DNA recovery by pressure cycling and its potential application to differential extraction

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Introduction

- NIJ is currently spending millions on DNA backlog reduction and recovery of degraded evidence
- A big issue is difficult extraction methods relying on specific buffers, sonification and other steps to isolate and remove DNA from substrates
- Efficiency and specificity of extraction is an important issue especially with sexual assault and touch samples.
- We are exploring pressure based extraction

What are the issues in extraction?

1. Removal of cells from a substrate – release from swabs, clothing.
2. Disruption of cell membranes and release of DNA – osmotic shock, detergents, enzymes
3. Isolation of DNA from cellular components - lipids, proteins – PCIA, chaotropic silica, Ion exchange
4. Differential extraction of male vs female cells

What about removal of cells?

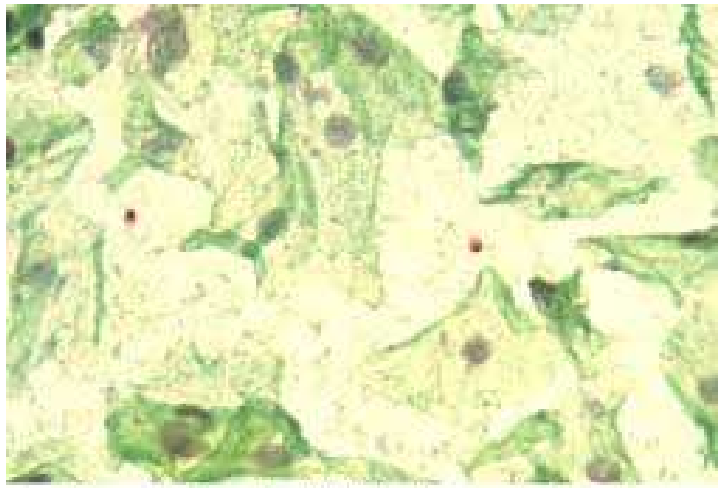
Cells are removed from swabs by soaking in isotonic solutions, followed by vortexing and centrifugation.

- Alternatively they are extracted directly from the swab.
- As little as 10% of cells on the swab may be removed depending on the procedure.
- These issues are exacerbated by large excesses of female cells or when few cells are present.

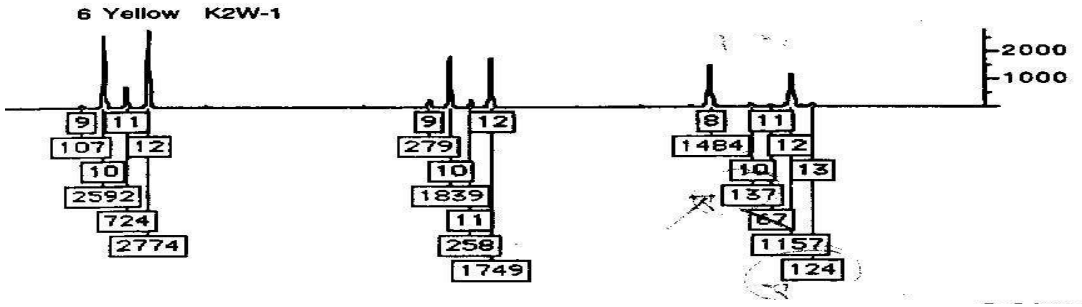
Voorhees, JC, Ferrance, J, and Landers, J, J. Forensic Sci., pp. 574-579, May 2006.

What problems exist with differential extraction?

1. The manual procedures are cumbersome and time consuming.
2. Sperm cells may lyse during initial digestion.
3. Female cells may not completely digest and contaminate sperm pellet

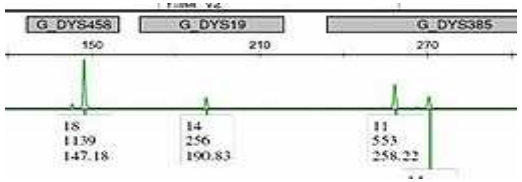


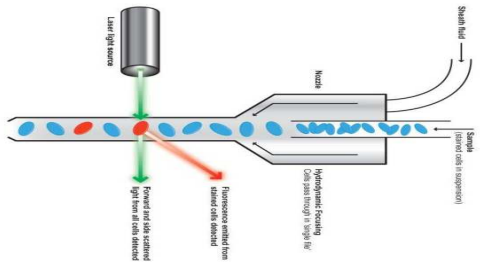
Sperm heads and epithelial cells as viewed under a high powered microscope using Christmas tree stain



Alternatives to differential extraction

- Y STR typing
- Laser microdissection
- Cell sorting via microdevices or flow cytometry
- Pressure cycling





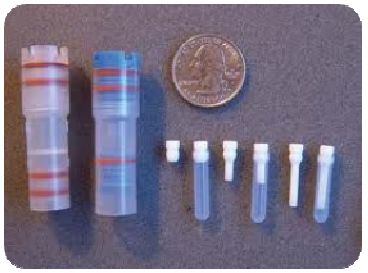
What is pressurized extraction?

- Cycles of pressure pulses are used to disrupt cell membranes and combined with specific buffers to isolate individual cell components.

a) Pressures can range from 5-45K PSI with up to 100 cycles.

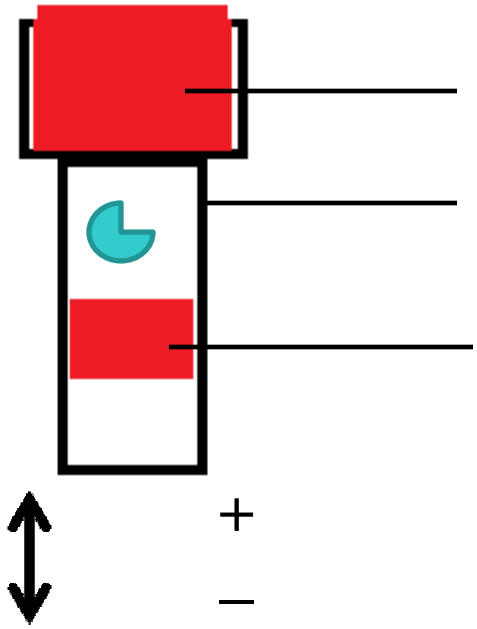
b) Samples are placed in special "pulse " tubes which permit application to individual samples.

c) Temperature and buffer conditions are varied to optimize release and digestion of cells.



Pulse Tubes

Cycles of pressure and release are applied by a ram to a pulse tube.
Duration, # of pulses can be controlled.



Fixed cap

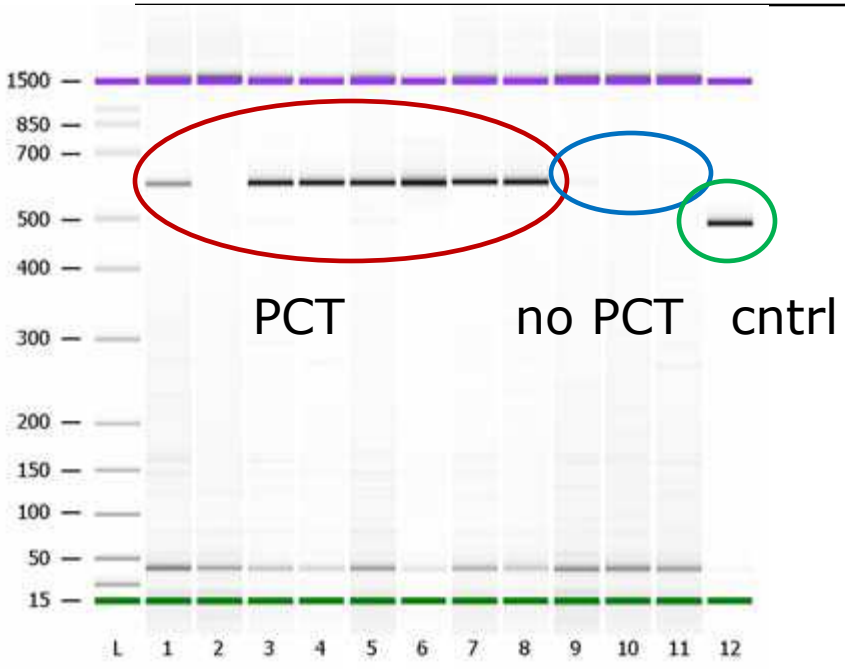
Pulse tube

Ram

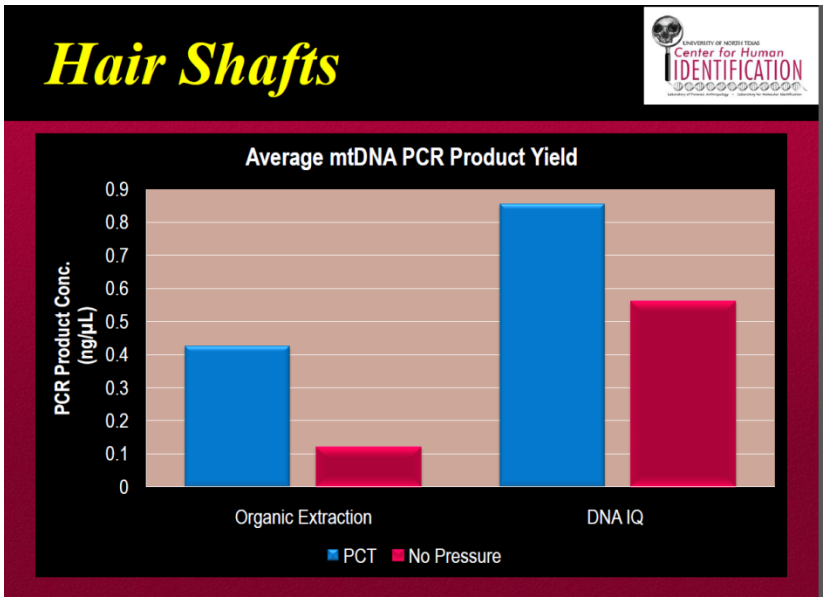
- Moveable Ram transfers pressure to sample ( )

What has been achieved thus far?

DNA from Bone



DNA from hair



PCR products from pig bone extracts

Lanes 1-8 bone incubated with acetic acid/EDTA for 60 min and followed by 10 cycles of PCT at 4°C.

Lanes 9-11 were incubated with acetic acid for 1 hr but no PCT

PCR products from hair

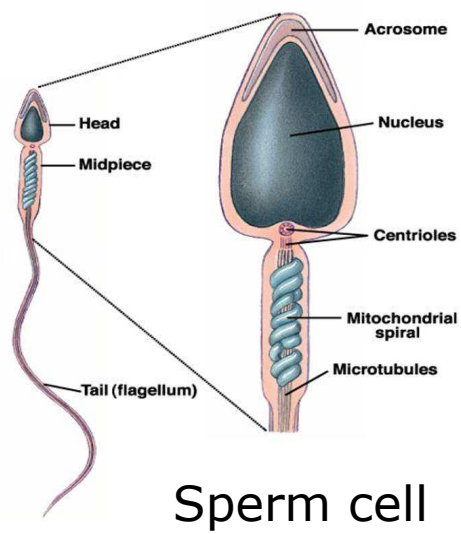
UNT data – organic vs DNA IQ w/ and w/out pressure

Note that the pressure treatment does not interfere with enzymatic digestion

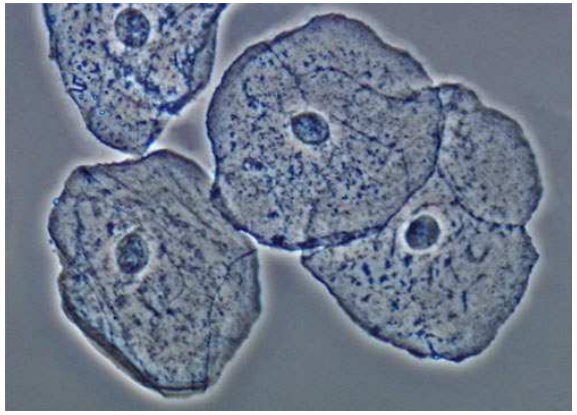
Our hypothesis:

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 as cross linked as protamines – less dense packing (0.2% cysteine)



