Fit for purpose experiments: Experience from bioreference material production

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Things to consider

- What is it you are trying to determine/deliver?
- What type of sample/s is needed?
- How are you collecting the sample/s?
- How are you going to measure the analyte/s of interest?
- How are you going to analyze the data?

What is it you are trying to determine/deliver?

- Presence or Absence

  If Present:

  - Copy number present/concentration
  - Form of the analyte:
    - Actual compound or a metabolite
- If Absent:
  - Detection limit (What does zero mean)

What type of sample/s is needed?

- Liquid
- Stain
- Swab
- Paper
- Cell pellet
- Extract

How are you collecting the sample/s?

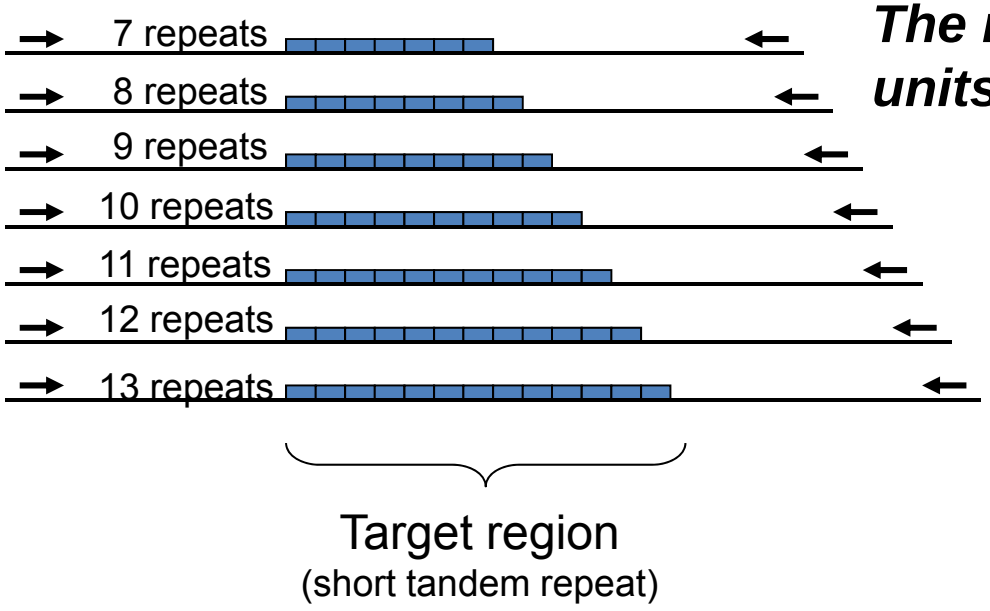
- Does the collection process provide:
  - a representative sample?
  - a reproducible sample?
  - a stable sample?
  - an analyzable sample (for all the methods of interest)?

Short Tandem Repeat (STR) Markers

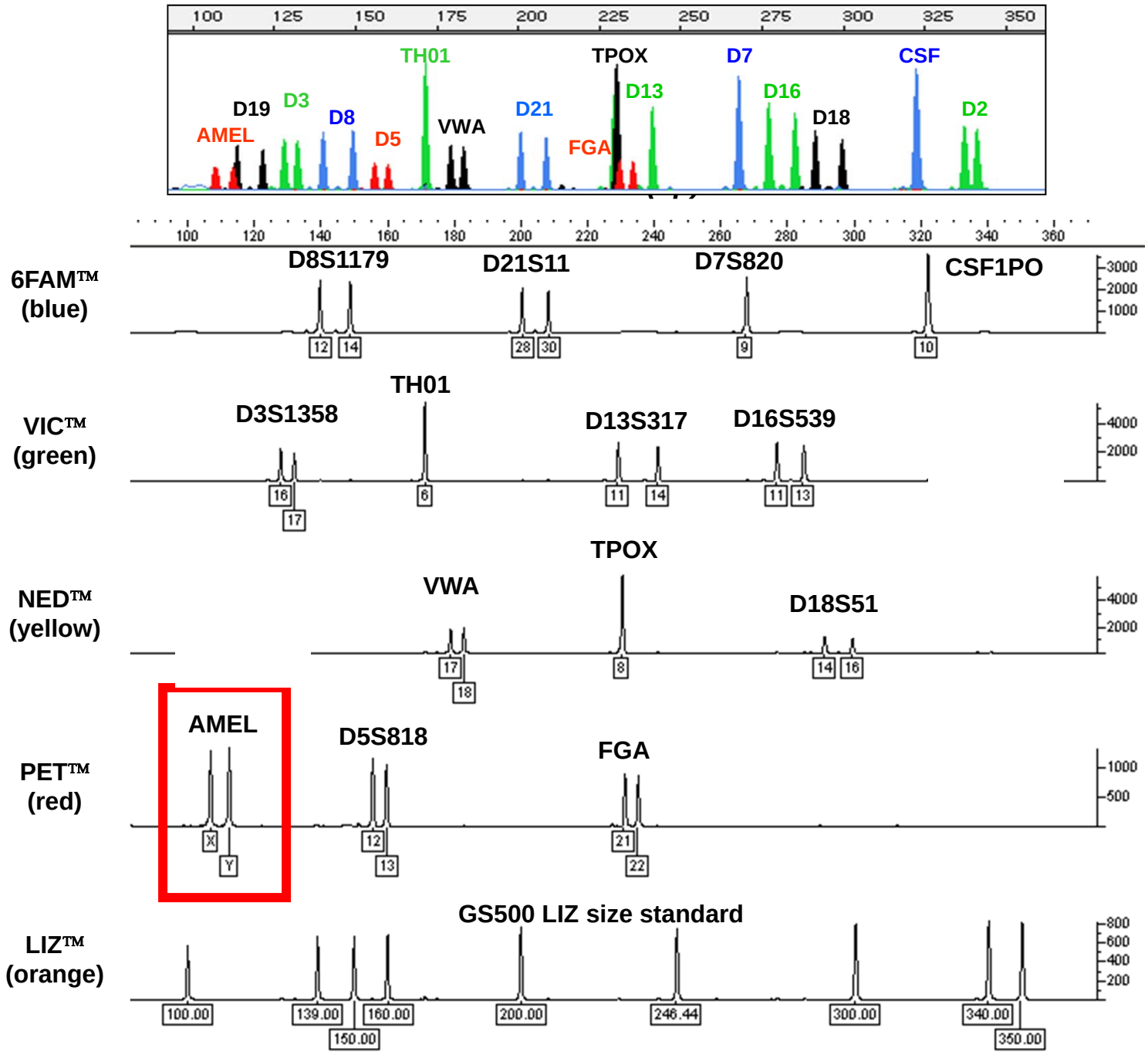
An accordion-like DNA sequence that occurs between genes

TCCCAAGCTCTTCCTCTTCCCTAGATCAATACAGACAGAAGACA
GGTG**GATAGATAGATAGATAGATAGATAGATAGATAGATAGATA**
GATAGATATCATTGAAAGACAAAACAGAGATGGATGATAGATACA
TGCTTACAGATGCACAC

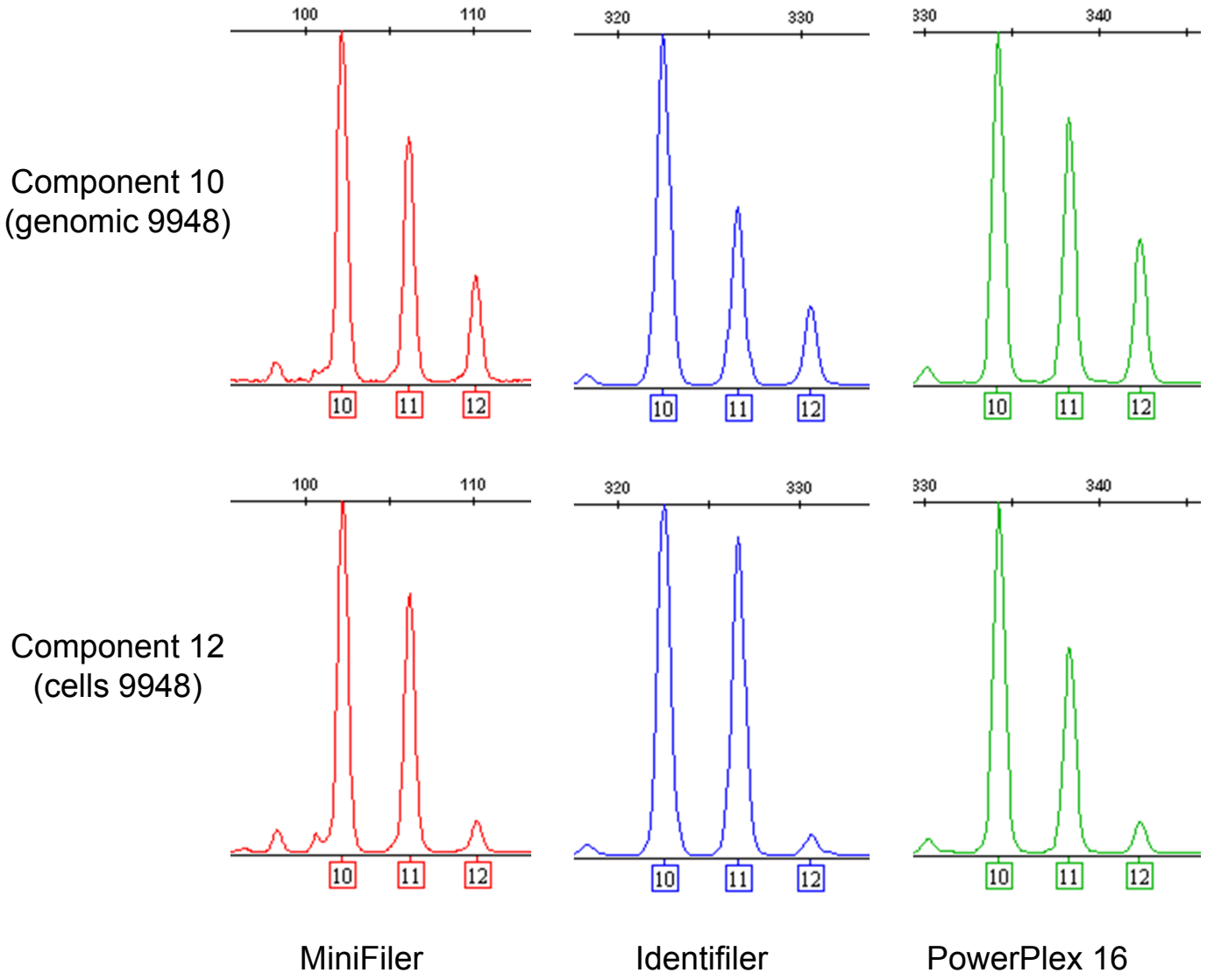
= 12 GATA repeats ("12" is all that is reported)



The number of consecutive repeat units can vary between people


100 125 150 175 200 225 250 275 300 325 350
AMEL
D19
D3
D8
D5
TH01
VWA
D21
FGA
TPOX
D13
D7
D16
D18
CSF
D2
100 120 140 160 180 200 220 240 260 280 300 320 340 360
6FAM™ (blue)
D8S1179
D21S11
D7S820
CSF1PO
12 14
28 30
9
10
3000
2000
1000
VIC™ (green)
TH01
D3S1358
D13S317
D16S539
16 17
6
11 14
11 13
4000
2000
NED™ (yellow)
TPOX
VWA
D18S51
17 18
8
14 16
4000
2000
PET™ (red)
AMEL
D5S818
FGA
X Y
12 13
21 22
1000
500
LIZ™ (orange)
GS500 LIZ size standard
100.00
139.00
150.00
160.00
200.00
246.44
300.00
340.00
350.00
800
600
400
200

A comparison of the tri-allelic profile of components 10 (genomic 9948) and 12 (cell line 9948) at the CFS1PO locus with MiniFiler, Identifiler, and PowerPlex 16



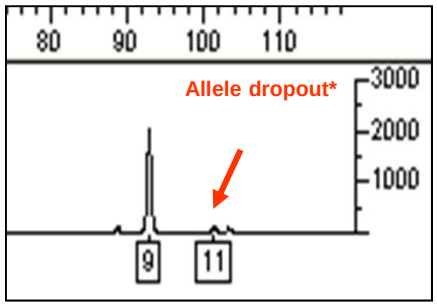
How are you going to measure the analyte/s of interest?

- Is the analysis method:
  - measuring what you wanted to measure?
  - adequately precise and unbiased?
  - adequately sensitive (dynamic range)?
  - robust to normal differences between samples?
  - robust to amount of sample collected?
  - affected by sample preparation?
  - relatively free from matrix affects (inhibitors, interferences,)?
  - validatable?

