


  
 EVROPSKÁ UNIE **esf** MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
   
 INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

---



Vzdělávání v oblasti forenzní genetiky  
 reg. č. CZ.1.07/2.3.00/09.0080

TENTO PROJEKT JE SPOLUFINANCOVÁN EVROPSKÝM SOCIÁLNÍM FONDEM A STÁTNÍM ROZPOČTEM ČESKÉ REPUBLIKY.

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

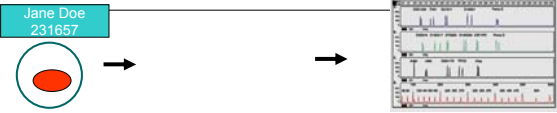
## DNA Typing and the Development of Methods for Determination of Degraded and Compromised Forensic Samples

**Bruce R. McCord**  
**International Forensic Research Institute**  
**Florida International University**  
[mccordb@fiu.edu](mailto:mccordb@fiu.edu)  
[www.fiu.edu/~mccordb](http://www.fiu.edu/~mccordb)





### Large Multiplex Kits provide Efficient and Rapid Analysis of Convicted Offender Samples

Jane Doe  
 231857


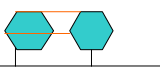
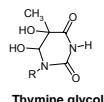


**But what about degraded DNA ?**

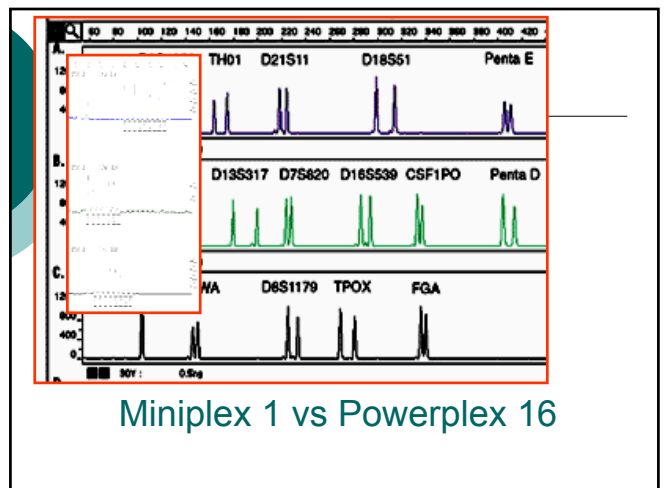
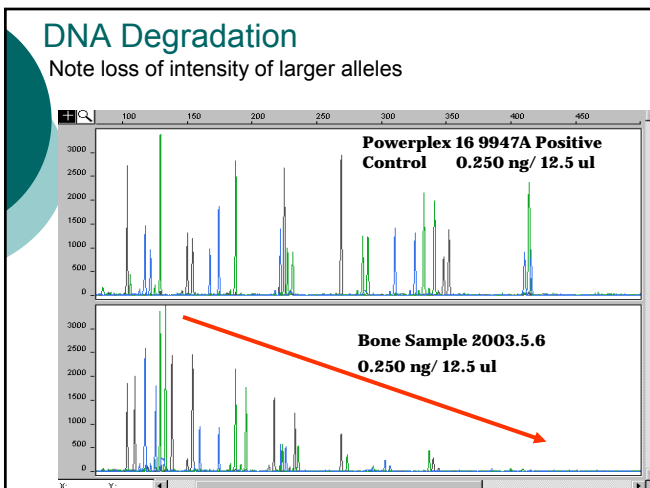


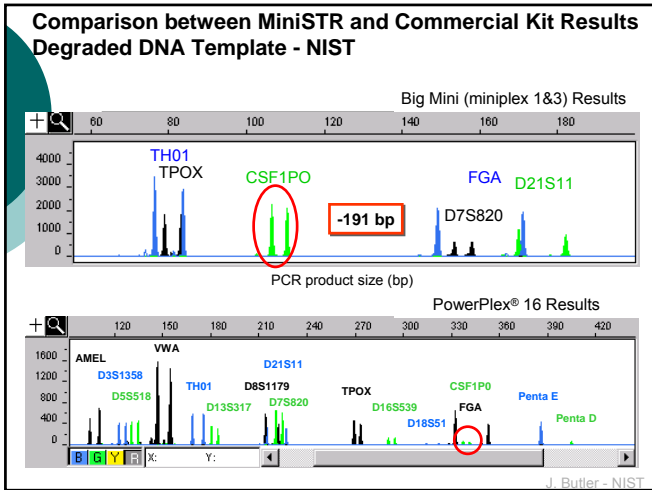
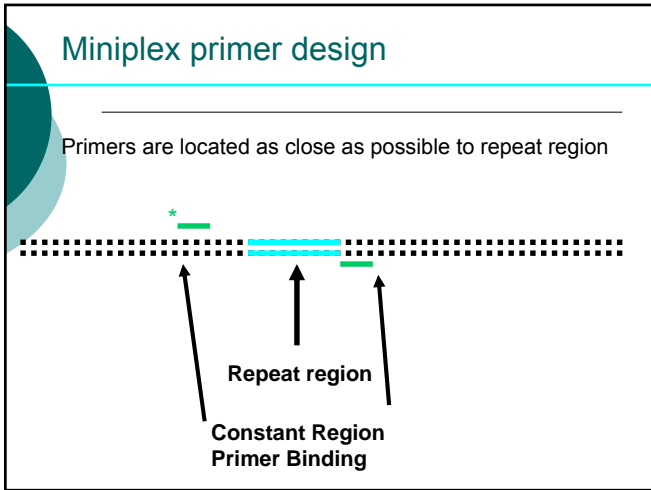
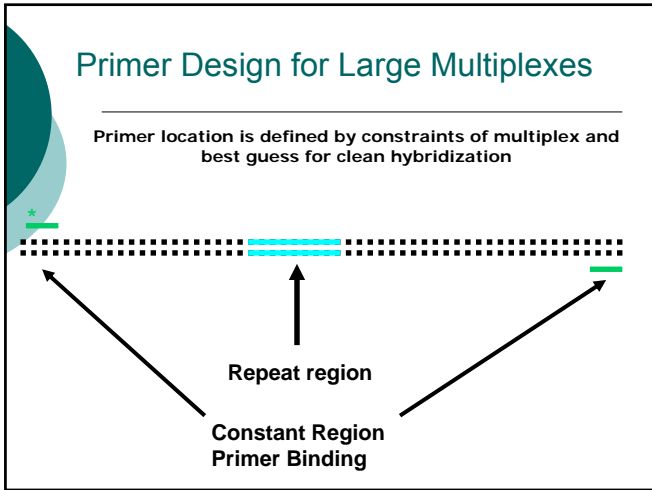
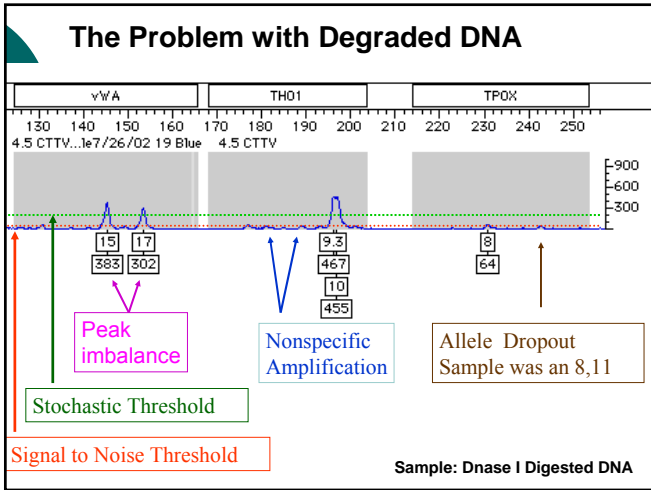
**Such samples present a special challenge**  
**Skeletal material being preped for extraction**

