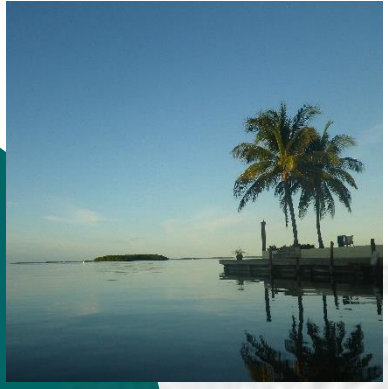
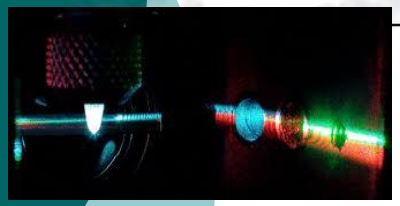
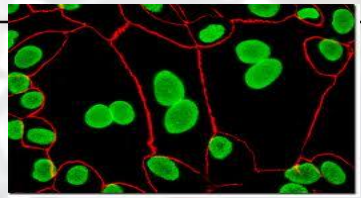


Differential extraction using Alkaline Lysis and Pressure cycling





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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.*

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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 derbis

$$K_{eq} = \frac{[DNA_{isolate}]}{[DNA_{cellular\ debris}]}$$

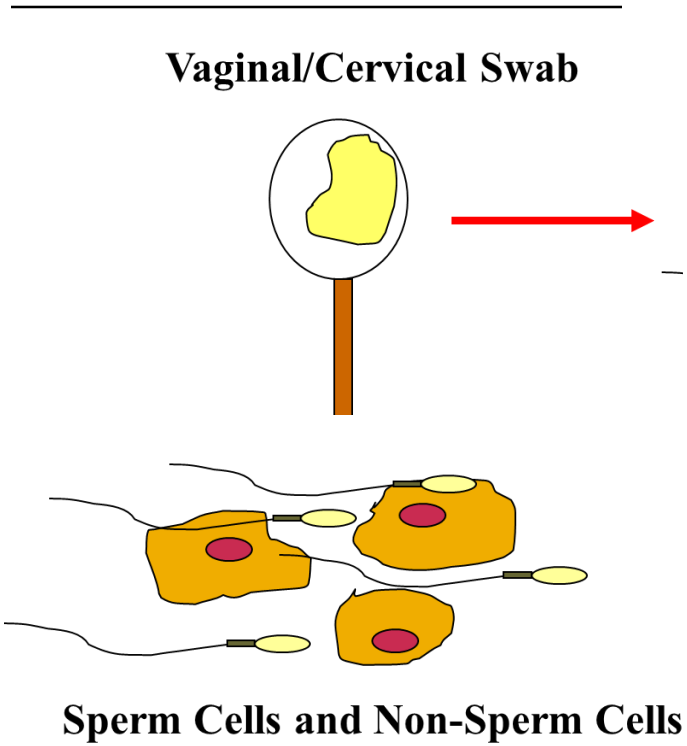
- Recovery
  - Cells from surface
  - Cells from swab
  - DNA from cell debris

$$\%Recovery = \frac{100x\ \ pg\ purified\ DNA}{pg\ total\ input\ DNA}$$

Differential Extraction

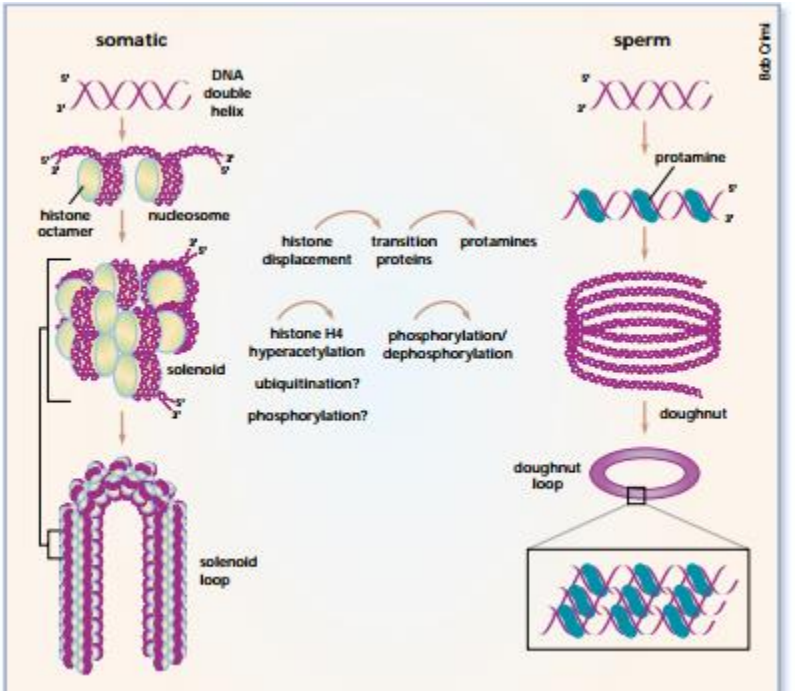
- First described by Gill, Jeffreys and Werrett in *Nature*, 1985.

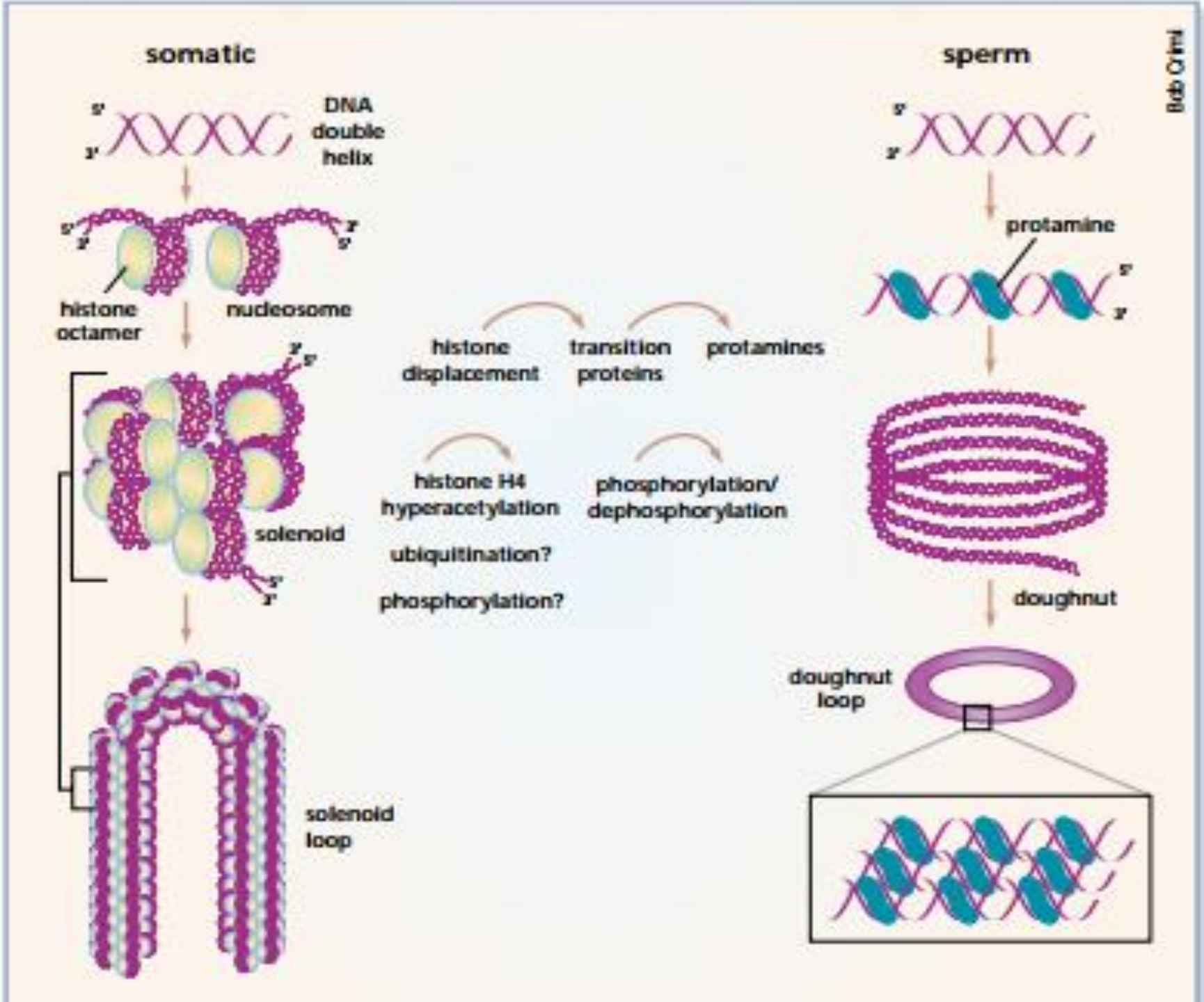
  - 1) DNA from swab is added to detergent and proteinase to lysis the epithelial cells without lysing the sperm cells.
  - 2) The sample is centrifuged to pellet intact sperm cells and supernatant removed.
  - 3) The pellet is subjected to additional washes to remove residual female DNA
  - 4) Sperm cells are treated with DTT to reduce protein disulfide bonds, lyse cells and release male DNA



Protamines

- 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 agenine 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

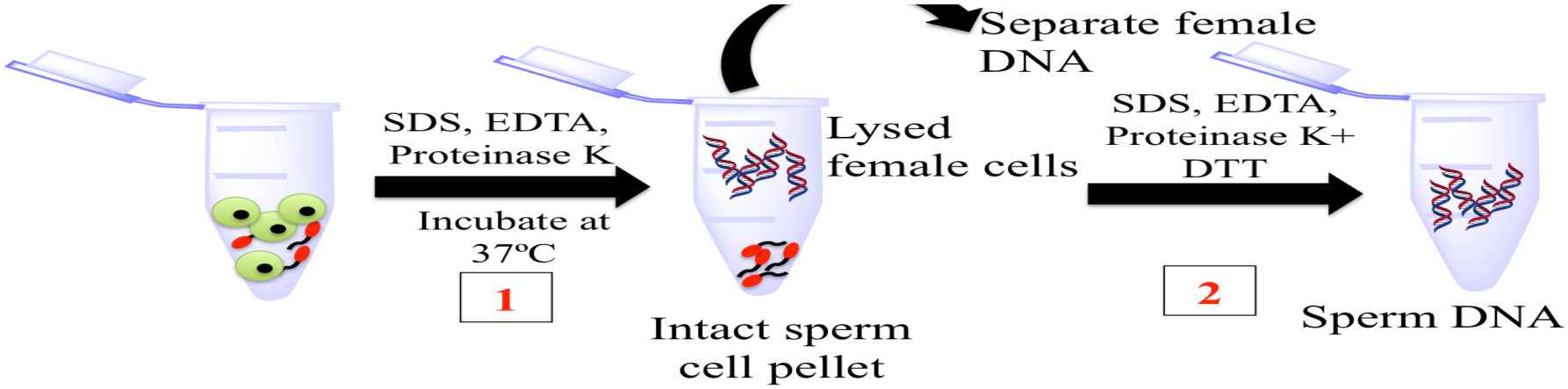



somatic
sperm
DNA
double
helix
protamine
histone
octamer
nucleosome
histone
displacement
transition
proteins
protamines
histone H4
hyperacetylation
phosphorylation/
dephosphorylation
ubiquitination?
phosphorylation?
solenoid
doughnut
doughnut
loop
solenoid
loop