Sperm cell



Buccal Epithelial cell

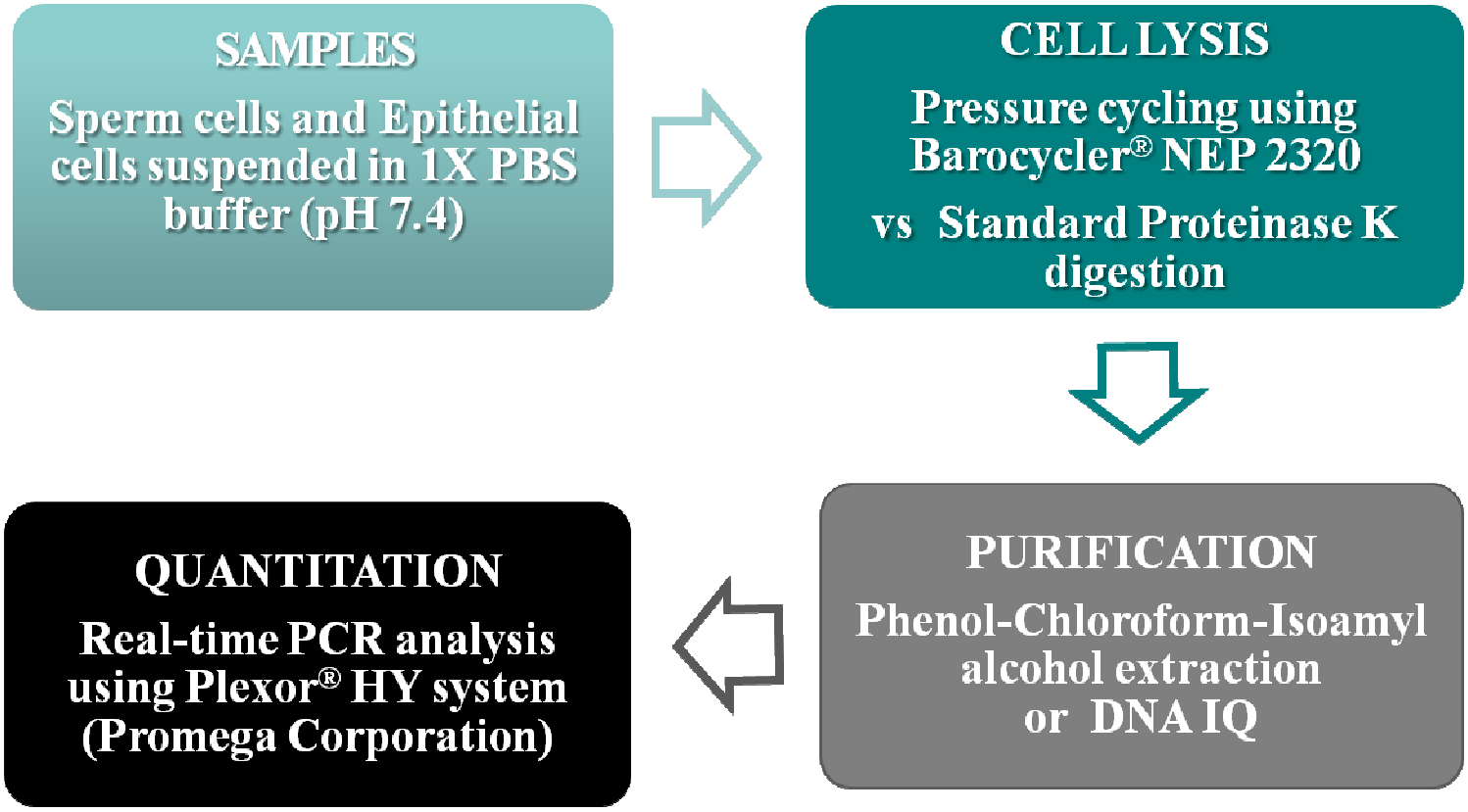
Experimental design

- Explore methods to preferentially lyse cells – leaving behind intact cells of specific type
- Alter physical parameters – pulse pressure, cycle number, temperature and time
- Utilize differences in buffer content – detergent, enzymes, DTT
- Monitor differential amplification by real time PCR and multiplex STR amplification



Experimental design

Key issue: controlling cell quantities and recovery of DNA



Use of real time PCR and extraction controls to provide confidence in results.

1st Goal: Examine the effect of increasing pressure on recovery of DNA

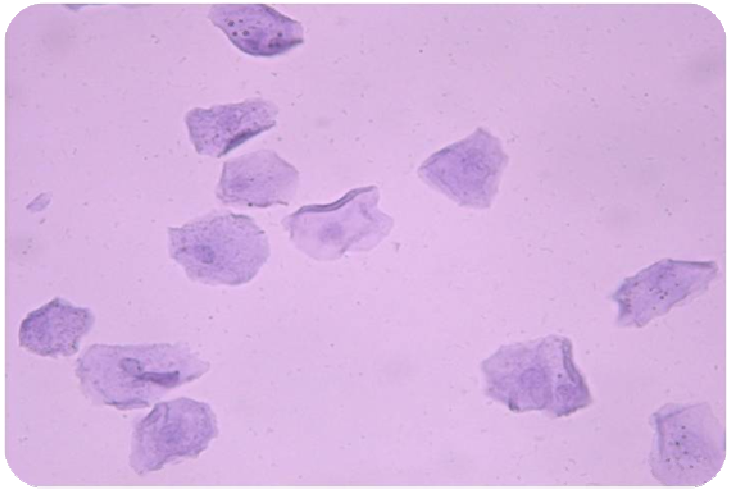
1. Dose response curve

- Buffer- 1X PBS (pH 7.4)
- Liquefied semen sample
  Vaginal epithelial cells extracted from a swab
- Develop similar cell count using hemocytometer- 2.5-3.0 x 10^5 cells/ml
- Compare total DNA in sample by ProK/PCIA with pressure cycling/PCIA using ALU/qPCR

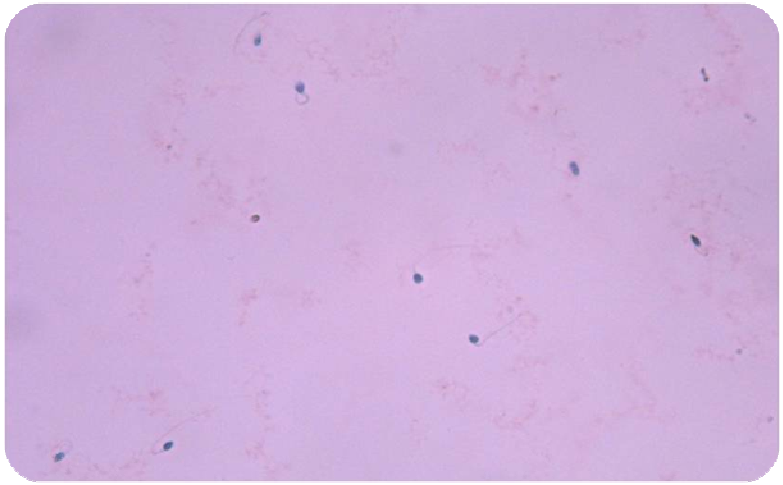
Initial PCT- Microscopic studies

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**



Vaginal epithelial cells

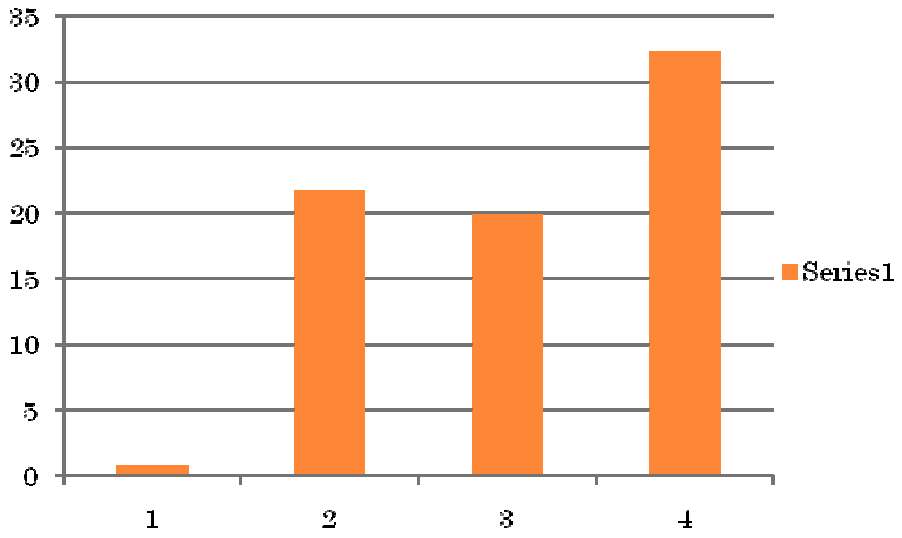


Sperm cells

Effect of pressure/PCIA extraction

- There is a high level of exogenous DNA in these samples ca 1- 2ng/ul
- This level does not change following pressure cycling and appears fairly reproducible
- Following PCIA, however, increasing numbers of cycles at max pressure (35,000 PSI) results in an increase in recovered DNA

ng/uL DNA

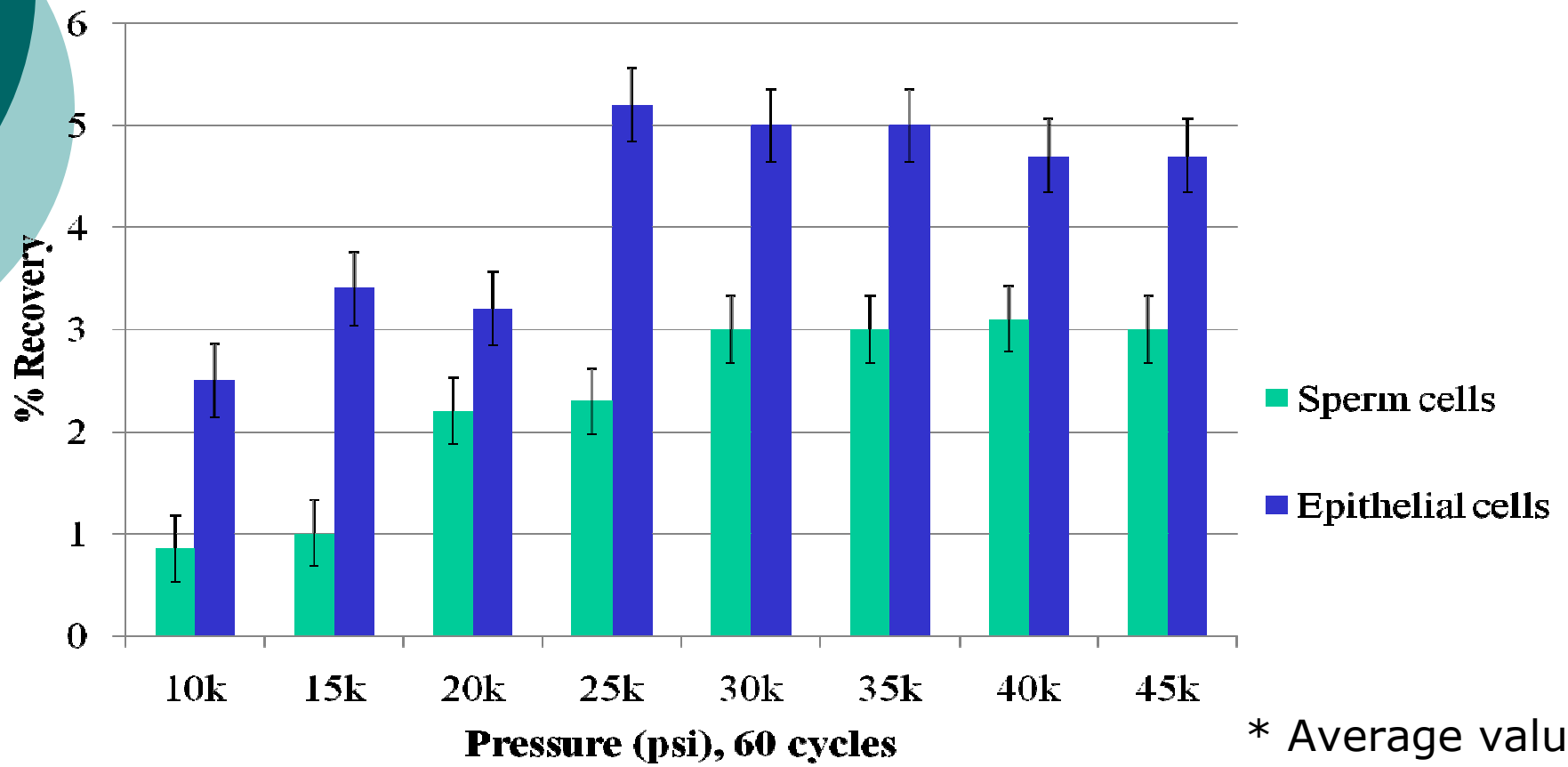


0 20 30 50 # cycles

Neat semen samples (1.5 mL) were dissolved in 1X PBS buffer (2.5 mL) Then subjected to - PCIA