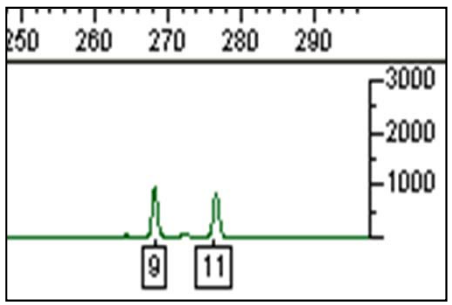
Different tests different answer

D16S539
SRM 2391b
Genomic 8

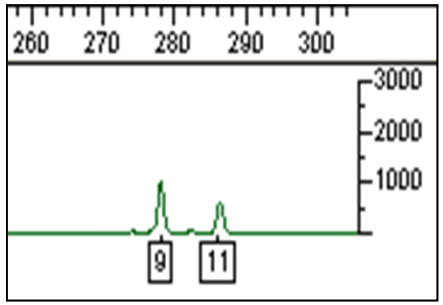
MiniFiler



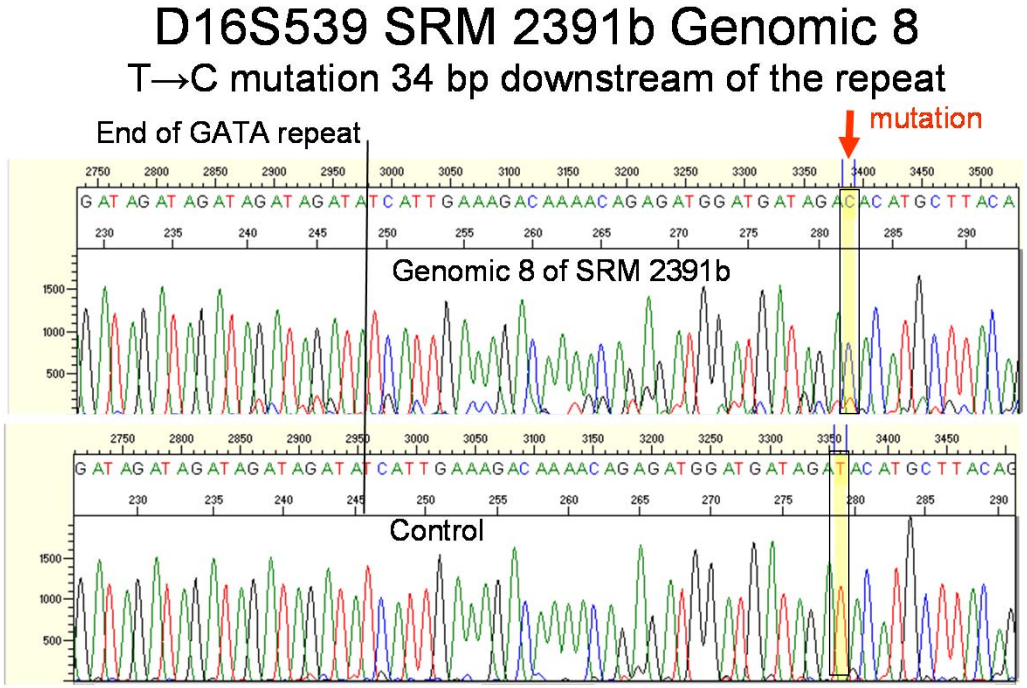
Identifiler



PowerPlex 16



**Due to primer binding site mutation*

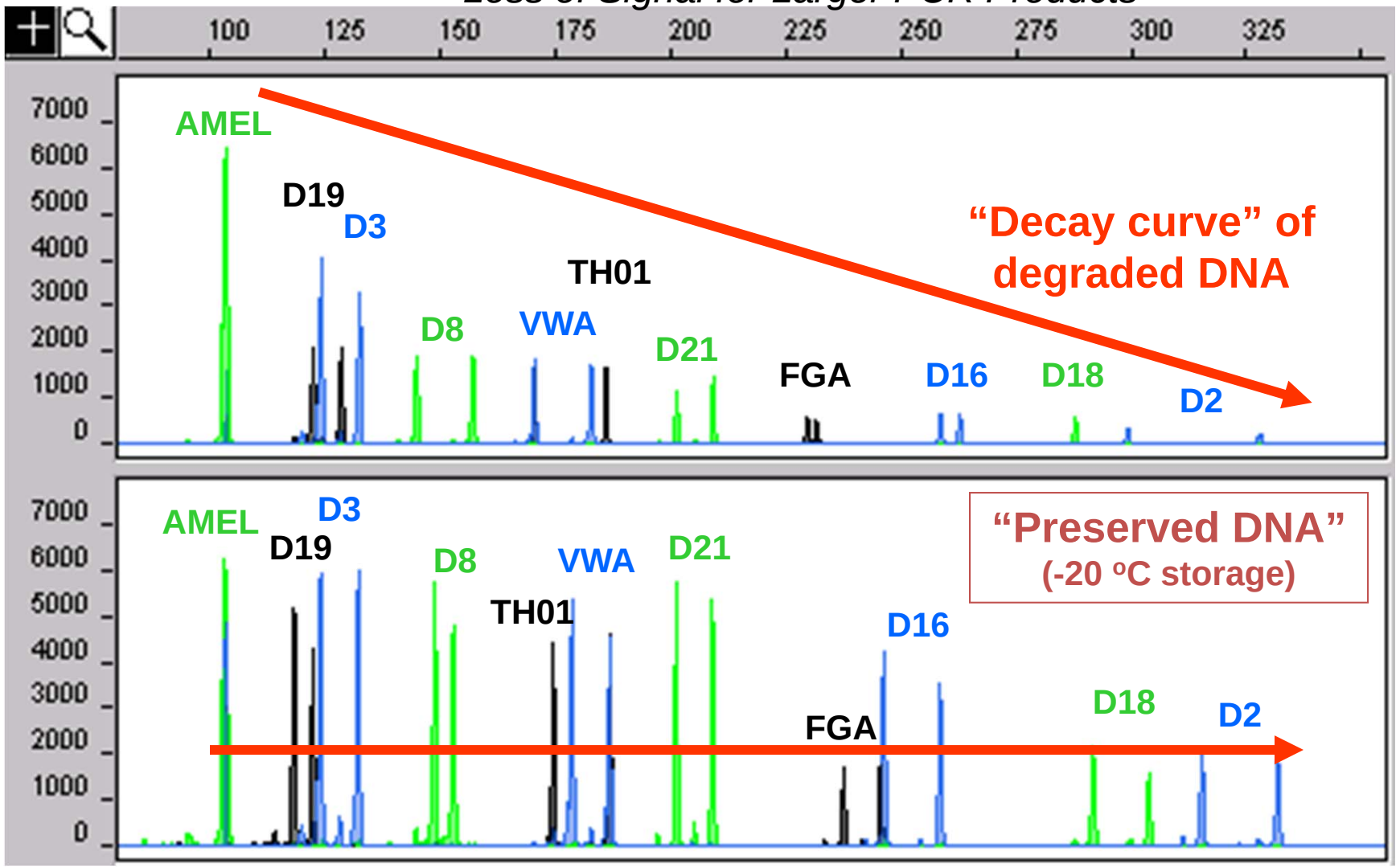


How are you going to analyze the data?

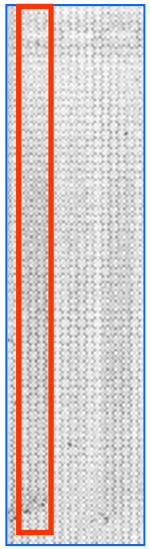
- Are the results of the measurement:
  - fit for purpose?
  - computer ready (spreadsheet, etc.)?
  - trackable?
  - traceable?

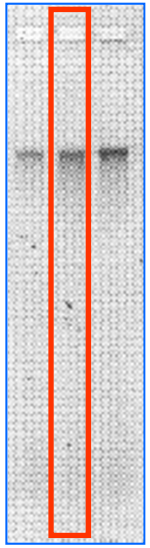
Degraded DNA

Loss of Signal for Larger PCR Products



Yield gel results

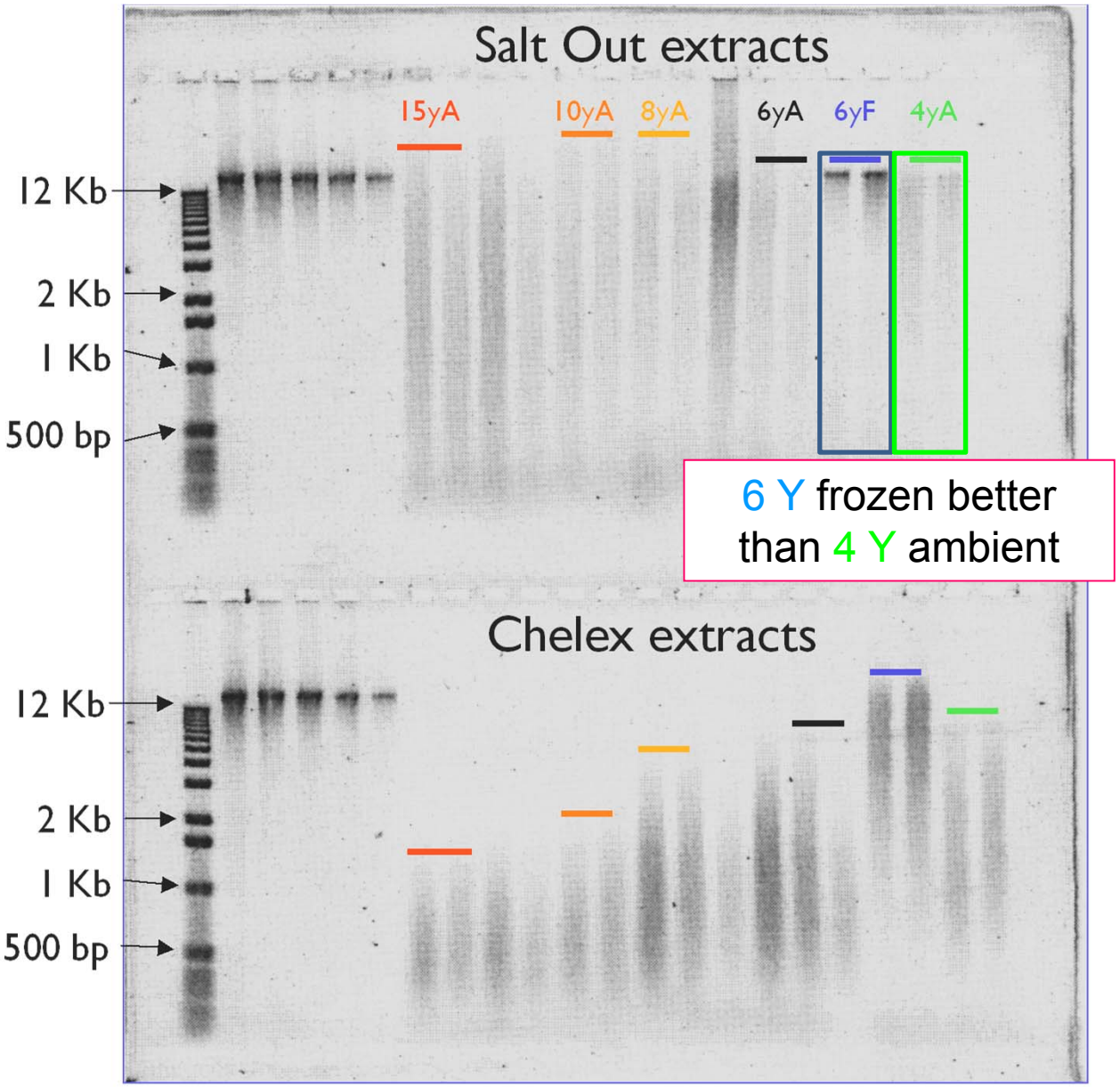




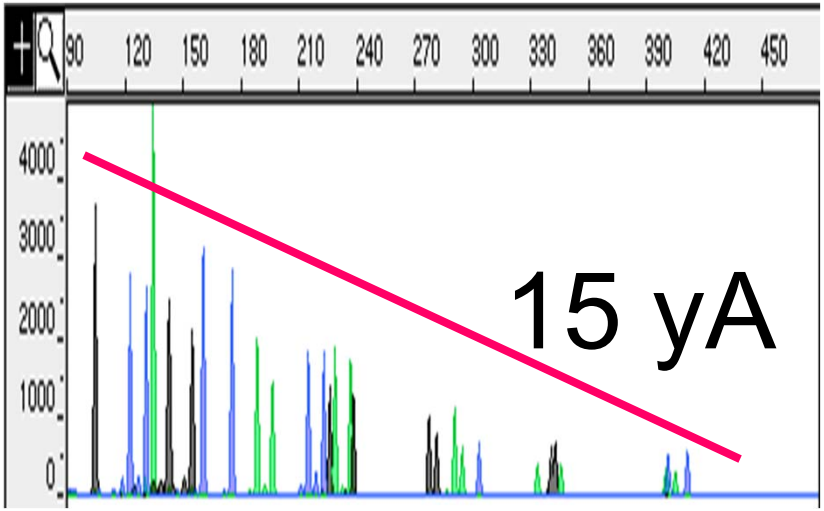
Results with SGM Plus STR kit (Applied Biosystems)

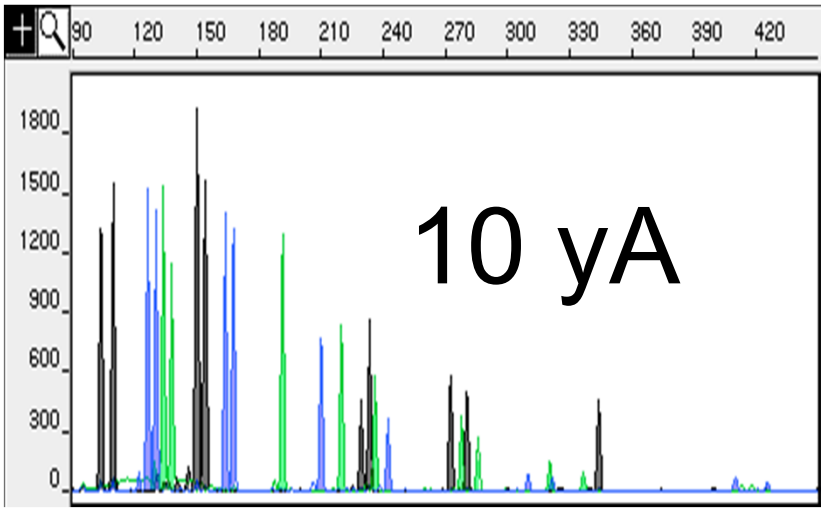
Data from a study done at NIST in May 2001