### DNA Degradation

1. polymer hydrolyzes (nucleic acids break apart) 
2. Pyrimidine dimers (bases X-link) 
3. Chemical oxidation (bases become unreadable) 

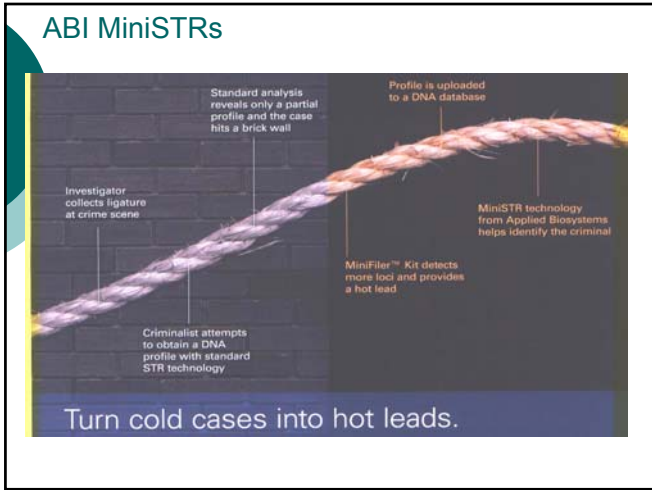
Thymine glycol





### Miniplex STR Primer Sets

	6FAM	VIC	NED
<b>Miniplex 1</b>	TH01	CSF1PO	TPOX
<b>Miniplex 2</b>	D5S818	D8S1179	D16S539
<b>Miniplex 3</b>	FGA	D21S11	D7S820
<b>Miniplex 4</b>	VWA	D18S51	D13S317
<b>Miniplex 5</b>	Penta D	Penta E	D2S1338
<b>"Big Mini"</b>	TH01, FGA	CSF, D21	TPOX, D7





## Application of Miniplexes

Locus	Jane	Daughter	LR	A
D5	11,12	12,12	1.465502	0.34118
D8	10,14	10,10	4.950885	0.10099
D16	8,12	8,14	13.72872	0.01821
WVA	17,18	17,17	1.776451	0.28146
D18	13,14	13,17	1.887505	0.13245
D13	11,13	10,11	0.736594	0.3394
TH01	9,3,9,3	9,3,3	1.360359	0.36755
CSF	13,13	13,13	10.41341	0.09603
TPOX	8,10	8,8	0.934981	0.53477
FGA	24,25	23,24	1.841485	0.13576
D21	30,32,2	30,30,2	0.898796	0.27815
D7	8,9	9,12	1.411233	0.17715
			7611.237	

- **Likelihood Ratio = 7,611**
- **Identify confirmed as a 54 year old woman missing since December 2000**

## Result



**Identified as Roberta Gile, Age 54  
Missing since December, 2000**

## Application of MiniSTRs in bone/bone reassociation Yugoslavia

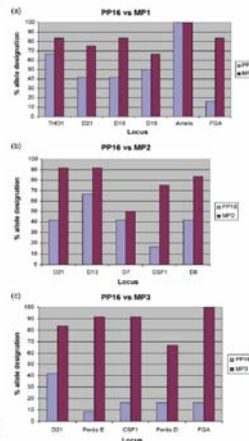
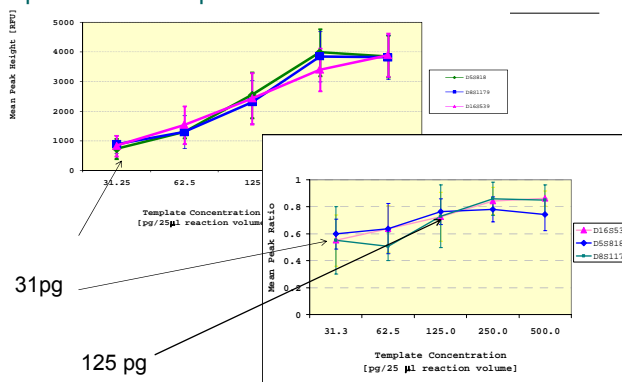


Fig. 1 An example of a bone from an association composed of right side of anthropologically unrepresentative skeletal remains recovered from the mass graves. Each set of remains contained under the skull bone, mandible, humerus, femur, tibia and ulna. The skull bone was used for DNA analysis. Description of the position of the bones and skull from the bone sets. Multiple typing of the other set of remains provided results to the identification of the person, allowing for a complete sample analysis to be conducted and related to the family.

Fig. 2 Preparation of replicable alleles recovered from either PP16 or MP1 or other MiniSTR amplicons obtained from a series of five highly degraded bone samples. (a) Results for MP1, (b) results for MP2 and (c) results for MP3.

Parsons et al, Forensic Science International: Genetics 1 (2007) 175-179

## MiniSTRs show the same effect In spite of the improved sensitivity, peak balance is poor at low template concentration



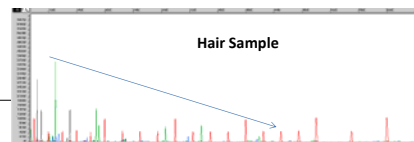
## The Down Side of MiniSTRs

MiniSTRs were developed to access degraded DNA.

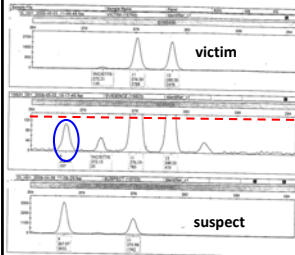
They do not solve the inherent low copy limitation of the PCR.

1. Short PCR primers amplify better.
2. Better amplification means laboratories can access extremely low levels of DNA
3. At such levels (1-20 cells) a **scientist cannot express a strong opinion about how DNA arrived at the site where it was recovered.**
4. This DNA could just as easily come from pre or post deposition as it could come from the suspect.