Differential extraction

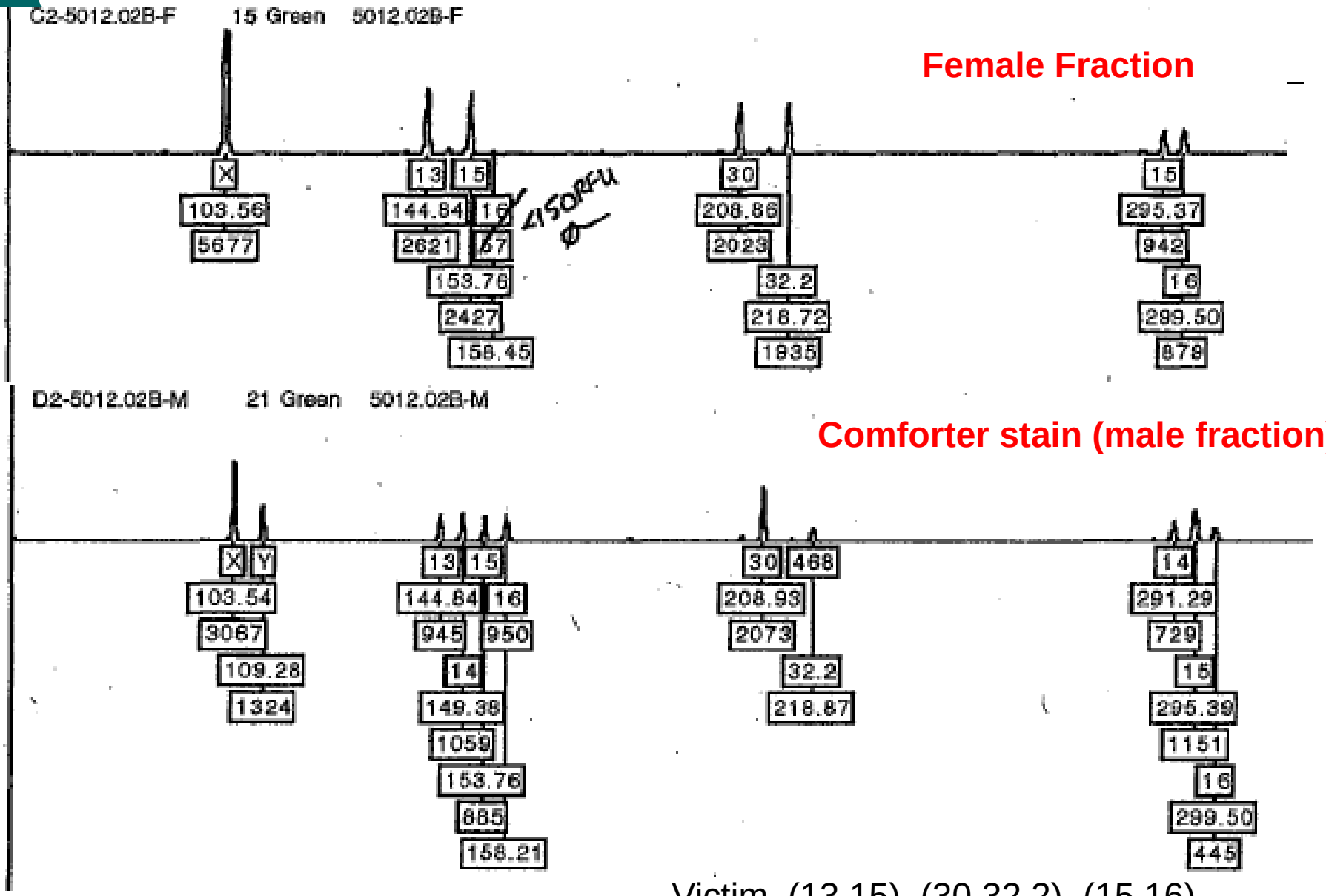
Step 1:removal of cells from swab
Step 2 :Lysis and separation of female epithelial DNA
Step 2: Lysis and purification of male sperm DNA

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



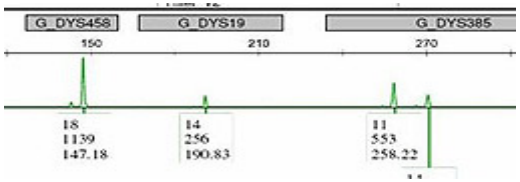
Victim (13,15), (30,32.2), (15,16)

The absence of sperm does not mean rape didn't occur

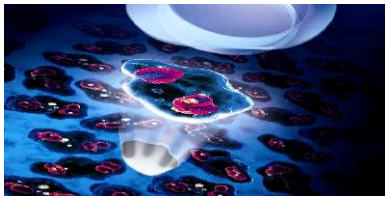
- Clinical evidence of sperm is absent in approximately 37% of rapes
- 1/3 of rapists exhibit some form of sexual dysfunction during their assaults, a finding consistent with the above
- Laws requiring the presence of sperm to confirm an act of rape fail to recognize that the assault is a pseudosexual acti motivated more by by feelings of hostility and power than sexual desire.
- https://www.ncjrs.gov/App/publications/abstract.aspx?ID=47007

Alternatives to differential extraction

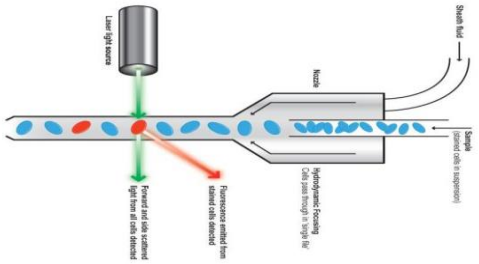
- **Y STR typing**



- **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

PULSE™ tubes

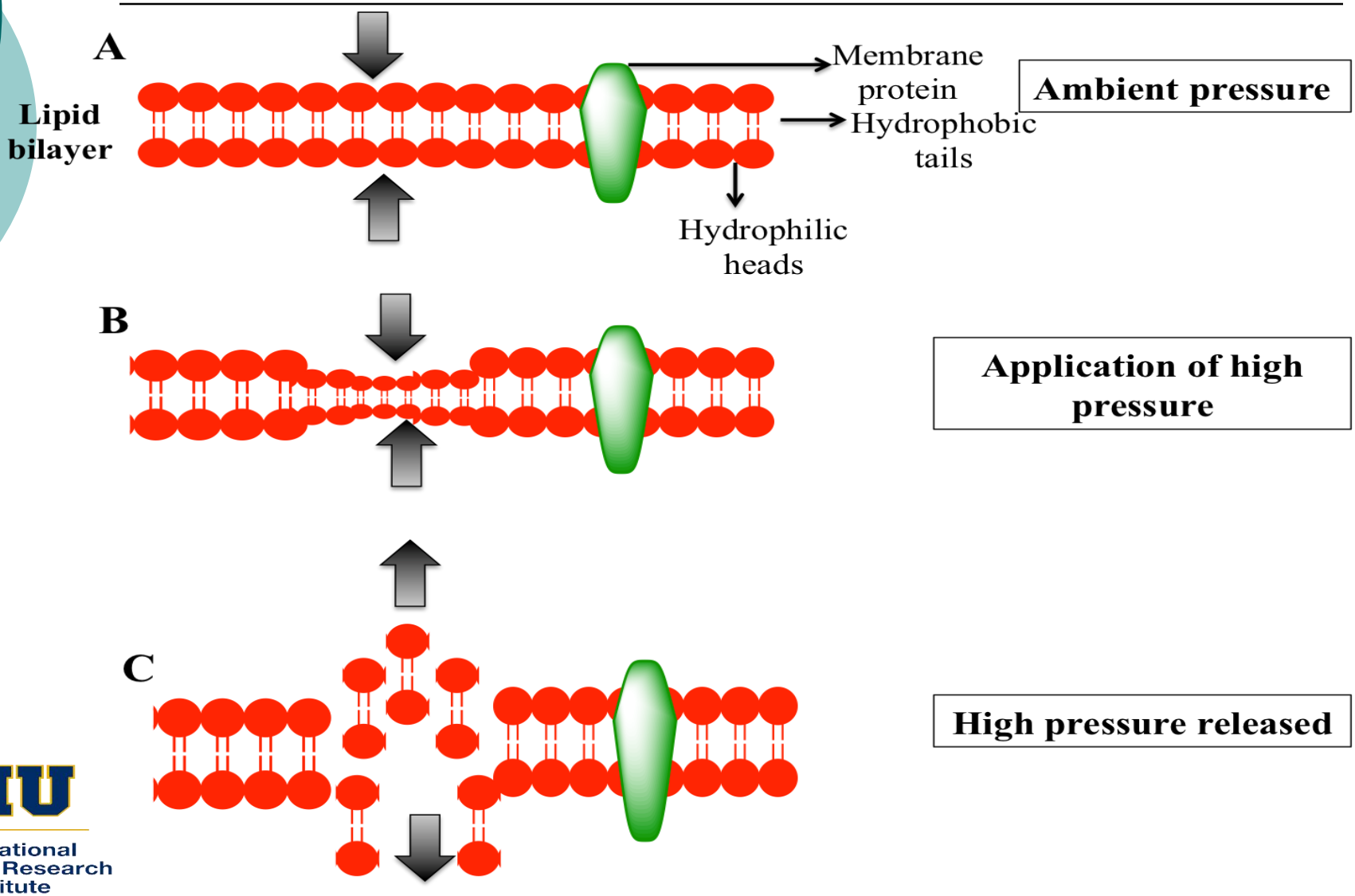
(up to 300 µl)

MicroTubes

(50-150 µl)

Mechanism of pressure-based lysis

lipids are highly compressible



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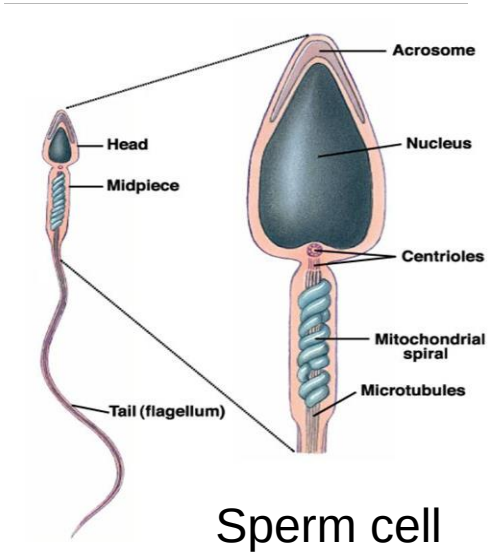
Hypothesis

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

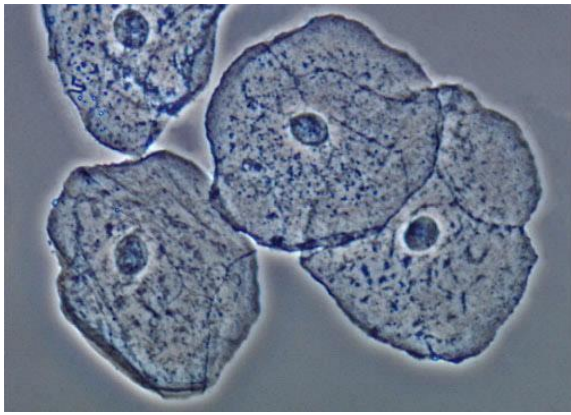
  - Epithelial cells are larger, with more diffuse structures. They should be more distorted by pressure, and thus more sensitive to its effects.

  - 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)



