Present and Future Trends for Analyzing Challenged Samples

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Sample Throughput

Swabs

LCM

TECHNICAL NOTE

David Sweet,[1] D.M.D, Ph.D.; Miguel Lorente,[2] M.D., Ph.D.; José A. Lorente,[2] M.D., Ph.D.; Aurora Valenzuela,[2] M.D., Ph.D., B.D.S.; and Enrique Villanueva,[2] M.D., Ph.D.

An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique

REFERENCE: Sweet D, Lorente M, Lorente JA, Valenzuela A, Villanueva E. An improved method to recover saliva from human skin: The double swab technique. J Forensic Sci 1997;42(2): 320–322.

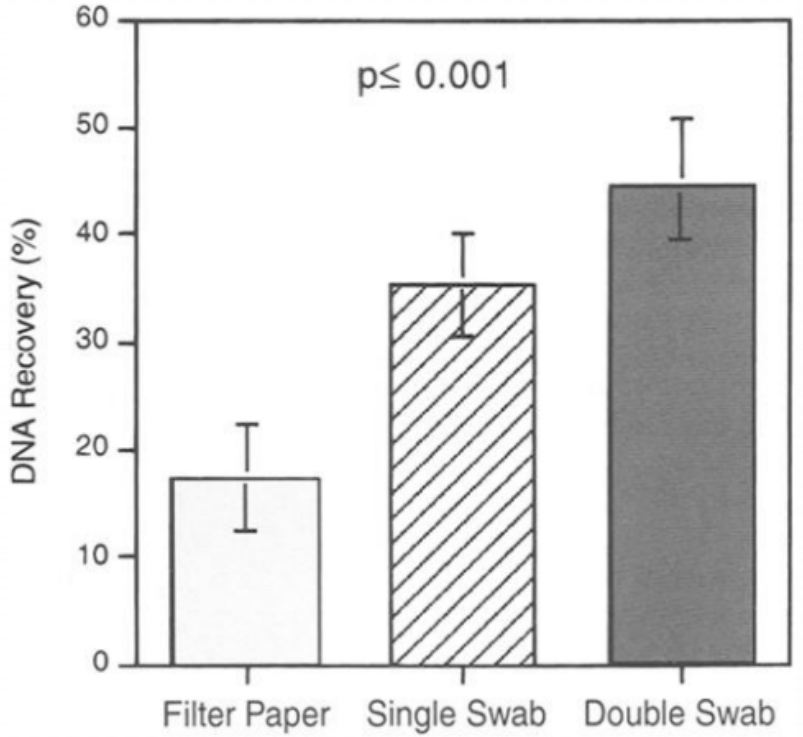


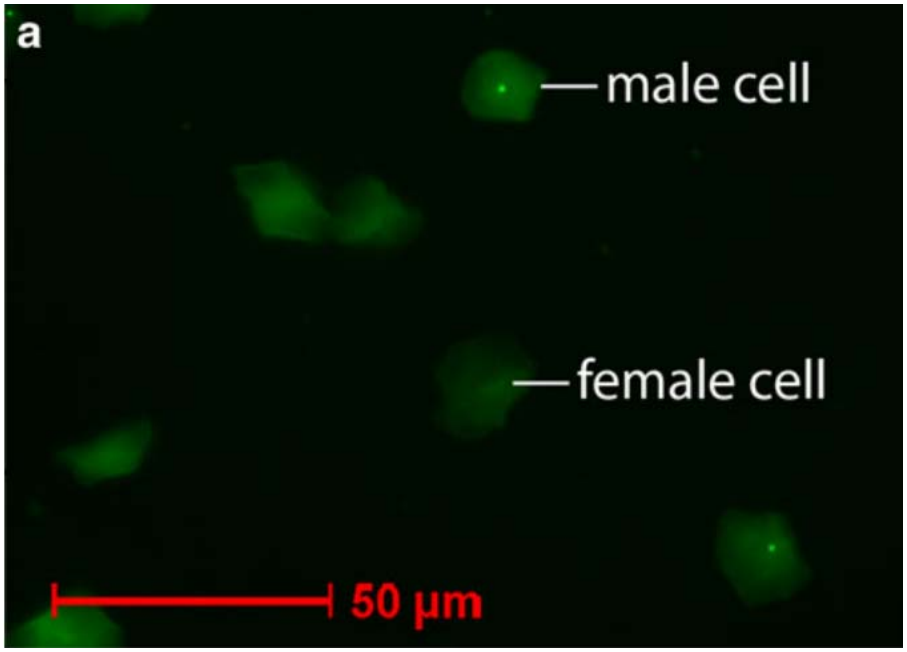
FIG. 1—*Comparison of the different methods to recover DNA from skin.*

Int J Legal Med
DOI 10.1007/s00414-010-0499-4

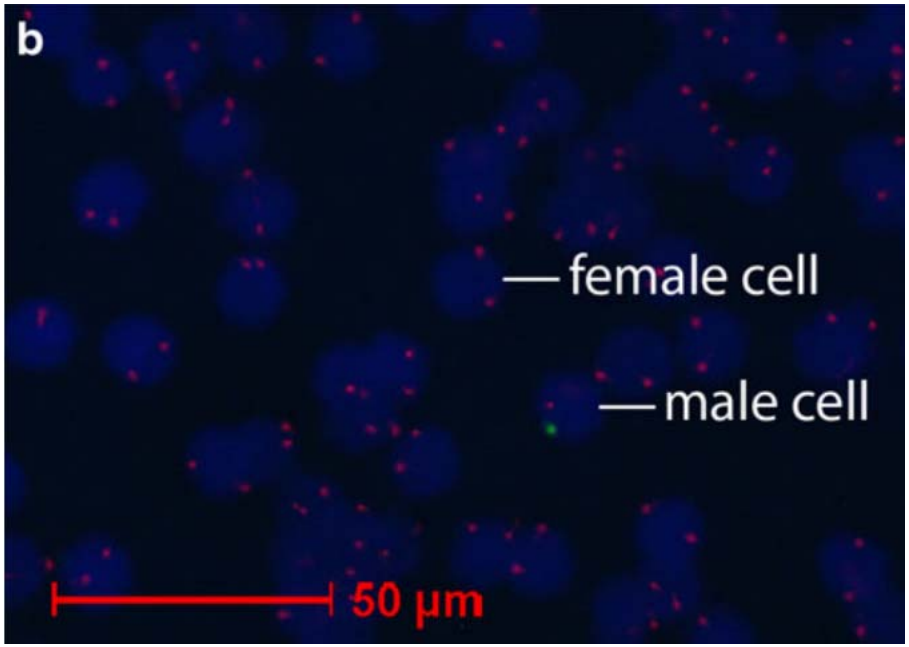
REVIEW ARTICLE

Laser capture microdissection in forensic research: a review

Mado Vandewoestyne · Dieter Deforce



Buccal Cells



Lymphocytes

Sample Throughput

Swabs

LCM

Organic

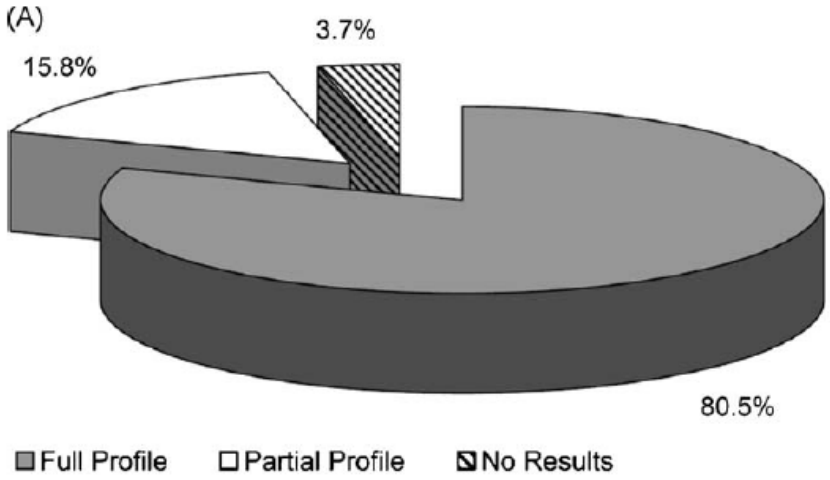
Silica

Resin

Magnetic Beads

Extraction Efficiency

- Defined using several different methods
  - Full vs. Partial STR Profiles
  - Number of loci successfully genotyped
  - Pass/Fail System



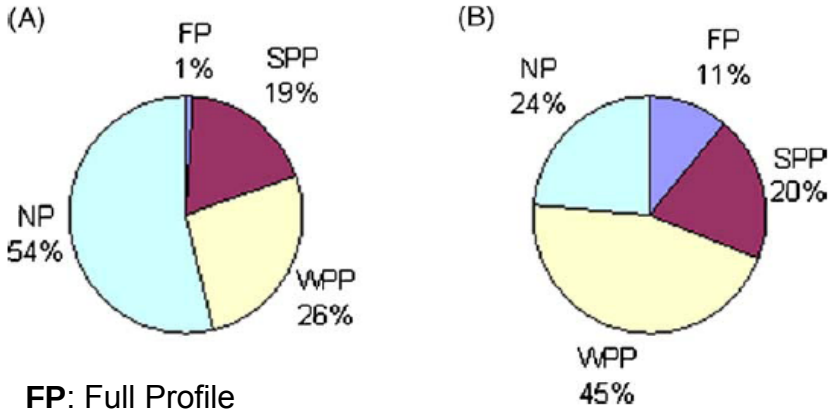
M. Stangegaard et al. “Automated extraction of DNA from reference samples from various types of biological materials on the Qiagen BioRobot EZ1 Workstation.” Forensic Science International: Genetics Supplement Series 2 (2009) 69–70

E. Milne et al. “Buccal DNA Collection: Comparison of Buccal Swabs with FTA Cards.” Cancer Epidemiol Biomarkers Prev 2006;15(4). April 2006

Typical Definition of Extraction Efficiency

- The number of observed full STR profiles

- Divided into three categories:
  1. Full Profile
  2. Partial Profile
  3. No Profile



FP: Full Profile
SPP: Strong Partial Profile
WPP: Weak Partial Profile
NP: No Profile