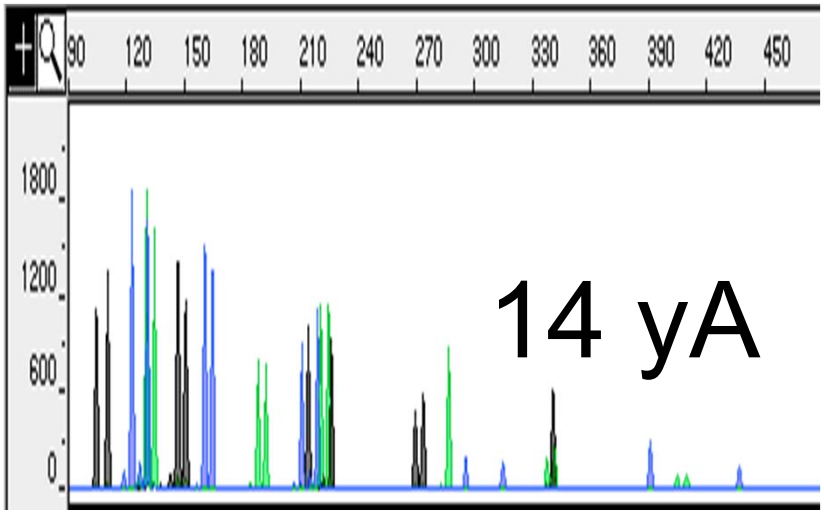
Quality of the DNA Extracted from Bloodstains Field Samples

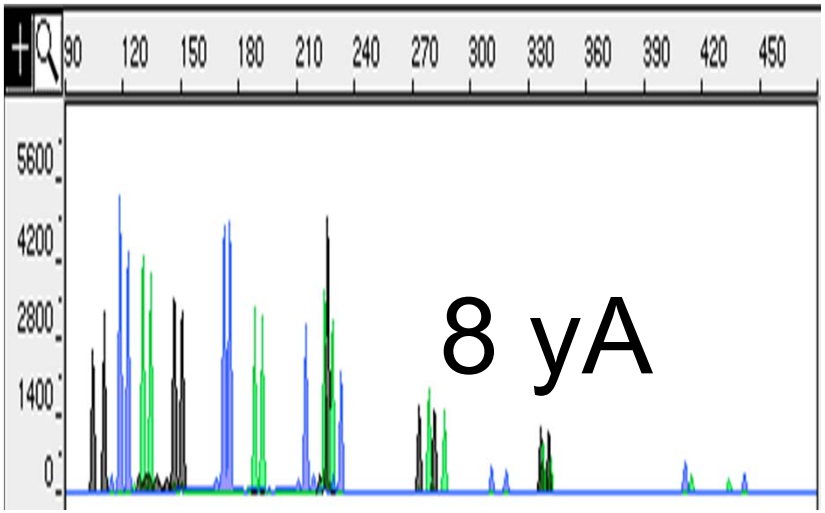


Ambient Stored Field Samples

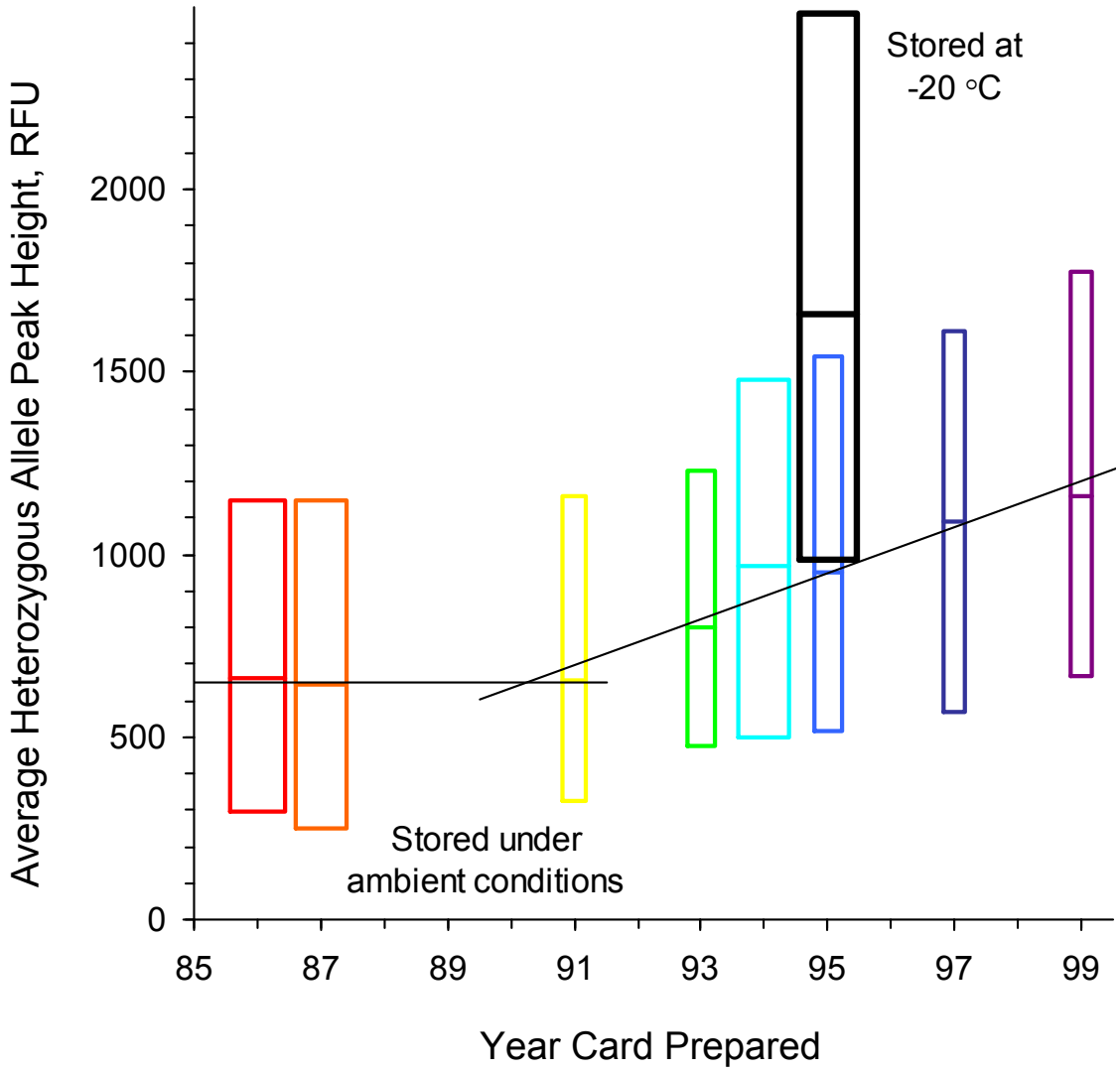








STR analysis results: Average Heterozygousity Peak Heights



Median allele height as a function of storage time.

Each “box” denotes the central 50% of each height distribution (25%, median, 75%). The box width is proportional to the number of alleles. The lines suggest a possible “plateau” in height loss after about a decade’s storage at ambient temperatures.

Study conducted in 2001

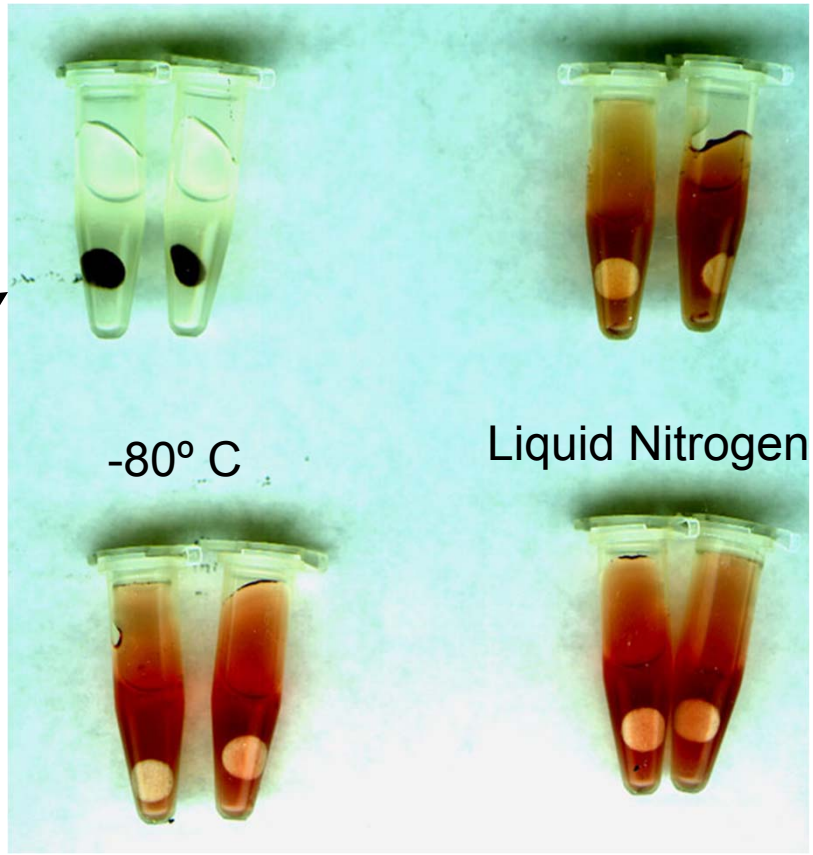
Bloodstain Stability Studies

- Degradation is a complex function of temperature and enzymatic actions
  - High temperatures may deactivate enzymes that would remain active at lower temperatures.

DNA Stability Study
6 year time point

903 paper

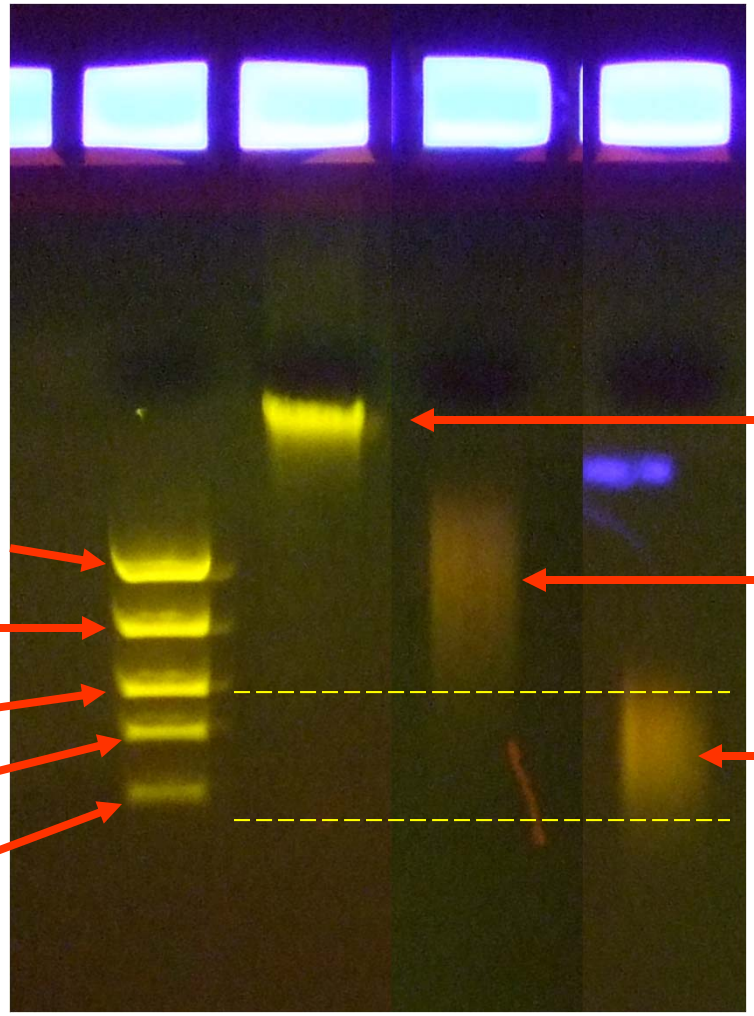
During Chelex extraction RT sample during water washes remains colored.



Degraded DNA Issue

- What is the difference in the qPCR results of “good” quality DNA verses degraded DNA?
- The Experiment:
  - Aliquot Control DNA into several different tubes
  - Hold one tube as a control.
  - Sonicate the remaining tubes for various lengths of time.
  - Quantify the samples and amplify based on quant values.
  - Check the results!