Sperm cell



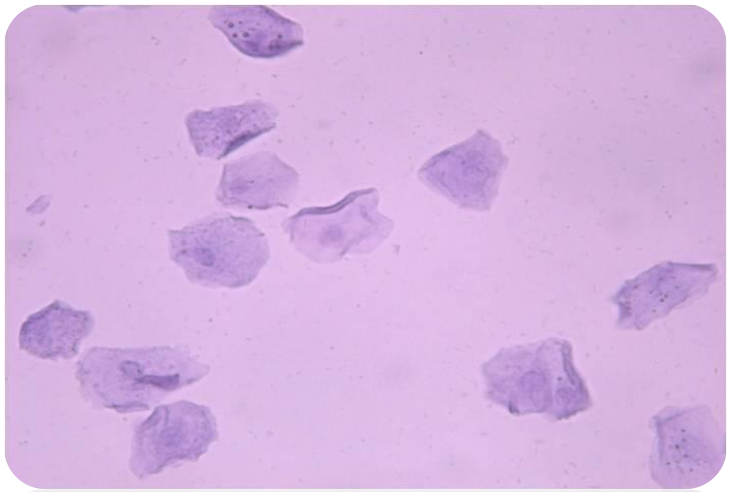
Buccal Epithelial cell

Initial work with pressure cycling Microscopic studies

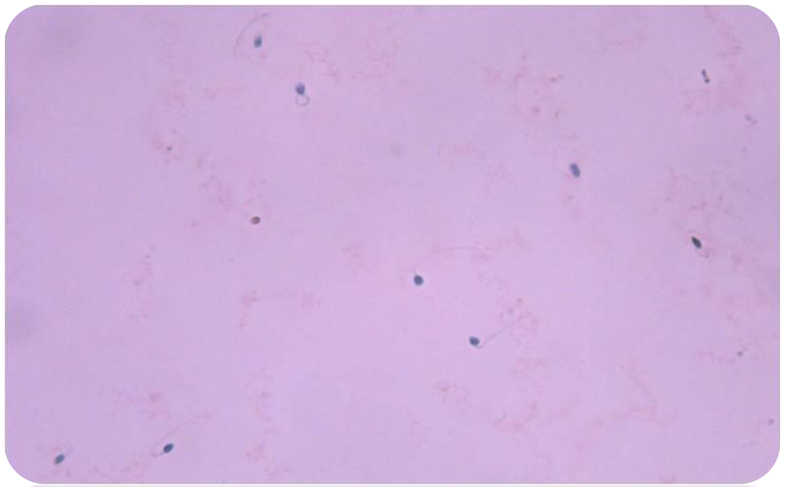
Cell Visualization in PBS

- 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



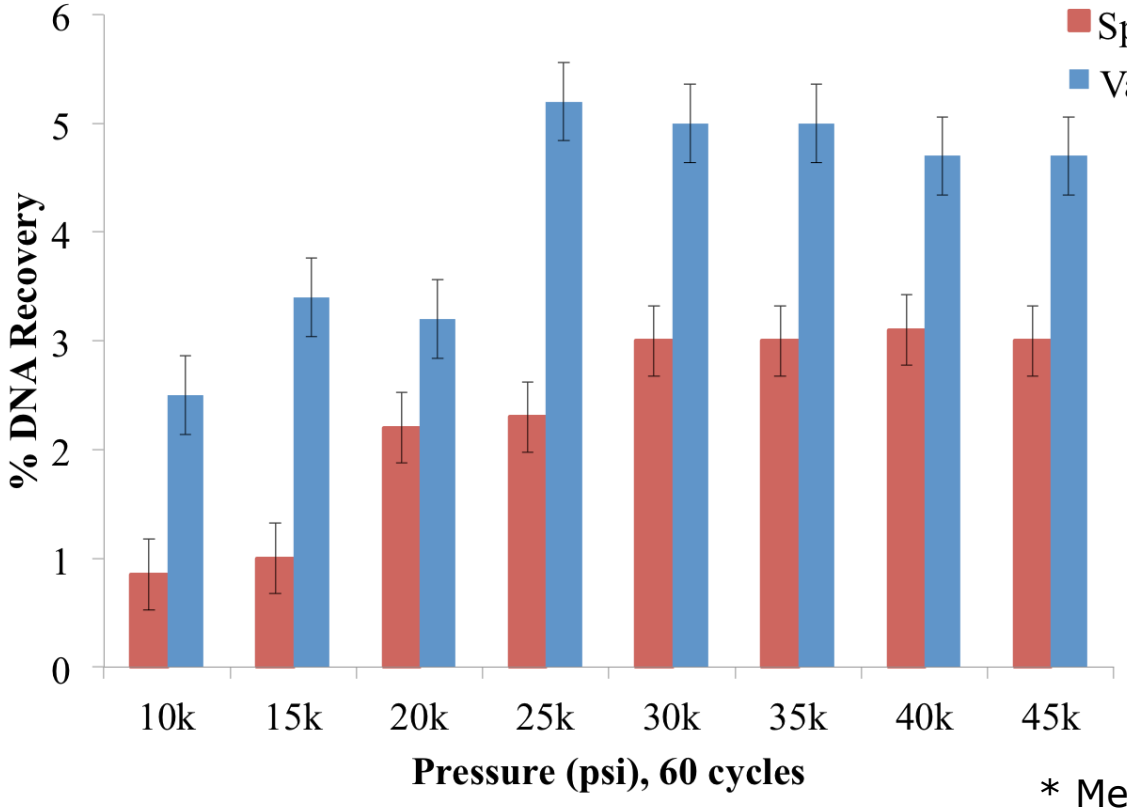
Vaginal epithelial cells



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

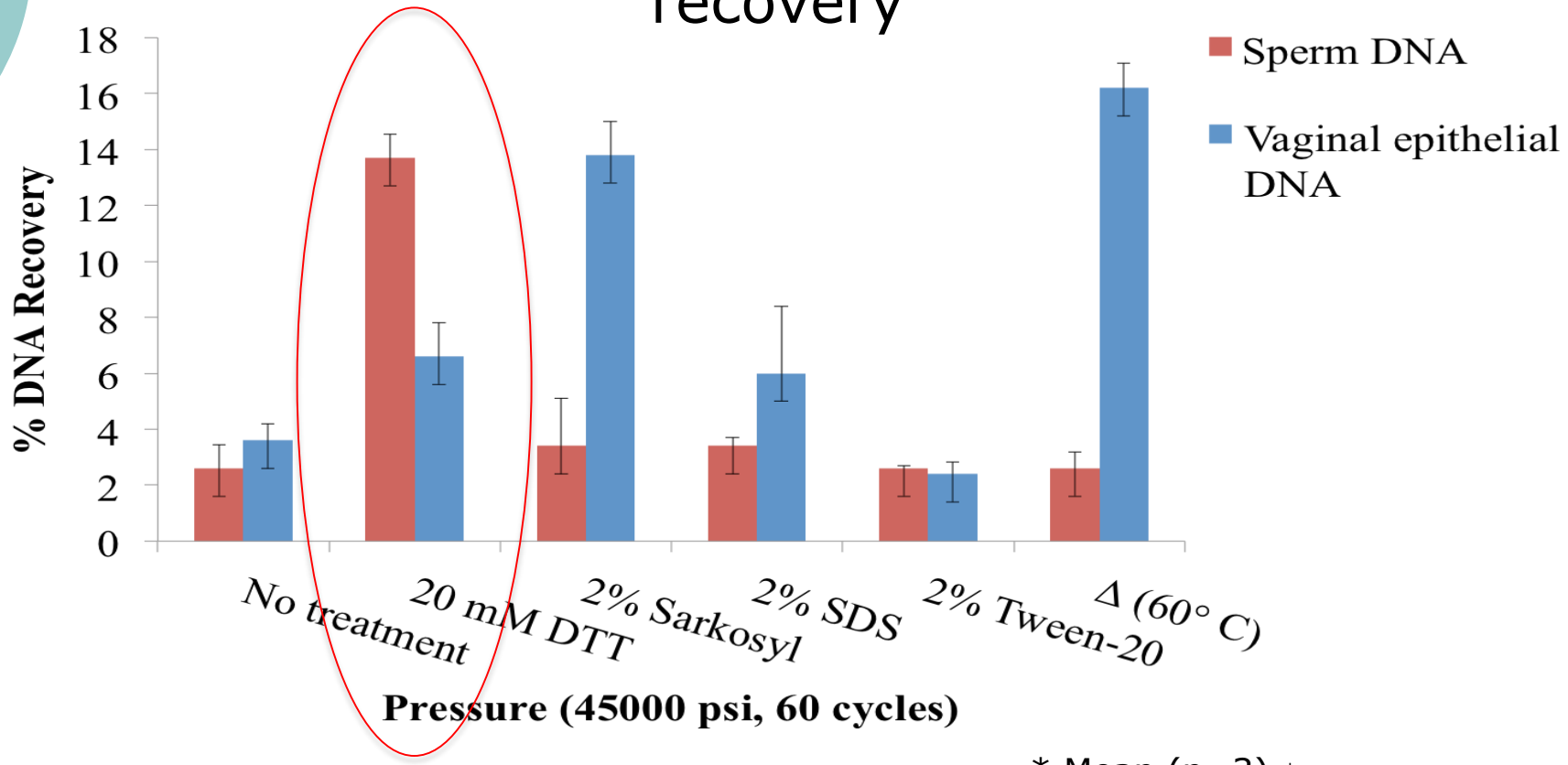


Low recovery

* Mean (n=3) ± SE

Improving DNA recovery

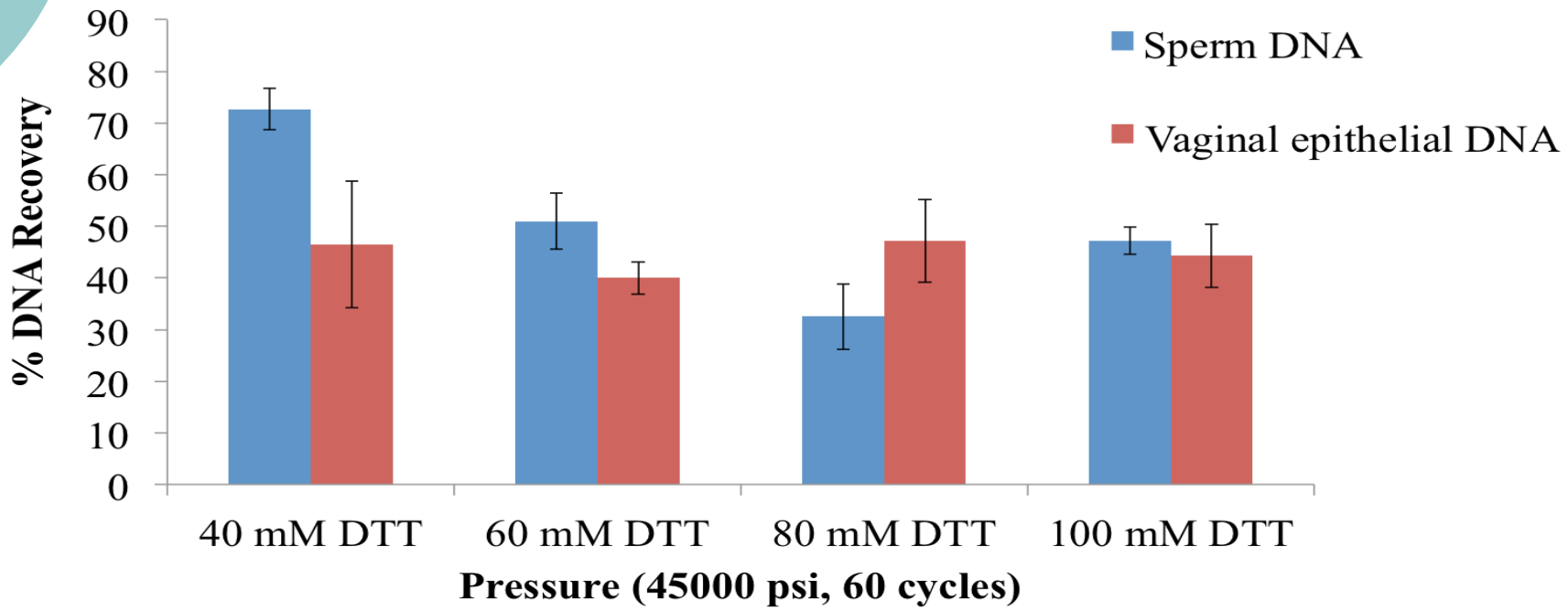
Reducing agent (DTT)- Sperm DNA recovery
Detergent, temperature- Epithelial DNA recovery



* 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

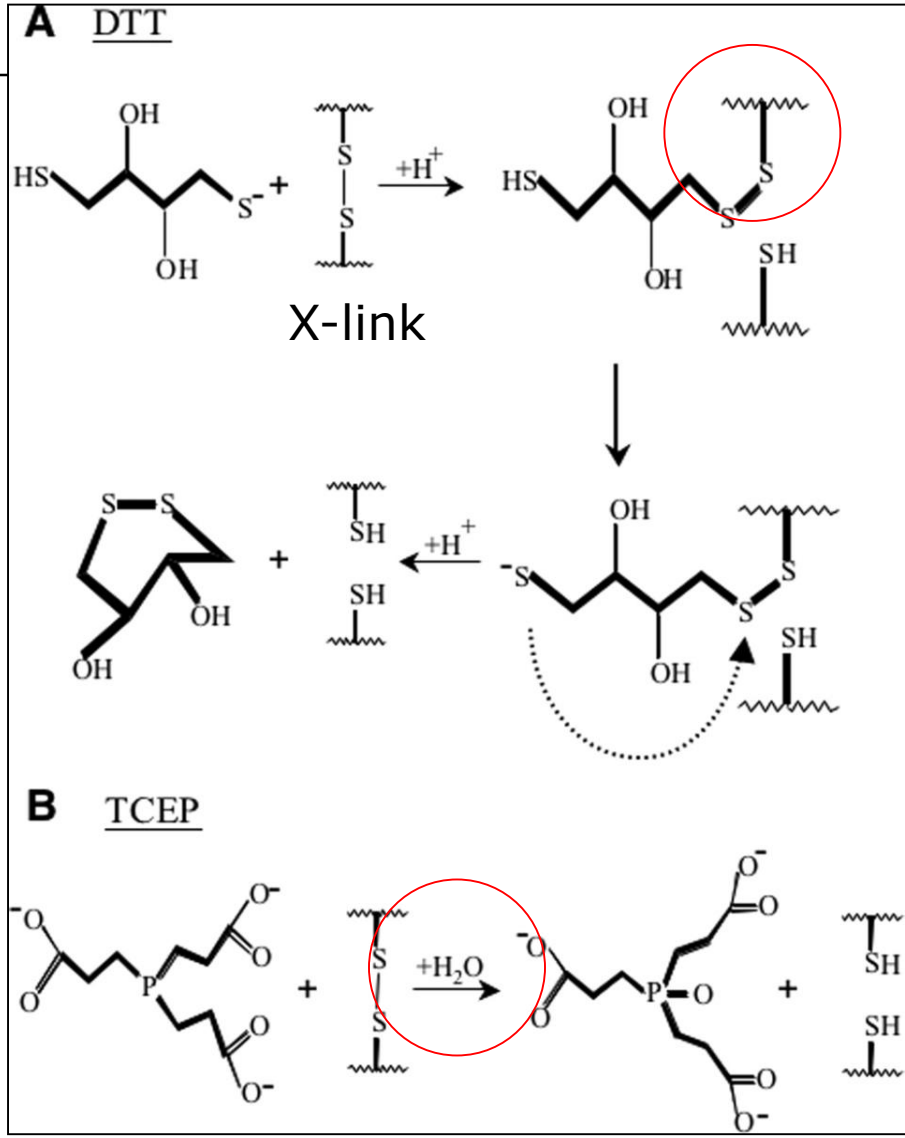
Goal: improve reduction of dithiol linkages for sperm protamines