## This sample needs MiniSTRs



This sample is problematic:



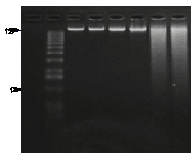
Duke Lacrosse player and evidence

**Lab interpretation threshold = 125 RFU**

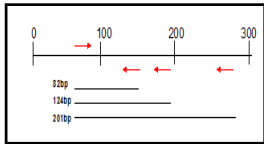
Non specific amplification was used to falsely include suspect

## DNA Quality by real time PCR

How do you tell if you need miniSTRs?



Standard Yield gels are not possible at low levels of DNA

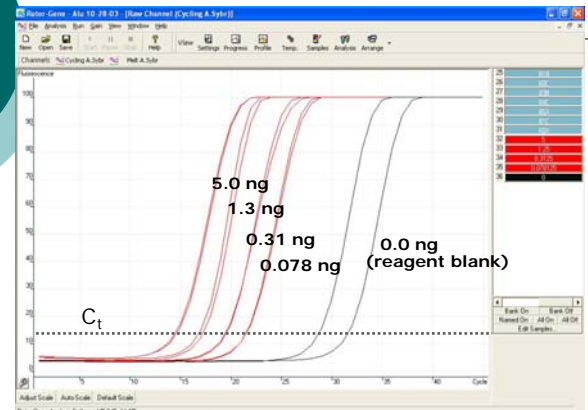


Primer design

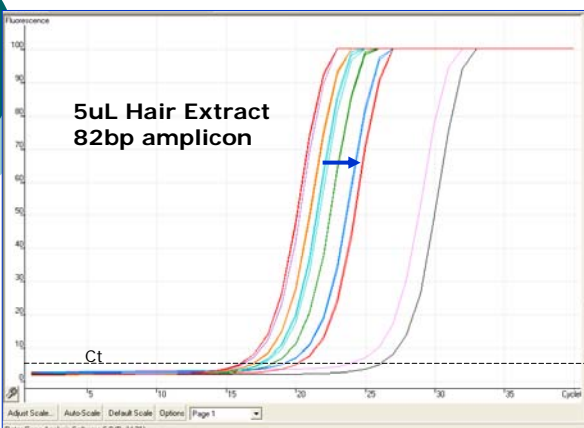


Virtual Yield Gel qPCR with qPCR

## Real Time PCR calibration

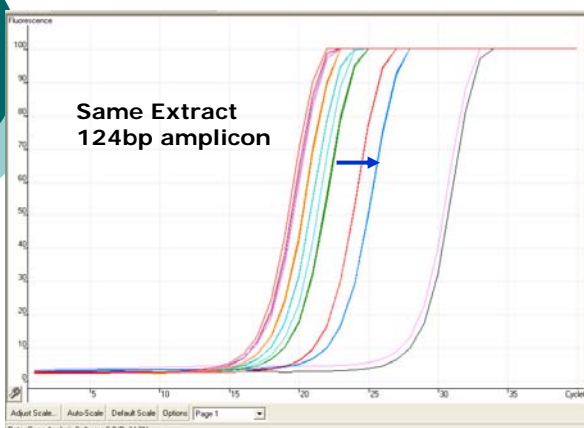


## Real Time PCR – 82 bp *Alu*



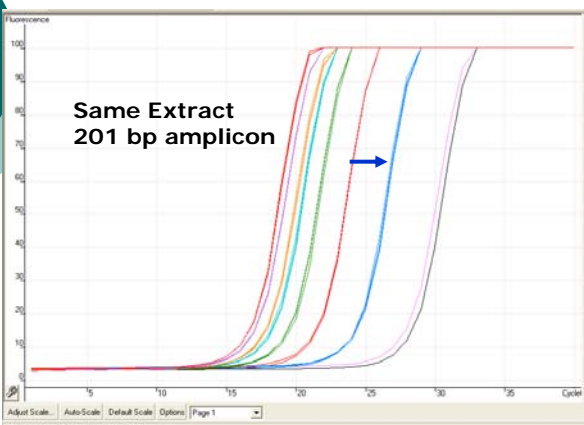
5uL Hair Extract  
82bp amplicon

## Real Time PCR – 124 bp *Alu*



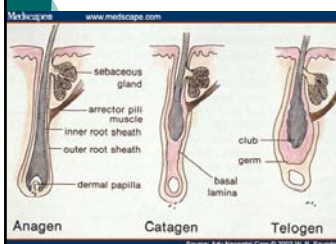
Same Extract  
124bp amplicon

## Real Time PCR – 201 bp *Alu*

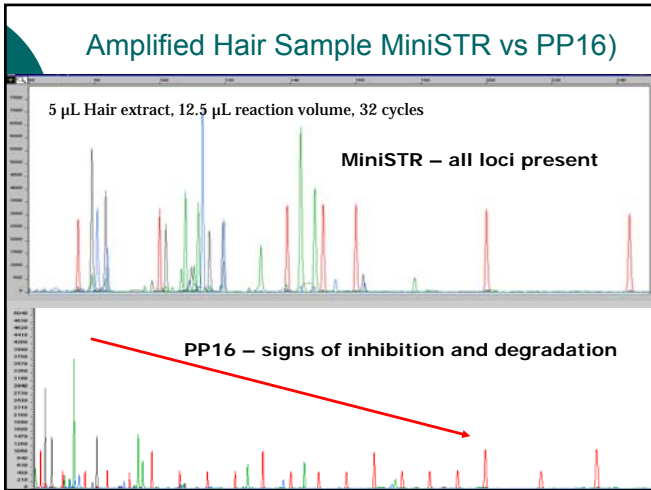
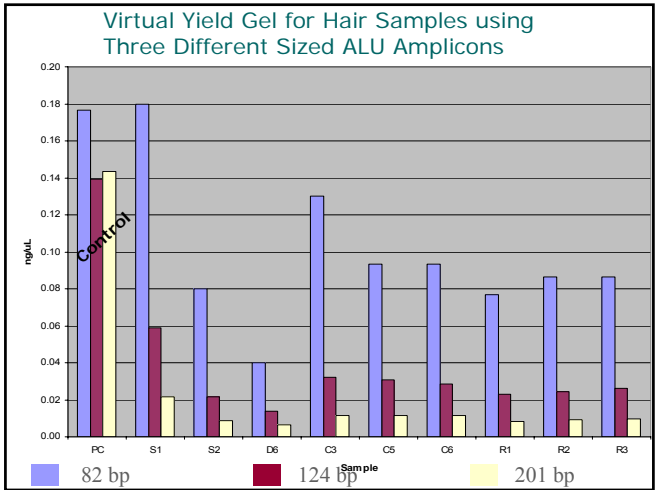
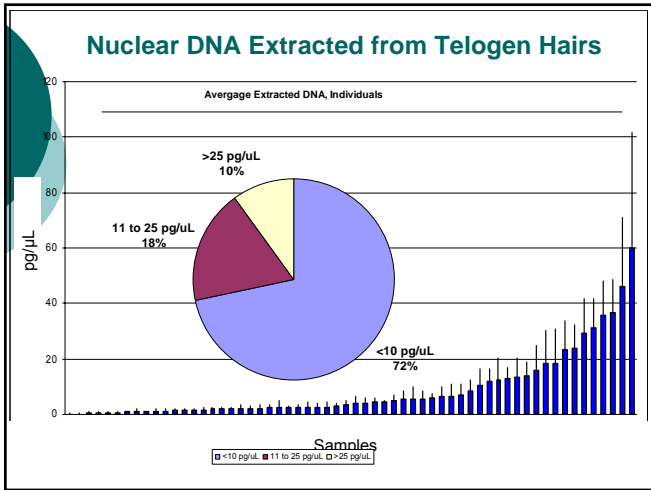