K.M. Horsman-Hall et al. "Development of STR profiles from firearms and fired cartridge cases." Forensic Science International: Genetics 3 (2009) 242–250

Typical Definition of Extraction Efficiency

- Recovery compared to another method of extraction (often organic)
- The comparison can be of STR loci recovered or by quantitation using real-time PCR methods
- This is a relative efficiency (practical use)

Limitations of Current Efficiency Metrics

- Measures end point - efficiency of STR genotyping
- Does not reflect the true efficiency of the extraction process
- Does not account for the initial amount DNA present in the sample
  - However, in case work samples the true amount of starting material is unknown

True Extraction Efficiency

- The ratio of the amount of DNA recovered (quantity) to the original amount of DNA (known) after extraction
- This offers the ability to evaluate the true efficiency of the extraction
- The original amount needs to be known

Testing True Extraction Efficiency

Placing a known amount of DNA into the extraction process and determine the amount recovered

- 3 sources of DNA
- 4 extraction methods
- Quantified with real-time PCR

Sources of DNA

1. Highly characterized extracted DNA
   - Known quant value: 52.44 ng/µL

2. Primary human cell lines*
   - MCF 10A: Human epithelial
   - Number of cells determined through flow cytometry

3. Whole blood*
   - Assumed white blood cell count of 4.0 million WBC/mL

*Assume 6 pg of DNA per cell

Qiagen EZ1 Advanced

EZ1 Advanced uses magnetic separation and multiple washes to purify DNA

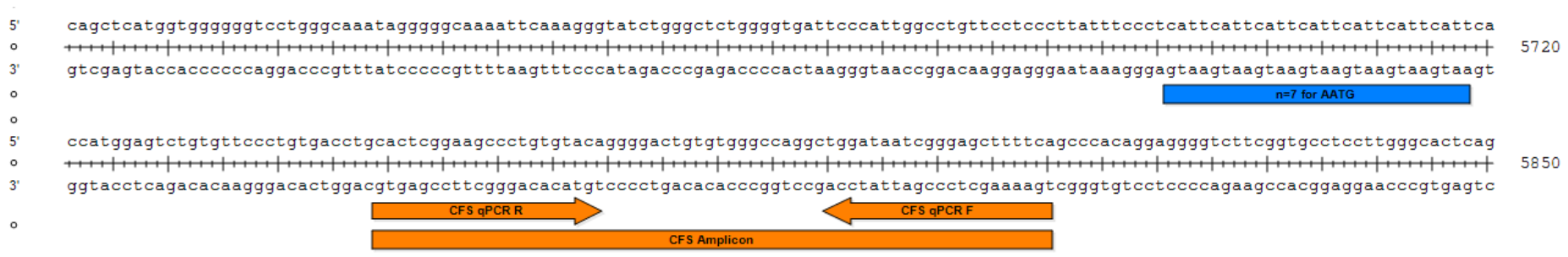
- Swabs & Stains: G2 Buffer and Proteinase K added to sample
- Incubated at 56°C for 15 minutes then 95°C for 5 minutes
  - Vortex periodically through incubation (~every 5 minutes)
- Blood: Total sample volume brought up to 200 µL with G2 Buffer

Modified Salt Out

- Manual extraction process
- Involves a Proteinase K digest
- Saturated Ammonium Acetate solution to separate DNA from other proteins
- Absolute Ethanol wash to precipitate DNA
- Rehydrated with 100 µL TE

DNA Quantitation Assay

- Targets the STR locus TH01
  - Chromosomal location: 11p15.5; intron 1 of human tyrosine hydroxylase gene
- Modified to run as a SYBR green assay
  - Run on ABI 7500



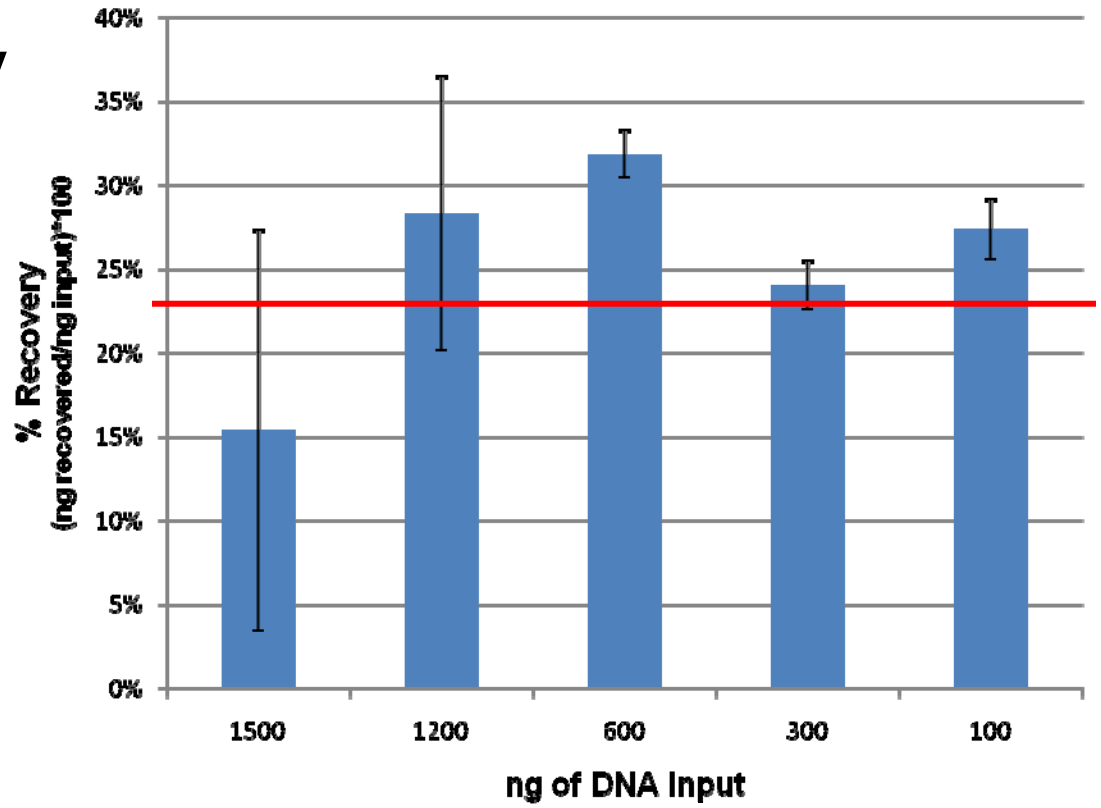
Richard, M.L., Frappier, R.H. and Newman, J.C. (2003) Developmental validation of a real-time quantitative PCR assay for automated quantification of human DNA. J Forensic Sci, 48 (5): 1041-1046

Extracted DNA Samples

- Varying amounts added to sterile swab (n=18 per quantity)
  - 1500 ng, 1200 ng, 600 ng, 300 ng, 100 ng
- Swabbing method using a Teflon tube
  - Simulated buccal swab being taken
- Allowed sample to dry in hood overnight

Extracted DNA Efficiency

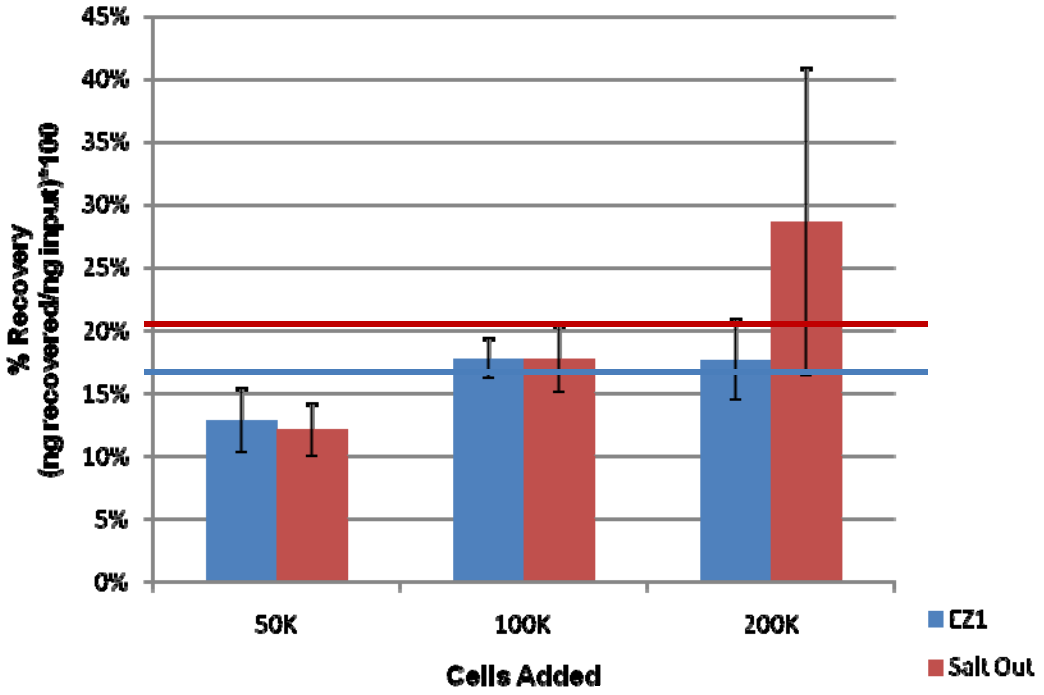
- Extraction with EZ1 from swabs
- n=18 per quantity



Extracted Cell Line Efficiency

Swabbed 100 µL of a solution containing human epithelial cells in a Teflon tube (n=12 per quantity)

- 50,000 cells (300 ng)
- 100,000 cells (600 ng)
- 200,000 cells (1200 ng)



Blood Extraction Efficiency

- Seven volumes of whole blood tested (n=2 per volume)*
  - 200 µL, 100 µL, 50 µL, 20 µL, 10 µL, 5 µL, 1 µL
  - Ranges from 4800 ng to 24 ng of DNA

- Liquid blood extracted without incubation
  - For EZ1 brought to a total volume of 200 µL with G2 Buffer
- For blood stains:
  - Blood spotted directly onto Whatman 903 paper
  - Cut into small pieces and placed into extraction tube