Advantages to TCEP

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

Disadvantage

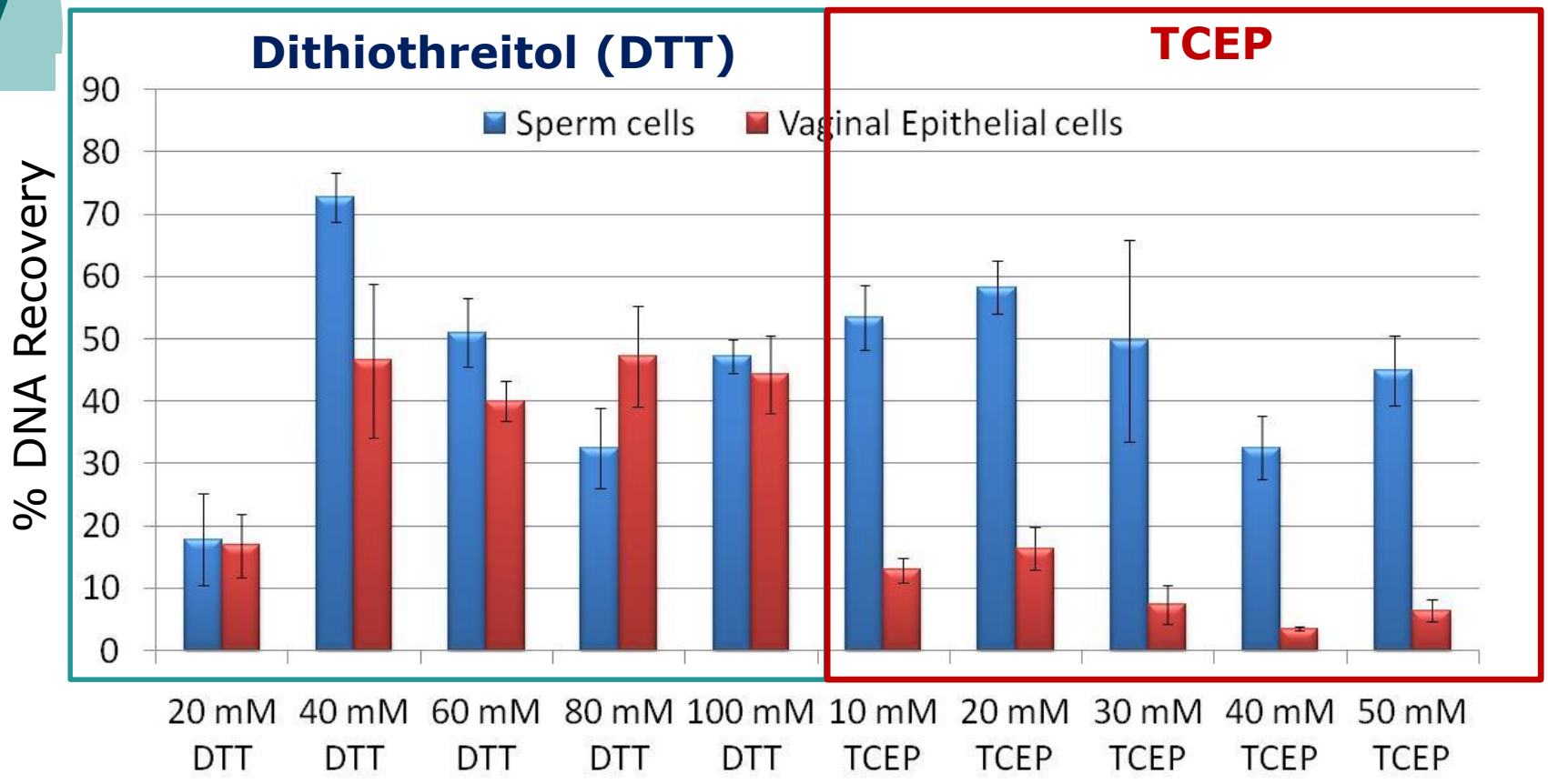
TCEP is not particularly stable in phosphate buffers



Bhasin, J Biological chem., 279 (44) 5865–45874, 2004.

A comparison: DTT vs. TCEP

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



Recovery- 73%±4
Selectivity- 1.5/1

Recovery- 58%± 4
Selectivity- 9/1

DNA recovery from swabs

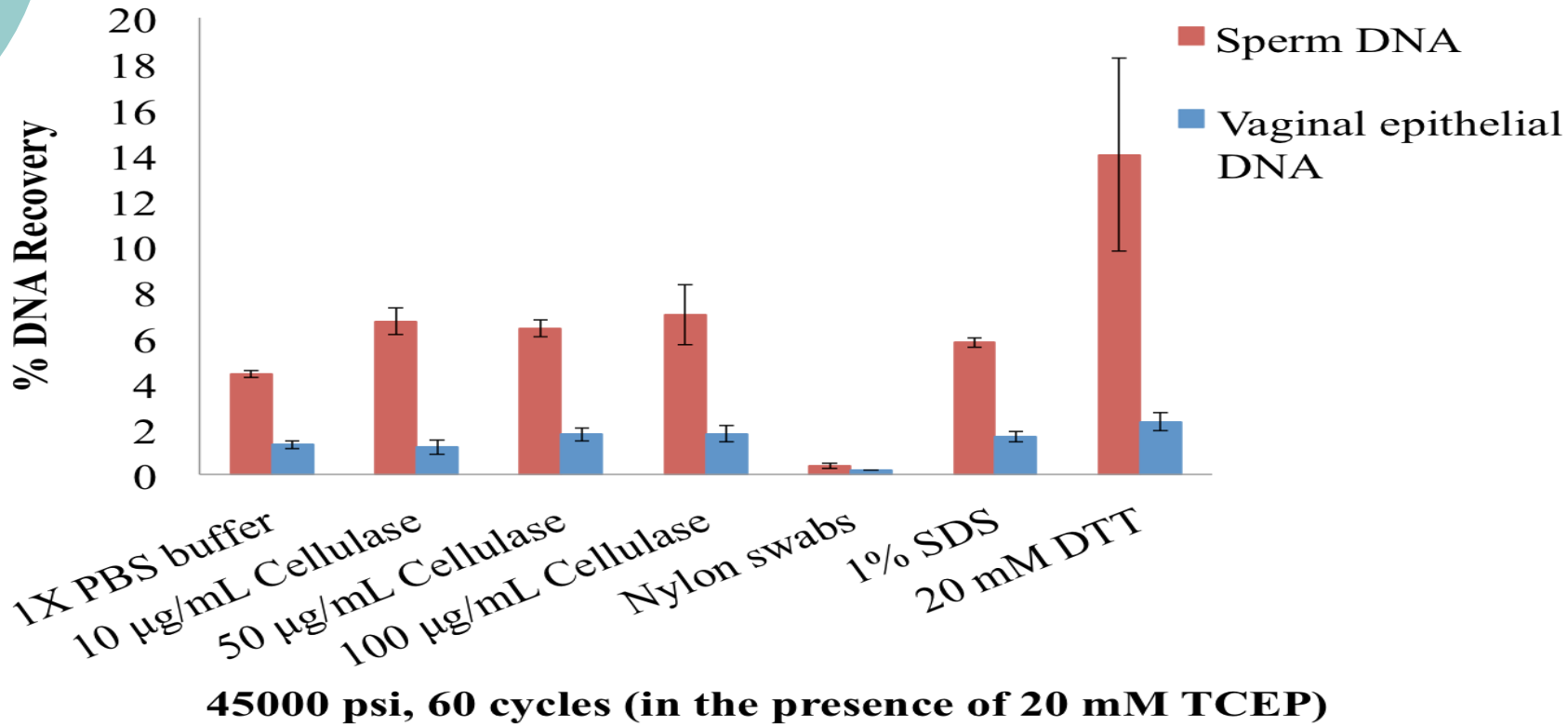
1. The next step was to access real samples and ideally combine cell removal and lysis using high pressure pulsing

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



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

* Mean (n=3) ± SE

Improving DNA recovery from cotton swabs using alkaline lysis

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence

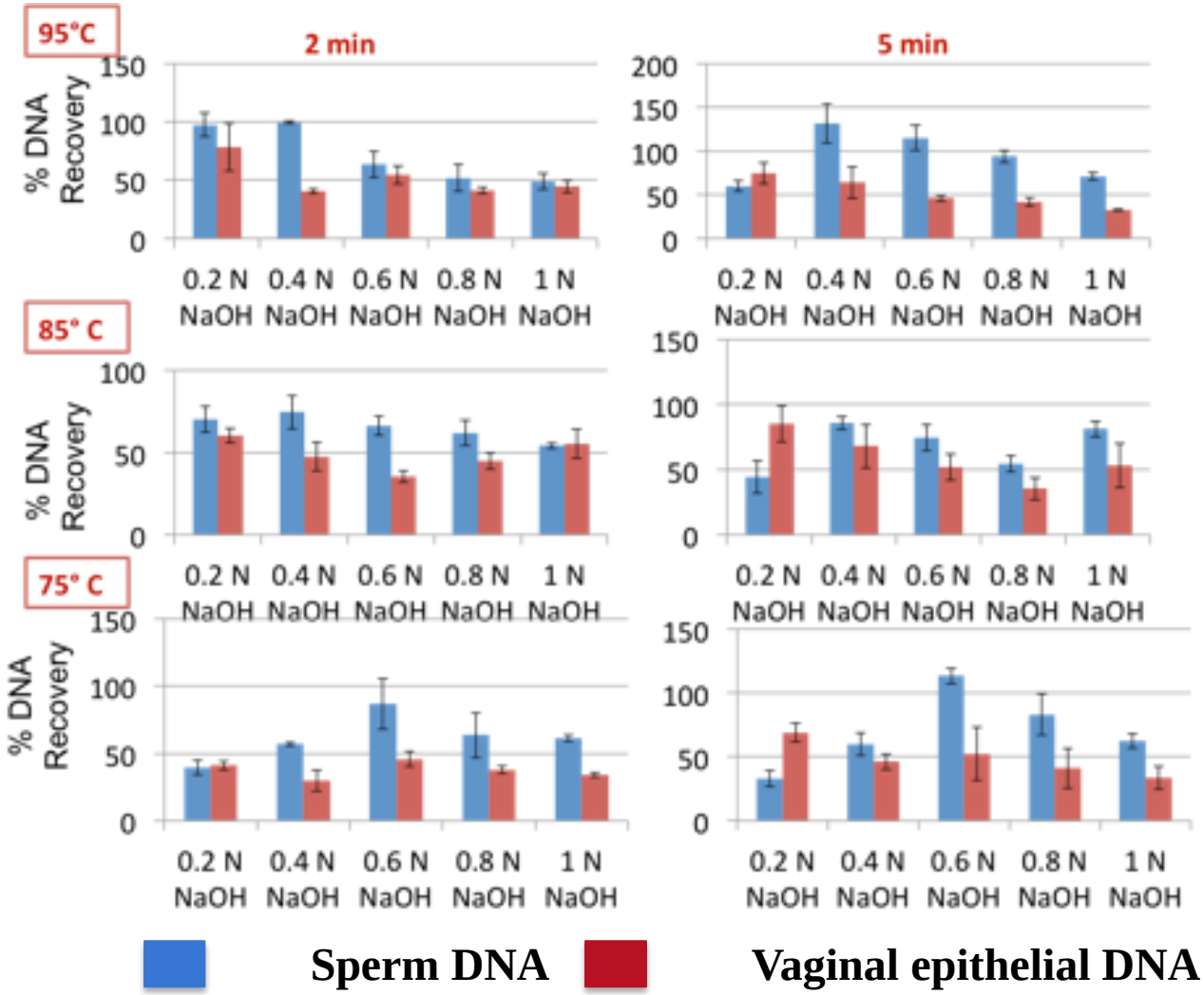
William R. Hudlow*, Martin R. Buoncristiani