Gel image of degraded DNA



Control DNA was pipetted into several tubes. The tubes were sonicated until various levels of degradation was observed

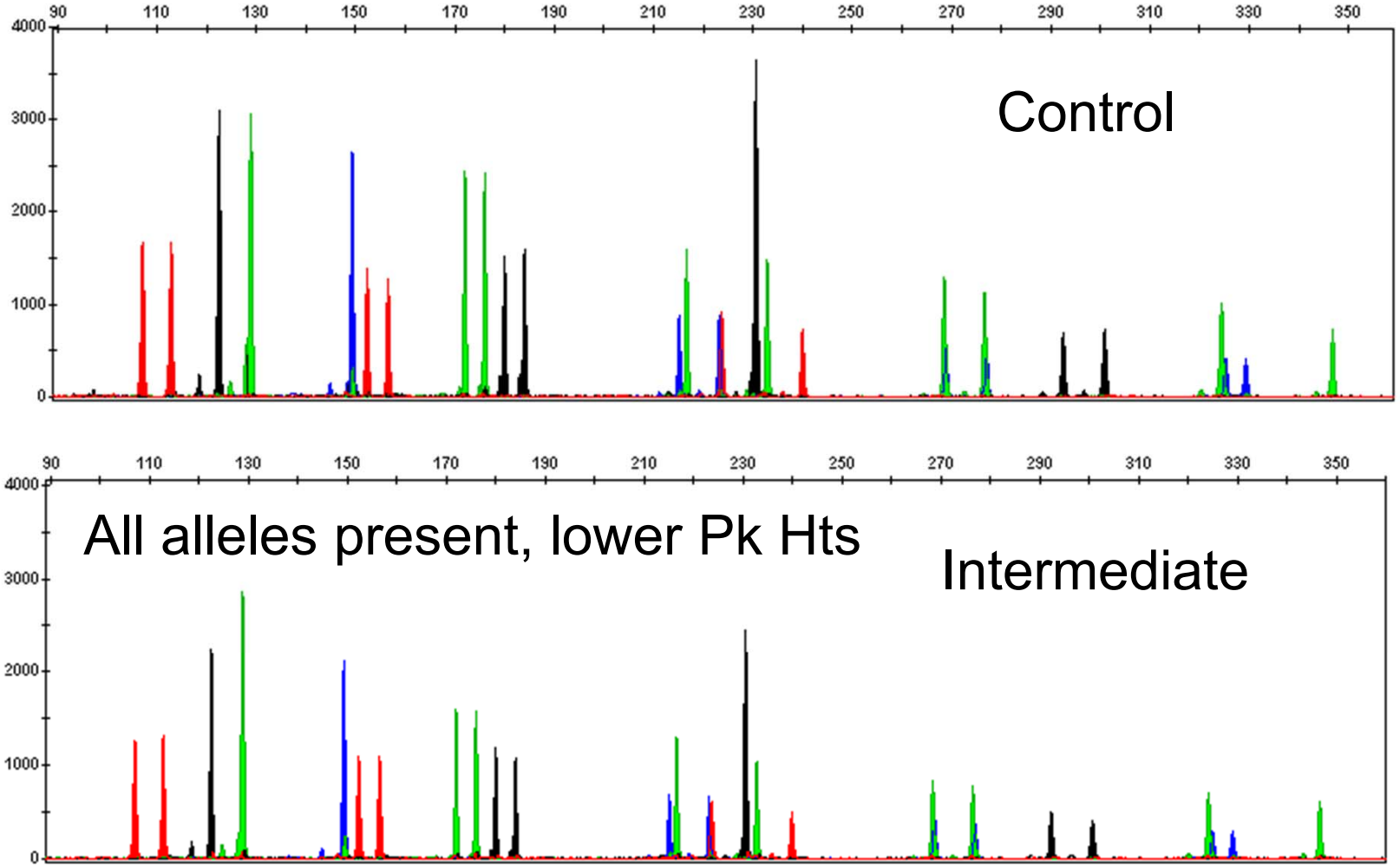
Control DNA

Intermediate Degradation

Degraded DNA

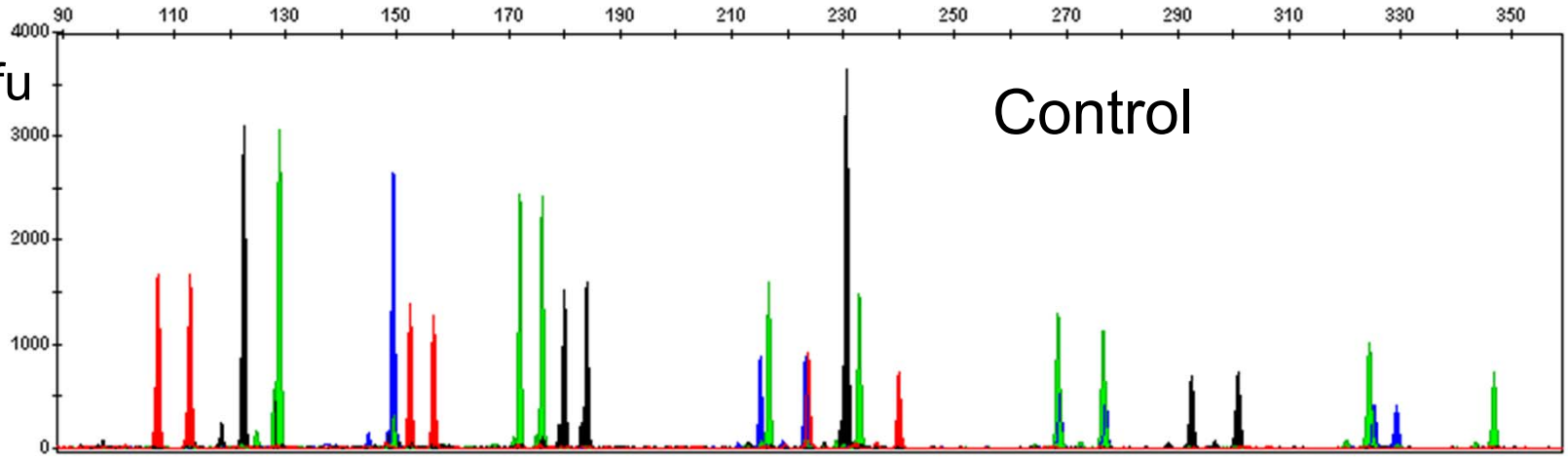
40 ng of DNA used for visualization, based on original [DNA].

“1 ng” Control / intermediate degraded DNA

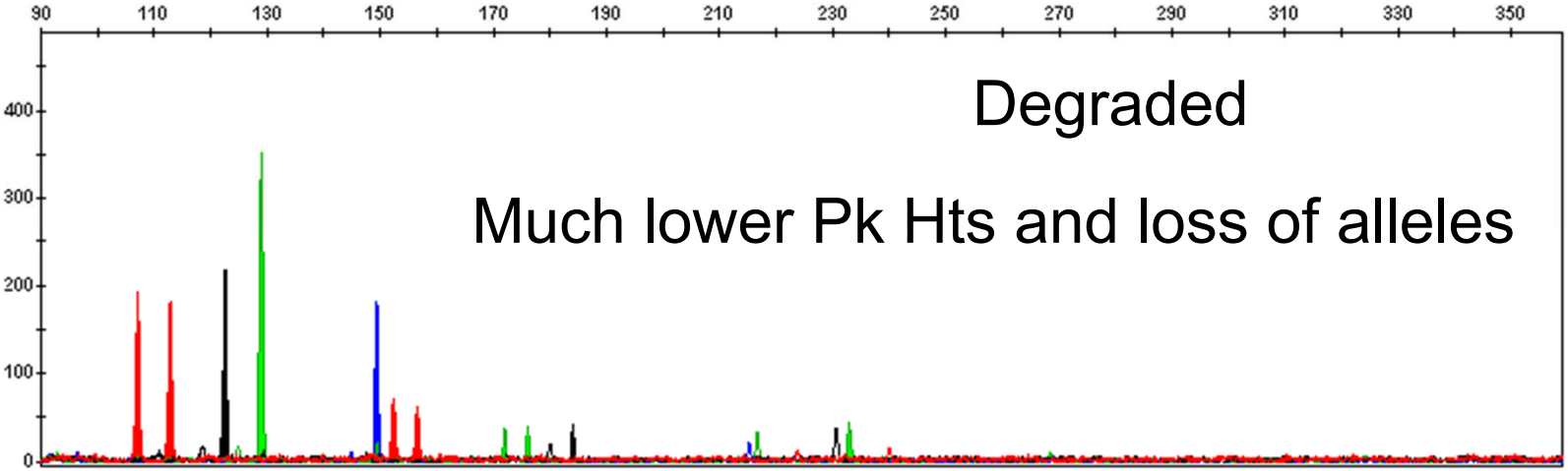


Typing “1 ng” degraded DNA

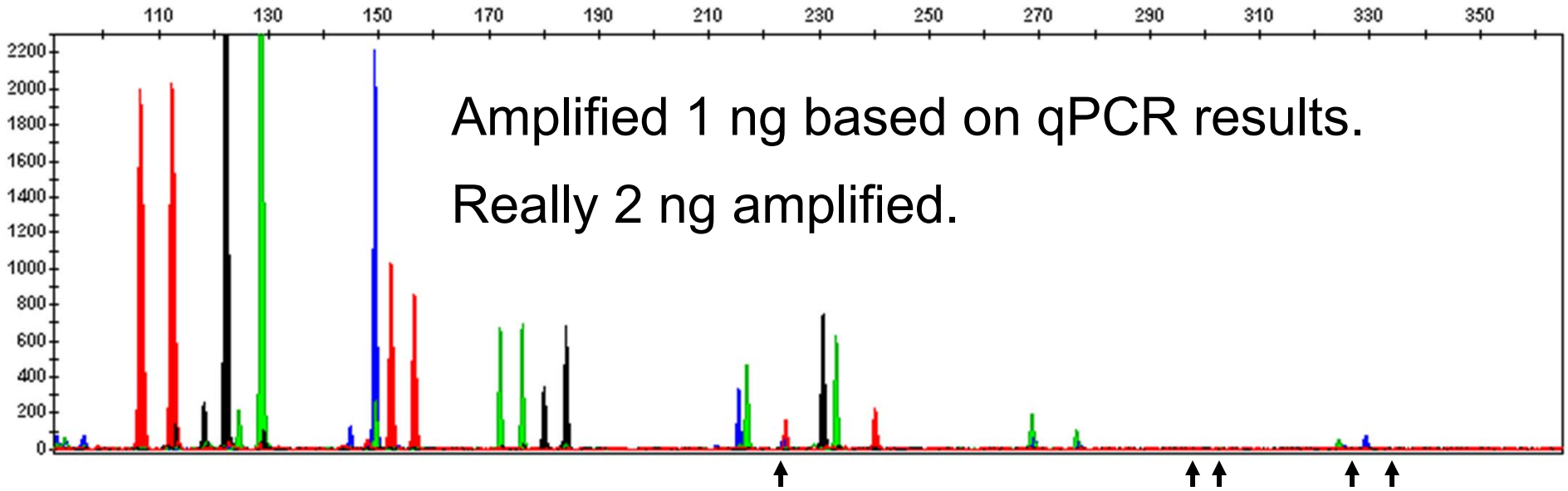
4000 rfu



600 rfu



Higher injection of Degraded DNA



Locus	# lost alleles	lost allele	True Genotype
D21S11	1	33.2	(31.2,33.2)
CSF1PO	1	11	(11,12)
D2S1338	1	25	(19,25)
D18S51	2	14,16	(14,16)

Elevated Temperature Studies

- Remember:
- What are you trying to measure?
- Is the elevated Temperature Realistic?
- Example: Proteinase K, used during DNA extraction, is active for:
  - 37 °C overnight;
  - 56 °C for 2 hours;
  - 65 °C for 15 minutes

  So if your storing dry bloodstains at 65 °C for accelerated stability - are you deactivating the enzymes responsible for DNA degradation?

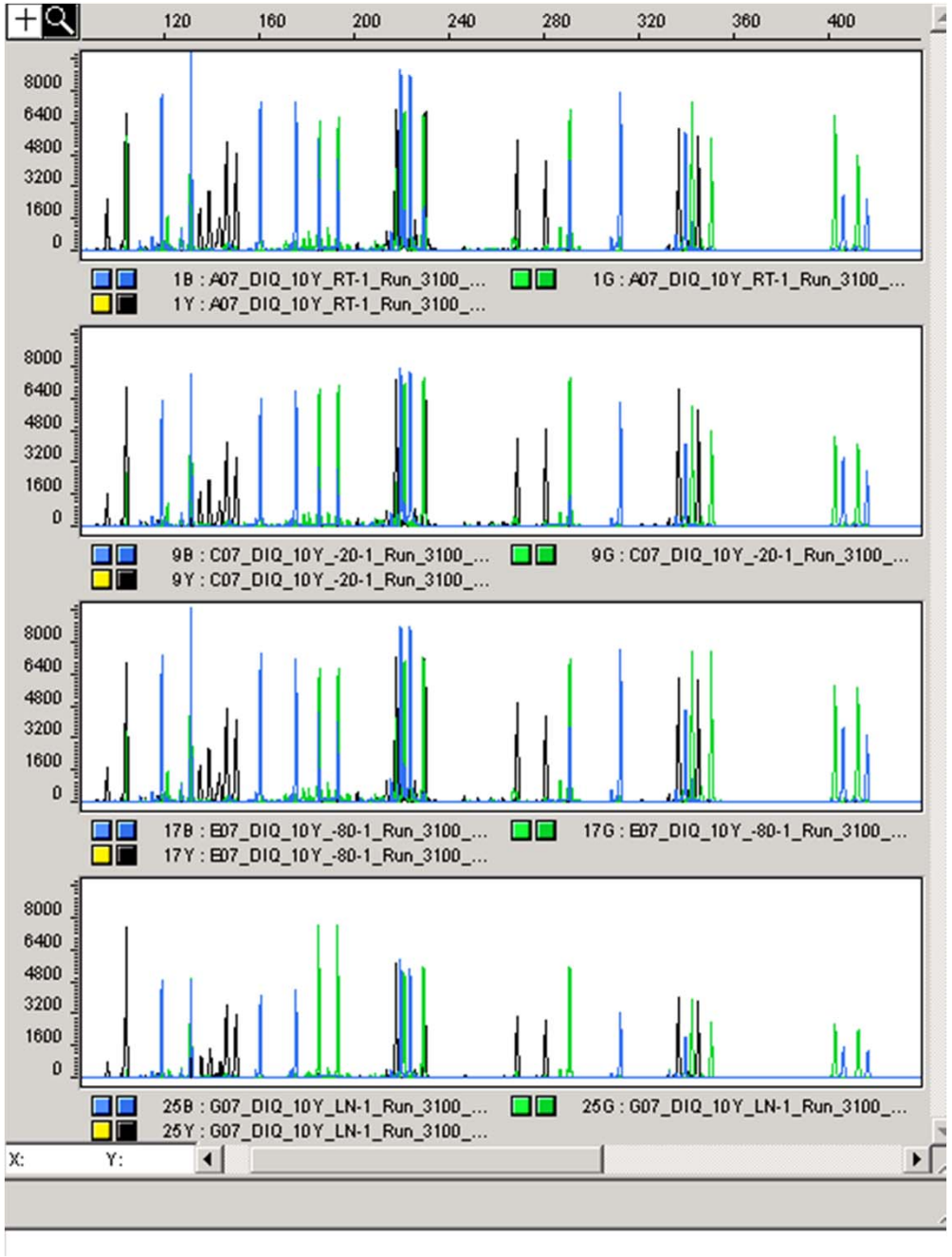
Bloodstain Stability Studies

- Extraction is a complex function the sample, the matrix, and the method.
  - Very cold storage (-80 °C, liquid N_2) may make the DNA harder to extract.