*Assuming 4.0 million WBC/mL and 6 pg of DNA per cell

n=2 per volume

Liquid Blood Extraction

EZ1 Extraction

µL Blood	ng DNA	% Recovery*
1	0.7	2.8%
5	30.9	25.7%
10	49.7	20.7%
20	108.3	22.6%
50	160.5	13.4%
100	133.5	5.6%
200	55.8	1.2%

Salt Out Extraction

µL Blood	ng DNA	% Recovery*
1	0.1	0.1%
5	1.0	0.8%
10	4.4	1.6%
20	58.5	12.2%
50	78.0	6.5%
100	11.6	0.5%
200	0.5	0.1%

*Assuming 4.0 million WBC/mL

n=2 per volume

Blood Stain Extraction

EZ1 Extraction

µL Blood	ng DNA	% Recovery*
1	1.9	8.0%
5	20.4	17.0%
10	47.0	19.6%
20	124.5	26.0%
50	292.0	24.3%
100	463.0	19.3%
200	347.5	7.2%

Salt Out Extraction

µL Blood	ng DNA	% Recovery*
1	0.2	1.0%
5	1.4	1.1%
10	3.1	1.3%
20	6.3	1.3%
50	3.4	0.3%
100	486.0	20.3%
200	559.0	11.7%

*Assuming 4.0 million WBC/mL

Extraction Efficiency Results in the Literature

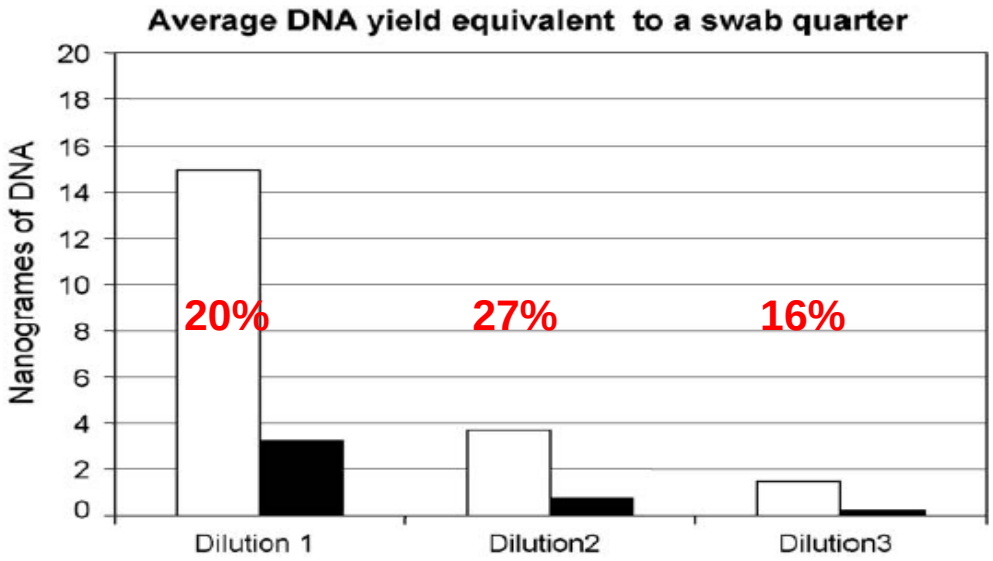


Fig. 1. The mean DNA input used to embed one quarter of swab (in white) is compared with the mean DNA yield recovered from the quarters of swab (in black).

A. Colussi et al. "**Efficiency of DNA IQ System in recovering semen from cotton swab.**" Forensic Science International: Genetics Supplement Series 2 (2009) 87-88.

R. Kishore et al. "**Optimization of DNA Extraction from Low-Yield and Degraded Samples Using the BioRobot EZ1 ad BioRobot M48.**" J Forensic Sci, September 2006, Vol. 51, No 5.

Liquid Blood Dilutions	Volume of Liquid Blood Extracted (µL)	BioRobot® EZ1, DNA (ng)	BioRobot® EZ1 with cRNA, DNA (ng)	Organic Extraction, DNA (ng)
1:10	0.1	8.025	10.000	7.900
1:50	0.02	0.213	2.250	1.840
1:250	0.004	0.050	0.260	0.263
1:1250	0.0008	0.000	0.040	0.038
1:2500	0.0004	0.000	0.013	0.000
		33%		**33%**

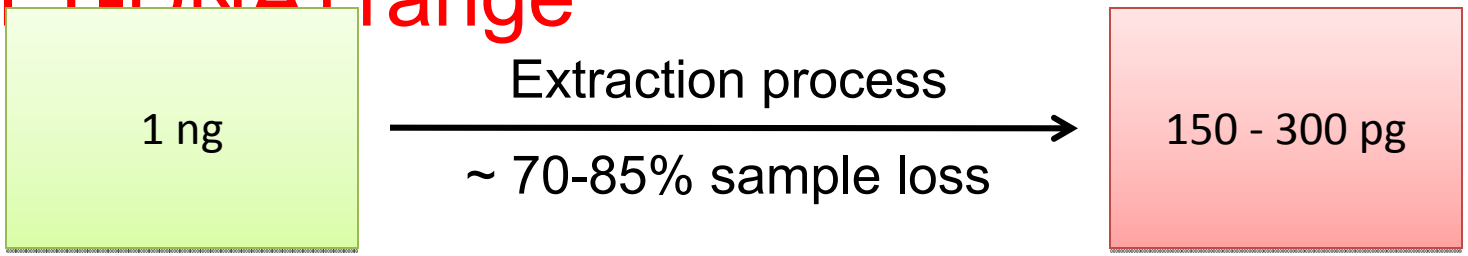
Summary of True Extraction Efficiency

- Literature studies: 16-33% true extraction efficiency

- Loss of about 70-85% of initial sample during the extraction process
- Loss is independent of extraction method or source of DNA (i.e. blood, cells, previously extracted)

The Net Effect…

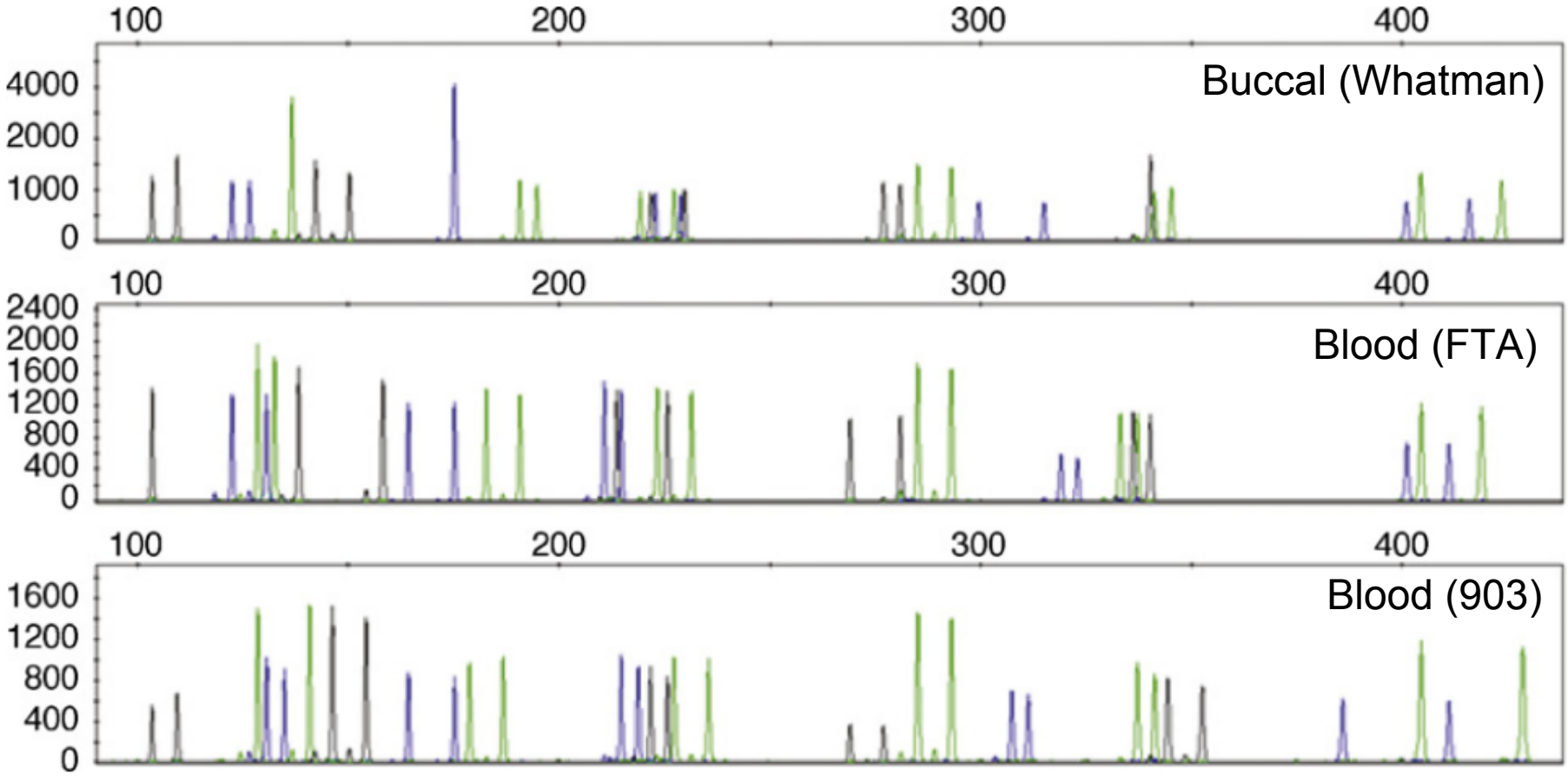
- A majority of sample is lost during extraction
  - Minimal impact on reference samples
  - Enough DNA is recovered for an STR profile
- Low extraction efficiency could lower sample quantity into the Low Template DNA (LT-DNA) range