California Department of Justice Jan Bashinski DNA Laboratory, 1001 W. Cutting Blvd., Suite 110, Richmond, CA 94804, USA

- 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

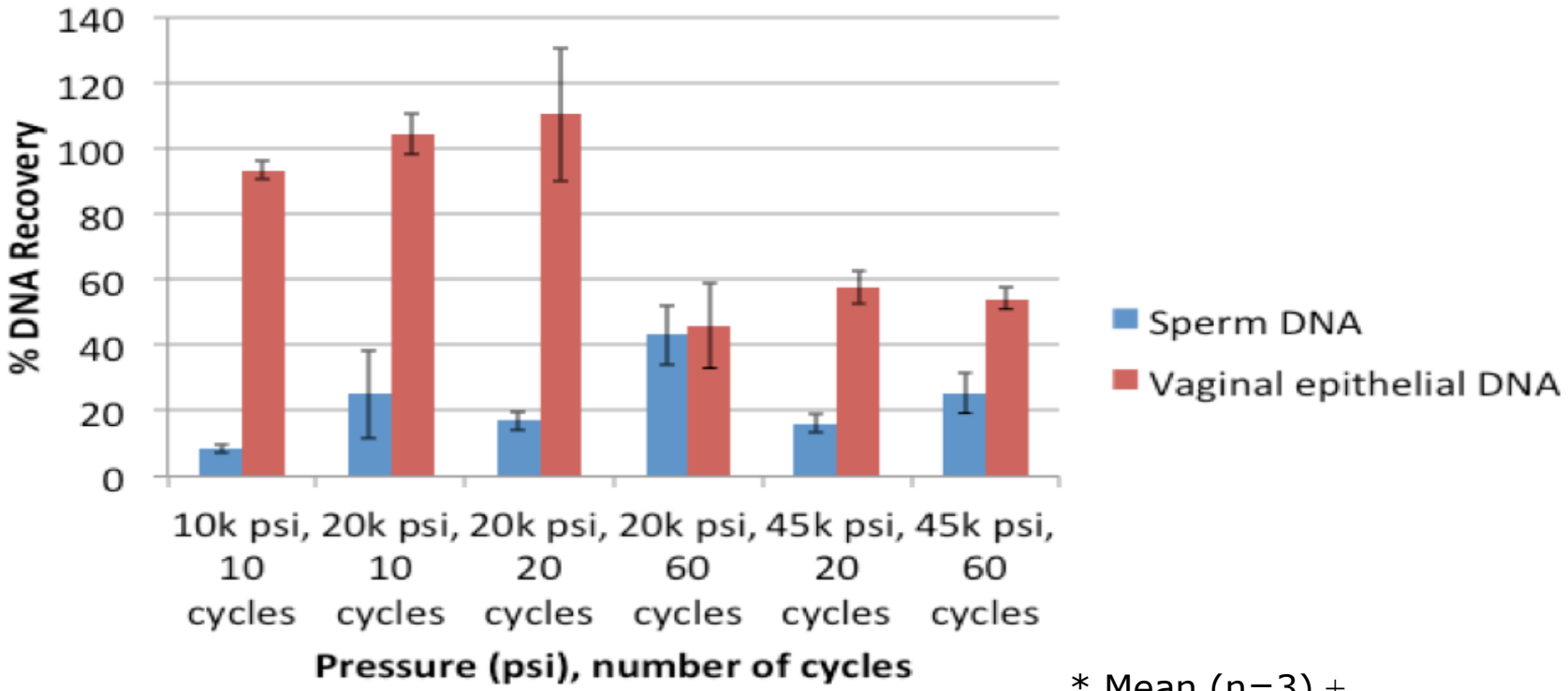


* Mean (n=3) ± SE

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

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

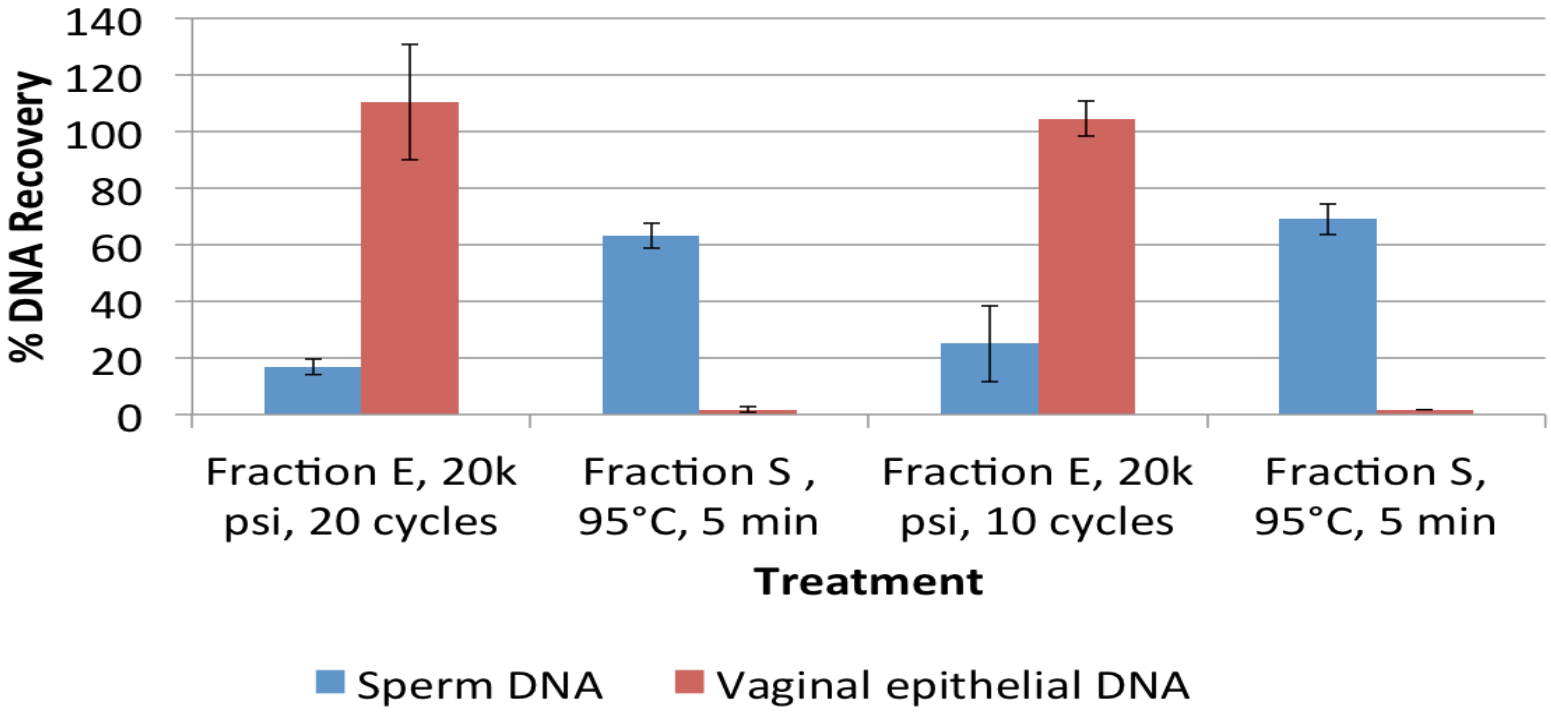
Purify liquid fraction with PCIA

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

Nori, D.; McCord, B. Analytical Bioanalytical Chemistry, 2015 published on-line 6/15

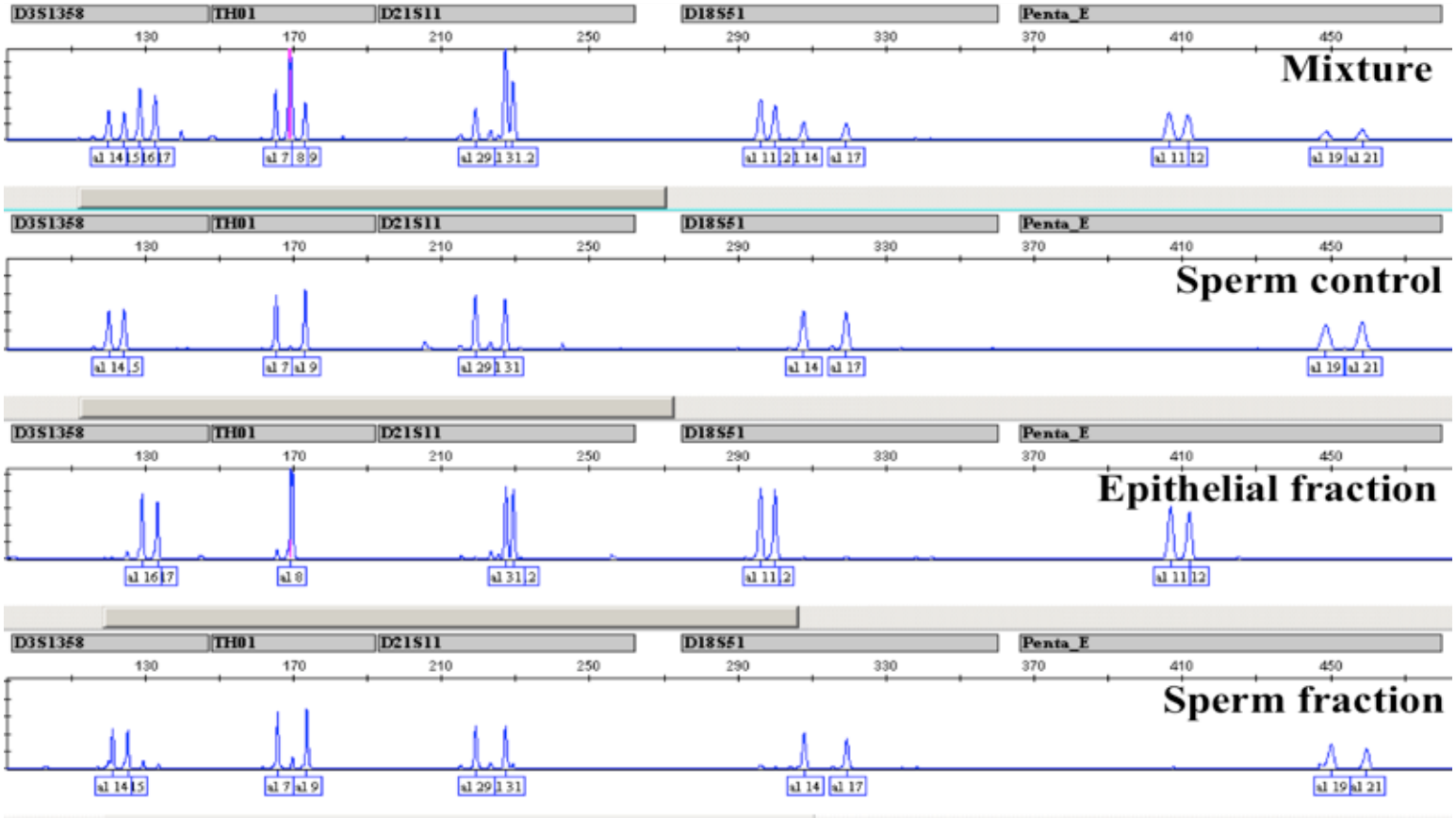
PCT treatment + Alkaline lysis

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



* Mean (n=3) ± SE

PCT treatment + Alkaline lysis - DNA profile of control mixture

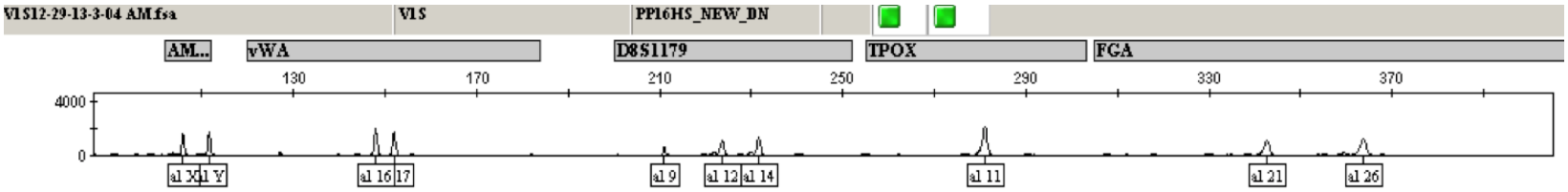


How does this compare to the current method?