DNA recovered from 10 year old bloodstains

Three different extraction methods used duplicated samples
Stains on 903 paper

Sample Storage	Extraction Method A	Extraction Method B	Extraction Method C
Lab Ambient	52 ng	75 ng	11 ng
-20 °C	49 ng	43 ng	13 ng
-80 °C	42 ng	45 ng	9 ng
Liq N_2	43 ng	37 ng	11 ng



RT

-20º C

-80º C

Liq N_2

Method A Extracts Controlled 10y Study

All loci present and equally amplified with PP16. No effort was made to balance the amount of DNA amplified

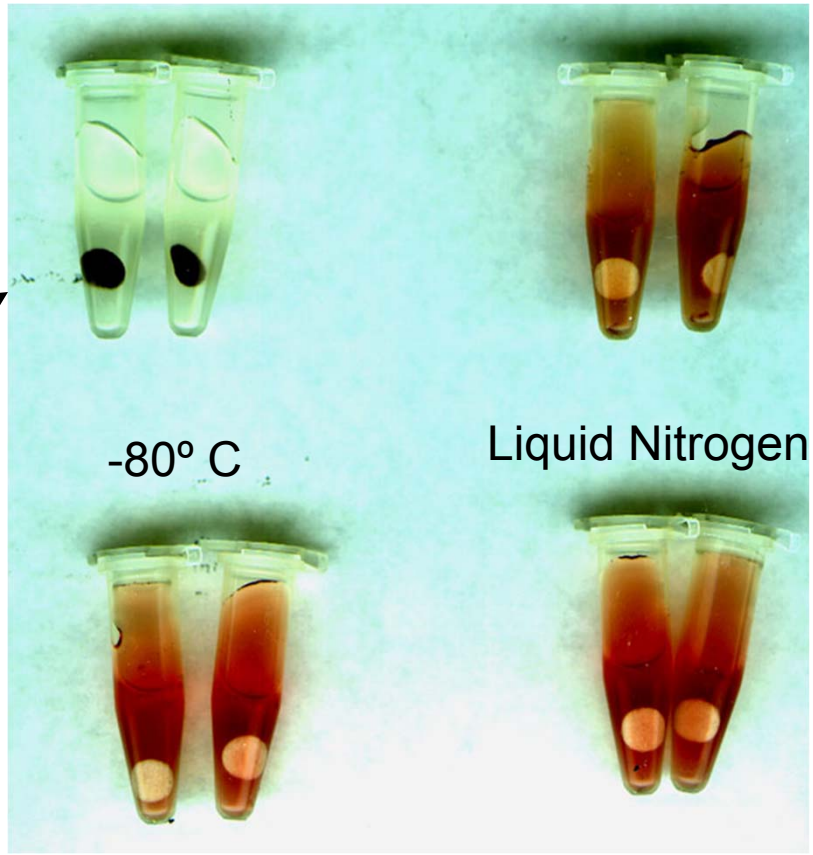
But how efficient is the extraction?

- Observations
  - The longer the bloodstain is stored at room temperature the harder it is to wash the heme away.
  - Heme washes away easily from stains stored at -20 °C.
- Theory:
  - If protein (i.e. heme) sticks to the paper what stops DNA from sticking?

DNA Stability Study
6 year time point

903 paper

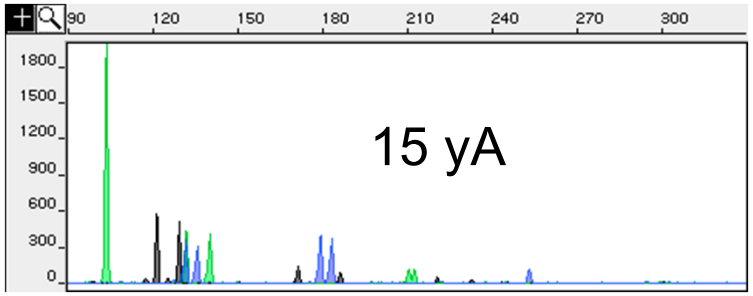
During Chelex extraction RT sample during water washes remains colored.

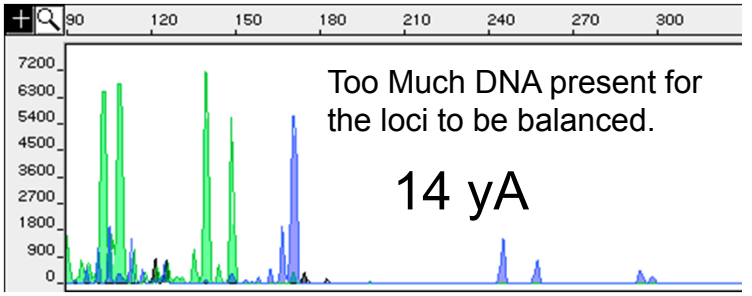


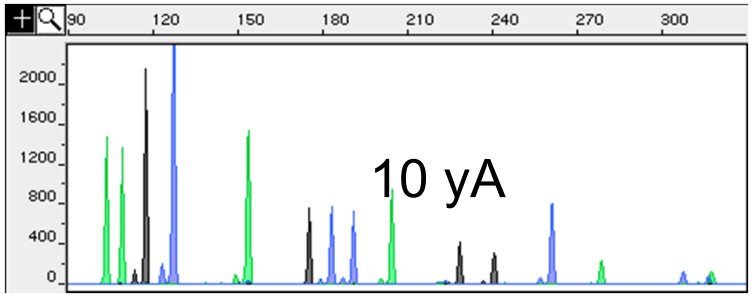
What's left on the paper?

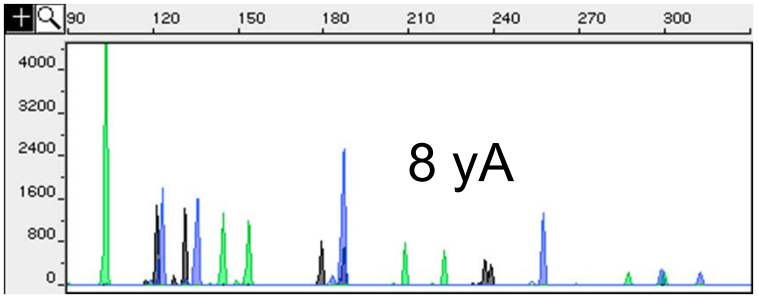
- Take bloodstain that has been Chelex extracted
- Wash with a Tris buffer
- Take a 1.2 mm punch of the washed stain
- Place the washed 1.2 mm punch in a PCR tube and amplify.

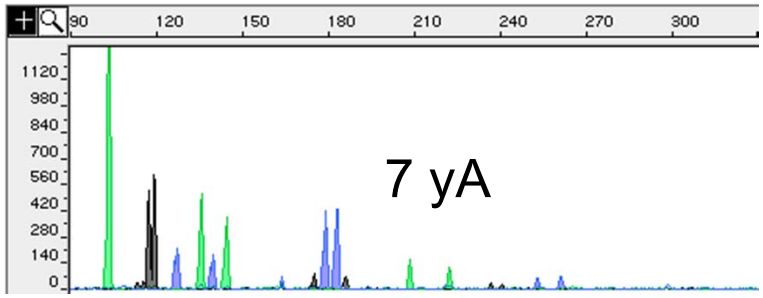
Amplification of the 903 spot after Chelex extraction. Sample Size: 1.2 mm