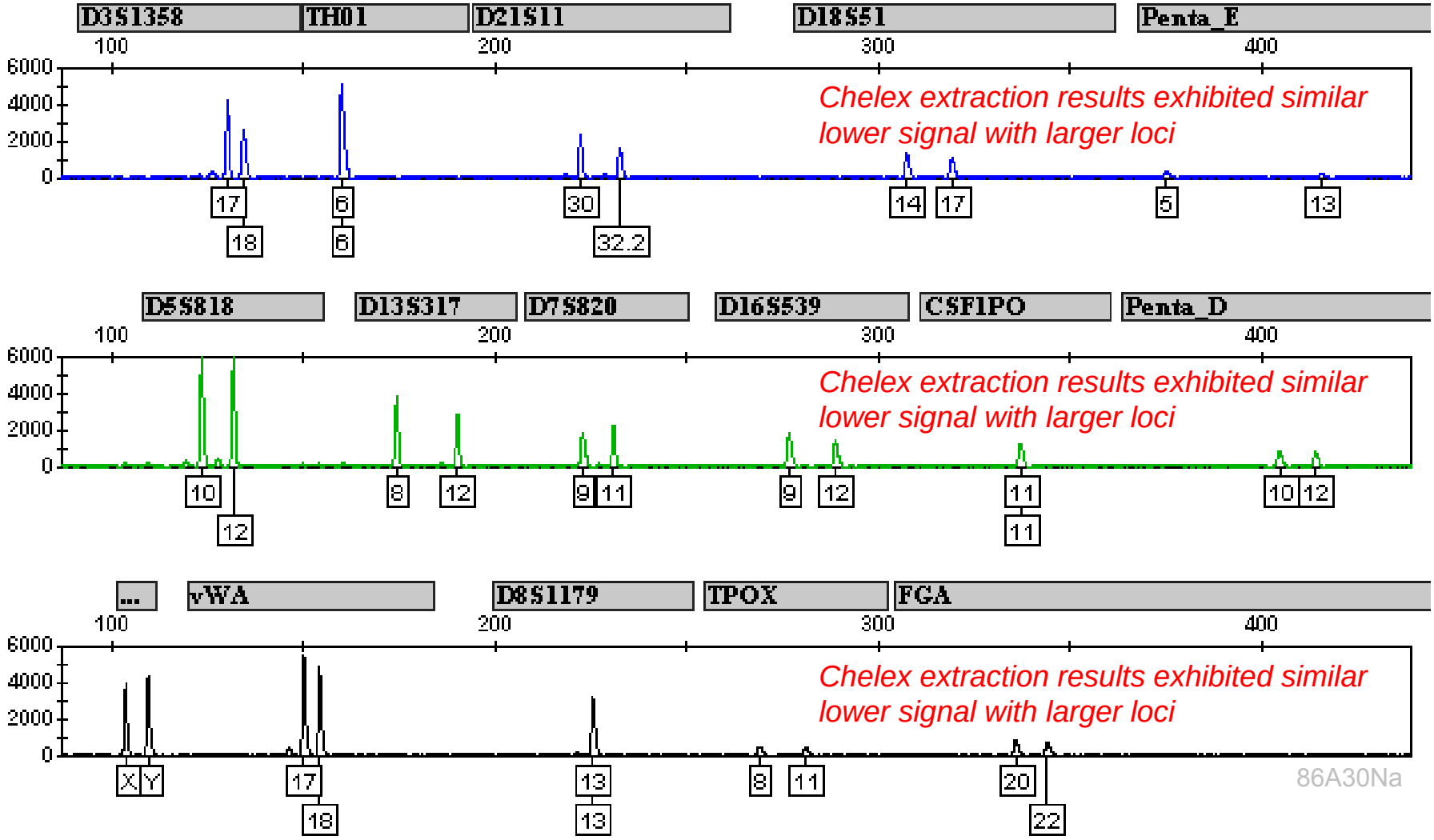
Developmental validation of the PowerPlex® 16 HS System: An improved 16-locus fluorescent STR multiplex

Martin G. Ensenberger[a,*], Jonelle Thompson[a], Becky Hill[b], Kristen Homick[c], Veronica Kearney[d], Kathleen A. Mayntz-Press[e], Paul Mazur[c], Amy McGuckian[f], Jelena Myers[d], Kelli Raley[e], Stewart G. Raley[e], Robin Rothove[g], Jonathan Wilson[h], Doug Wieczorek[a], Patricia M. Fulmer[a], Douglas R. Storts[a], Benjamin E. Krenke[a]

FSI-Genetics 4 (2010) 257–264.



Direct PCR (no DNA extraction) **using PP16 HS** from a 23 year old blood stain (room temperature storage)



1.2 mm punch (untreated blood stain card S&S 903) and PP16 HS (28 cycles)

Bone extraction

Extraction of Skeletal Remains

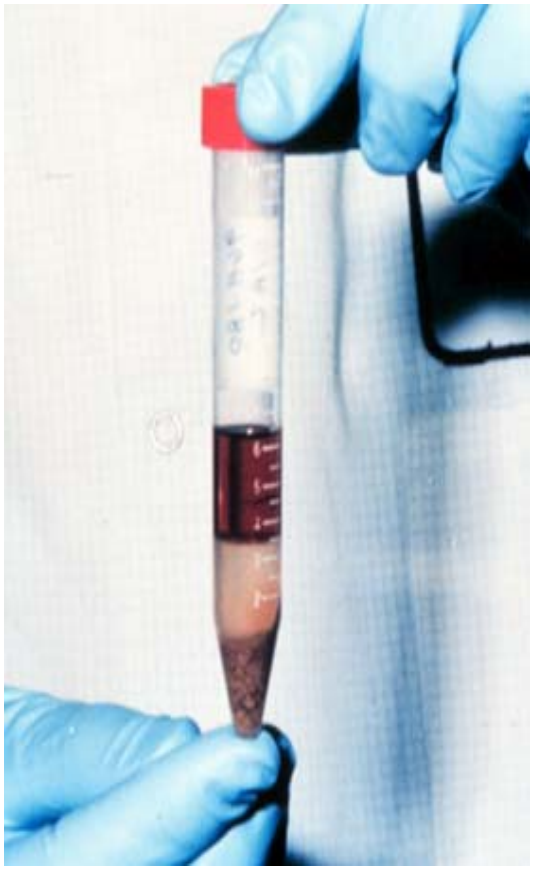
Use 2.0-2.5g of powdered bone

Incubated overnight in 3mL extraction buffer:

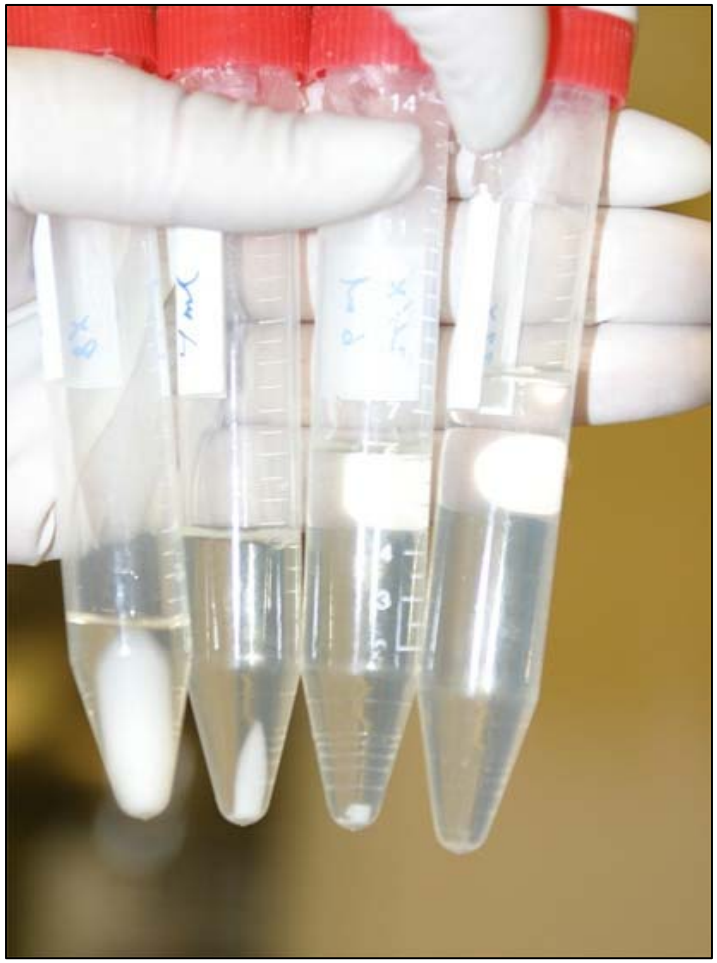
10mM Tris, pH 8.0,
100mM NaCl, 50mM EDTA, pH 8.0,
0.5% SDS
+100ul of proteinase K

Purified using PCIA and butanol washes

Concentrated in a centrifugal filtration column



Demineralization protocol (I)



- EDTA 0.5M, pH 8.5
- Detergent
- Proteinase K
- 1g powder

15ml extraction buffer

•Organic extraction (phenol-chloroform)
•Concentration and washes in Centricons.

Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics 1 (2007) 191–195
www.elsevier.com/locate/fsig

Short communication

High efficiency DNA extraction from bone by total demineralization ☆

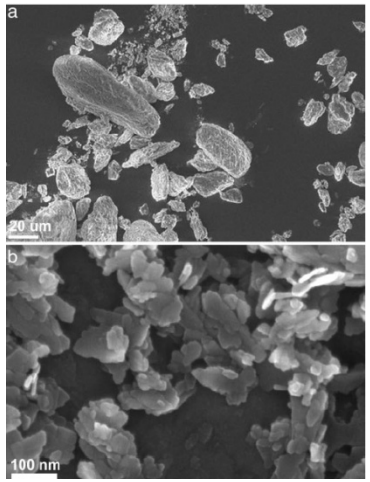
Odile M. Loreille *, Toni M. Diegoli, Jodi A. Irwin, Michael D. Coble, Thomas J. Parsons [1]

Armed Forces DNA Identification Laboratory, 1413 Research Blvd., Bldg. 101, Rockville, MD 20850, United States

Received 24 January 2007; accepted 3 February 2007

Better quality DNA ?