Initial response to pressure treatment

Suspended cells in PBS following pressure treatment vs standard Proteinase K as measured by qPCR

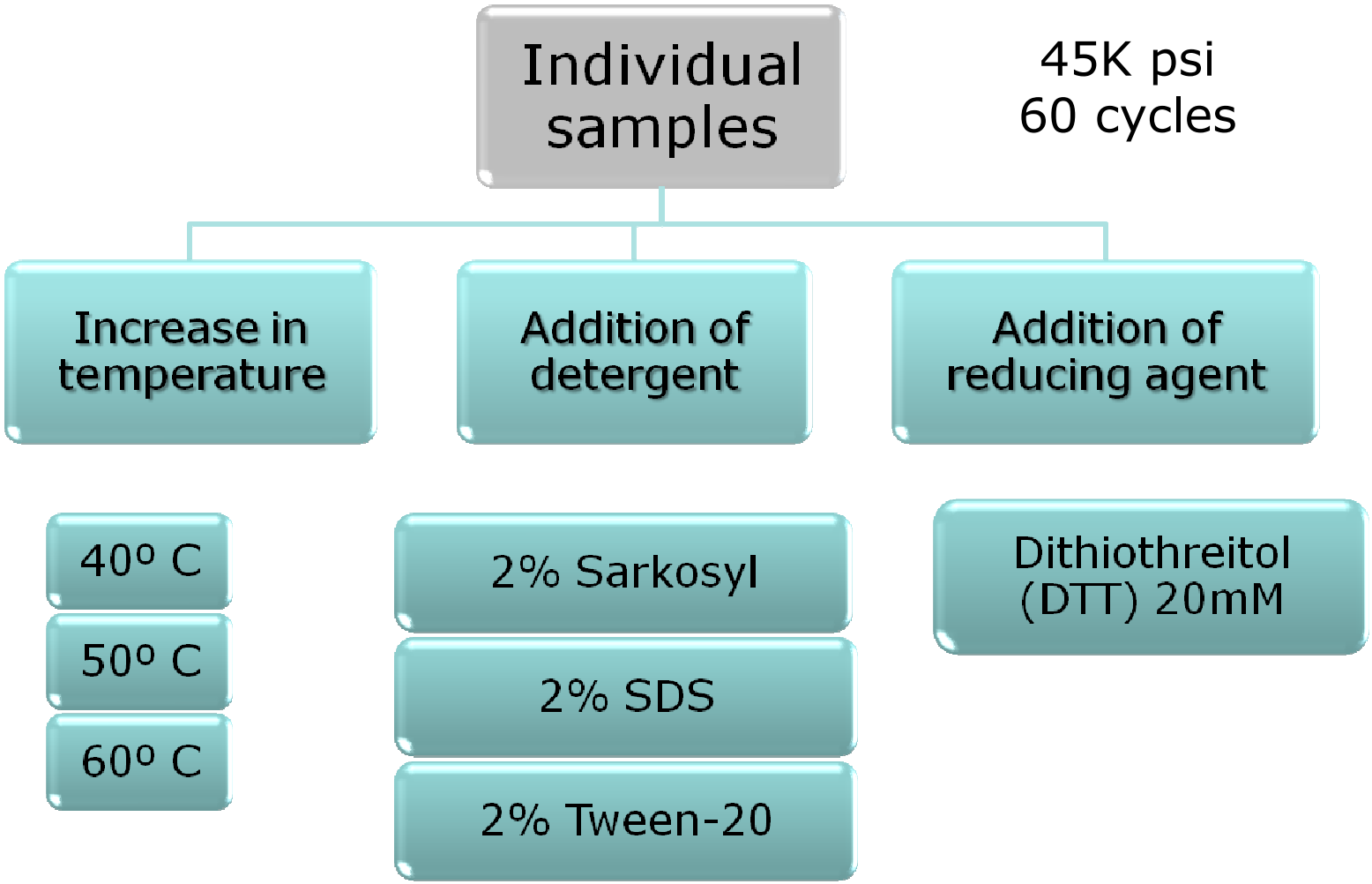


* Average value of triplicates

Results indicate optimal results at 30 PSI with differential extraction at lower pressures
Recovery is relatively low

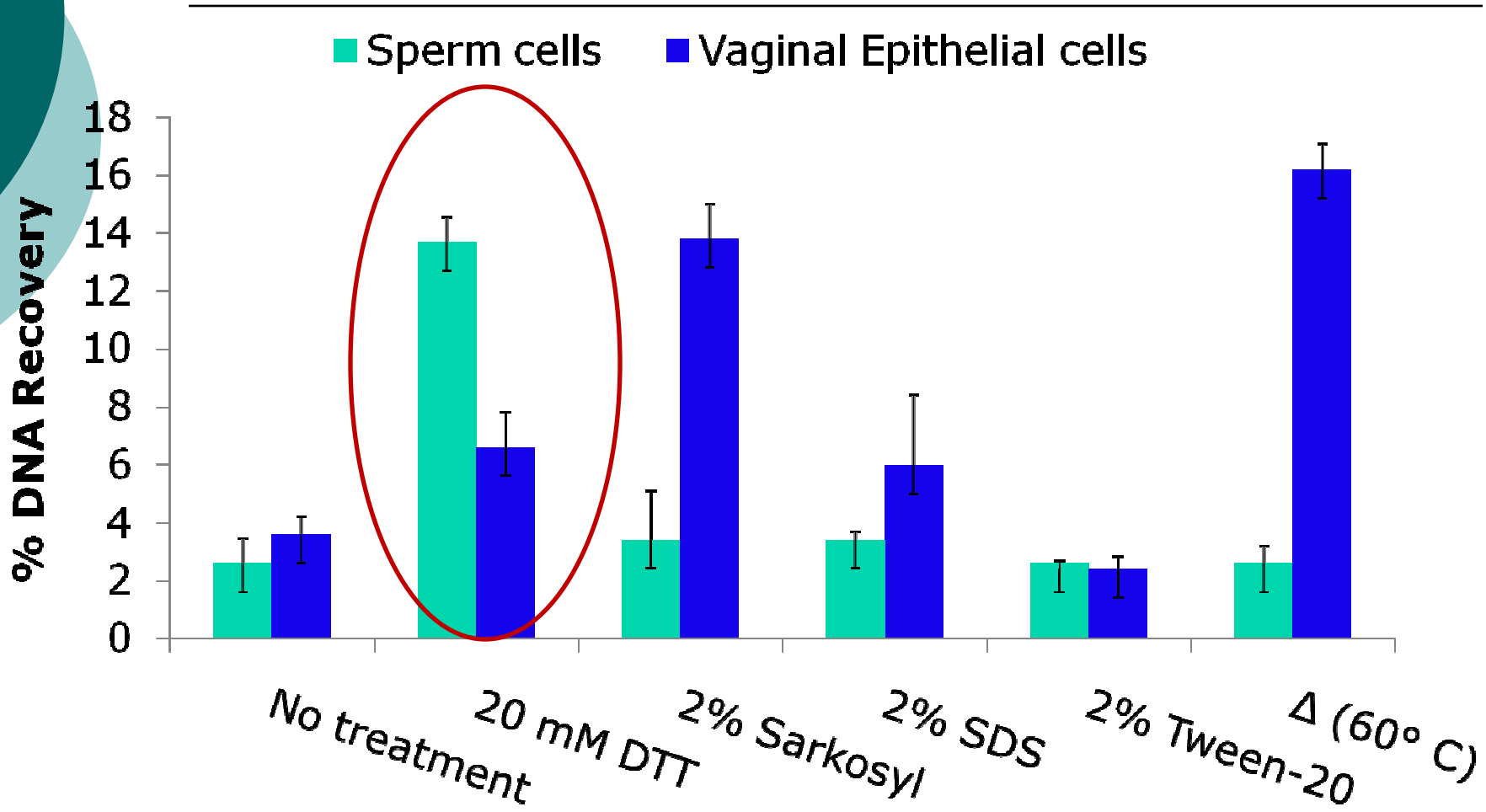
Improving recovery

Effects of temperature, detergents and reducing agent



Effect of temperature and added reagents

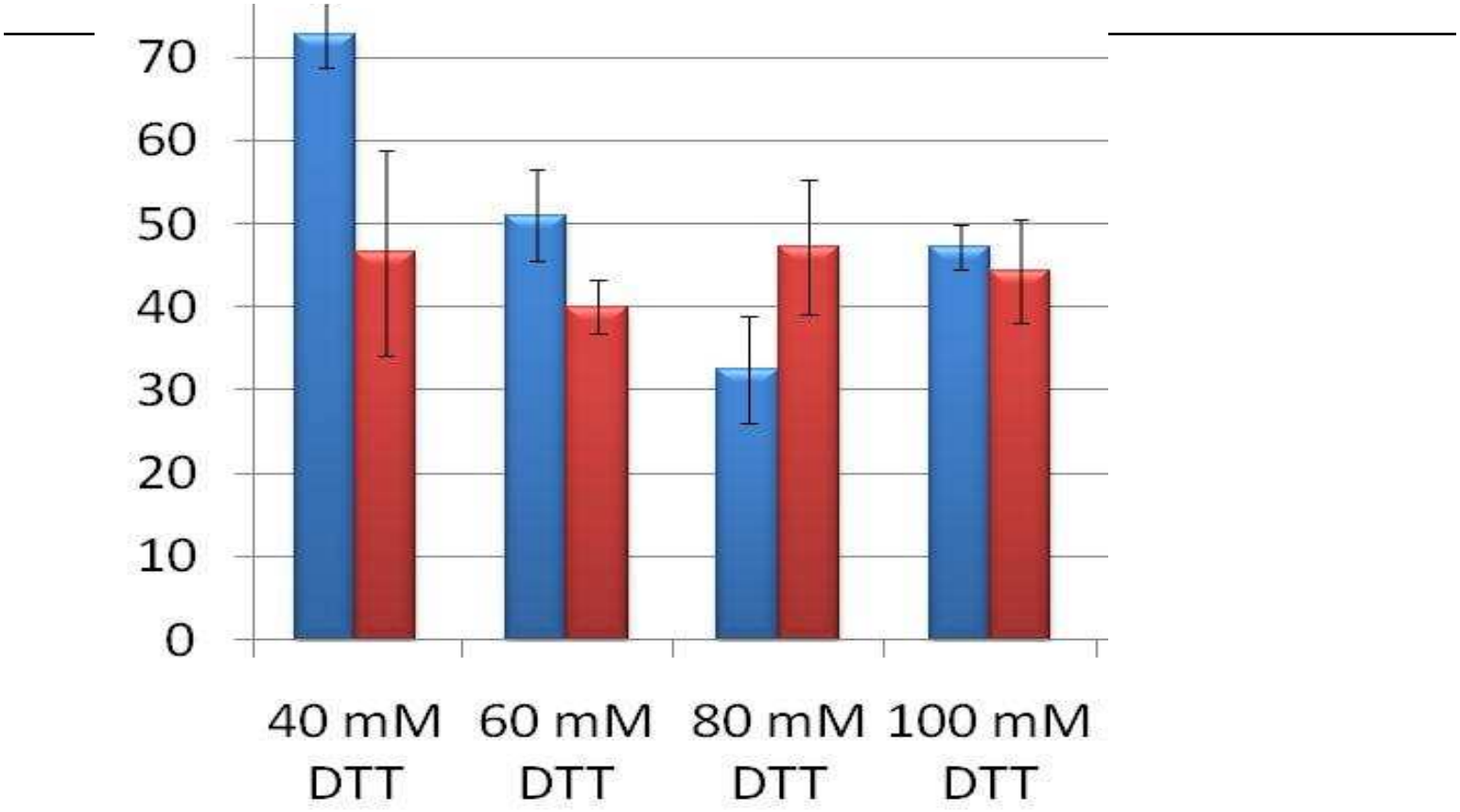
Marked improvement in recovery and interesting selectivity with DTT, temp, sarkosyl