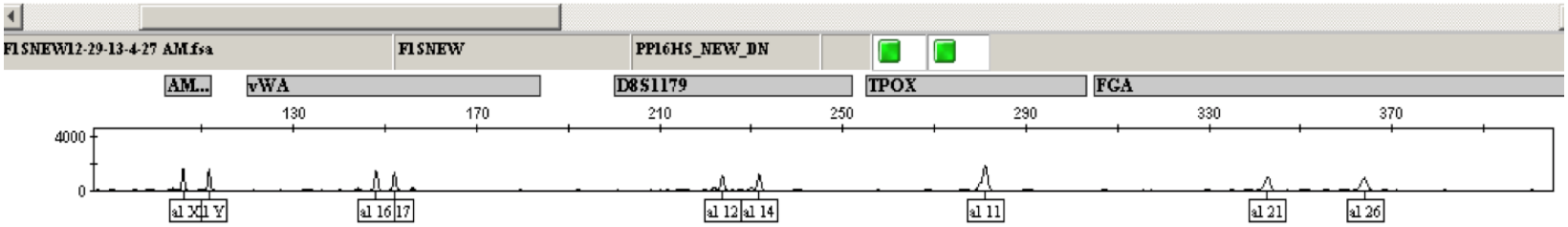
- Broward Sheriff's Office (BSO) crime lab protocol

vs. Alkaline lysis and PCT Post coital sample

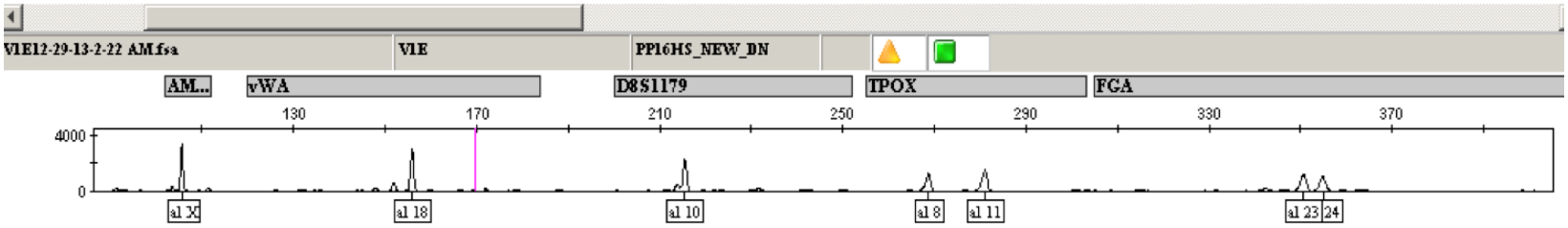
Sperm (BSO)



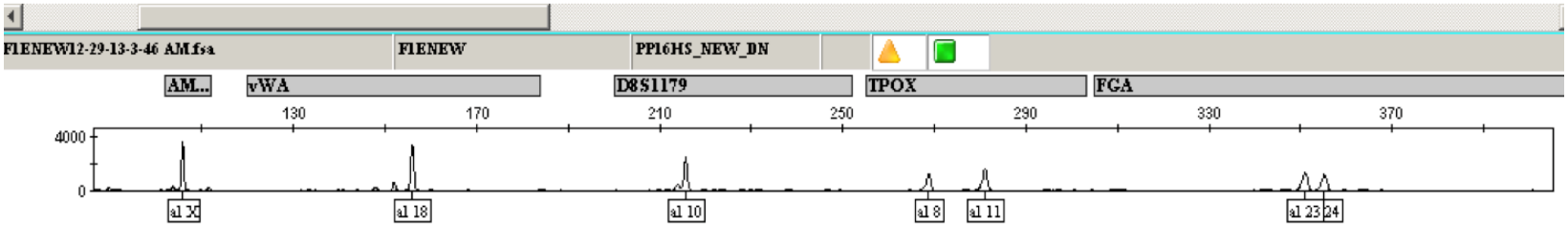
Sperm (PCT)



Epi (BSO)



Epi (PCT)



Mixture Ratio Study

- Varying ratios of male to female cells

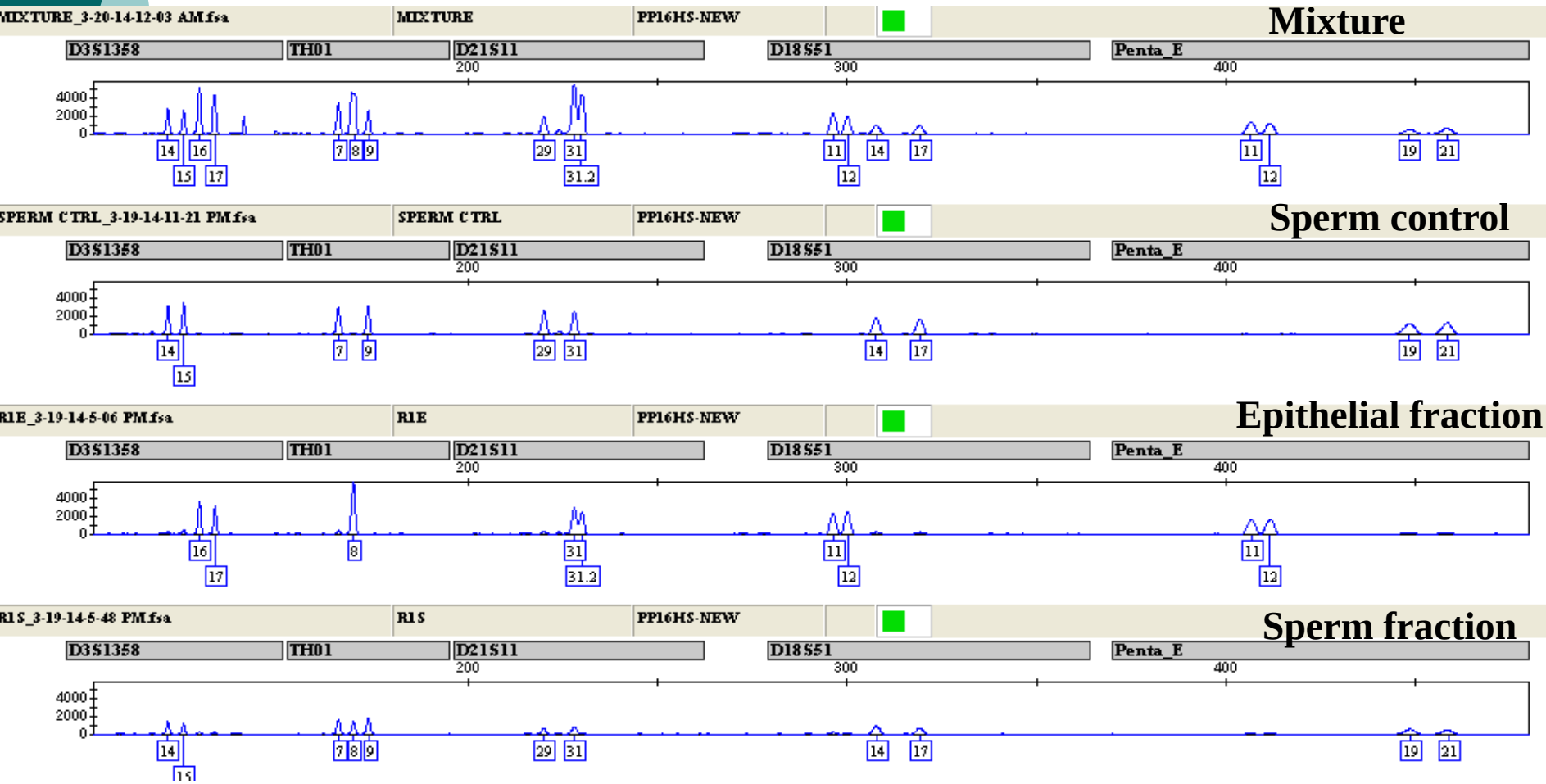
Ratio of male: female	Number of sperm cells	Number of vaginal epithelial cells
1:1	96000	96000
1:2	48000	96000
1:5	19200	96000
1:10	9600	96000
1:50	3000	150000

Some Validation Studies

- Mixture ratios
- Substrates
- Environmental and PCR inhibition
- Post coital samples and comparison with standard procedures

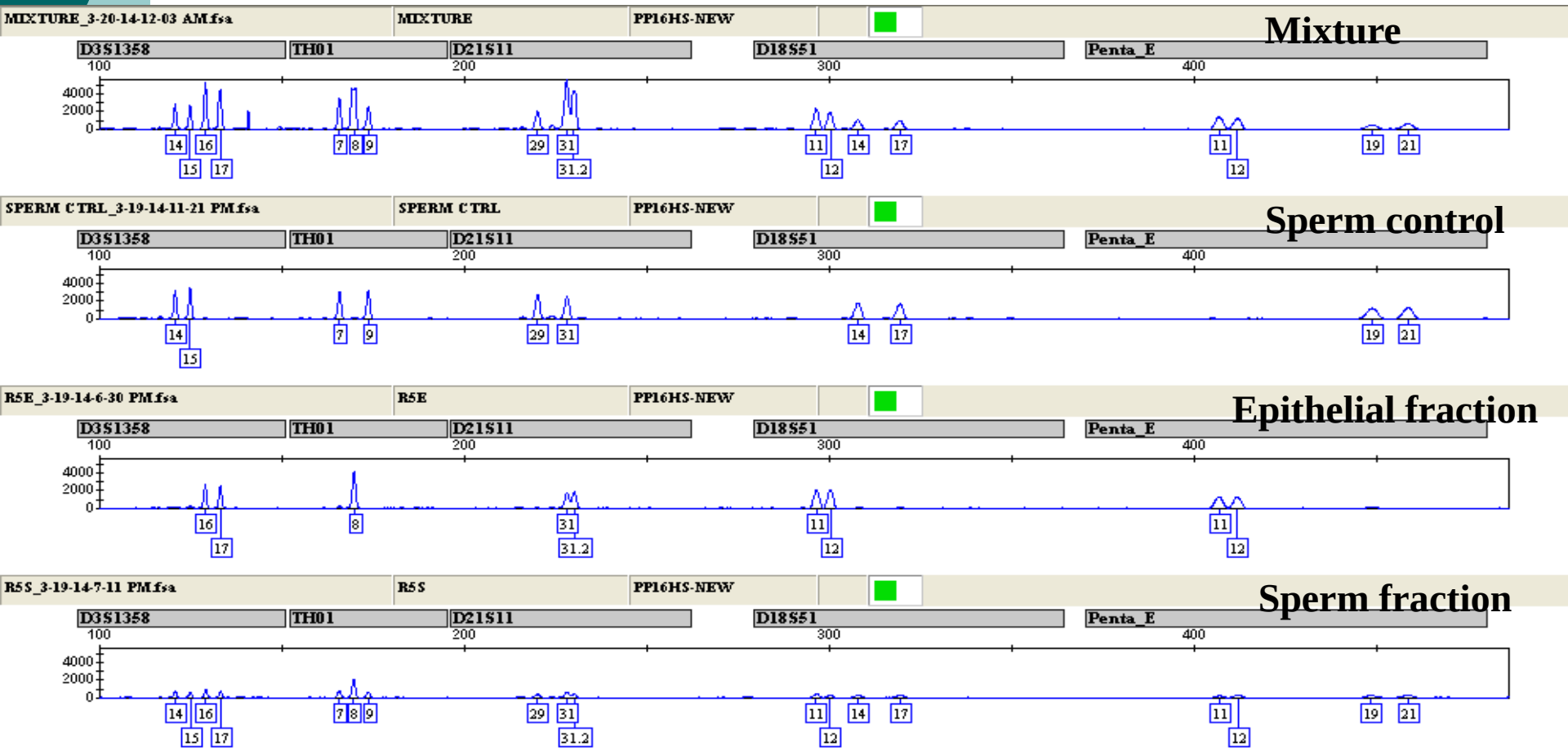
Sensitivity studies- 1:2 male to female cells