Sample	Extraction	Bone Powder	Real-time Data
A	Demin.	0.2	2.83
	Casework	1.96	0.16
		1.65	0.16
B	Demin.	0.2	28.56
	Casework	2.02	0.34
		1.29	1.33
C	Demin.	0.2	5.95
	Casework	1.93	0.18
		2.16	0.33
D	Demin.	0.2	31.42
	Casework	2.04	1.19

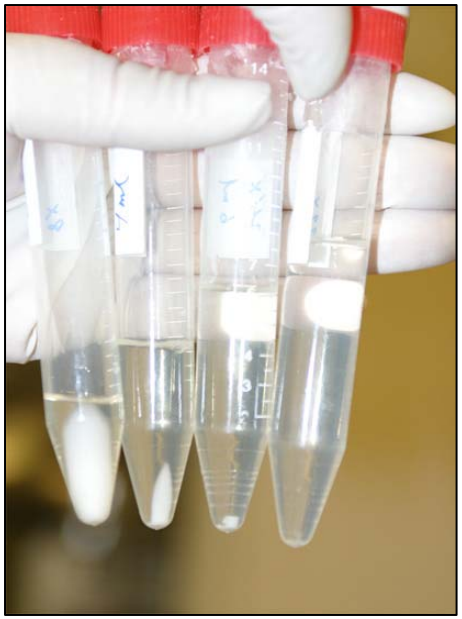


Salamon et al

Demin I - Limitations

- Loss of DNA during the Phenol-Chloroform extraction stage (toxicity).
- The Phenol-Chloroform stage and centricon spins are time consuming.
- Centricons are now extinct.

Demineralization Protocol II



Demin. Buffer

Ultra 4

Qiagen
Mini Elute

Forensic Science International: Genetics 4 (2010) 206–212

Volume reduction solid phase extraction of DNA from dilute, large-volume biological samples

Carmen R. Reedy [a], Joan M. Bienvenue [d], Lisa Coletta [a], Briony C. Strachan [a], Naila Bhatri [d], Susan Greenspoon [e], James P. Landers [a,b,c,*]

[a] *Department of Chemistry, University of Virginia, Charlottesville, VA 22904, United States*
[b] *Department of Mechanical Engineering, University of Virginia, Charlottesville, VA 22904, United States*
[c] *Department of Pathology, University of Virginia Health Science Center, Charlottesville, VA 22908, United States*
[d] *Armed Forces DNA Identification Laboratory, Rockville, MD 20850, United States*
[e] *Virginia Department of Forensic Science, Richmond, VA 23219, United States*

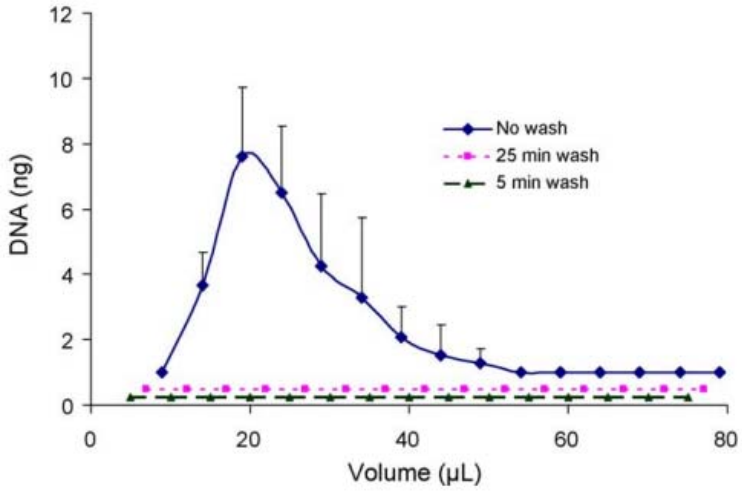


Fig. 3. Elution profiles representing optimization of time necessary for 80% IPA wash step during vrSPE extraction procedure for dilute whole blood samples. No 80% IPA wash step was found to be the optimal wash time providing the greatest quantity of recovered DNA.

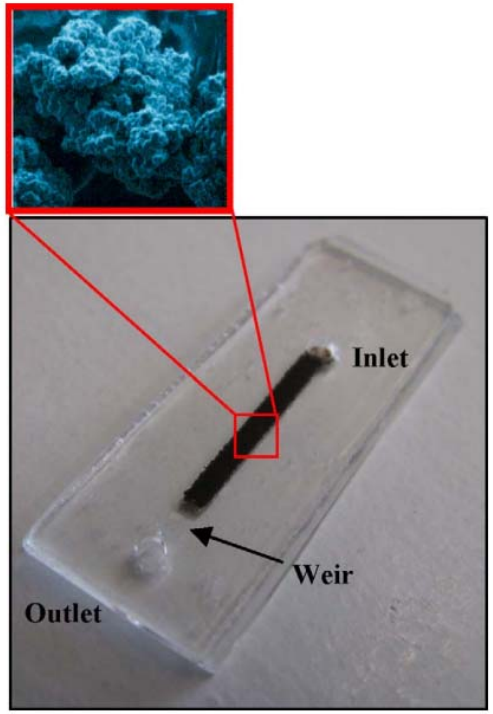


Fig. 1. vrSPE device (1 cm to weir, 1 mm line width, 200 µm deep, 20 µm weir depth) packed with MagneSil™ solid phase. SEM image of a MagneSil™ particle is shown enlarged [22].

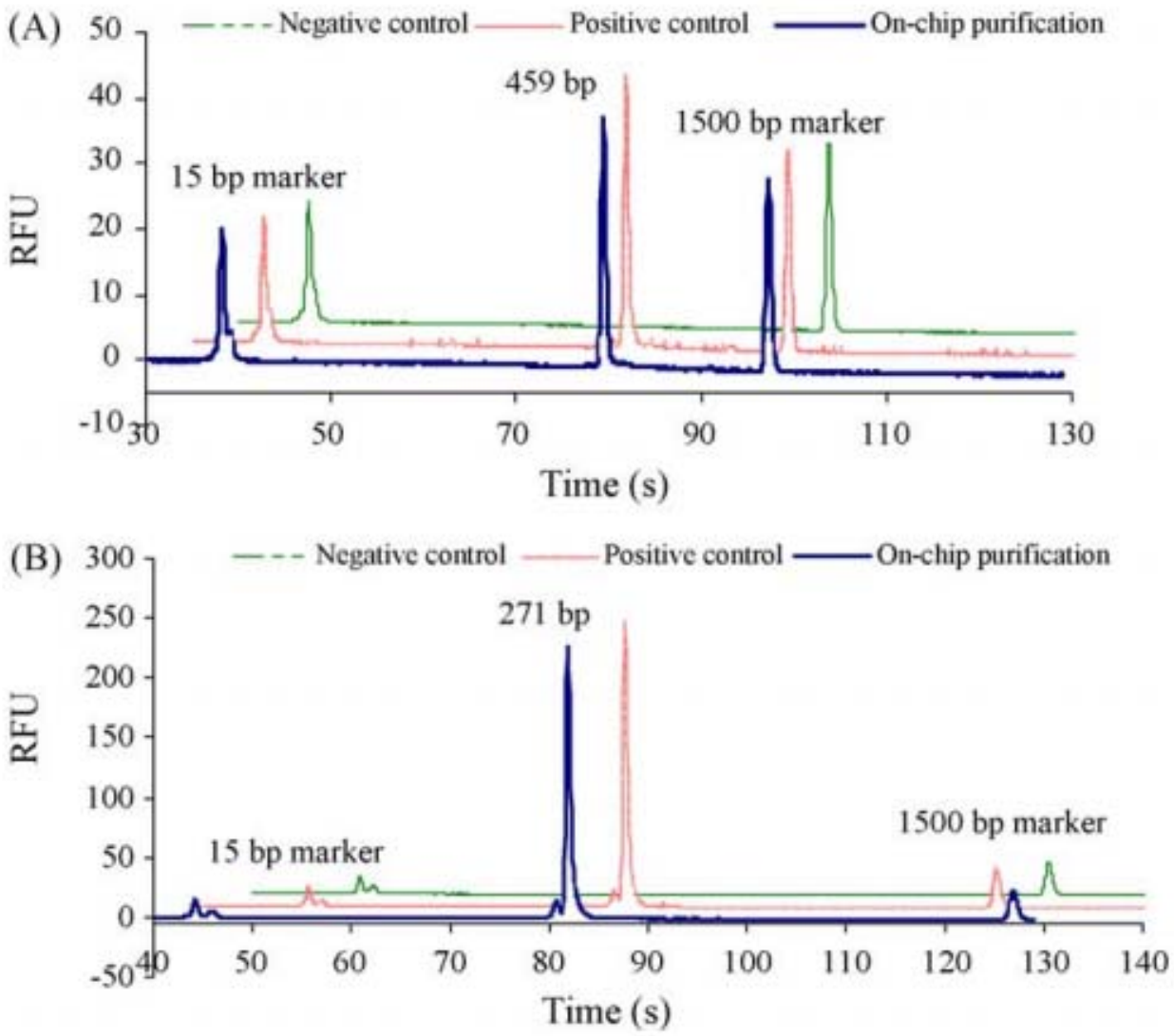
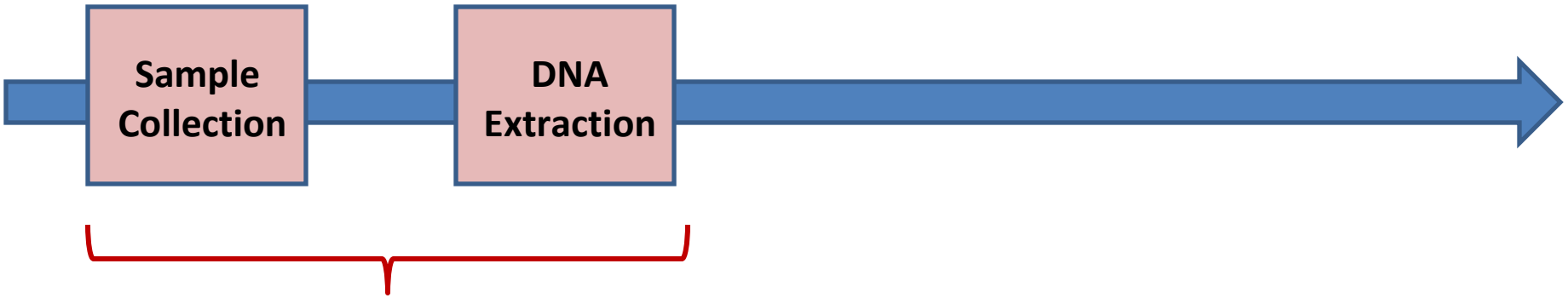