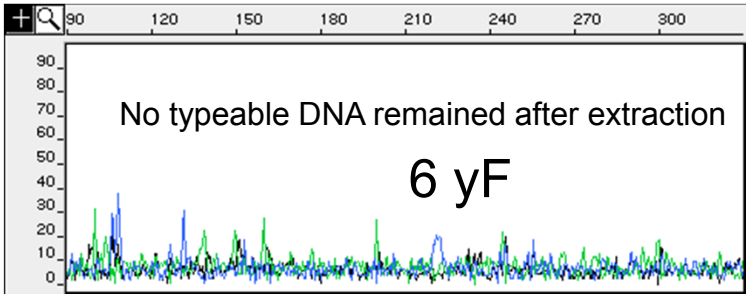


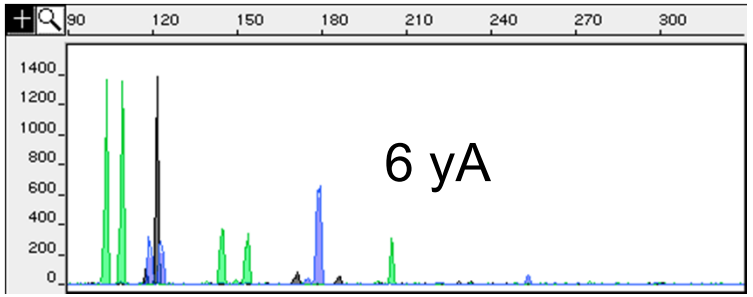


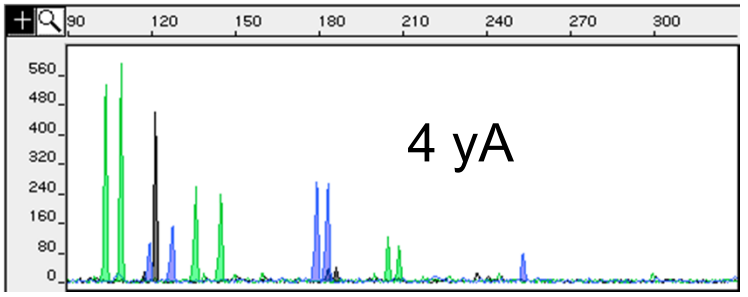




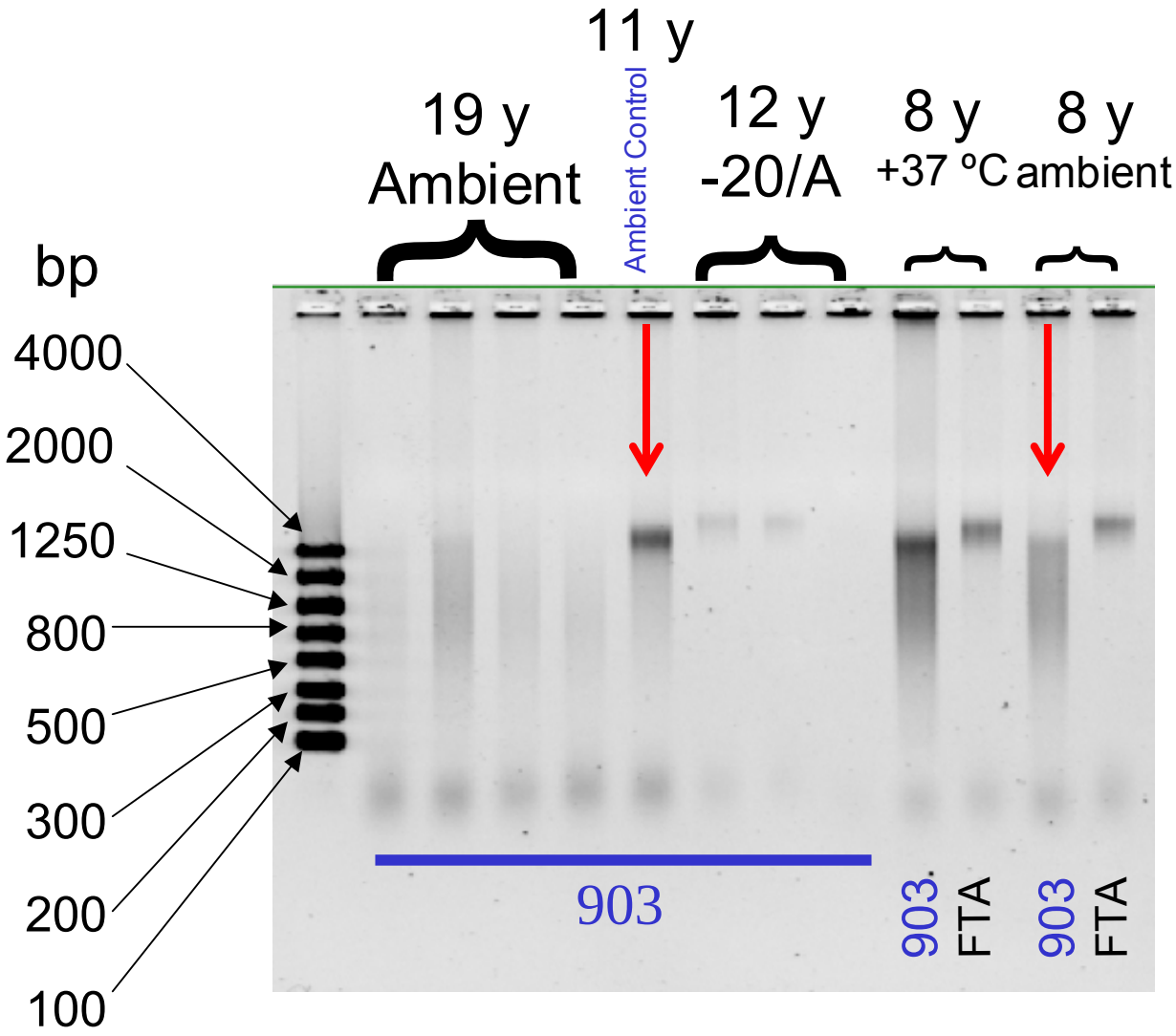




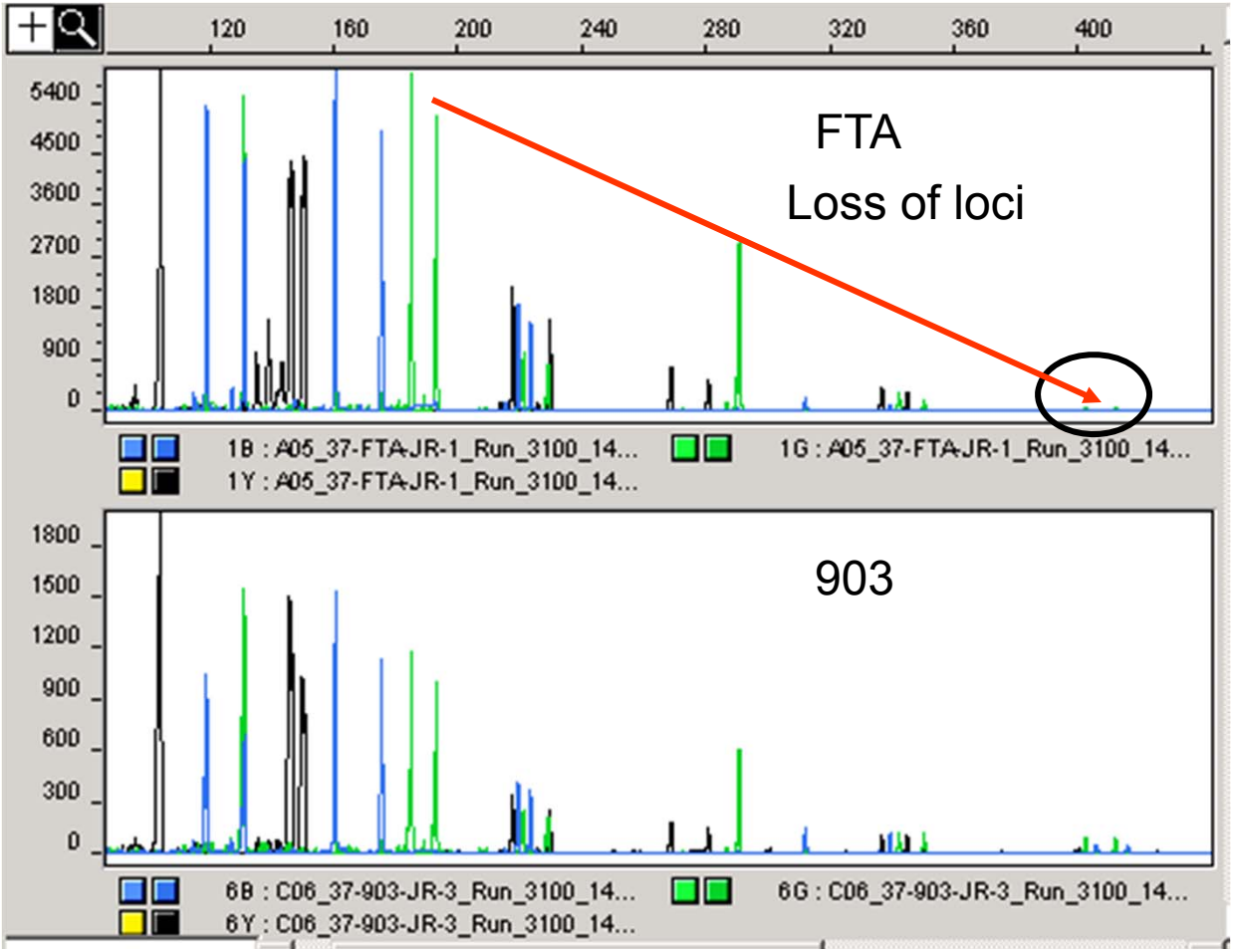




Sample Matrix: FTA, 903

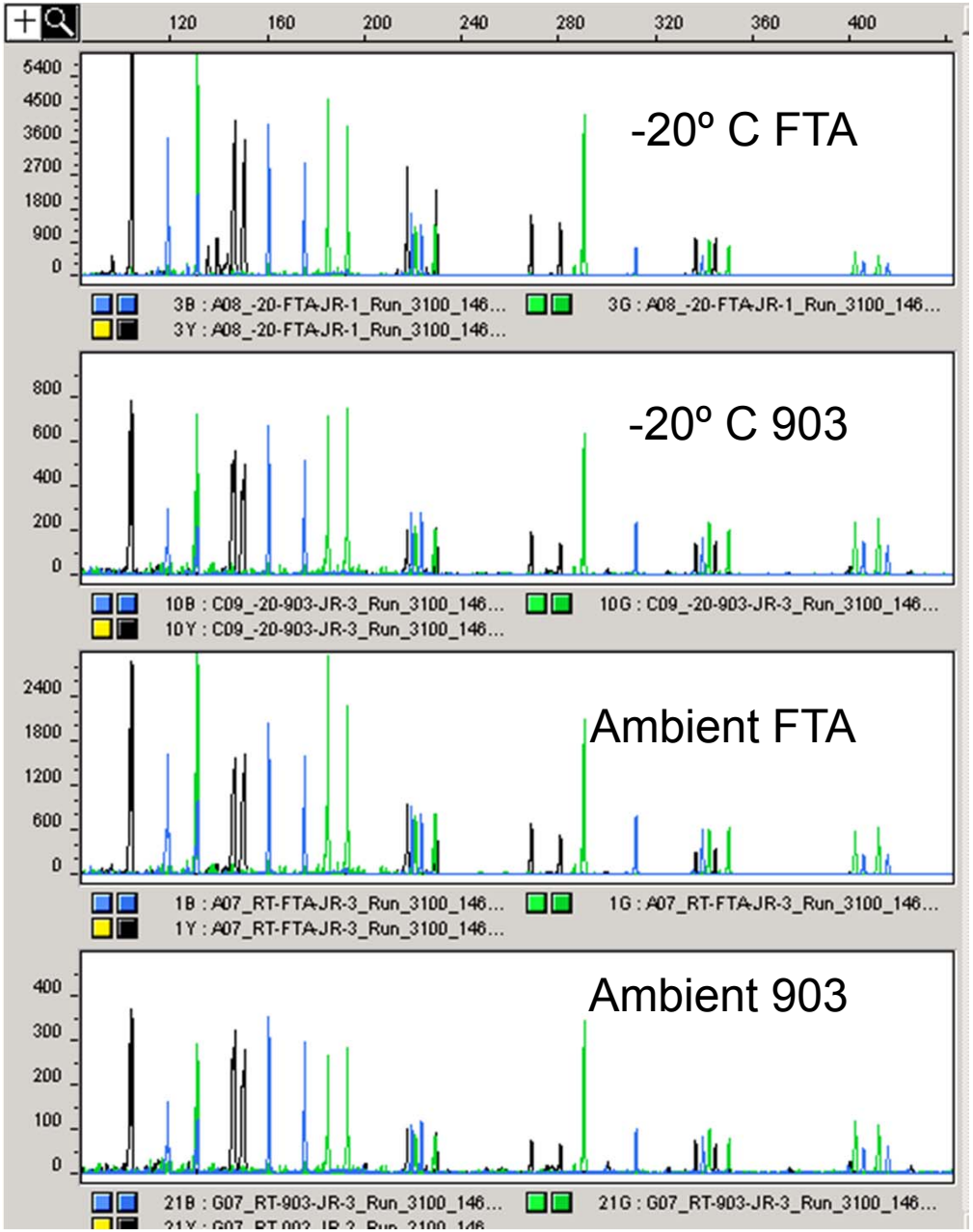


37º C FTA and 903 papers at 8 years



Over amplification of the smaller loci with loss of the larger loci with the FTA paper

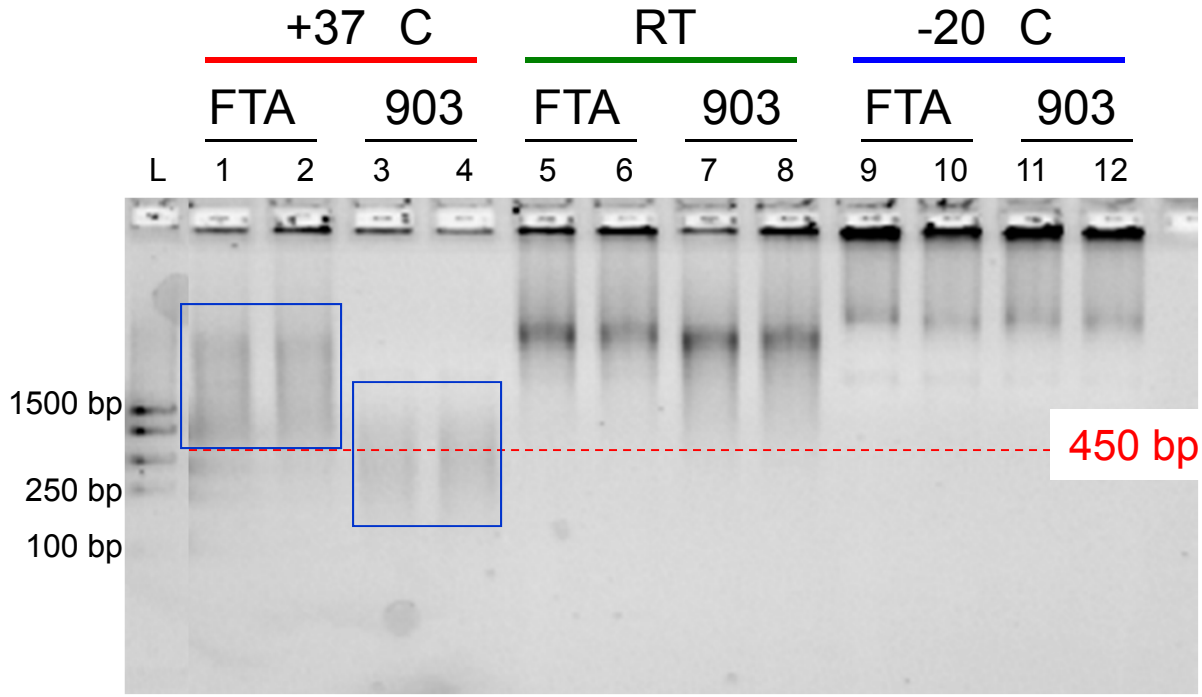
903 large loci weak but still typeable. Smaller loci are not “over amplified.



-20º C FTA & 903 8 years

All loci present and equally amplified with PP16

Quality of the Extracted DNA

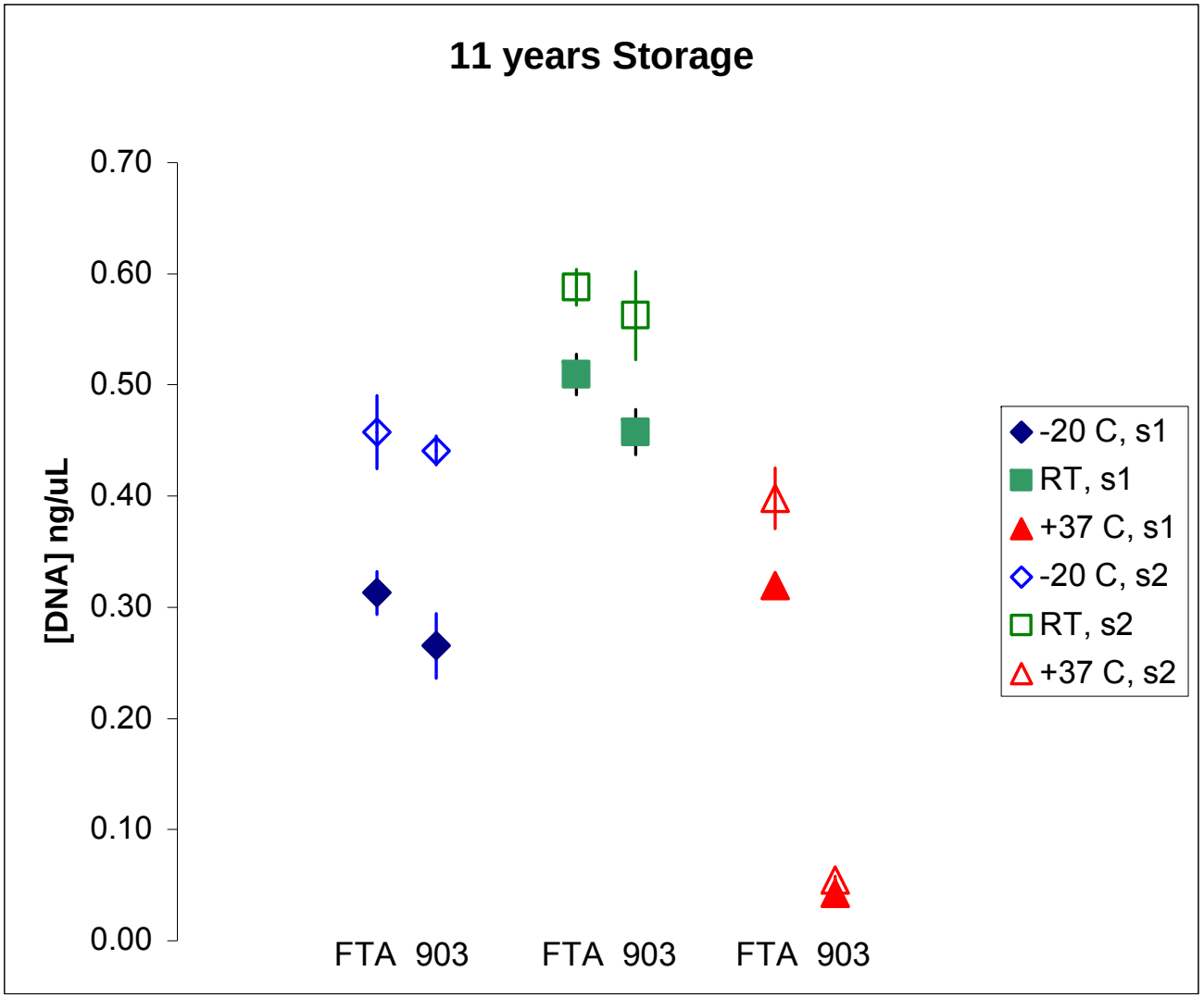


Most STR typing kits have products that are less than 450 bp

L ladder with 250 bp, 400 bp, 800 bp and 1500 bp bands visible
Lanes 1, 2: + 37 C FTA; Lanes 3, 4: + 37 °C 903;
Lanes 5, 6: RT FTA; Lanes 7, 8: RT 903;
Lanes 9, 10: -20 C FTA; Lanes 11, 12: -20 °C 903;

After 11 years of storage at 37 C both FTA and 903 show signs of degradation, the FTA samples exhibit DNA with slightly higher molecular weight than the 903 samples.