Same Extract  
201 bp amplicon

## Application to Telogen Hairs found at Crime Scenes



- Generally hair samples are poor samples for nuclear DNA typing
- Telogen hair contains little tissue
- DNA is poorly incorporated in hair
- Hair contains melanin, a PCR inhibitor
- mtDNA is the usual method due to these problems



### Results for telogen hairs

- <100 pg – Mini 2 (D5, D8, D16)
  - 60% 2 loci (D5 and D16)
  - 80% at least 1 locus (D16)
- 100-500 pg Mini 2 + Mini 4 (vWA, D18, D13)
  - 70% 3 or more loci
  - 30% 1 loci or less
- >500 pg all 3 sets
  - 70% 4 loci or more
  - 40% 6 loci or more

Sometimes you can run telogen hairs

### Mini STRs for use in Microchips

#### Agilent Bioanalyzer

A disposable analytical platform  
That works right out of the box.  
Fieldable for mass disaster?  
but resolution is too low for 4base STRs

How to improve resolution?

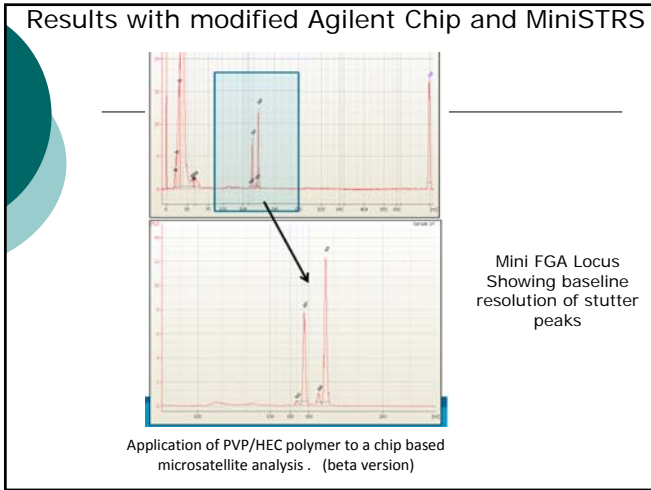
1. Use mini STRs – increase resolution
2. Switch to denaturing polymer

### Agilent Bioanalyzer (size of a postage stamp)

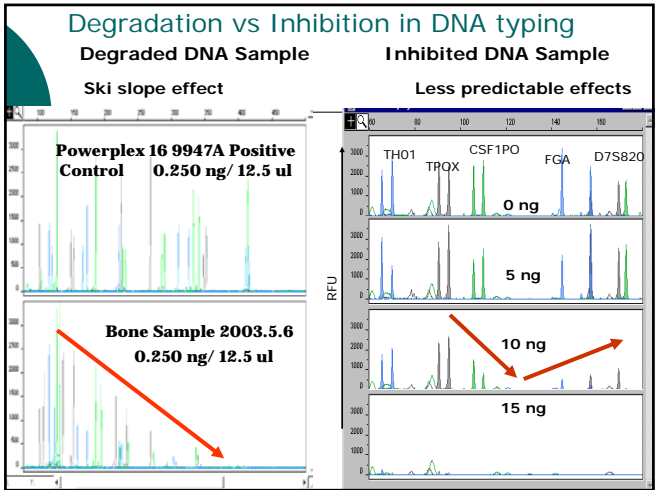
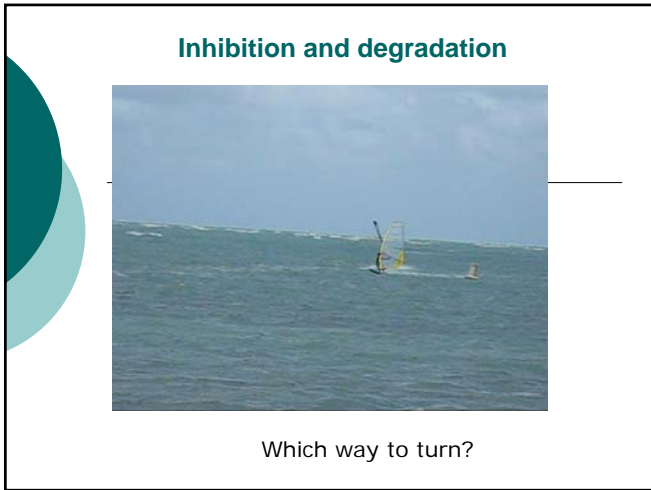
Kit-on-a-Chip Speeds Cell Assays

Micro-channels are filled with a sieving polymer and fluorescence dye

- Sample injection
- Analysis starts
- Components separate
- Fluorescence detection



- ### Conclusions
- MiniSTRs are for degraded DNA
  - Validation data reveals a robust and sensitive multiplex amplification
  - Virtual yield gel using qPCR helpful for proper analytical results
  - Stochastic effects still occur for samples under 125pg
  - Improved results are possible for bone and telogen hair
  - Microfluidics can also benefit from this approach



- ### The Issue:
- With increasing interest in the analysis of environmentally challenged samples, better interpretation of electropherograms is needed
    - We need to determine the relative effects of DNA degradation and inhibition on peak height ratios.
    - We need to understand effects of different types of inhibitors
    - We need to be able to predict the usefulness of different cleanup procedures

### PCR Inhibitors

- Substances co-extracted in forensic samples that affect amplification of template DNA
- Theories
  - Inhibitors bind with the polymerase
  - Inhibitors interfere with polymerase by binding to DNA
  - Polymerase affected during primer extension

Competitive inhibition

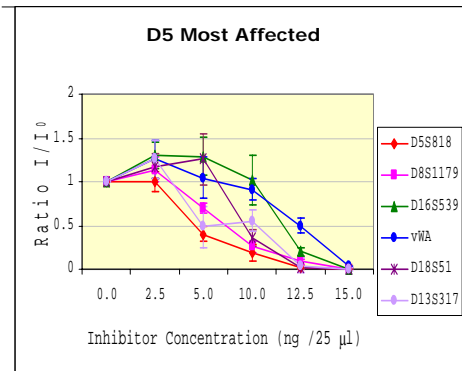
Noncompetitive inhibition

<http://www.cat.cc.md.us/courses/bio141/lecguide/unit4/genetics/protsyn/regulation/comp.html>