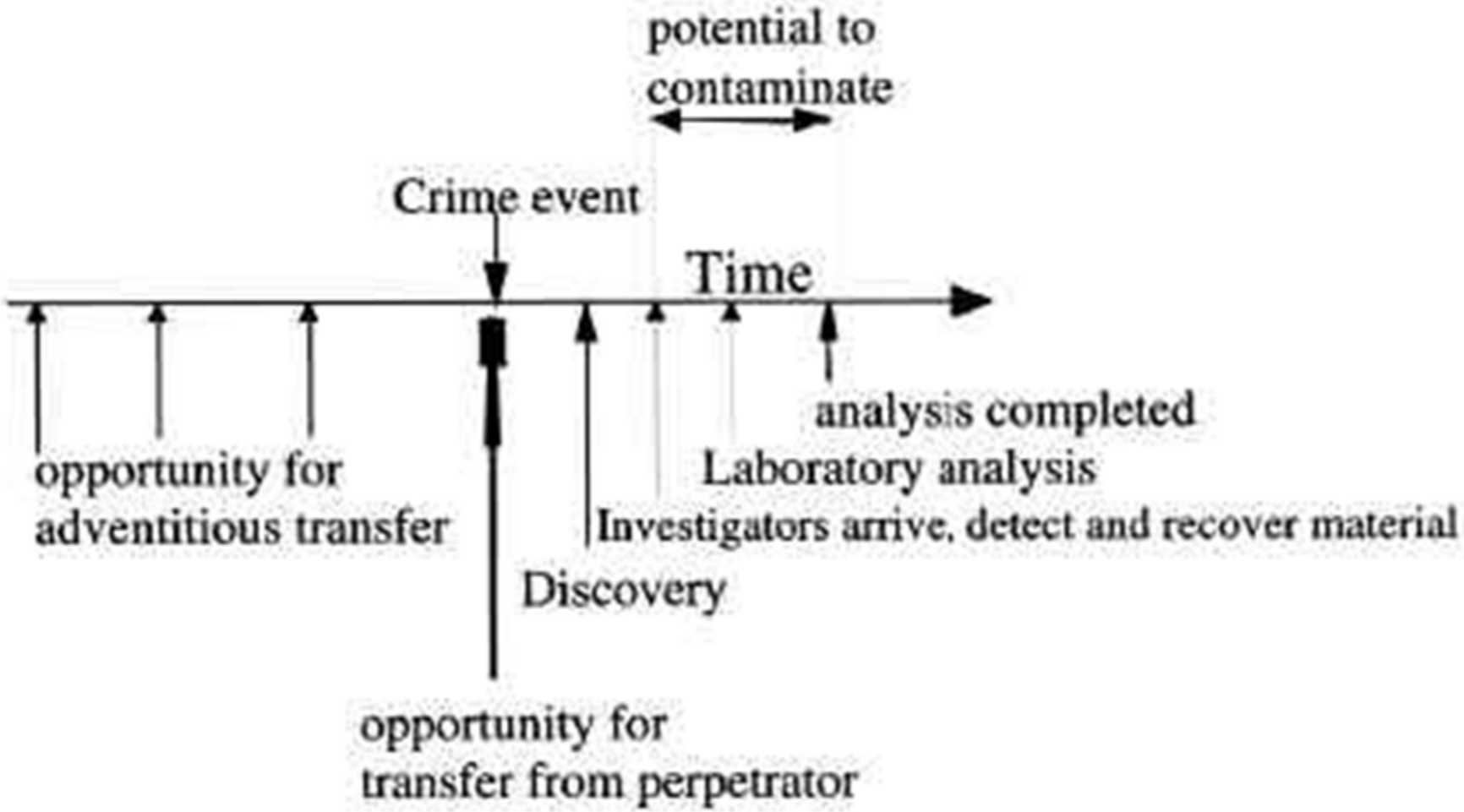
Fig. 6. Electropherograms indicating successful amplification of a portion from the HV1 region of the mtDNA genome (HVI primers from VDFS and MPS 2B or PSII primers from AFDIL) after on-chip vrSPE of mtDNA from (A) dilute whole blood and (B) a degraded blood stain.

Closed system to reduce contamination

Sample Throughput

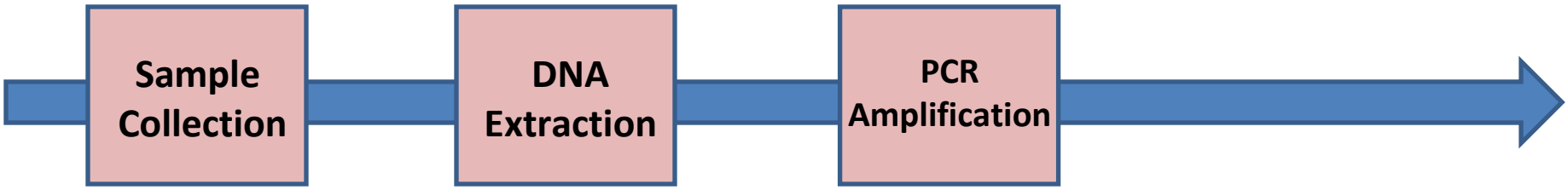


Sensitivity to Contamination



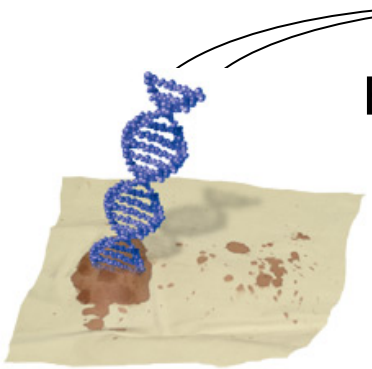
Peter Gill, Presentation at ISHI (2009)

Sample Throughput

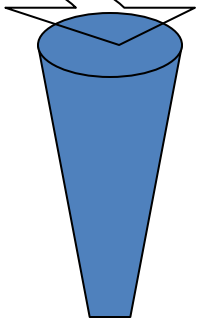


Sample Collection	DNA Extraction	PCR Amplification
Swabs	Organic	LT-DNA
LCM	Silica	New Kits
	Resin	New Markers
	Magnetic Beads	

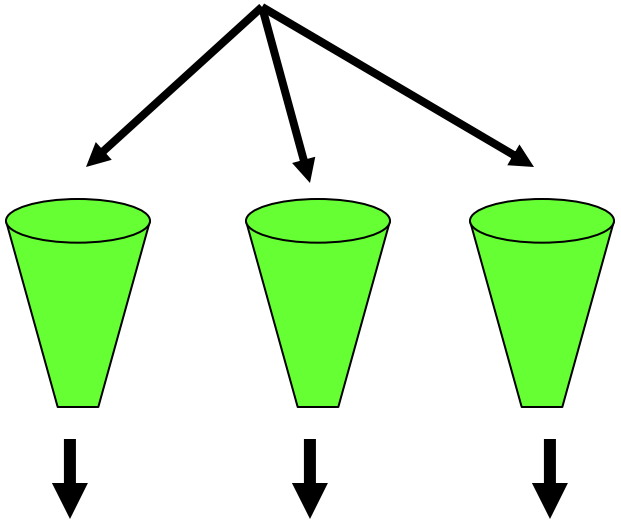
Typical LT-DNA Analysis Procedure



Extract DNA
from stain



Quantify Amount of DNA Present



Perform 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile

(based on replicate consistent results)

Comparison of Approaches

What "LCN Labs" Are Doing

Replicate Amplification with Consensus Profile

Low amount of DNA examined

Stochastic effects

Amplification #1
Amplification #2
Amplification #3

Consensus Profile Developed
(from repeated alleles observed)

Interpretation Rules Applied
(based on validation experience)
e.g., specific loci may dropout more

Result can be and usually is Reliable & Reproducible

Single Amplification

Low amount of DNA examined

Stochastic effects

Amplification #1
(only a single test)

Result can be Unreliable

Individual results may vary but a consensus profile is reproducible
(based on our experience with sensitivity studies and replicate amplifications)

Consensus Profiles

G Model
FSIGEN-613; No. of Pages 13
ARTICLE IN PRESS

Forensic Science International: Genetics xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Forensic Science International: Genetics

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

Forensic population genetics—original research

Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results

Corina C.G. Benschop [a], Cornelis P. van der Beek [b], Hugo C. Meiland [c], Ankie G.M. van Gorp [a], Antoinette A. Westen [a], Titia Sijen [a,*]

[a] Netherlands Forensic Institute, Department of Human Biological Traces, P.O. Box 24044, 2490 AA The Hague, The Netherlands
[b] Netherlands Forensic Institute, Department Dutch DNA database, P.O. Box 24044, 2490 AA The Hague, The Netherlands
[c] Leiden Institute of Advanced Computer Science, Leiden University, Niels Bohrweg 1, 2333 CA Leiden, The Netherlands

Benschop et al. (*in press*)

