

Purify liquid fraction with PCIA

Suspend the pellet in 1X TE buffer (Epithelial DNA)

Suspend the pellet in 1X TE buffer (Sperm DNA)

PCT treatment + Alkaline lysis

- Predominantly female DNA recovered post-pressure treatment
- Clean male fraction obtained following exposure to high temperature

* Mean (n=3) ± SE

![Graph showing % DNA Recovery](image)

- Fraction E, 20k psi, 20 cycles
- Fraction S, 95°C, 5 min
- Fraction E, 20k psi, 10 cycles
- Fraction S, 95°C, 5 min

Legend:
- Sperm DNA
- Vaginal epithelial DNA

* Mean (n=3) ± SE
PCT treatment + Alkaline lysis - DNA profile of control mixture
How does this compare to the current method?

- Broward Sheriff’s Office (BSO) crime lab protocol vs. Alkaline lysis and PCT

Post coital sample
## Mixture Ratio Study

- **Varying ratios of male to female cells**

<table>
<thead>
<tr>
<th>Ratio of male: female</th>
<th>Number of sperm cells</th>
<th>Number of vaginal epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>96000</td>
<td>96000</td>
</tr>
<tr>
<td>1:2</td>
<td>48000</td>
<td>96000</td>
</tr>
<tr>
<td>1:5</td>
<td>19200</td>
<td>96000</td>
</tr>
<tr>
<td>1:10</td>
<td>9600</td>
<td>96000</td>
</tr>
<tr>
<td>1:50</td>
<td>3000</td>
<td>150000</td>
</tr>
</tbody>
</table>
Some Validation Studies

- Mixture ratios
- Substrates
- Environmental and PCR inhibition
- Post coital samples and comparison with standard procedures
Sensitivity studies - 1:2 male to female cells

- Clean male autosomal profile obtained
Sensitivity studies - 1:5 male to female cells

- Mixed profile
- (complete male autosomal STR profile observed)
Sensitivity 1:10 male to female cells

- Mixed profile - major contribution by female
- Complete male STR profile obtained
Sensitivity 1:50 male to female cells

- Loss of male alleles observed
Varying ratios of male: female cells
Percent contribution in sperm fraction

- Mean (n=7) ± SE
STABILITY STUDIES

- Environmental insults - exposure of swab with mixed to outside environment for 7 days

- Chemical insults
  i. Exposure of swab to inhibitor mix (12.5 mM Indigo, 0.5 mM Hematin, 2.5 mg/ml Humic acid) and
  ii. Extraction of dried stain from denim and other substrates
Stability studies

A complete male STR profile was obtained from all samples.

Sperm control
Inhibitor mix
Denim

S1E2 - Outside environment
Additional samples

**Sperm control**

**Denim**

**Cotton panty**

**Bed spread**

**Socks**

**Colored t-shirt**
CORRELATION STUDIES

- 10 Post-coital samples
- Comparison of extraction protocols
- Broward sheriff’s office (BSO) differential digestion protocol
- Alkaline lysis and pressure cycling technology (AL+PCT)
Correlation studies post coital samples

- The results show equal or higher levels of Y STR recovery with alkaline lysis when compared with standard techniques.

![Graph showing comparison between BSO and AL + PCT with error bars for each sample.](image)
Concluding remarks

1. We have developed a novel differential extraction method that involves selective digestion of sperm and female epithelial cells using alkaline lysis and pressure cycling.

2. The procedure involves first lysing and removal of female cells via pressure cycling in 0.4N NaOH followed by lysing male cells in 0.4N NaOH at 95°C.

3. The procedure is rapid (10 min) and results in high recovery of cells directly from cotton swabs.

4. The procedure works well on a variety of substrates and recovers complete male profile for ratios up to 10 to 1.

5. Future work involves improving selectivity for high female/male ratios.
Acknowledgements

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The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music.

-Lewis Thomas