45000 psi, 60 cycles

N=3, error bar= 1 SD

Effect of higher concentrations of DTT

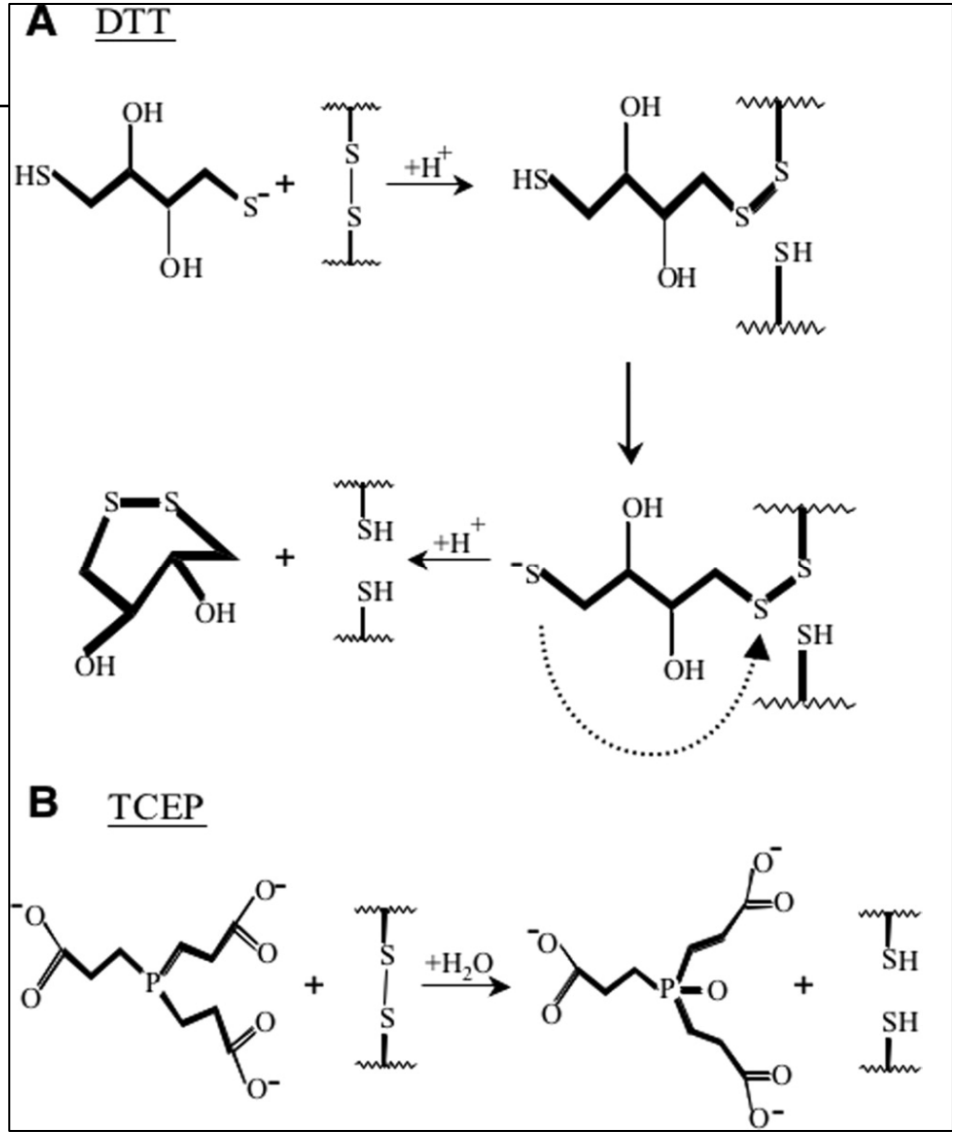


Alternate reducing agent - TCEP

Goal: improve reduction of dithol linkages for sperm protamines

- <u>Advantages to TCEP</u>
  - water solubility
  - odorless
  - Wide pH range
  - Resistant to air oxidation
- <u>Disadvantage:</u>

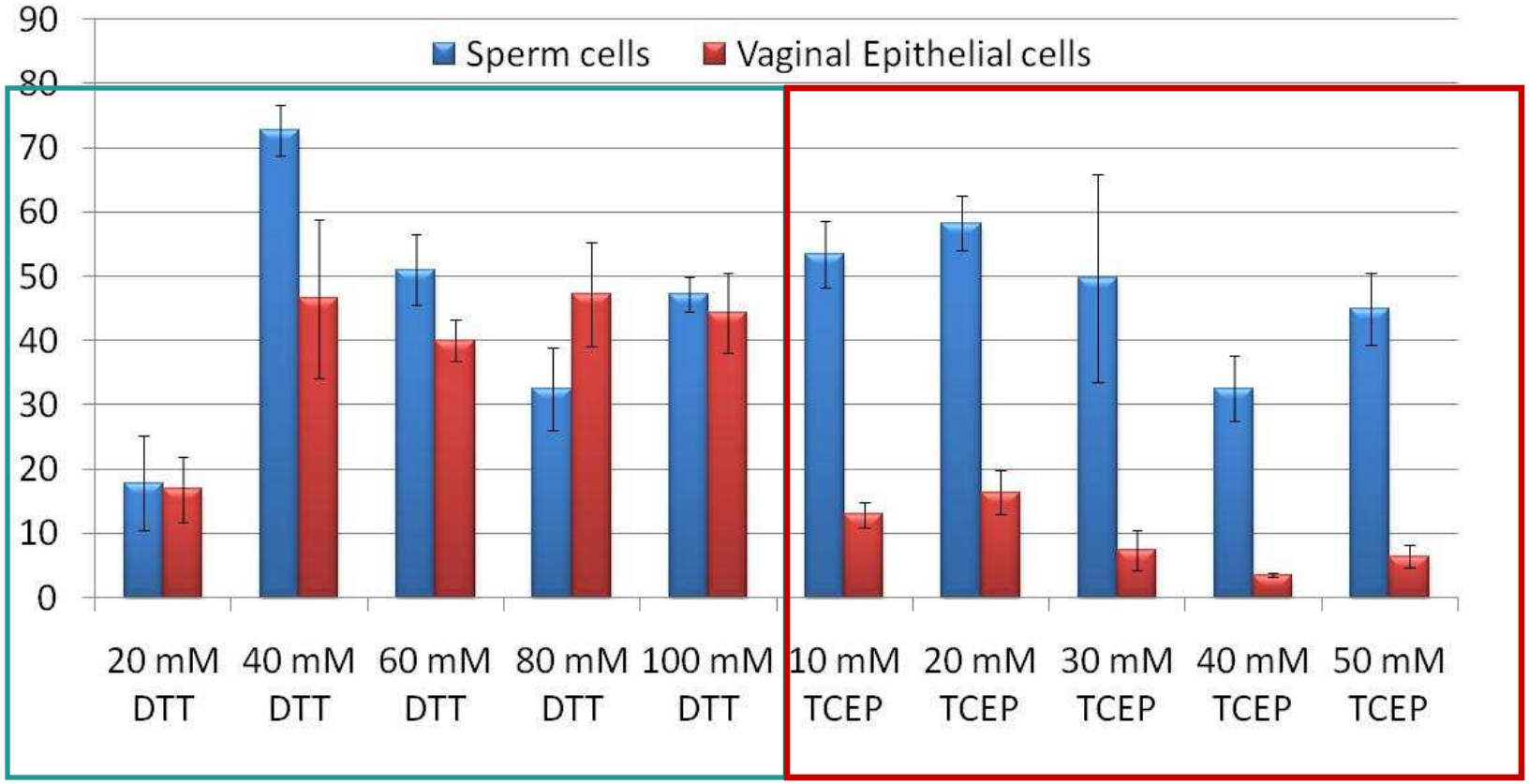
  TCEP is not particularly stable in phosphate buffers prepare fresh daily.



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

A comparison: DTT vs TCEP

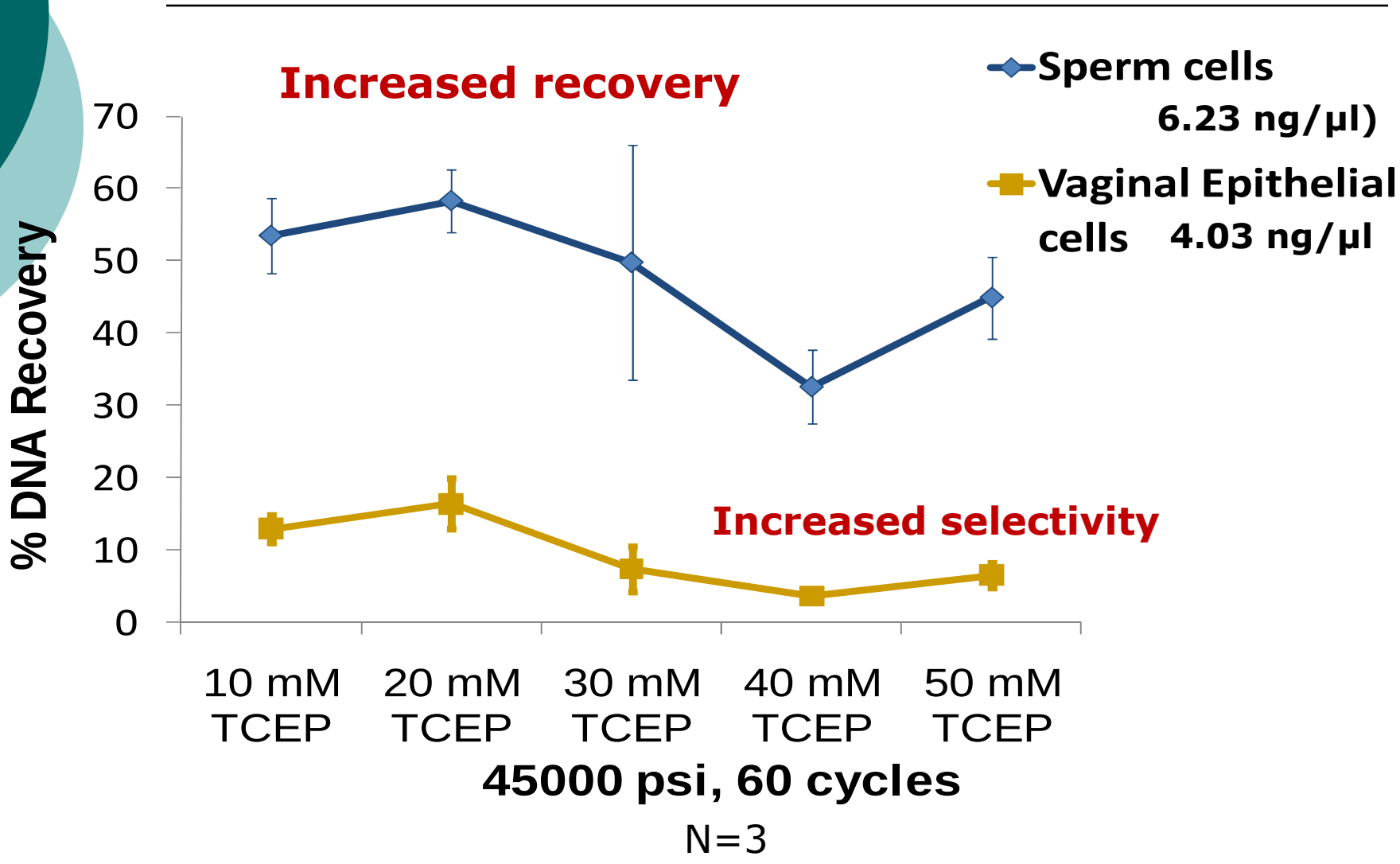
Switching to **TCEP** caused an increase in selectivity between sperm cell and epithelial cell lysis



Dithiothreitol (DTT)