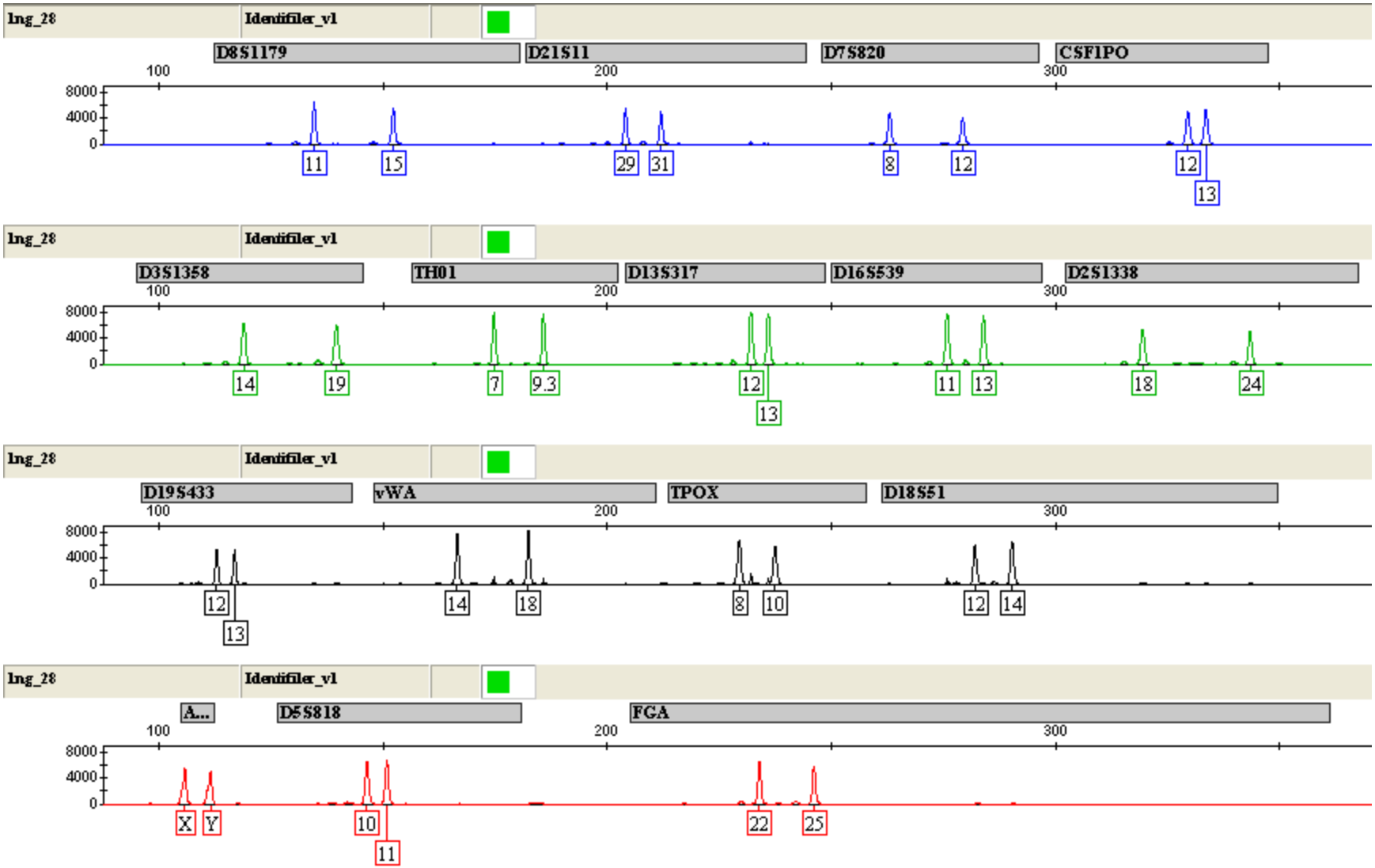
Table 1
Consensus profiles are generated using varying numbers of PCR replicates ($n=2$ to $n=6$) with a variable level of requested reproducibility ($x=1$ to $x=5$). For two LT methods (9 kV and 28 + 6 cycles) the twenty-three single donor samples result in 6578 consensus profiles and the five two-person mixtures result in 1430 consensus profiles.

Level of requested reproducibility (x)	Number of PCR amplifications (n)				
	$n=2$	$n=3$	$n=4$	$n=5$	$n=6$
Composite	$x=1$	$x=1$	$x=1$	$x=1$	$x=1$
$n-1$[a]	$x=2$	$x=2$	$x=3$	$x=4$	$x=5$
$n/2$[a,b]	$x=2$	$x=2$	$x=2$	$x=3$	$x=3$
2×	$x=2$	$x=2$	$x=2$	$x=2$	$x=2$
# of methods for each (n)	2	2	3	4	4
# of combinations out of 6 replicates	15	20	15	6	1
# of consensus profiles per sample	30	40	45	24	4
Total # of consensus profiles per sample per LT method			143		

[a] Since these methods are based on reproducibility to include an allele, the minimum number for x is two.
[b] Rounded up (e.g. for three replicates $n/2$ becomes two).

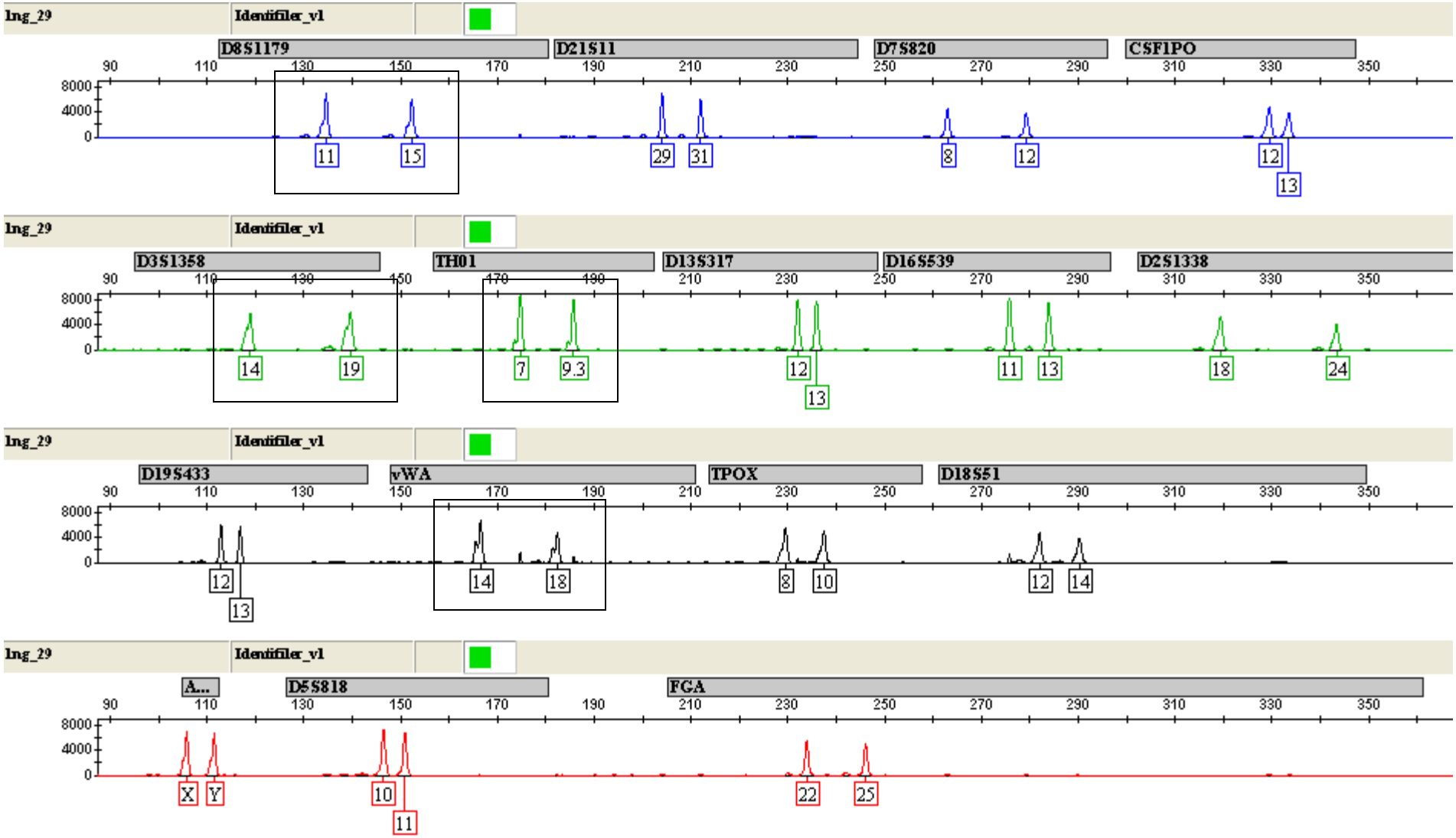
Composite	**n - 1**	**n / 2**	**2X**
10	10 15	10 15	10 15
12	10	10	10
	10 15	15	15
	15	12	9 12
10,12	10,15	10,15	10,15
	10, F		

Identifiler Plus with 1 ng, 28 cycles (Standard Protocol)