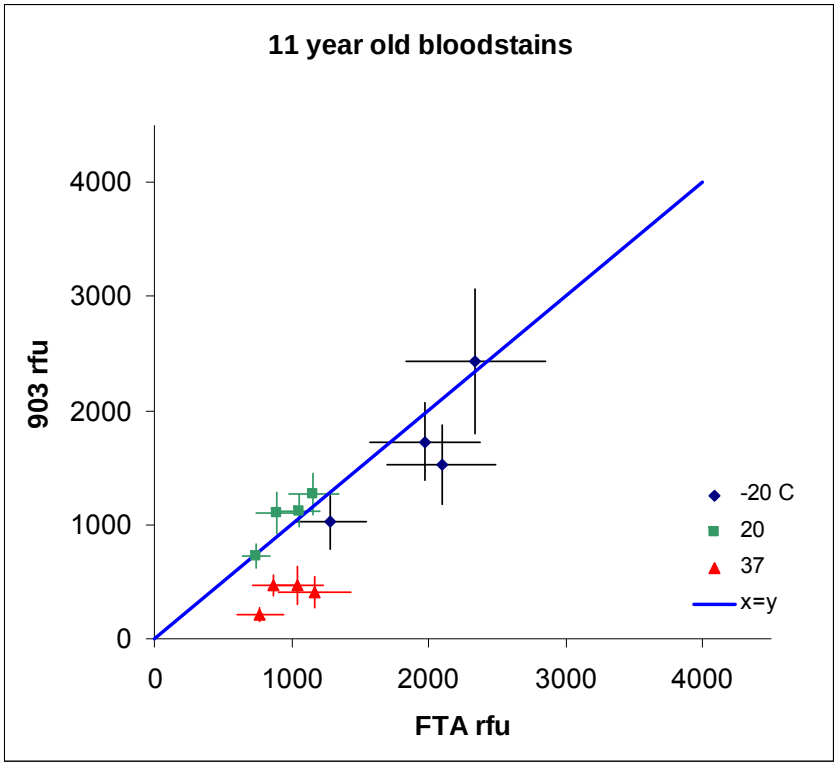
Extracted DNA Quantitation



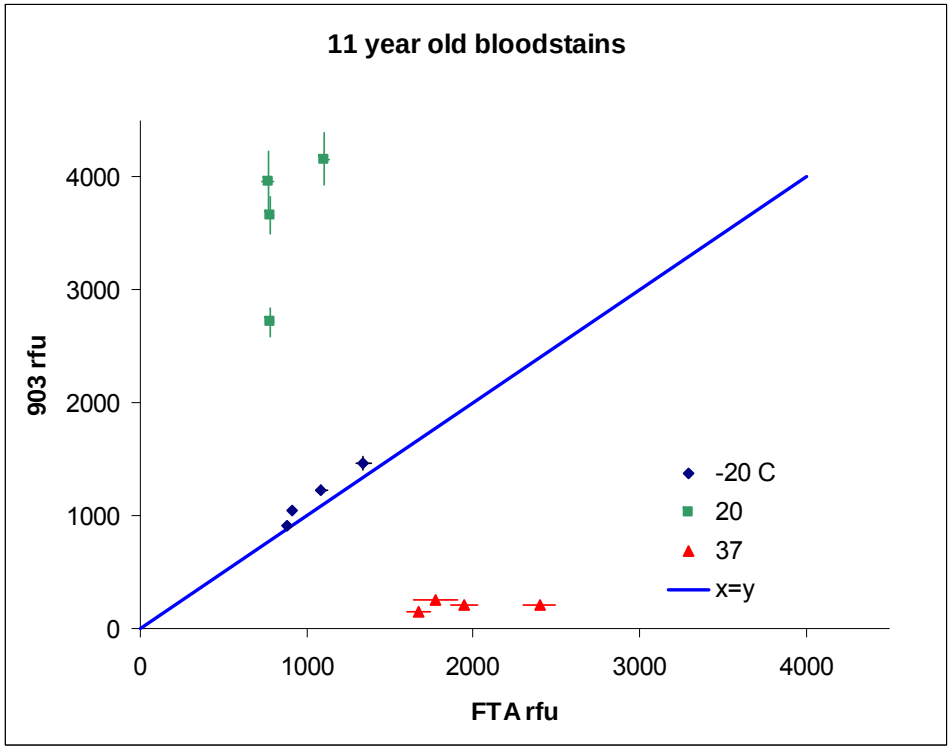
Stains from 2 different individuals

Comparison of the Idfiler Peak Hts FTA vs 903

Sample 1

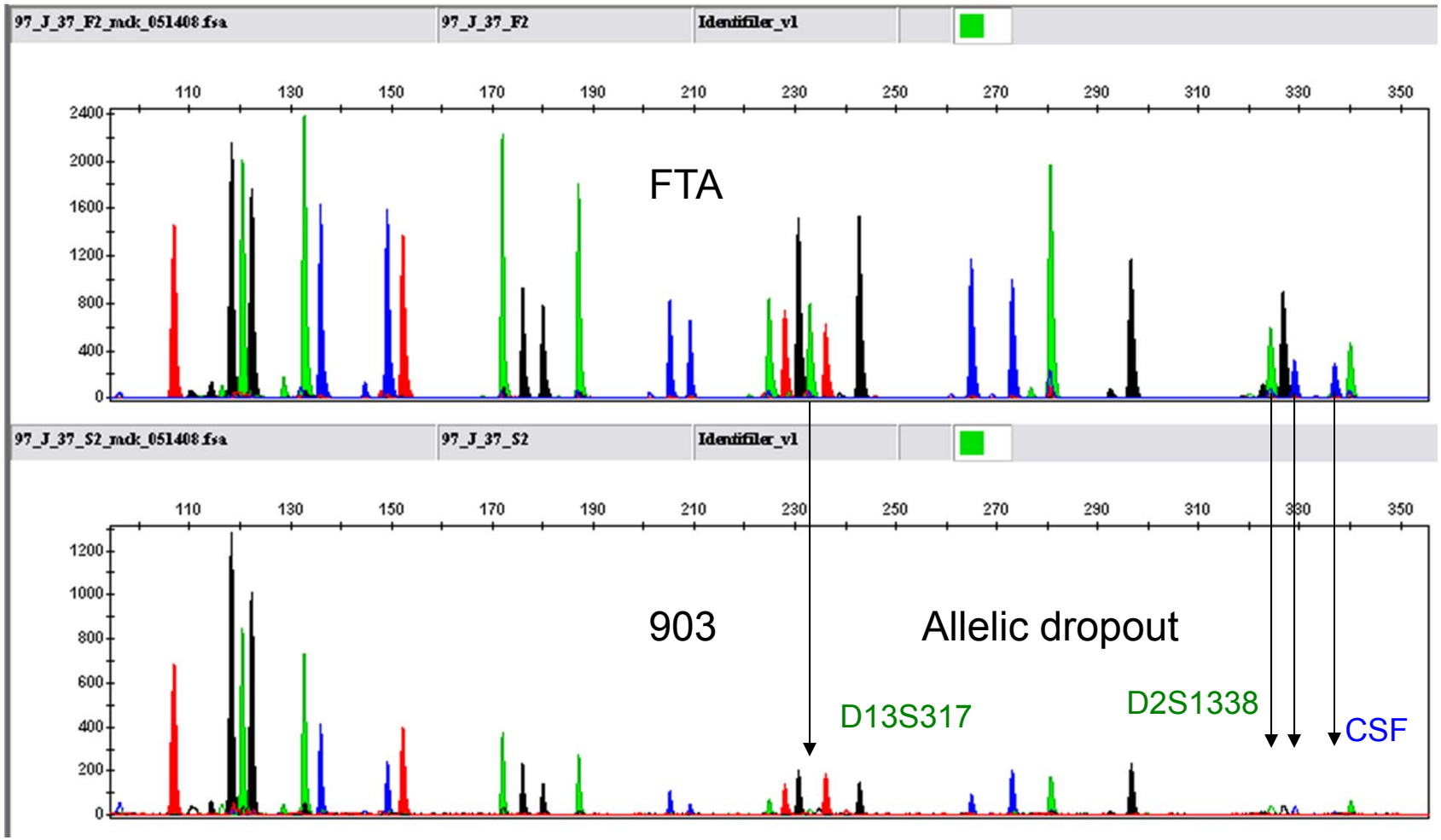


Sample 2

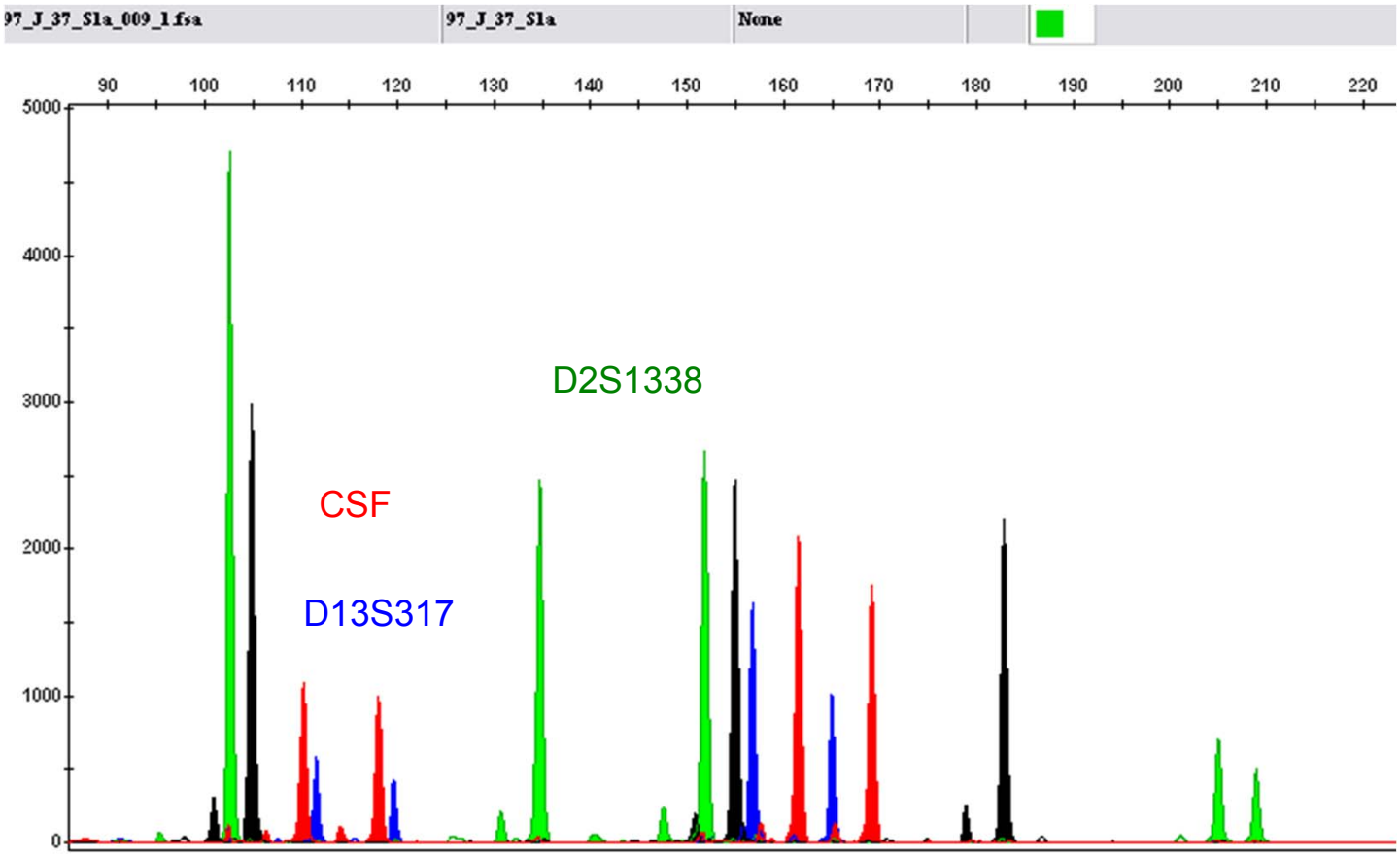


10 µL Idfiler reaction volume with 0.2 – 0.6 ng Extracted DNA amplified
Allelic dropout seen in CSF, D16 and D2 in some of the +37 °C stored samples.
Those alleles were recovered with Mnifiler.