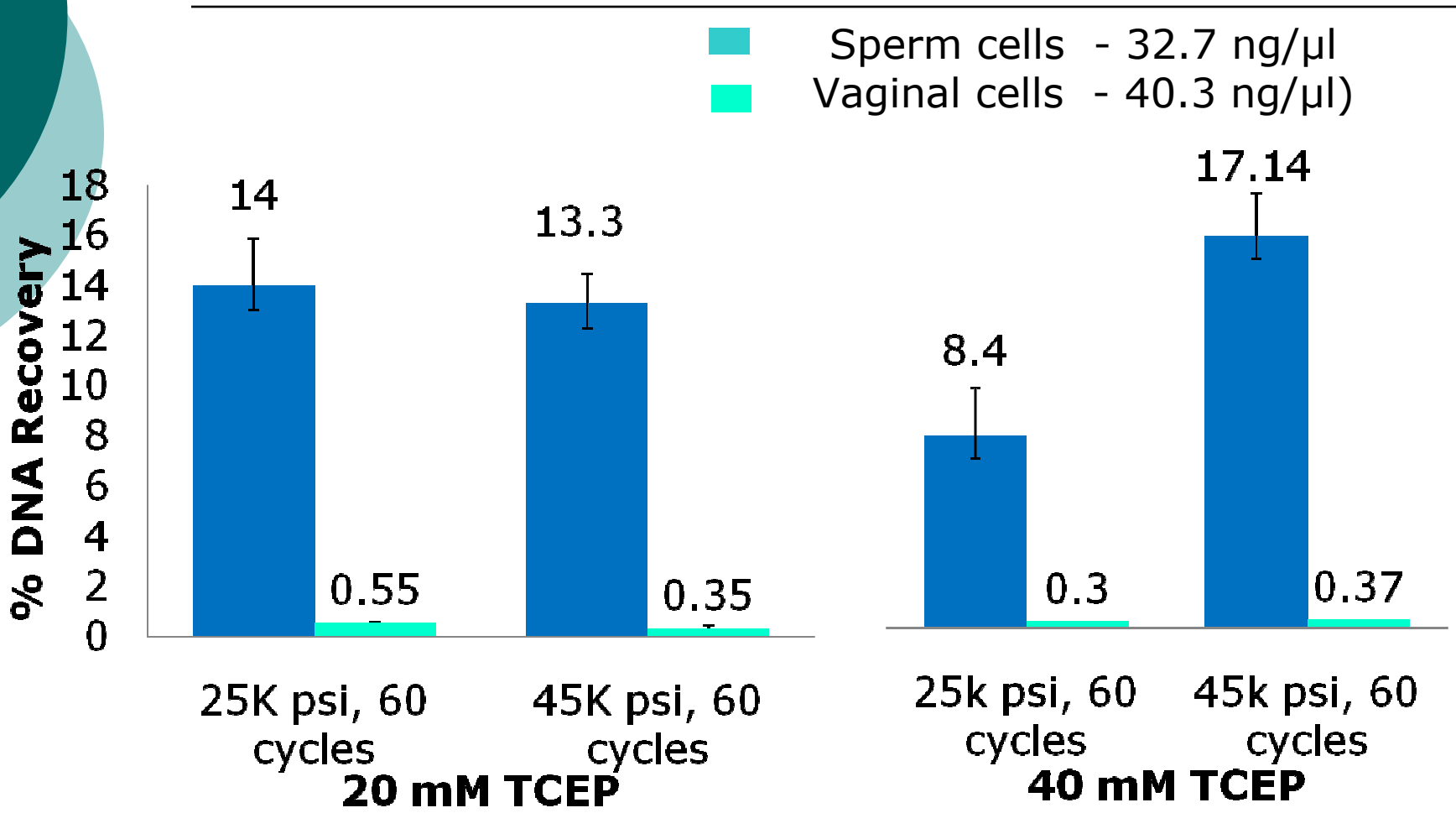
Tris (2-carboxyethyl)phosphine (TCEP)

TCEP studies



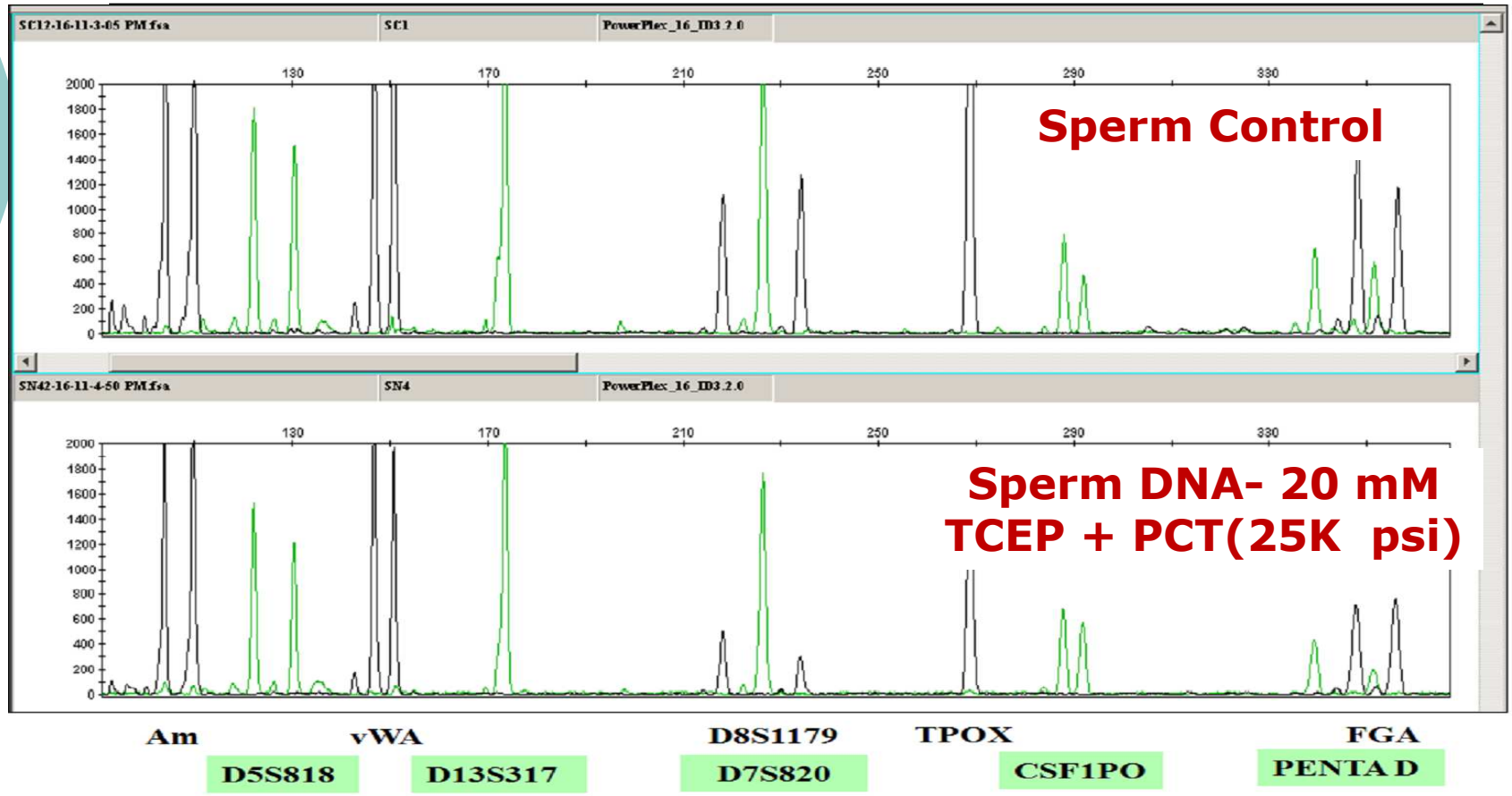
Effect at higher cell concentrations:

Some loss in recovery, but selectivity is increased



* N=3

Does TCEP and pressure cycling affect STR amplification?

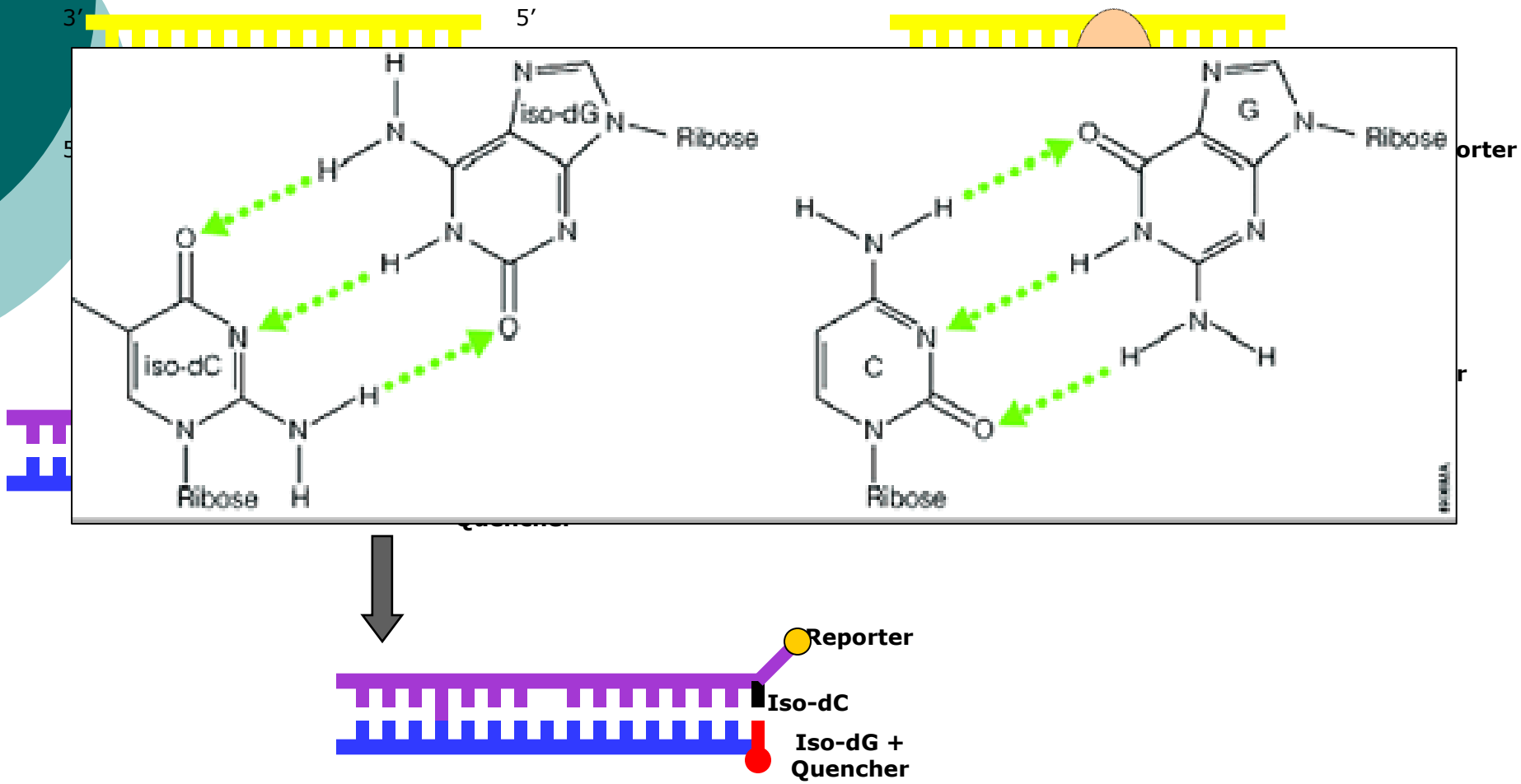


PowerPlex® 16 HS preliminary results

Next step:
Mixtures and dried stains

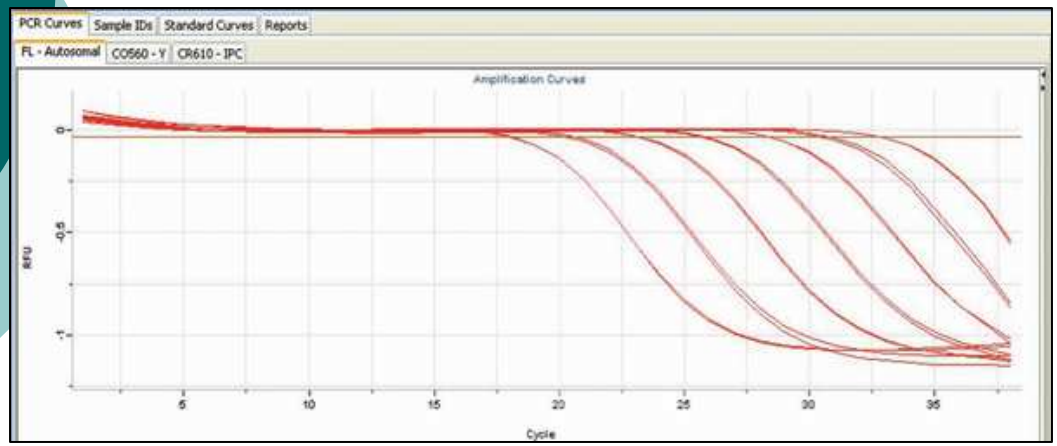
- Key issues
- Switch to Plexor HY to permit simultaneous quantification of both autosomal and Y DNA
- Examination of liquid and dried samples.
- Key issues: Developing accurate quantification, reoptimization of method for dried stains.