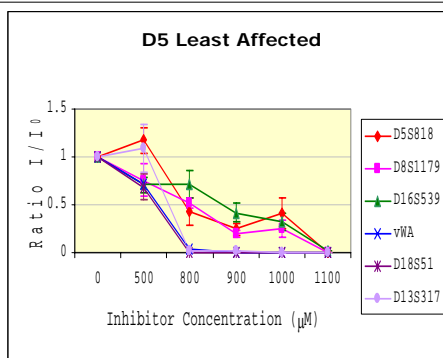
## PCR Inhibitors

- In the PCR process, the enzyme moves along the DNA strand, adding complementary bases
- If inhibitors are present the PCR process fails- why?
- In our studies, the failure seems to be more a function of sequence than of amplicon size.
- Mechanisms for inhibition may vary however, and in many cases- soil and bone- for example, both degradation and inhibition may be present

## MiniSTR Amplification w/ increasing Humic Acid



## MiniSTR Amplification w/ increasing calcium



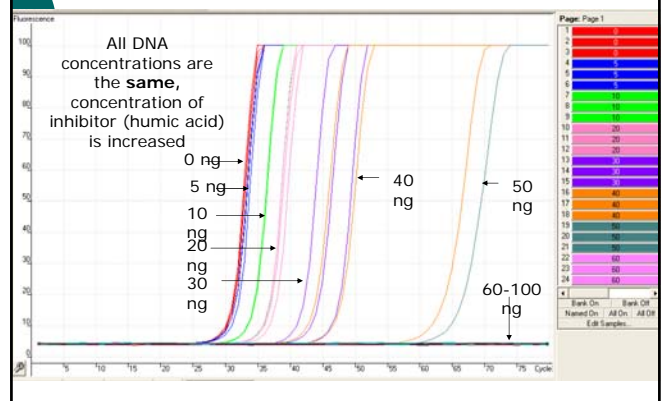
## Results – Inhibition Thresholds MiniSTRs

Inhibitor	Big Miniplex	Miniplex 2	Miniplex 4
Hematin	1 µM	0.8 µM	0.8 µM
Indigo	280 µM	320 µM	300 µM
Melanin	0.16 ng/µL	0.2 ng/µL	0.2 ng/µL
Humic Acid	0.5 ng/µL	0.6 ng/µL	0.6 ng/µL
Collagen	24 ng/µL	32 ng/µL	24 ng/µL
Calcium	1100 µM	1100 µM	800 µM

## PCR Inhibition: Observations

- Inhibitors act in many ways – The most worrisome are those which co-extract with DNA
- These inhibitors produce various effects on data including- peak balance problems, locus specific dropout, enhanced stutter, and poor sensitivity
- Mechanisms appear to vary with type of inhibitor and sequence of amplicon
- It is important to understand concentration effects and mechanisms so that inhibition cannot be confused with degradation, dropout and mixture effects

## Effect of Inhibitors on qPCR



## Tests for PCR inhibition using Realtime PCR with high resolution melt

- Compare inhibition for a single locus (TH01) with primers of various lengths and melting temperatures
- Determine the effect of length and sequence on PCR inhibition
- Examine melt temperatures of amplicons in inhibited samples
- Classify inhibitors by effect on PCR
- Determine mechanism of different PCR inhibitors

## Experimental Design

Locus – HUMTH01 STR  
DNA – Homozygous 9.3 allele

### Primers

- 3 lengths (~100, 200, 300 bp)
- 3 Tm (58, 60, 62° C)
- Amplified product (one product)

### qPCR conditions

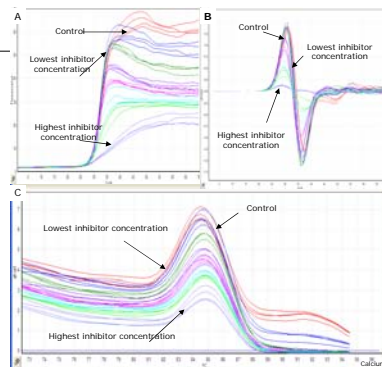
- Standard conditions for *Alu* quantification (Nicklas et al. 2003)
  - No BSA
  - Lower Primer Concentration
  - Reduced Taq

### Inhibitors

- Calcium, humic acid, hematin, collagen, melanin, tannic acid

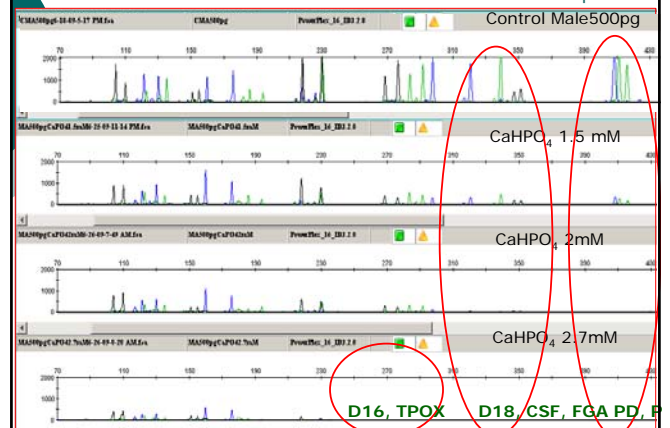
## qPCR Calcium Inhibition

- No shift in take off cycle
- No change in melting curve
- Efficiency of amplification affected
- No difference for size or Tm



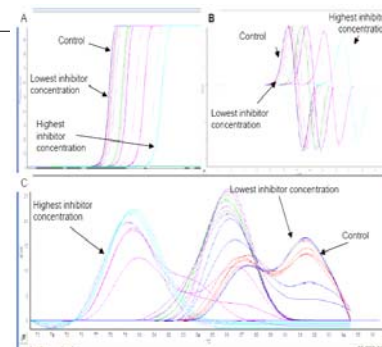
Conclusion: Taq Inhibitor

## Inhibition of PP16 with CaHPO<sub>4</sub>



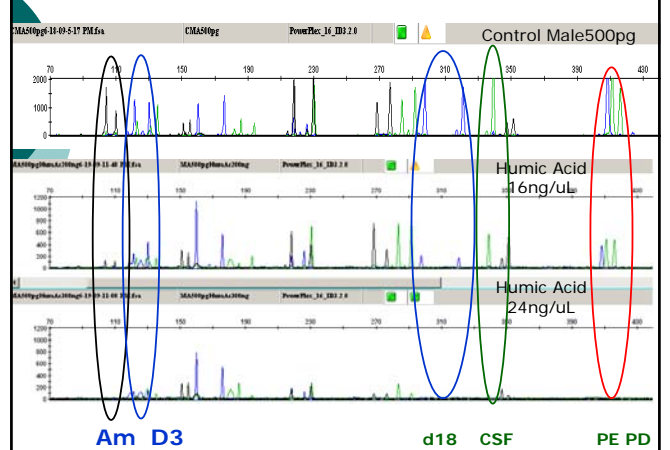
## qPCR Humic Acid Inhibition

- Shift in take off cycle
- Change in melting curve
- No efficiency of amplification change
- Size effects on melt curve