FTA – 903 +37 C Storage Idfiler

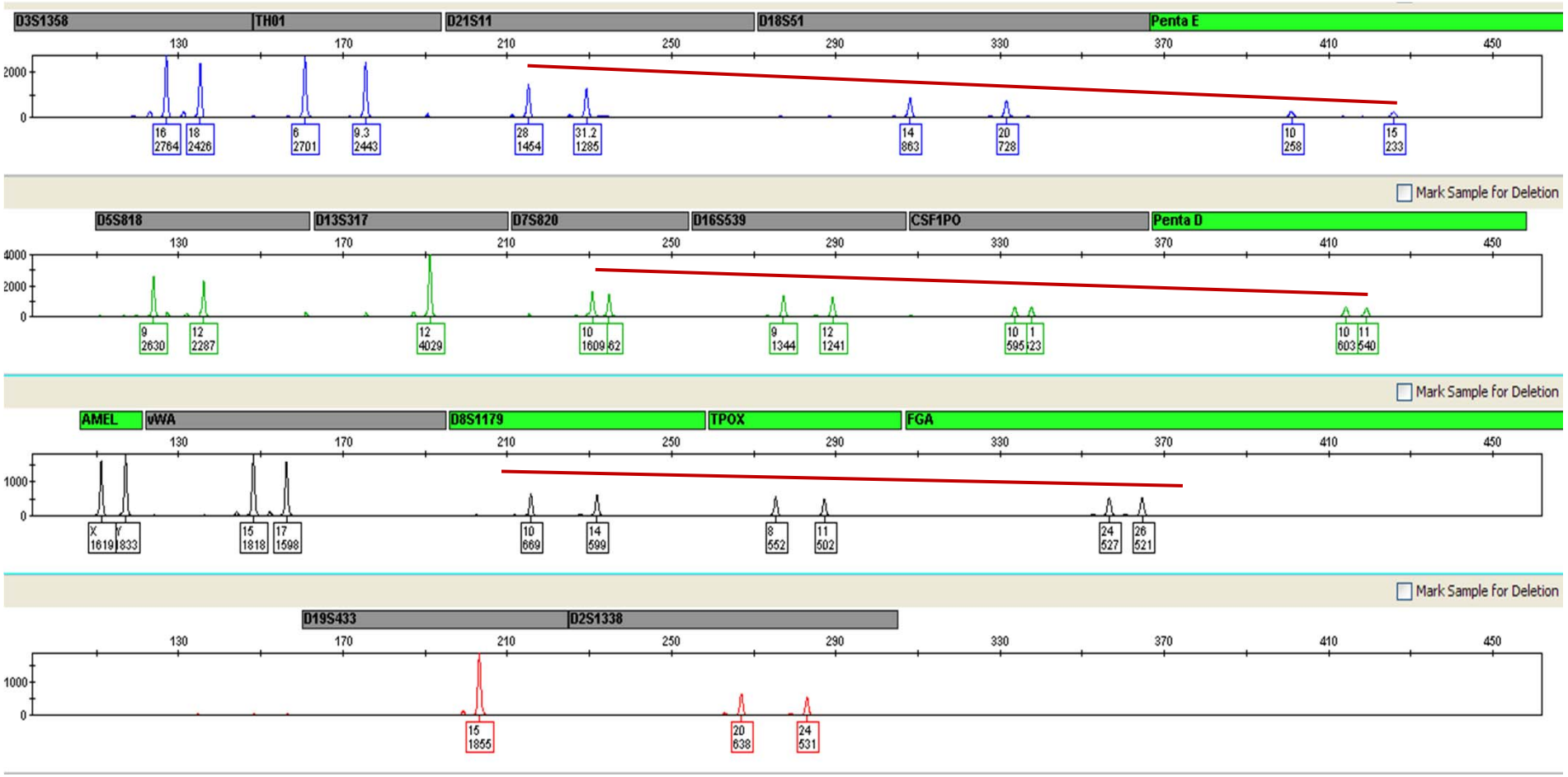


903 +37 C Storage Minifiler amp



Alleles dropping out with Idfiler amplification are recovered with Minifiler

25 year old Bloodstain(1986) PowerPlex 18D (903 paper)

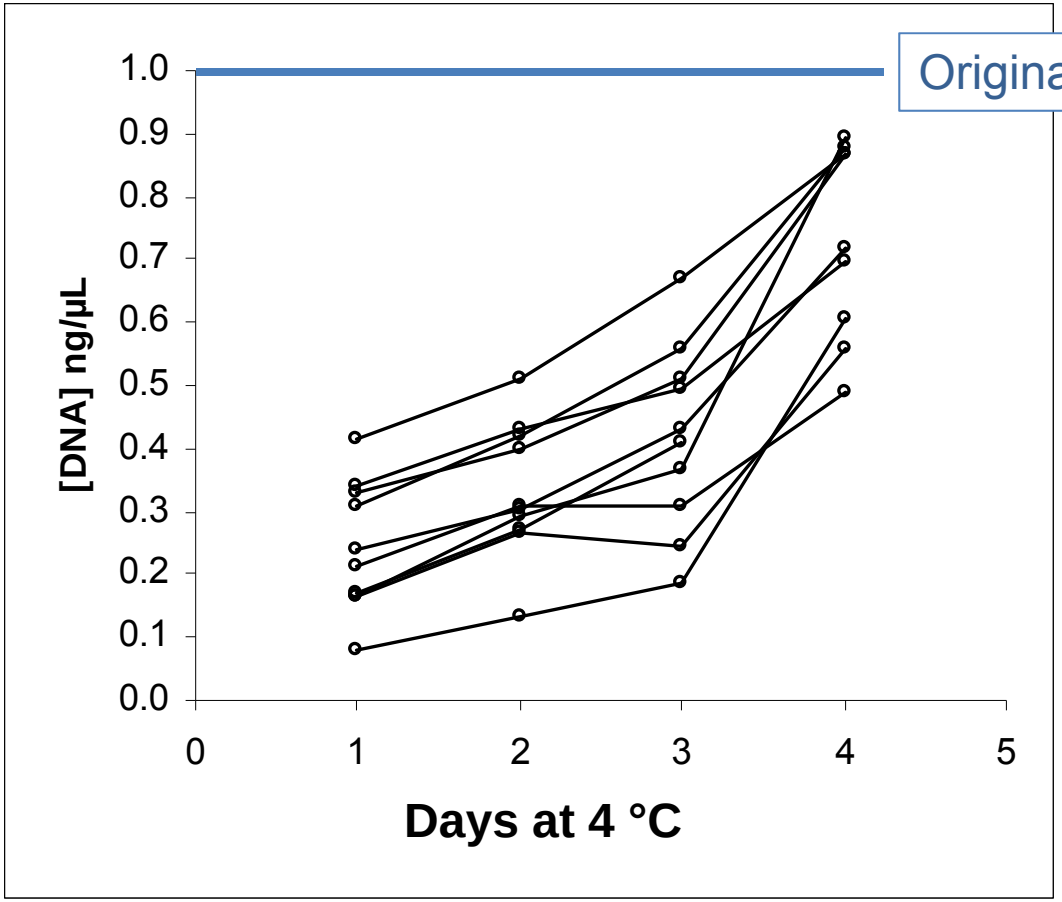


Single 1.2 mm punch stored at room temperature

No Extraction

Data from Pete Vallone and Erica Butts

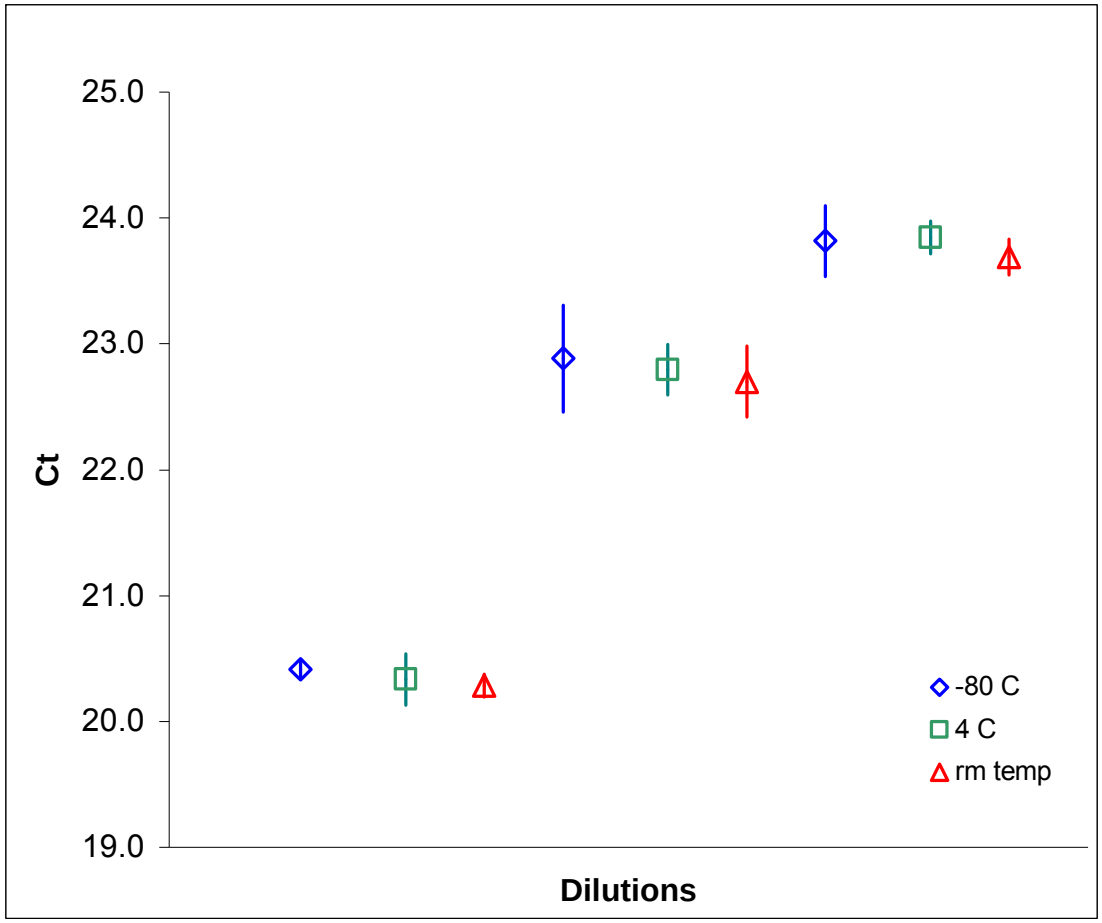
DNA sticking and releasing from tube walls



DNA stored in tubes for 5 years at -20 °C then moved to 4 °C

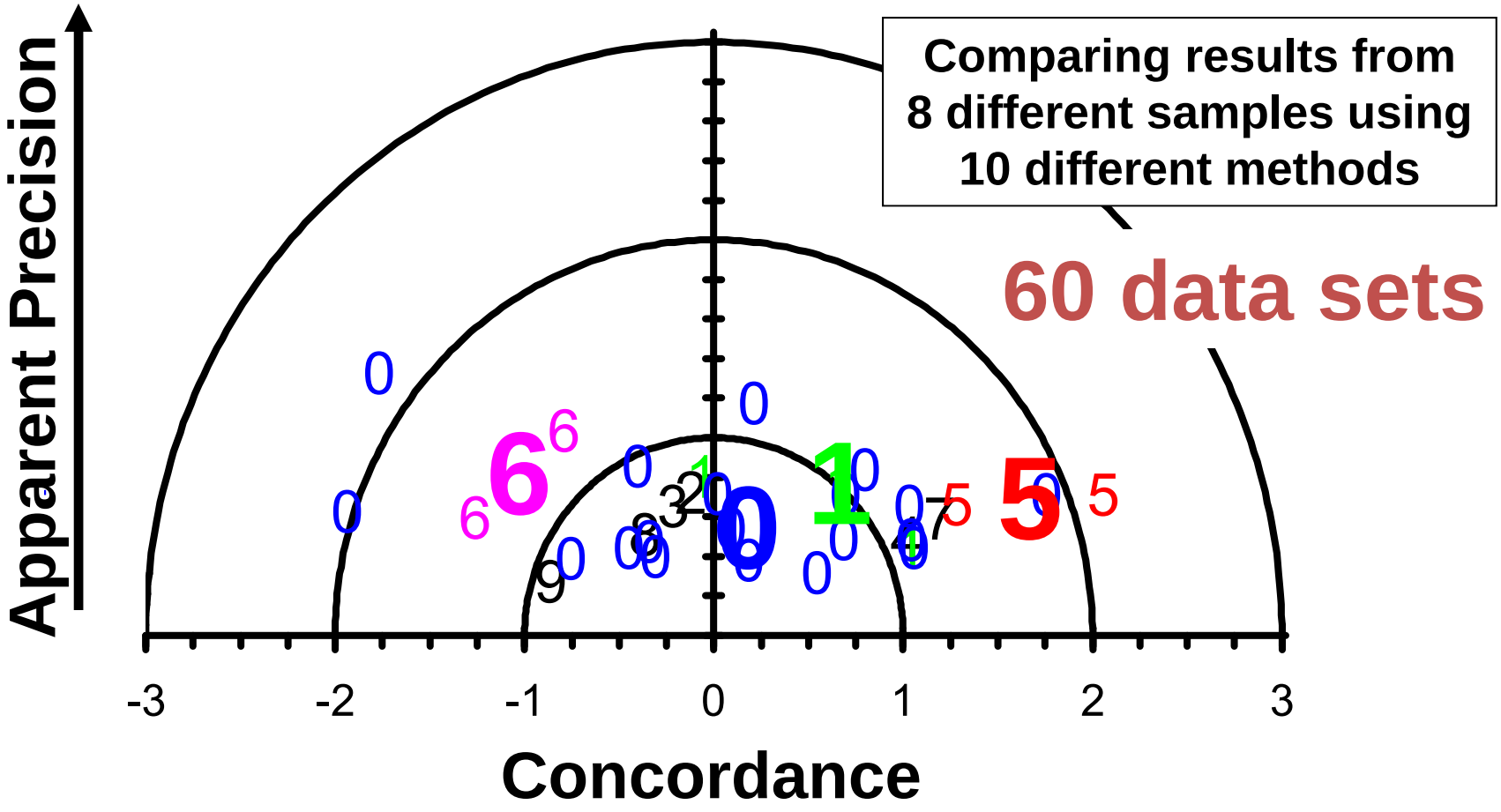
Each line represents an SRM 2391b component and the points along the lines (open circles) correspond to DNA concentrations obtained in this analysis. Several components (2, 8, 9,and 10) were approaching the original nominal DNA concentration of 1 ng/µL at the last time point of 4 days.

6 year Extracted DNA Stability in PFA Tubes



Data from DNA extracts stored in teflon tubes at -80 °C, 4 °C, and room temperature for 6 years.
Each storage temperature had three DNA concentrations: neat, 1→5, 1→10.
qPCR results using Quantifiler Human, triplicate aliquots used from each sample.
Error bars represent 2 sd.
There is no difference as a result of temperature storage after 6 y.
Experiment started 29 October 2003. Date of analysis 29 October 2009.

Interlaboratory Comparison of qPCR Methods



0 = Quantifiler 1 = Alu RT-PCR 5 = BRCA1 6 = CFS-HUMRT

Kline, *et al.* (2005) *J. Forensic Sci.* 50(3):571-578

Thank you for your Attention!!

Acknowledgments

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