Plexor™ for Real-Time PCR
(Promega Corp)

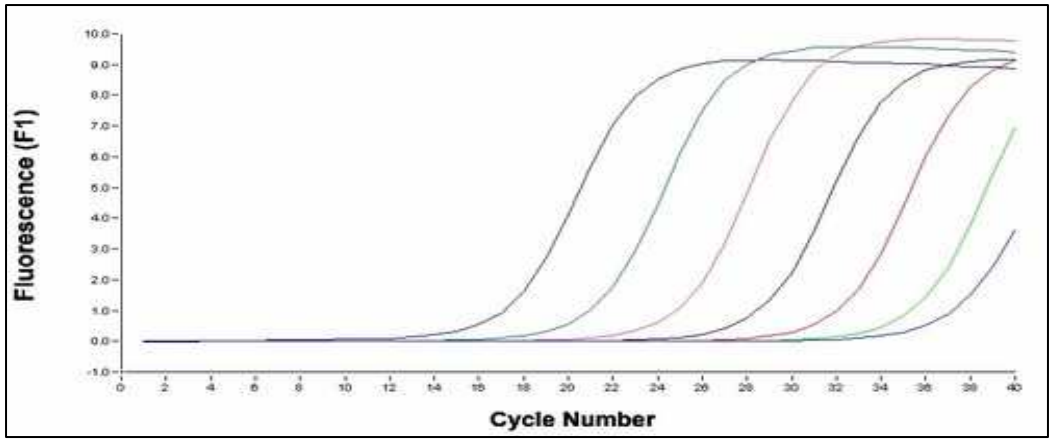


Quenching of the fluorescent signal by dabcyl during product accumulation

Amplification Curves
Plexor™ vs. Standard techniques



http://www.promega.com/plexorhy/system.htm



http://www.capitalbio.com/life_sciences/bioanalysis_kit/capitalbio__real-time__qpcr__universal__kits

- Plexor
  - fluorescence↓ as amplification↑

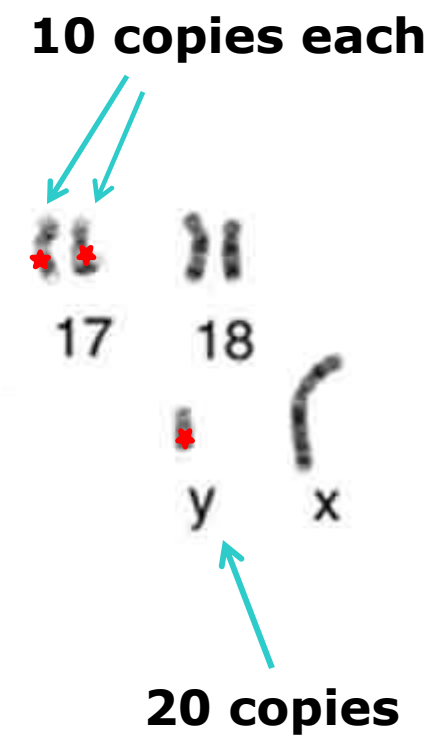
- Standard real time techniques
  - fluorescence ↑ as amplification↑

Plexor HY Setup

Plexor uses copy number variants for enhanced sensitivyt

There are 10 copies of the target on chromosome 7 locus RNU2m making for a total of 20 copies on the paired chromosome

- There are 20 copies of the Y target - locus TSPY making the total number of amplified copies equivalent
- The copy number variants make the procedure abo an order of magnitude more sensitive than single copy methods.
- However, at times, there can be mutations, making the ration between X variable and Y



Plexor also permits the relative detection of autosomal to Y DNA. Because the test uses multilocus probes, care must be taken to evaluate the possibility mutations which will affect ratio of autosomal to Y STRs

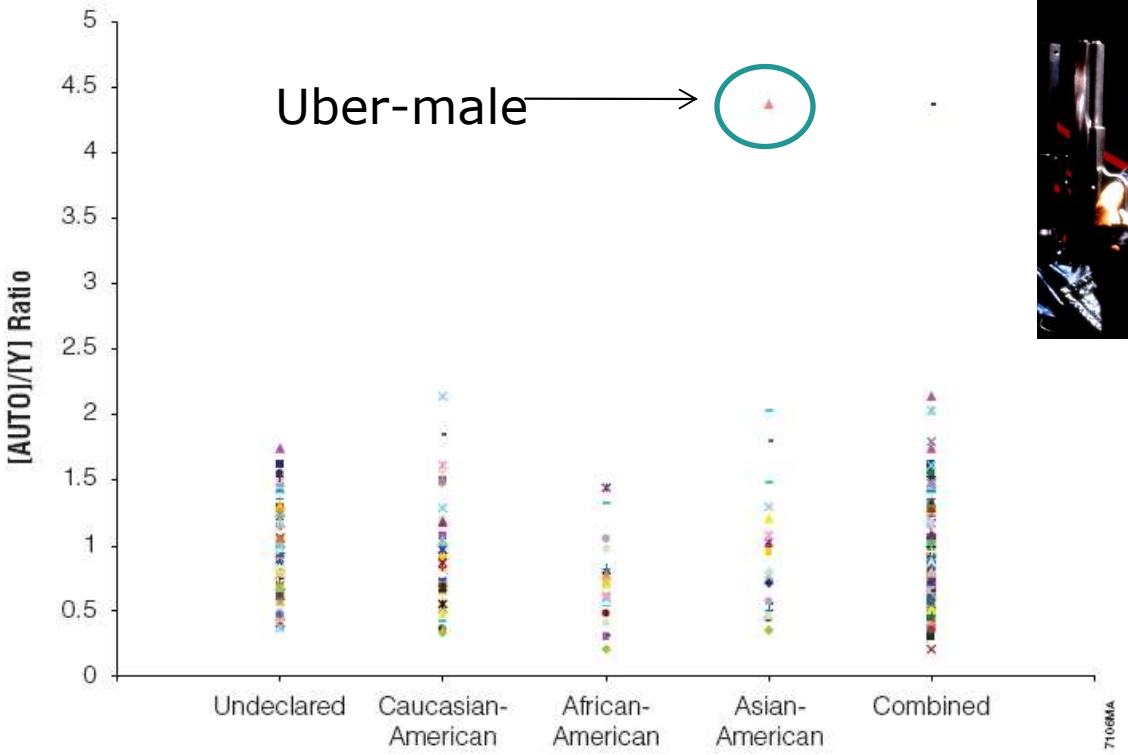
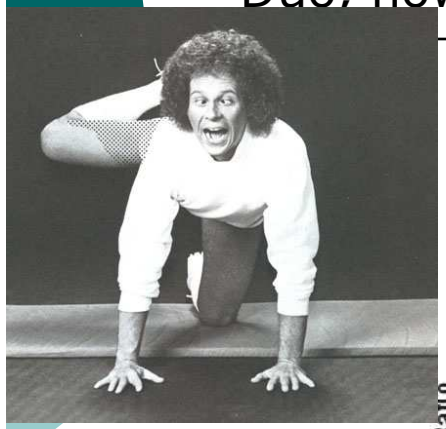


Figure 7. [AUTO]/[Y] ratios for four groups of male samples. For African-American, n = 20; Caucasian-American, n = 40; Asian-American n = 20 and undeclared, n = 80.

Plexor also permits the relative detection of autosomal to Y DNA. Because the test uses multilocus probes, care must be taken to evaluate the possibility of Y chromosomal effects. Similar concern may exist with Duo, however such mutational events may be difficult to track.



Girley Man

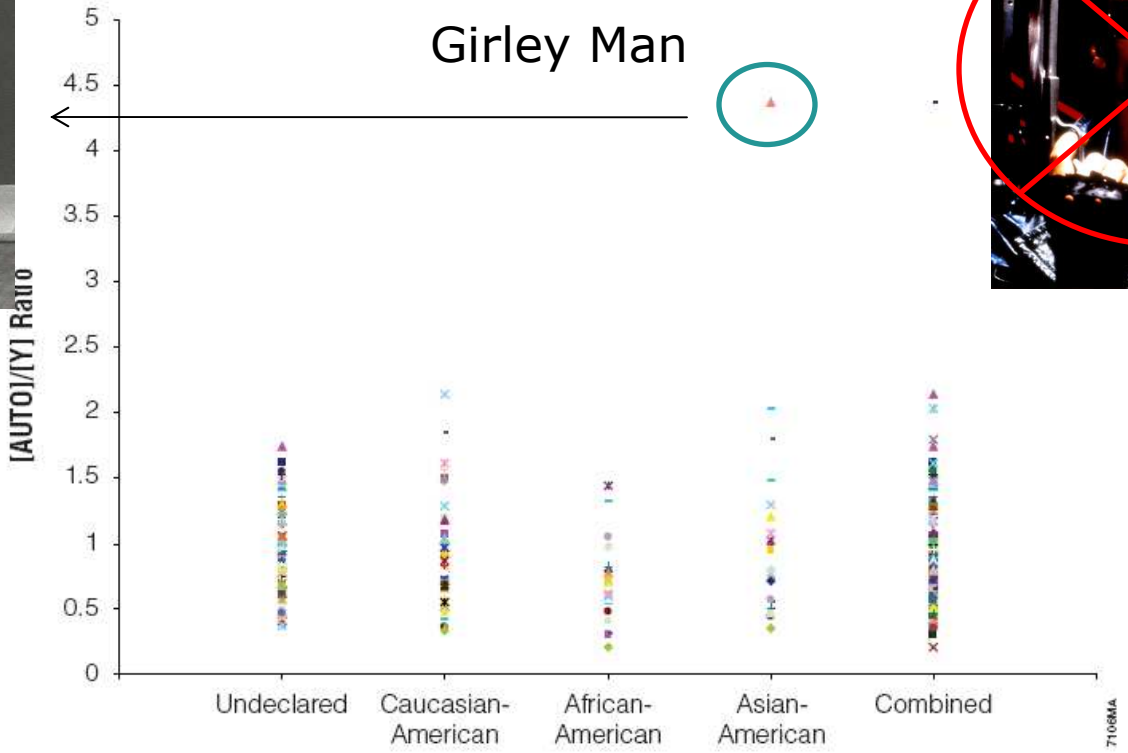
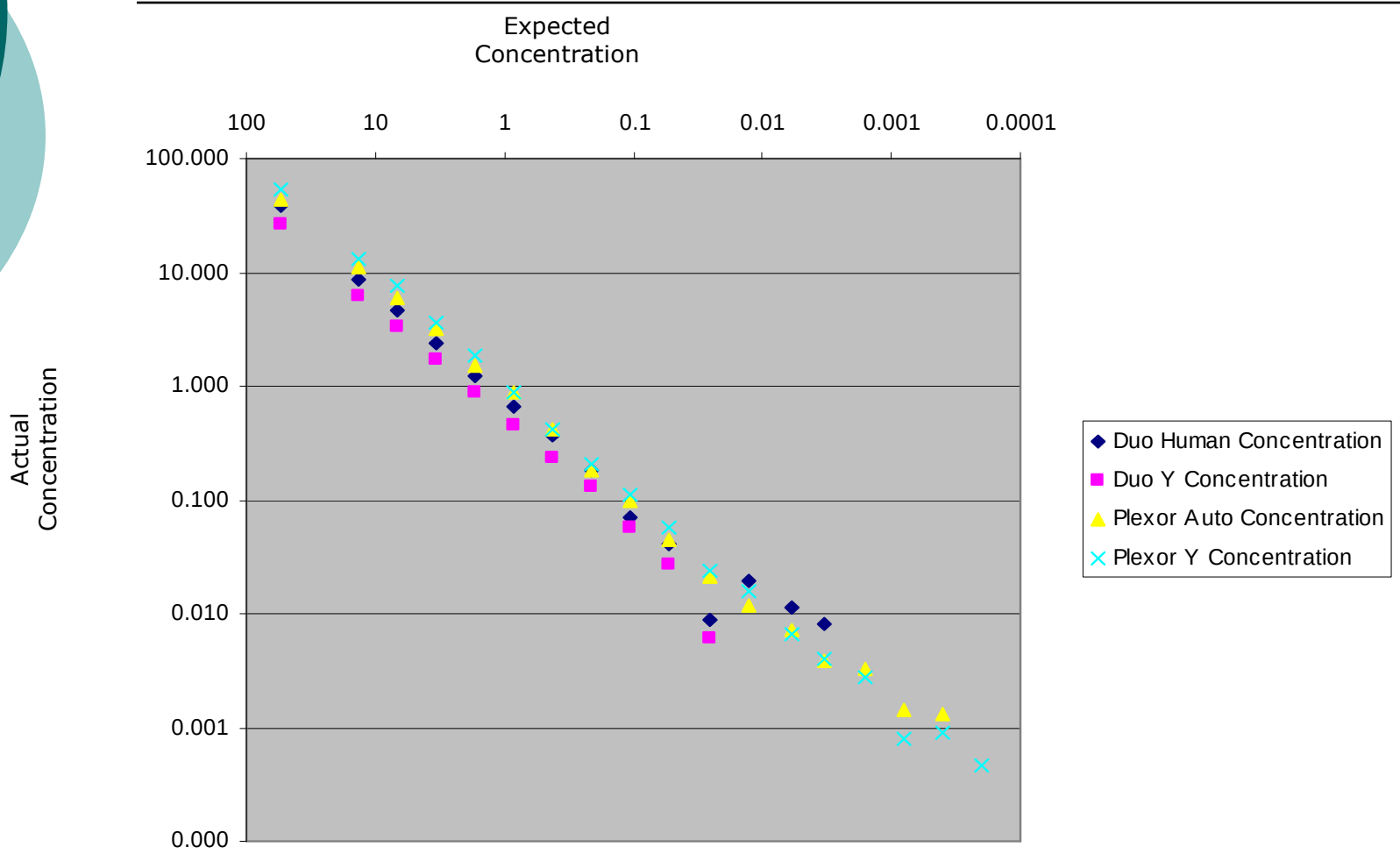