- Clean male autosomal profile obtained



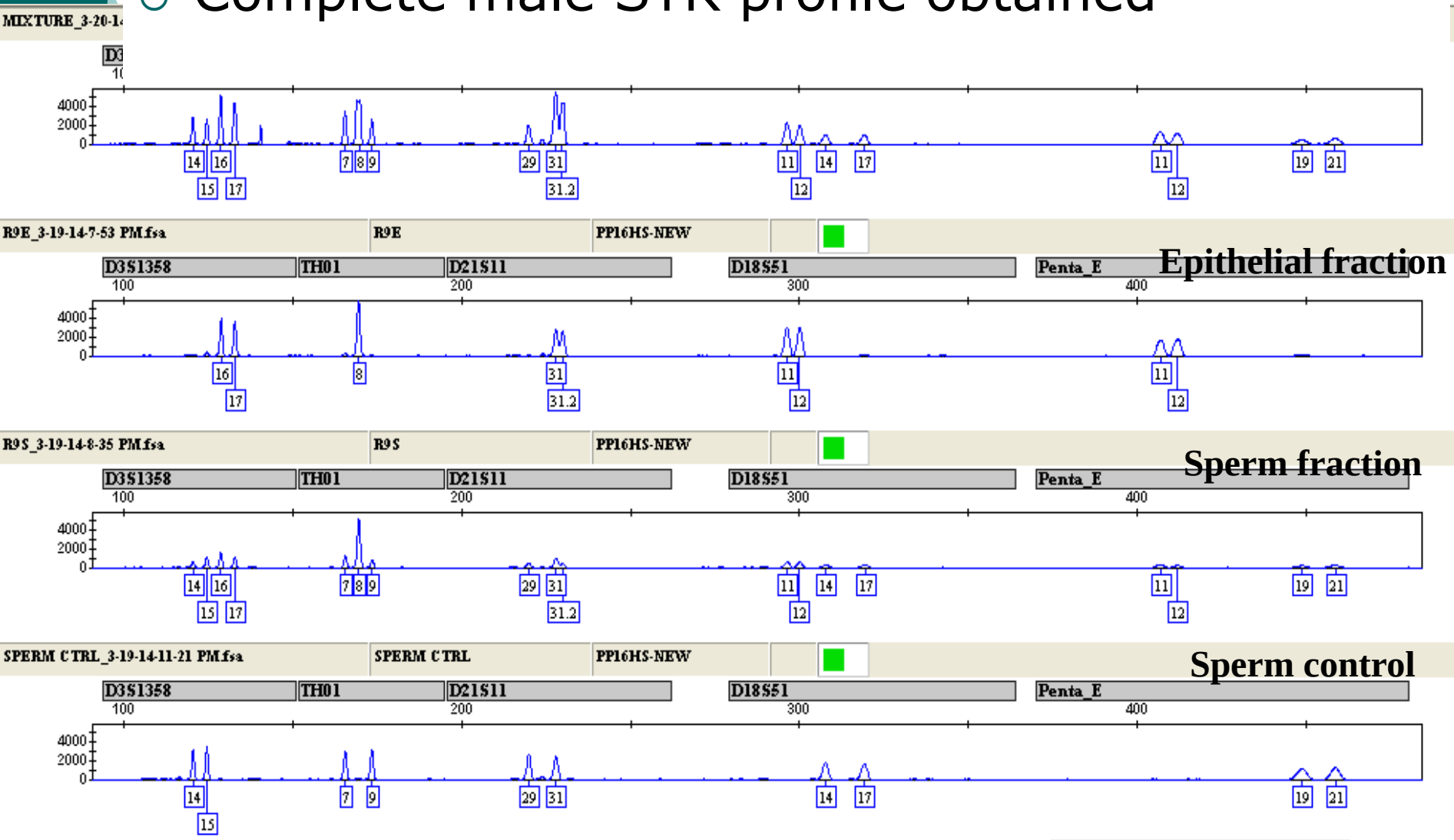
Sensitivity studies- 1:5 male to female cells

- Mixed profile
- (complete male autosomal STR profile observed)



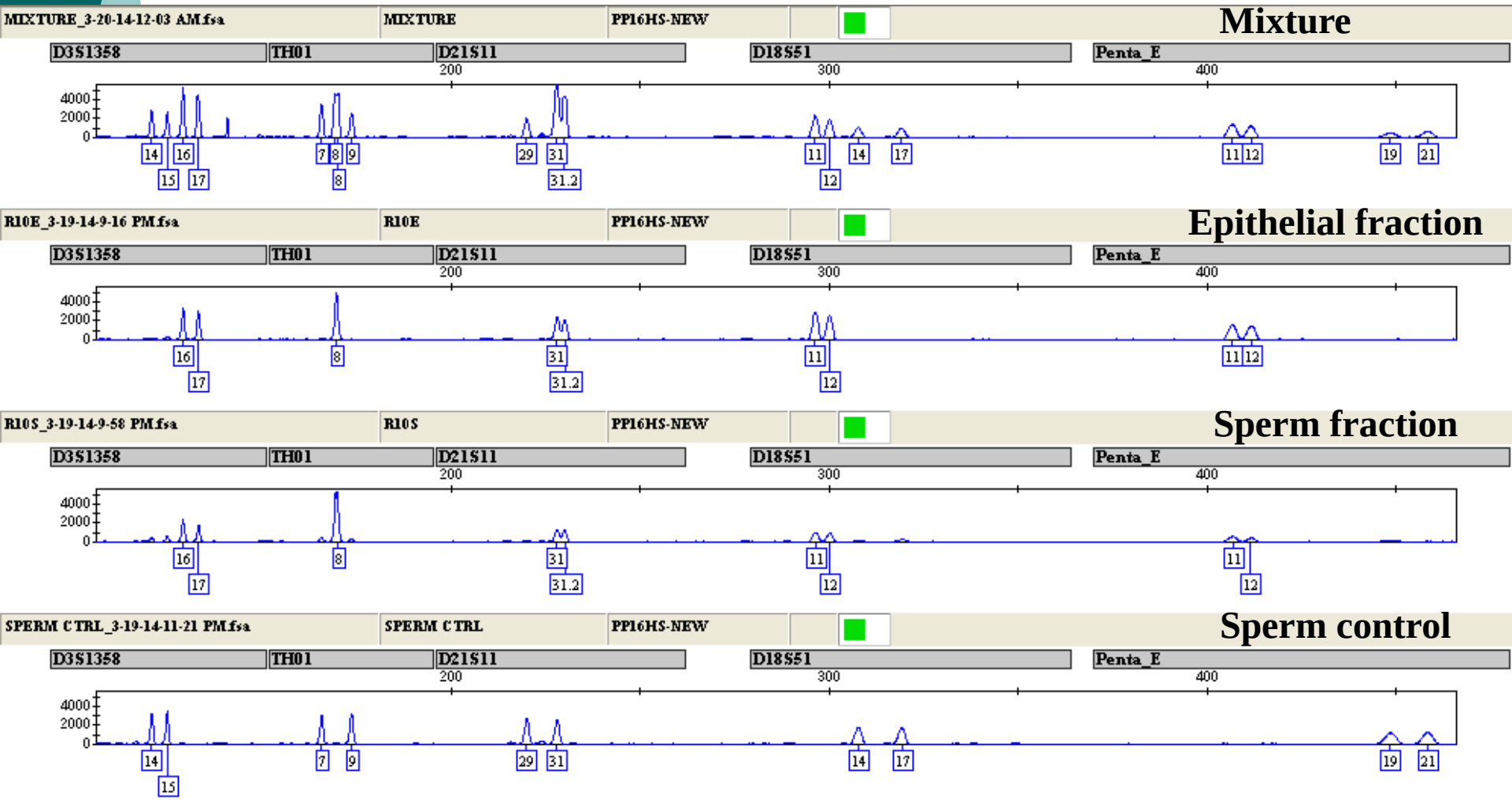
Sensitivity 1:10 male to female cells

- Mixed profile- major contribution by female
- Complete male STR profile obtained