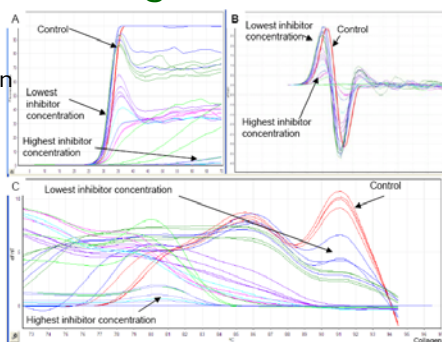
Conclusion: Sequence specific Inhibitor

## Inhibition of PP16 with Humic Acid



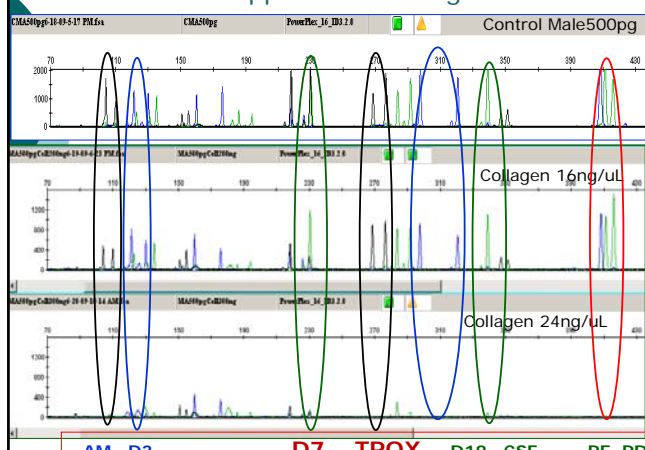
## qPCR Collegen Inhibition

- Minimal shift in take off cycle
- Change in melting curve
- Change in efficiency of amplification



**Conclusion: Taq inhibitor through binding DNA**

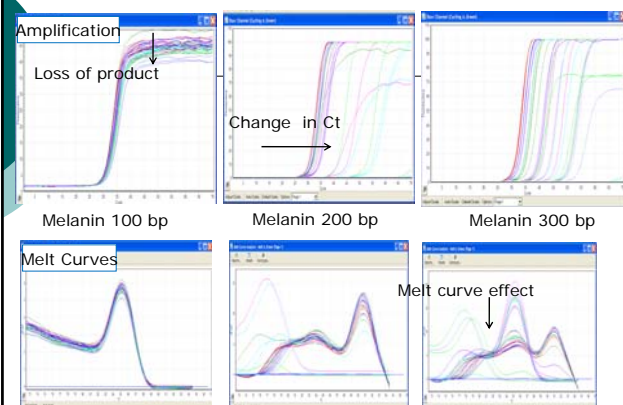
## Inhibition of pp16 with Collagen



## Size Effects

- Inhibition tests examined both concentration and amplicon size of THO1 target – 100, 200, 300bp
- Results indicate melt curve effects are reduced for smaller amplicons for many inhibitors.
- 300bp amplicons show the effect best.

## Effect of Amplicon length on Inhibition: Increasing Melanin



**Larger amplicons tend to show DNA inhibitor binding effects  
Small amplicons not effected. Loss of product evident.**

## Important point for these results

- When used for real time PCR, short amplicons may be unable to pick up inhibitors that only affect large STRs
- This provides a reason why in some circumstances, qPCR internal control sequences do not pick up inhibition
- Thus when designing qPCR systems, amplicon size and inhibition effects should be considered

## Conclusions

- DNA typing is a complex process involving PCR amplification of STRs, Laser induced fluorescence, and capillary gel electrophoresis
- Degraded Samples (bone and hair) can be accessed through redesigned Primers, however stochastic effects must be considered
- Sample quality and quantity can be assessed using real time PCR
- PCR inhibition can be tracked and understood using real time PCR melt curves.

## Acknowledgements


- o **McCord DNA Research Group –**
- o **Collaborators:**
  - George Duncan- BSO
  - Eric Buel -Vermont
  - John Butler – NIST
  - Jiri Drabek – Czech R.
- o **Funding:**
  - National Institute of Justice
  - National Science Foundation
  - FBI Laboratory
  - Agilent Technologies
  - Promega Corporation

**Post Docs:** Yin Shen, Jiri Drabek, Maximilien Blas, Maribel Funes, Silvia Zoppis, Jing Wang

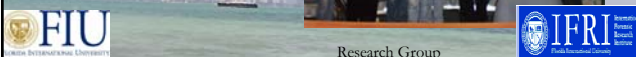
**PhD Students:** Denise Chung, Brittany Baguley, Kerry Opel, Maurice Aboud, Deepthi Nori,

**MS Students:** Ada Nunez, Rayna Hebard, Sarah Hall, Robyn Thompson, Tanya Madi


**UG Students:** Stefano Boulas, Oscar Cabrices, Willaim Kennedy, Justin Jans, Ramone Alatorre



Research Group

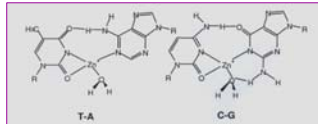


## Thank you



## Transition metal ions

Metal cations present in degraded samples represent a different type of inhibition

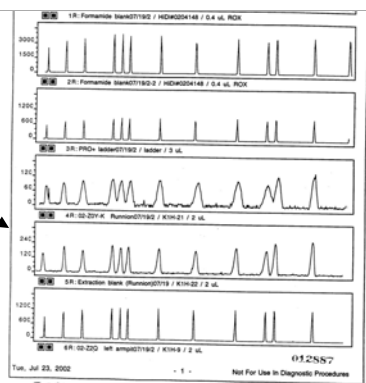


Zn<sup>2+</sup>, Co<sup>2+</sup>, and Ni<sup>2+</sup> form DNA-metal ion complexes, termed M-DNA at pH conditions above 8,