Figure 7. [AUTO]/[Y] ratios for four groups of male samples. For African-American, n = 20; Caucasian-American, n = 40; Asian-American n = 20 and undeclared, n = 80.

Calibration curves: single and multicopy qPCR NIST QSRM C serial dilutions



Bottom line: use single copy for accuracy, CNV for sensitivity

Initial mixture studies using Pressure Cycling and Plexor quantification

I. Mixed samples suspended in PBS

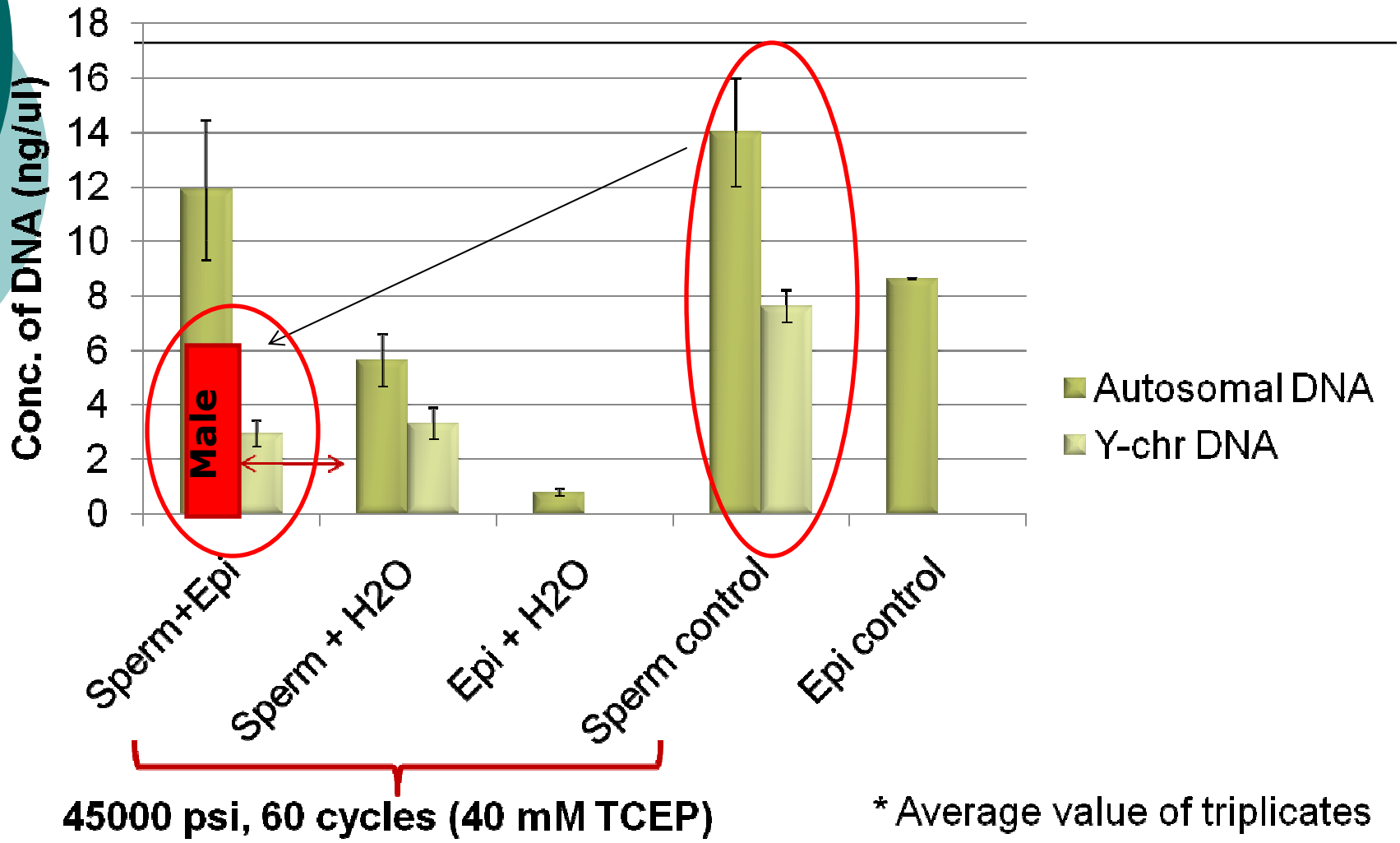
Conditions: 40 mM TCEP or
40 mM TCEP + 20 mM DTT

Pressure- 45 kpsi

No. of cycles- 60 or 99

Trial # 1: Liquefied samples - Results

Note: our donor showed a 2/1 ratio Autosomal/Y
Poor recovery of male DNA in this initial test.

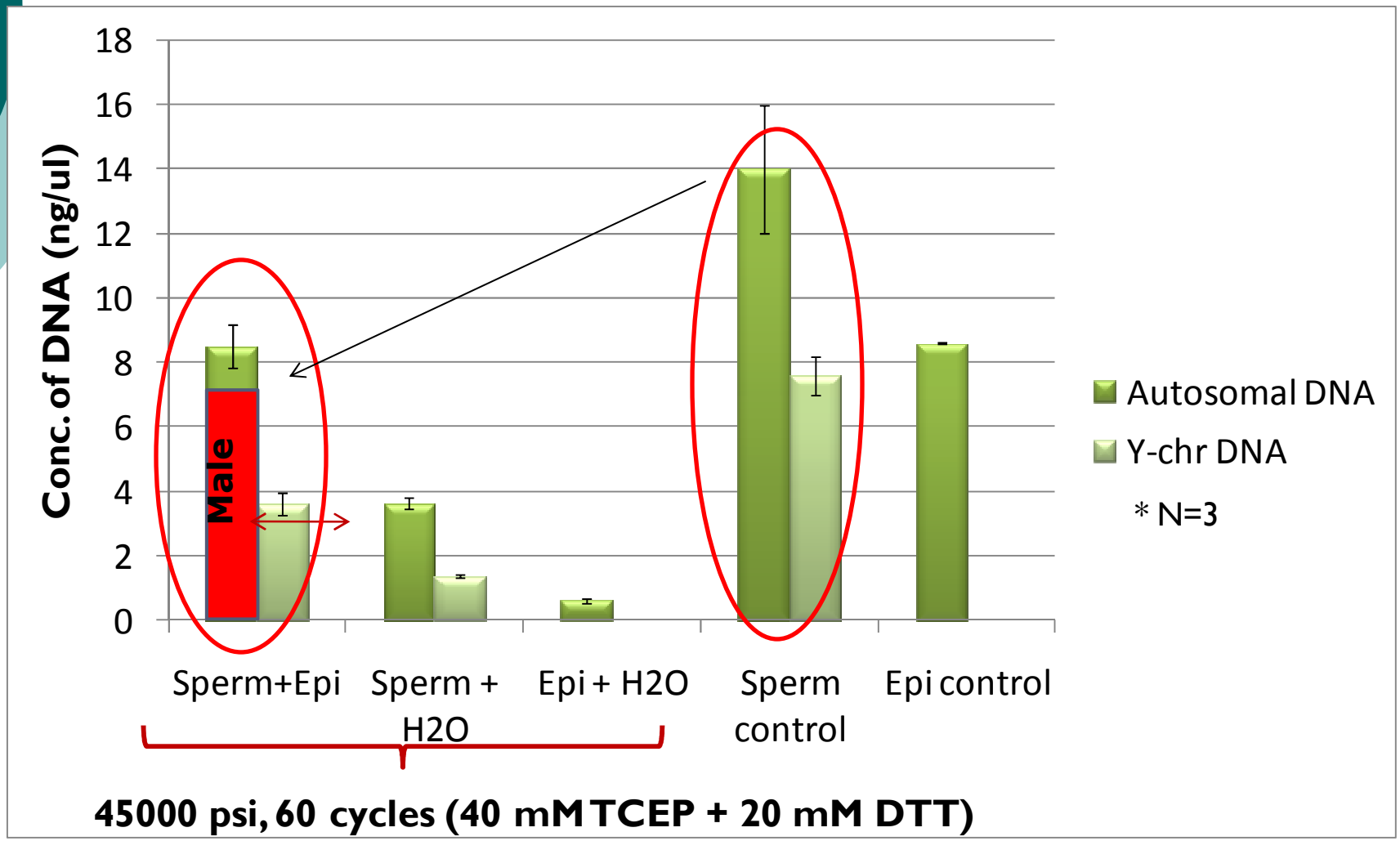


* Average value of triplicates

Sperm cell control DNA- 14 ng/ul (chr: 7.6 ng/ul)
Epithelial cell control DNA- 8.6 ng/ul