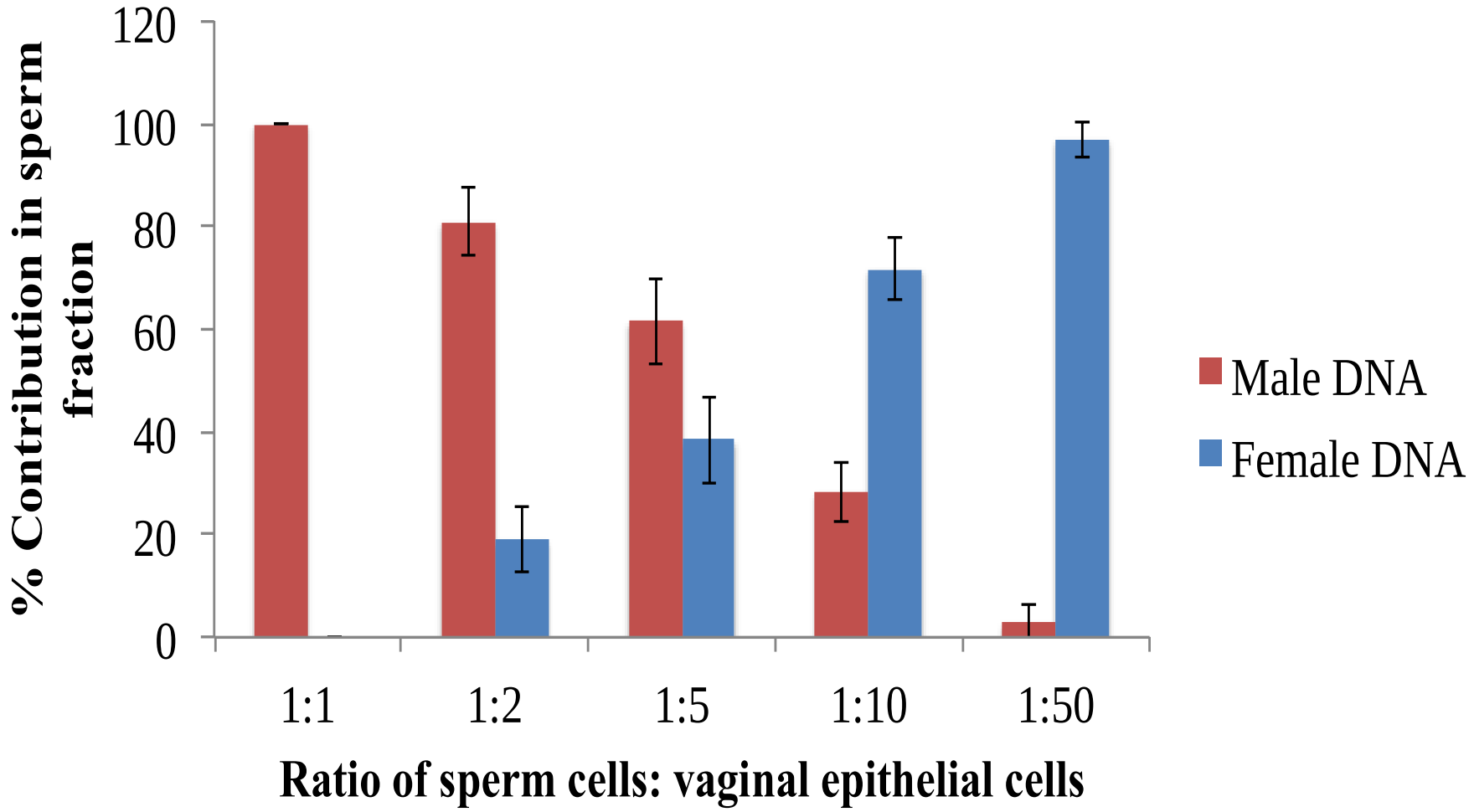


Sensitivity 1:50 male to female cells

- Loss of male alleles observed



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



* Mean (n=7) ± SE

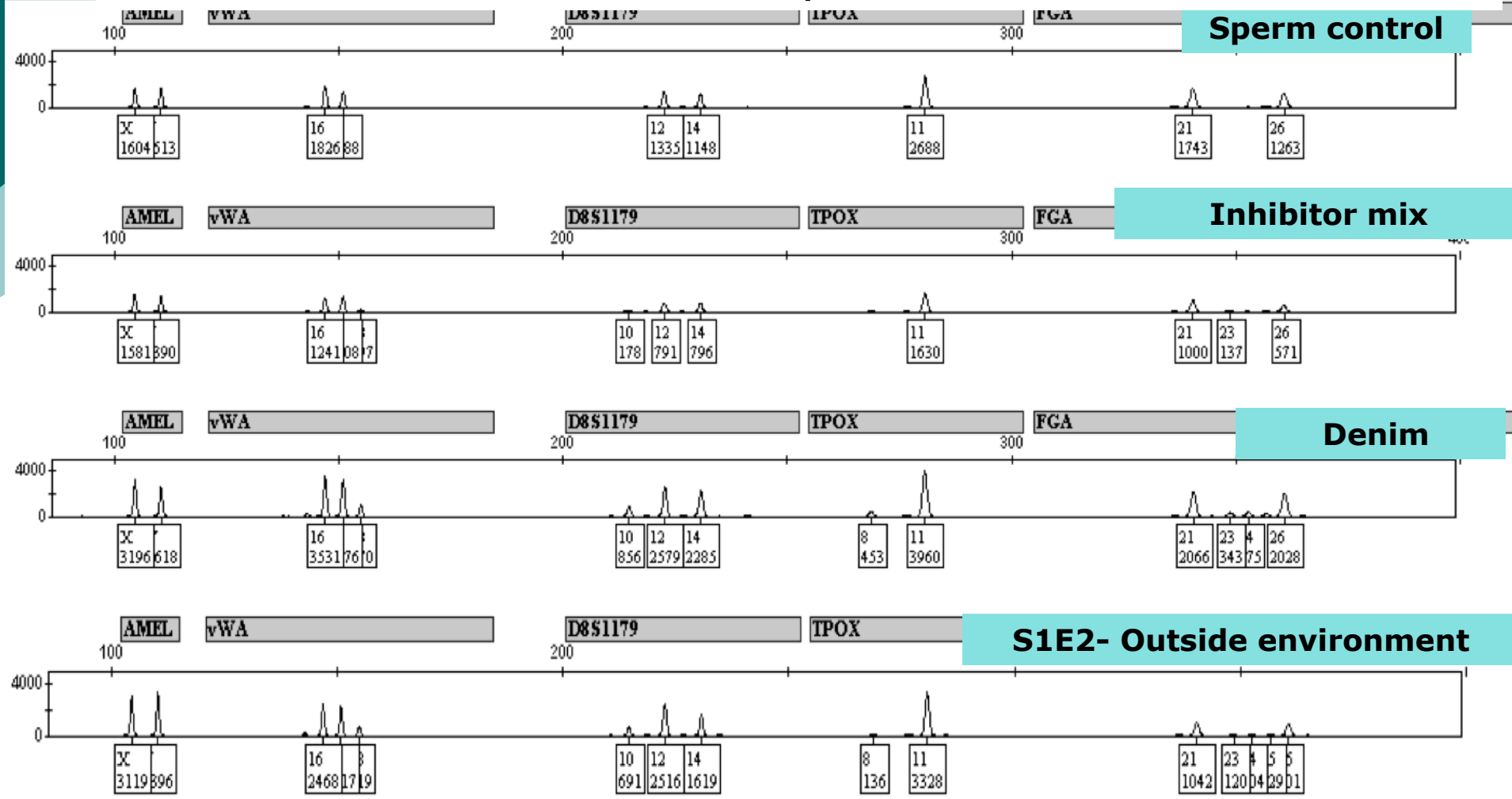
STABILITY STUDIES

- 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

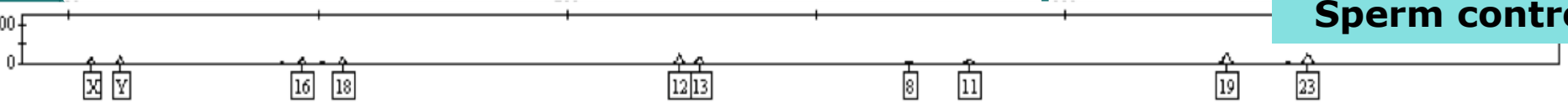
Stability studies

A complete male STR profile was obtained from all samples

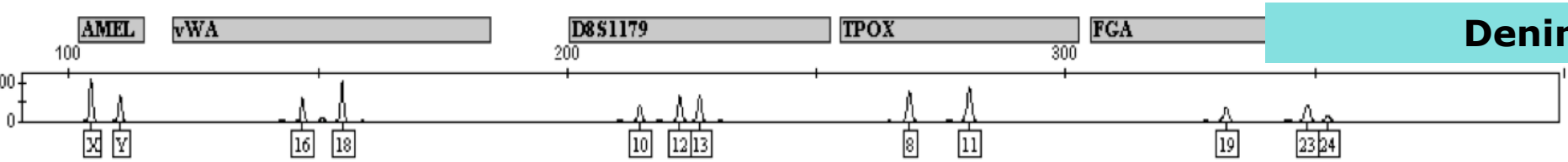


Additional samples

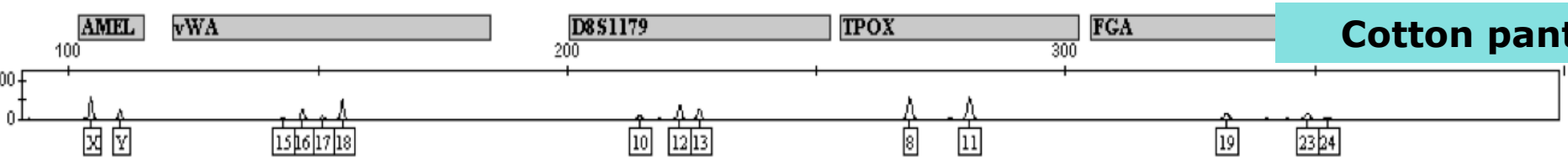
Sperm control



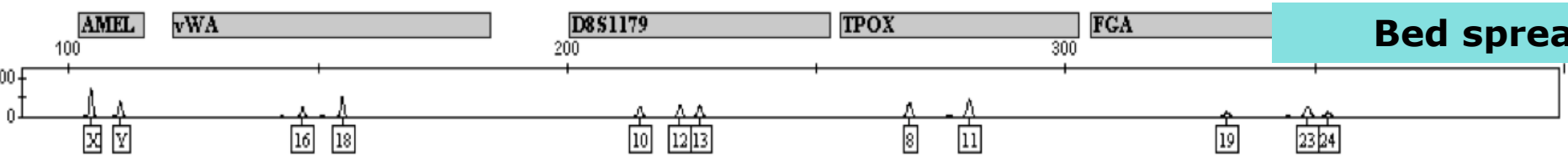
Denim



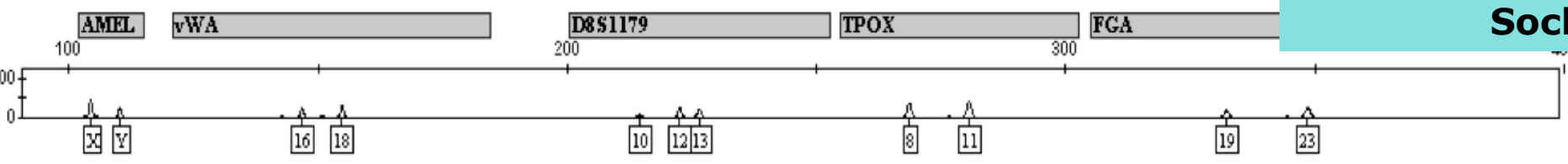
Cotton panty



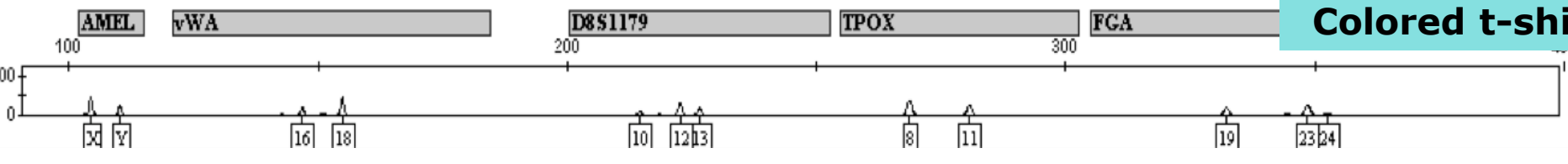
Bed spread



Socks



Colored t-shirt

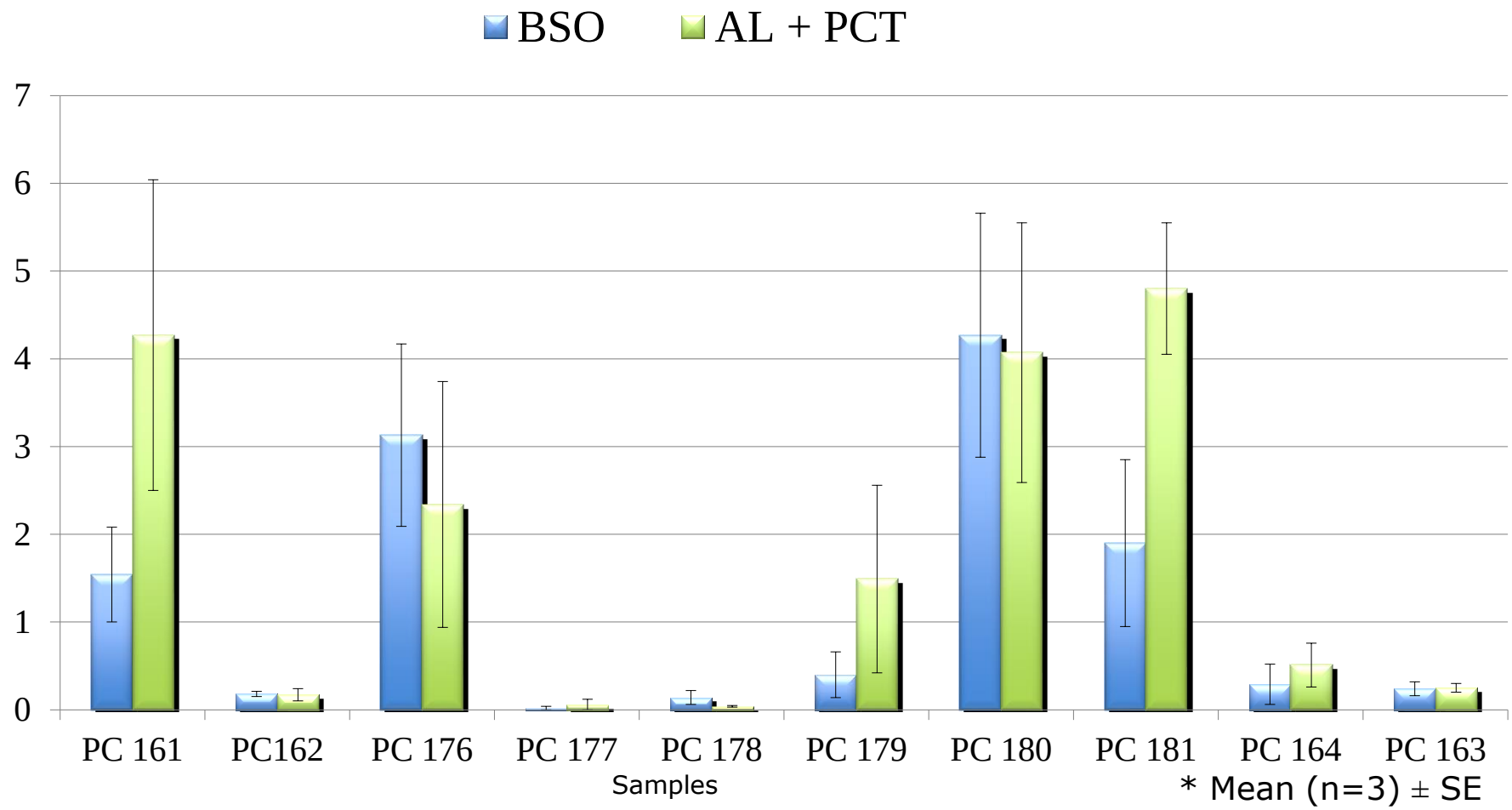


CORRELATION STUDIES

- 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 w/ standard techniques



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

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Questions?

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