These cations produce severe effects in CE injection and analysis

Hartzell and McCord, *Electrophoresis*, 2006

## Effect of contaminant in reference sample



Note problems in subsequent analyses

Effect is transitory

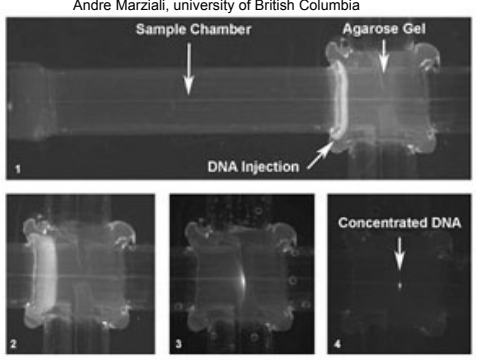
## Clean-Up of PCR Inhibitors

- o Bovine serum albumin (BSA)
  - Make enzyme more efficient and binding certain inhibitory compounds
- o Microcon
  - Wash smaller inhibitors from larger DNA
- o Low-melting temperature agarose/sephadex/filtration
  - capture large polymers like DNA, releasing smaller inhibitors
- o Electrophoretic Purification
  - Inhibitors move at different rates under applied fields
- o Addition of higher concentrations of Taq polymerase/Mg
  - Overwhelm inhibitors that bind to taq/decrease selectivity
- o Dilution of Sample
  - DNA still amplifies, inhibitors are less concentrated and bind to taq and/or other reaction components
- o Destruction of inhibitors w/ NaOH
- o Improved enzymes/increased Taq
- o Use magnetic bead extraction

## Multidimensional Electrophoresis in low melting agarose is another possibility

SCODA

Andre Marziali, university of British Columbia



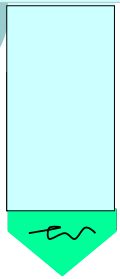
Sample Chamber      Agarose Gel

DNA Injection

Impure DNA

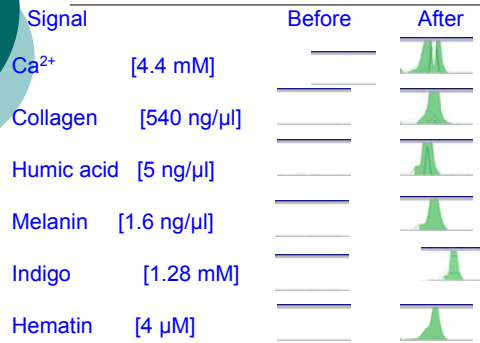
Concentrated DNA

## LMT Agarose Method



- Use 1.6% LMT
- Add DNA with inhibitors to 5 ul of LMT
- 300 ul of TE buffer and let shake overnight
- Pipet supernatant out and wash again
- Melt and elute DNA

## Results of Cleanup with LMT Agarose



## Other methods

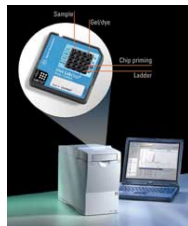
- Microcon may cause DNA losses, may not release DNA binding
- Rinse with NaOH
  - Sample added to microcon, rinsed twice with 0.4 N NaOH and collected in Tris-HCl buffer
  - Increased handling of sample
  - 20-50% loss of DNA, not useful for low yield samples
- Dilution of sample
  - Decrease concentration of inhibitors
  - Also decreases DNA, not useful for low yield samples
- All of these procedures can be tested with various levels of inhibition once thresholds are known

## Conclusions and future work

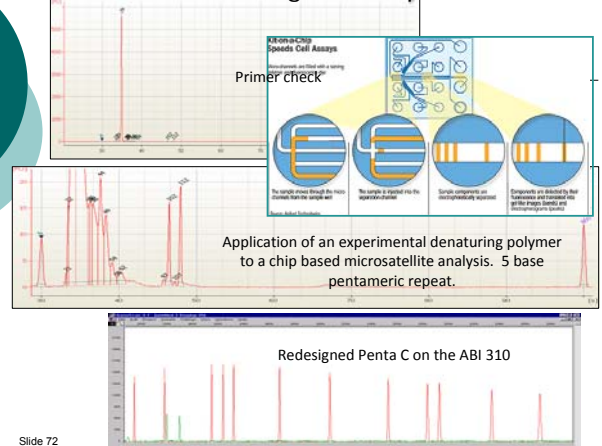
- DNA may be degraded, inhibited or at low copy or all three
- Subtle clues exist to tell the difference
- MiniSTRs can help with DNA degradation but not LCN
- QPCR can predict inhibition and degradation through amplicon size and melt curve detection
- Further testing is needed to classify forms of inhibition and determine mitigation