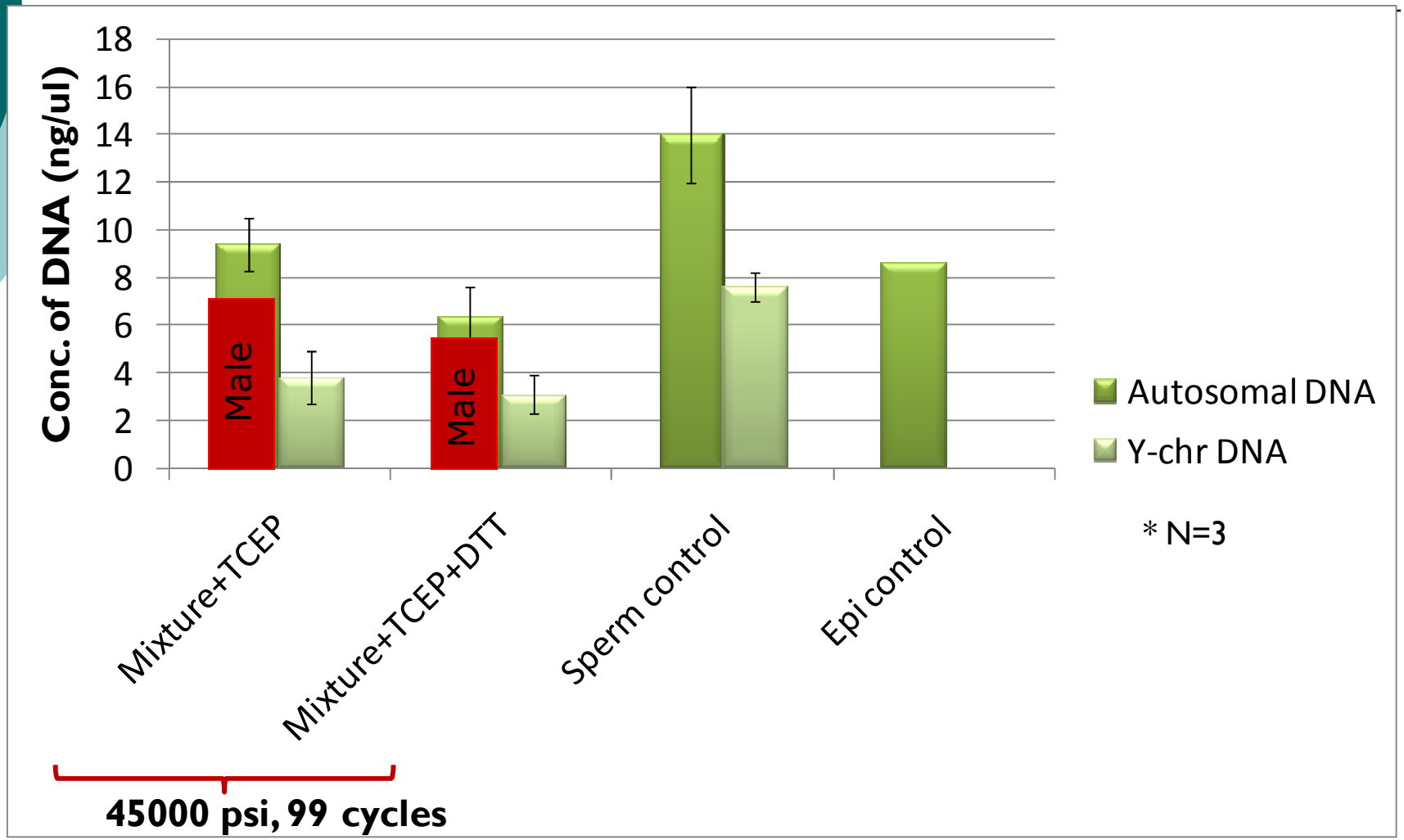
Trial # 2: PBS suspension - Results

Addition of DTT to TCEP improved selectivity of extraction
Much improved recovery

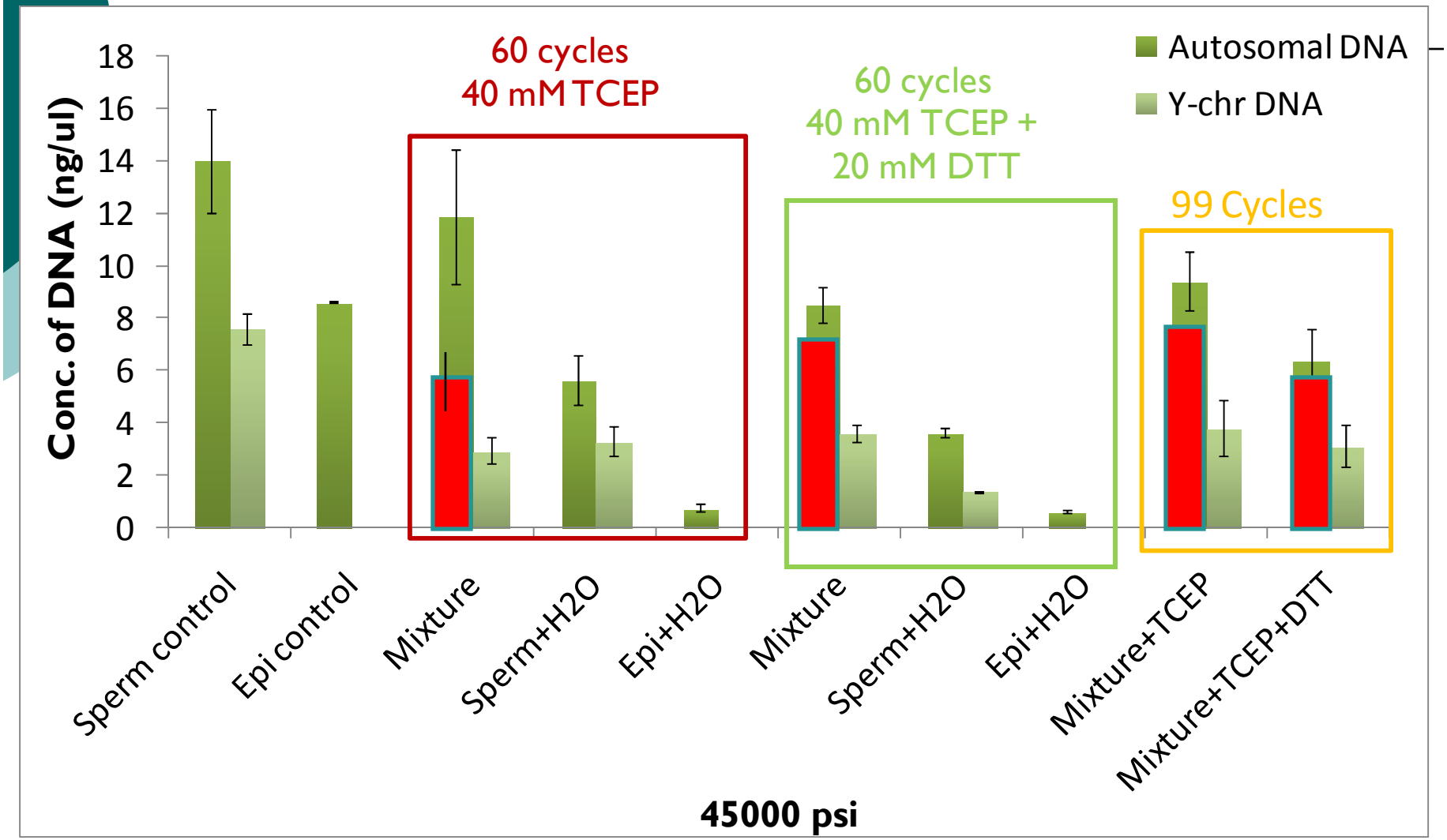


Trial # 3: Increasing cycle number from 60-99

Little improvement in yield, selectivity still best with DTT/TCEP



Results- mixed suspended cells



Conclusions

- PCT treatment can produce selective extraction of epi and sperm cells
- Depending on buffer component epi or sperm cells can be selectively lysed
- TCEP produces improved selectivity of sperm extraction
- Combining TCEP and DTT further improves yield in case of mixed samples
- Increasing pressure cycles above 60 has no effect on yield

Future work

- Solubilizing and examining dried stains – measuring yield for removal of cells from swabs and fabric samples
- Using experimental design techniques to further optimize yield by examining temperature and buffer concentration
- Increasing the numbers and types of samples for validation

Acknowledgements

FIU

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Vera Gross

Promega Corporation

Len Goren

Broward Sheriff's Office DNA Laboratory

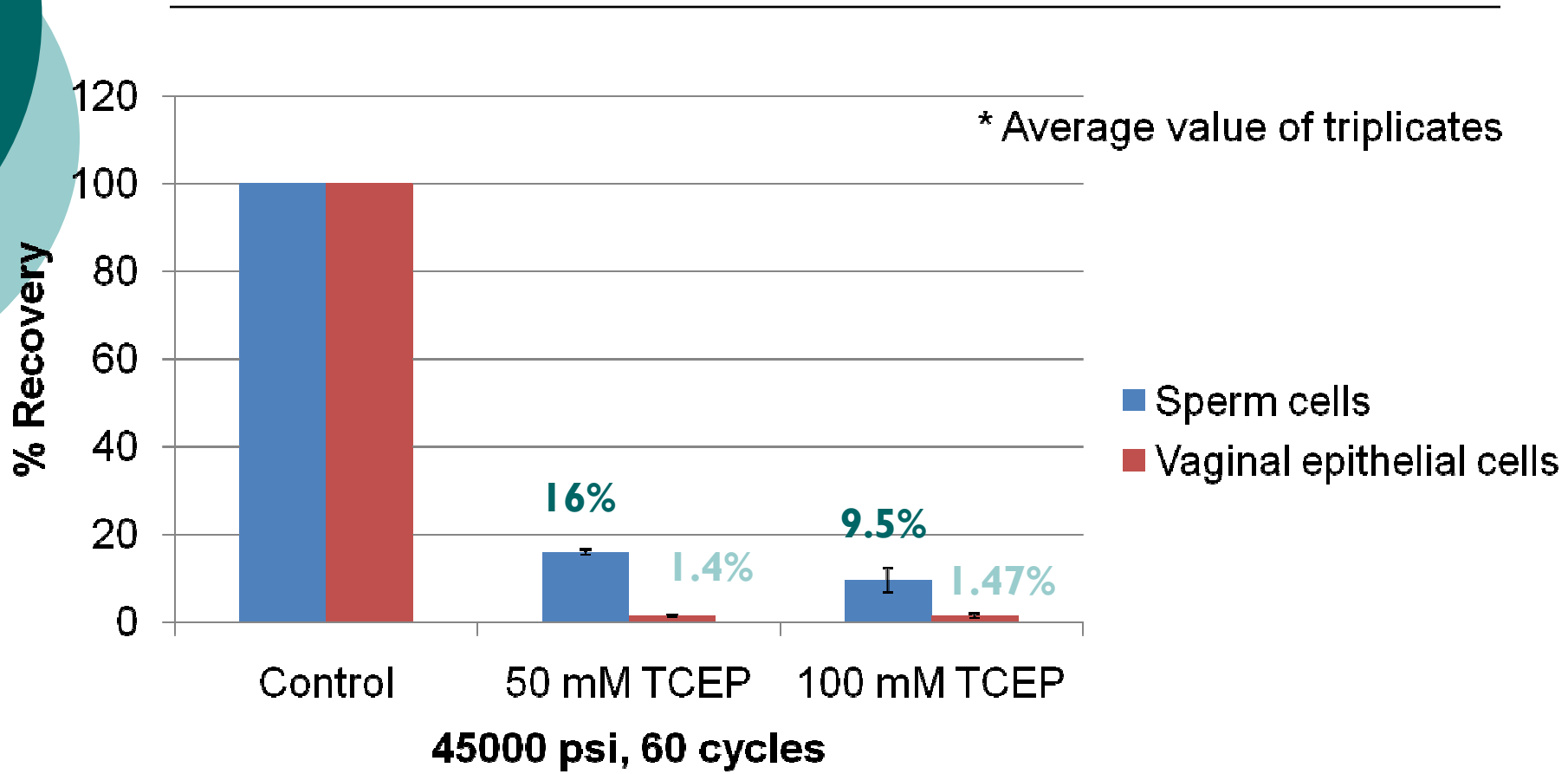
Dr. George Duncan

McCord Research Group

- Major support provided by
- Pressure Biosciences, Promega

Thank you

Effect of Higher TCEP concentrations:



- Control: standard digestion/PCIA extraction
- Sample Pressure digestion/PCIA extraction

Purification procedure

- Add equal volume (130 µL) of Phenol/Chloroform/ Isoamyl alcohol to the sample
- Add 40 µL of protein precipitation solution (consisting of Sodium acetate and Glacial acetic acid)
- Centrifuge at 13,000 rpm for 10 min
- Transfer the aqueous supernatant to a microcentrifuge tube
- Add 20 µL of protein precipitation solution and 500 µL of absolute ethanol
- Vortex, spin and leave at -20ºC for 1 hour
- Centrifuge at 13,000 rpm for 10 minutes and decant the solution
- Wash the pellet with 70% ethanol
- Centrifuge at 8,000 rpm for 8 minutes
- Repeat the wash and air dry the pellet
- Add 30 µL of de-ionized water to the pellet and put it in a water bath (56º C) for 15 minutes for the pellet to dissolve
- The sample is ready for further use.