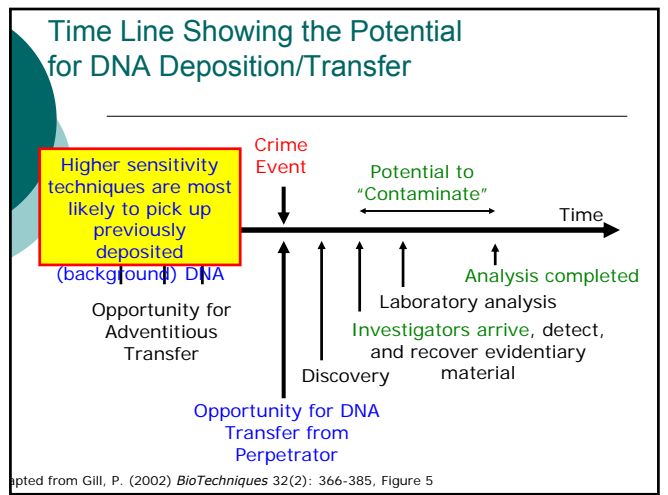
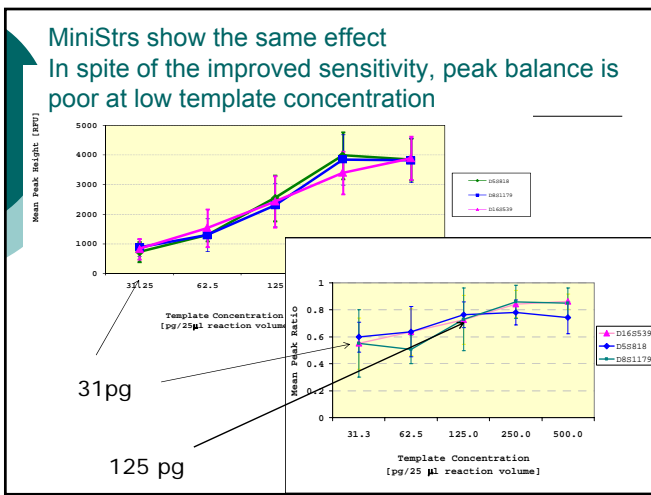
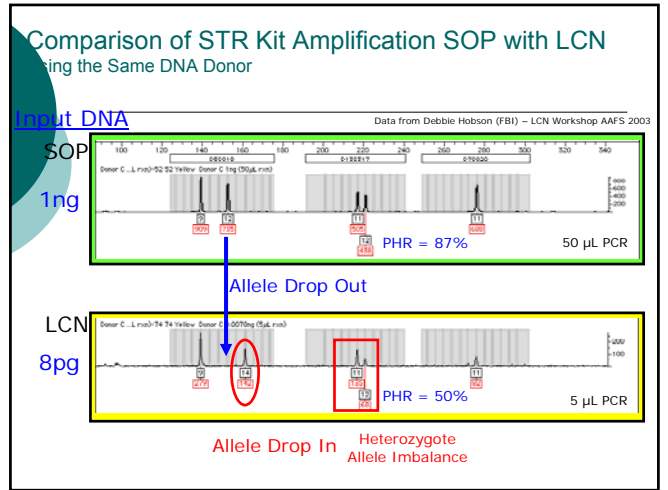
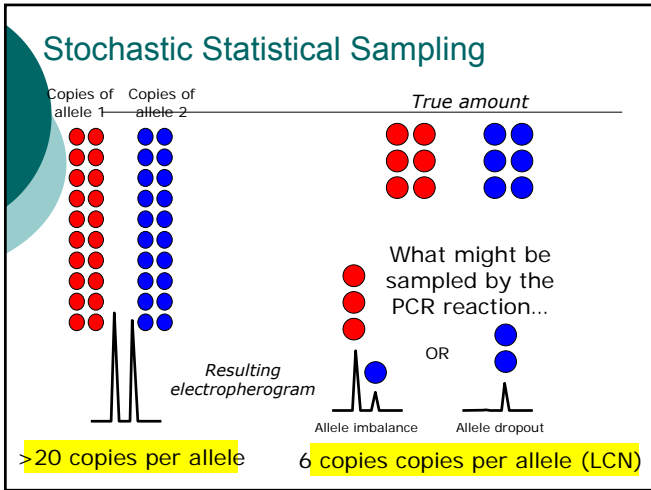
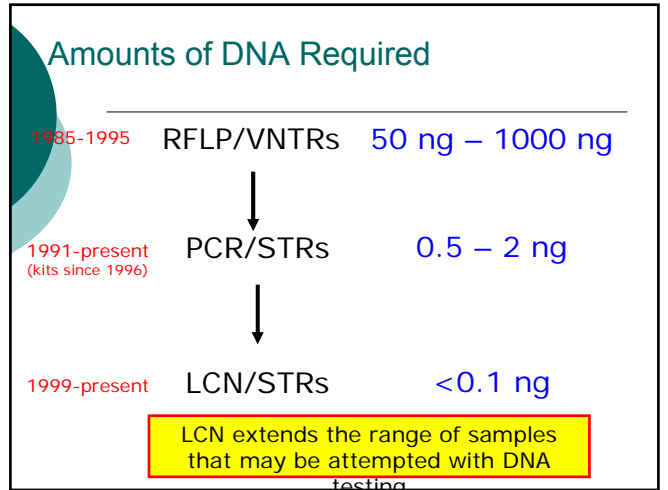
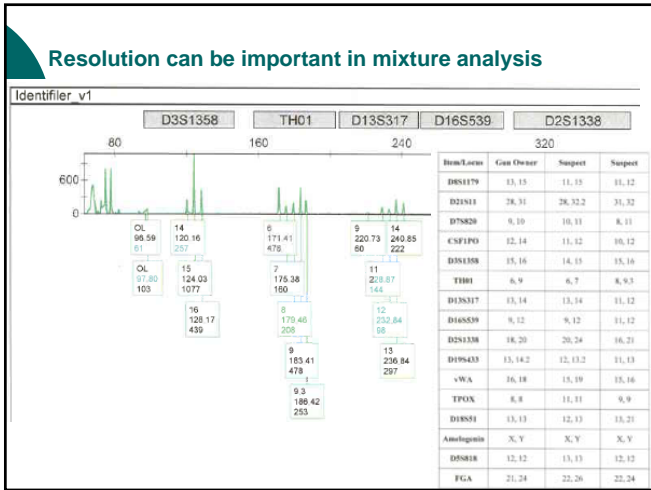
## The Agilent bioanalyzer is a commercially available chip system (it works like an agarose gel)

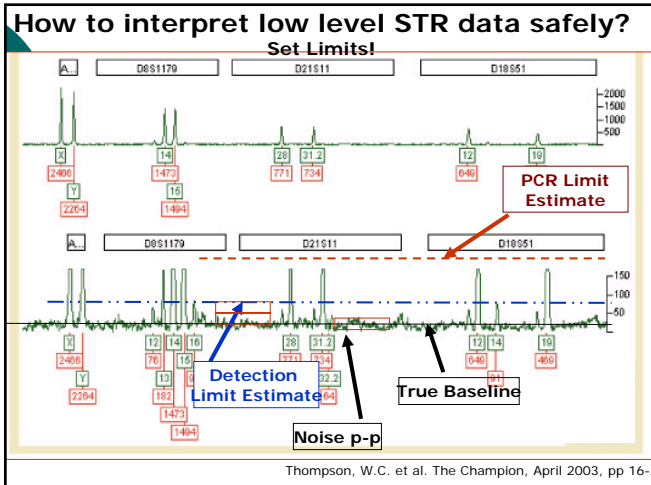
- A disposable analytical platform That works right out of the package. Fieldable for mass disaster?
- but resolution is too low for 4base STRs
- How to improve resolution?
  1. Use mini 5 base penta STRs
  2. Switch to denaturing polymer



## Results with modified Agilent Chip and Penta STRs







### What to look for in the QPCR results.

B1	STD 1	Quantifier Human	Standard	24.48	0.046	5.0
		IPC	Unknown	(31.83)	1.539	
B2	STD 2	Quantifier Human	Standard	26.11	0.048	16.7
		IPC	Unknown	25.93	0.668	
B3	STD 3	Quantifier Human	Standard	27.72	0.053	5.58
		IPC	Unknown	28.41	0.353	
B4	STD 4	Quantifier Human	Standard	28.12	0.002	1.85
		IPC	Unknown	29.26	0.21	
B5	STD 5	Quantifier Human	Standard	30.74	0.064	6.29E-01
		IPC	Unknown	29.34	0.176	
B6	STD 6	Quantifier Human	Standard	32.3		2.10E-01
		IPC	Unknown	29.37	0.152	
B7	STD 7	Quantifier Human	Standard	33.92	0.309	6.80E-02
		IPC	Unknown	29.39	0.222	
B8	STD 8	Quantifier Human	Standard	35.01	0.526	2.30E-02
		IPC	Unknown	29.38	0.251	
B9	NTC-4	Quantifier Human	NTC	Undetermined		
		IPC	Unknown	29.16		
C1	EBC042407HN3	Quantifier Human	Unknown	Undetermined		
		IPC	Unknown	29.38		
C2	07-0212QE	Quantifier Human	Unknown	37.22		6.83E-03
		IPC	Unknown	29.31		

Sample is at a low level, nominally 6pg/uL but outside the calibration range.

- ### Conclusions
- MiniSTRs are for degraded DNA
  - Validation data reveals a robust and sensitive multiplex amplification
  - Virtual yield gel using qPCR helpful for proper analytical results
  - Stochastic effects still occur for samples under 125pg
  - Improved results are possible for bone and telogen hair
  - Degradation is still a problem

### Recovery of DNA from degraded Samples UT Forensic Anthropology Center

Implications for Mass Disasters

And Questions about Recovery of Ancient DNA