Peak Heights <8000 RFUs, no adenylation issues, well balanced peak height ratios

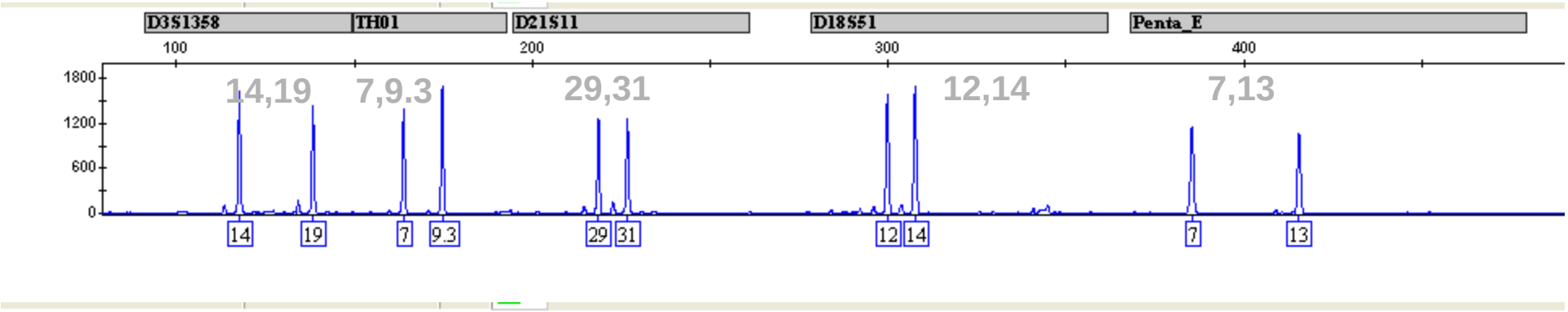
Identifiler Plus with 1 ng, 29 cycles

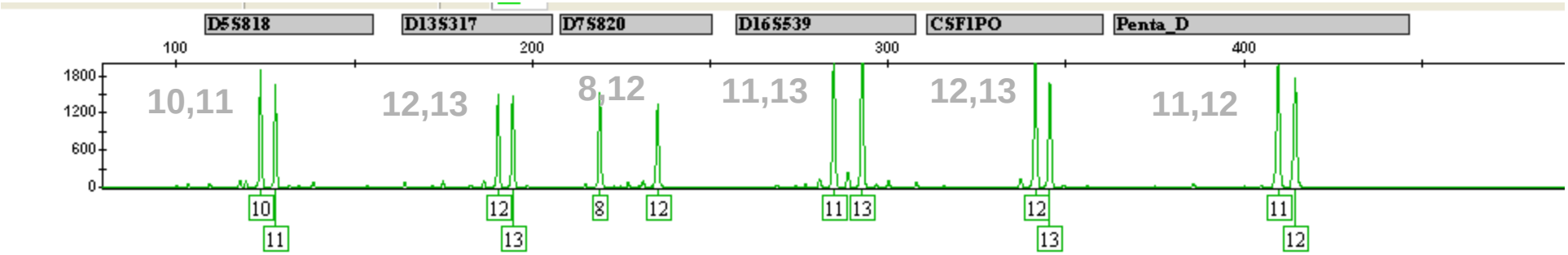


Peak Heights <9000 RFUs, incomplete adenylation, bleed through

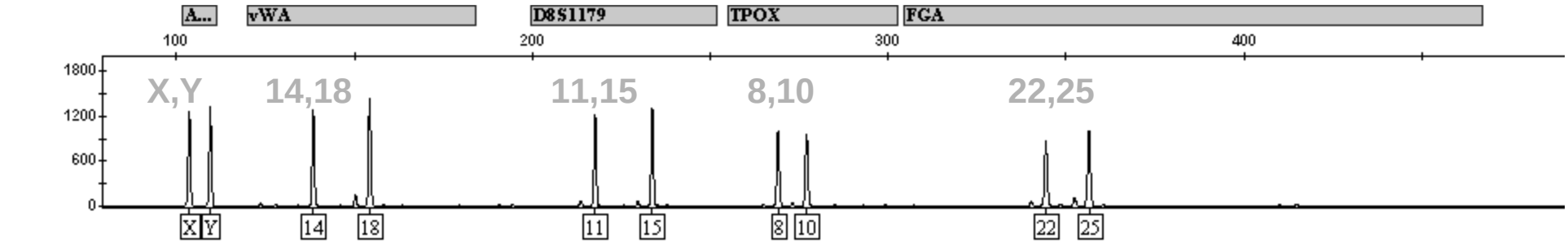
PowerPlex 16 HS (½ Reaction)
1 ng @ 30 cycles

High signal, balanced peak heights (>0.80), no artifacts, low stutter

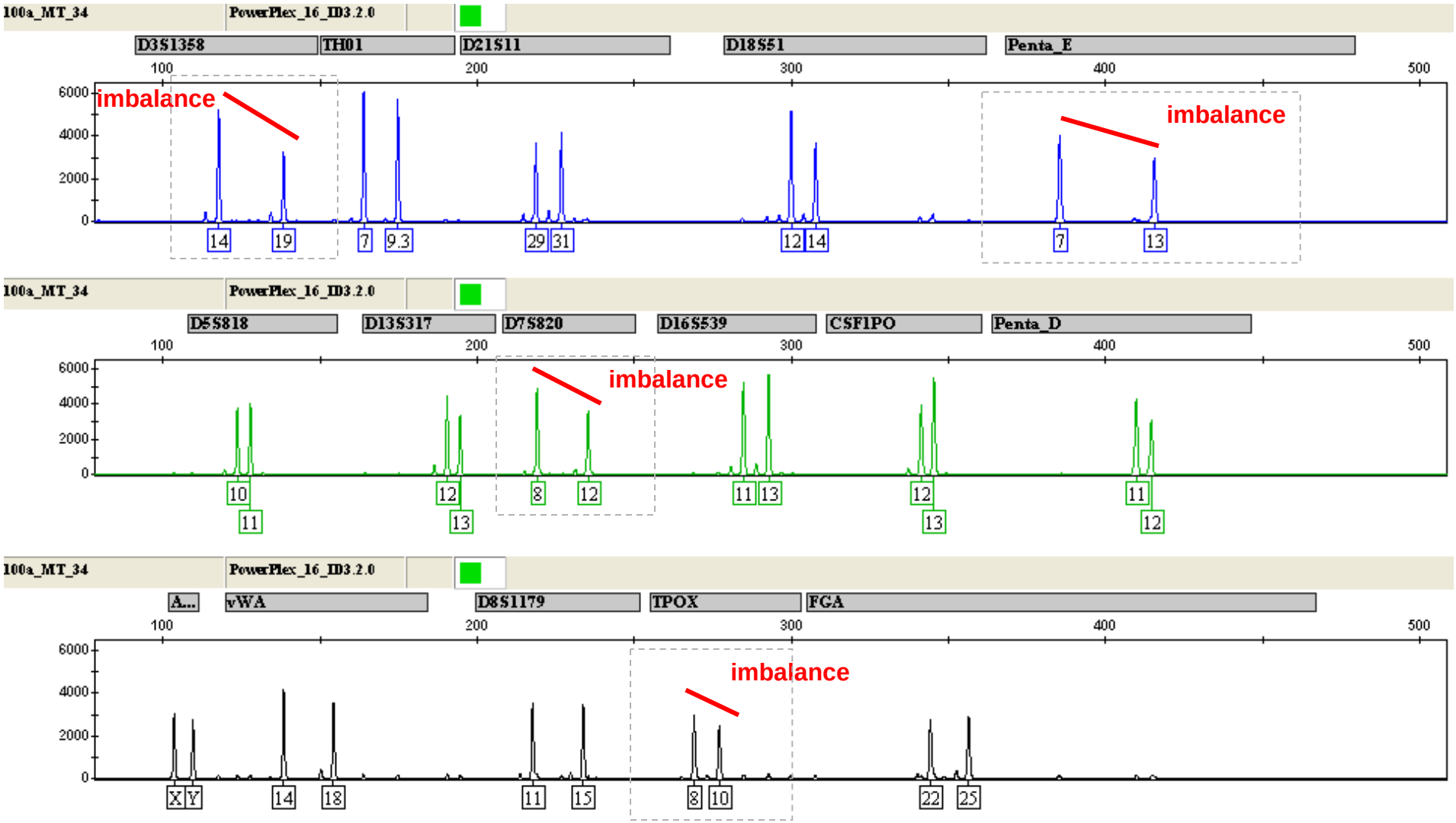




A Fully Heterozygous Sample (2 alleles for each locus)

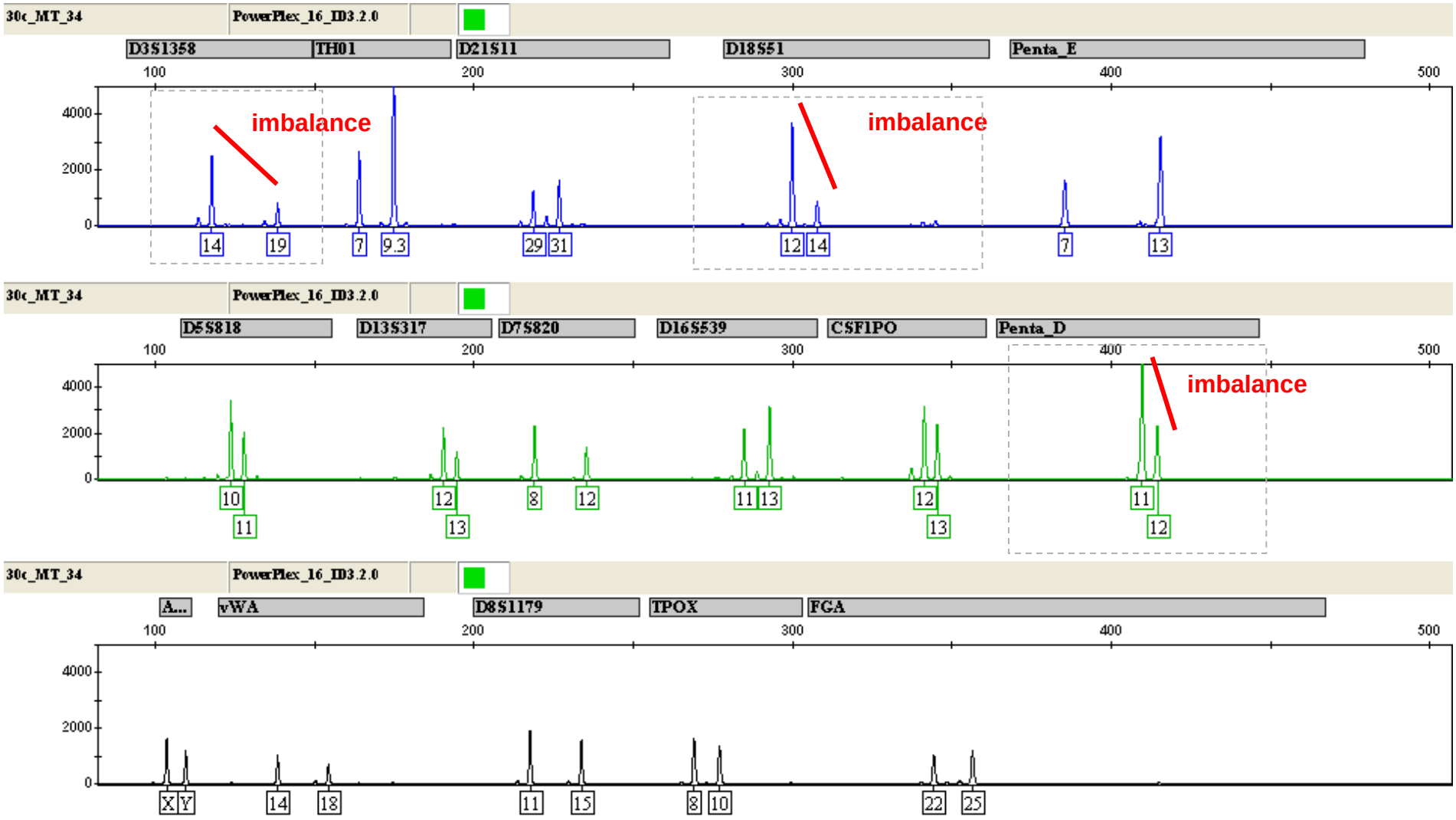


PowerPlex 16 HS, 100 pg @ 34 cycles, ½ Reaction



***No drop-out, slight peak height imbalance, full profiles in all replicates**

PowerPlex 16 HS, 30 pg @ 34 cycles, ½ Reaction

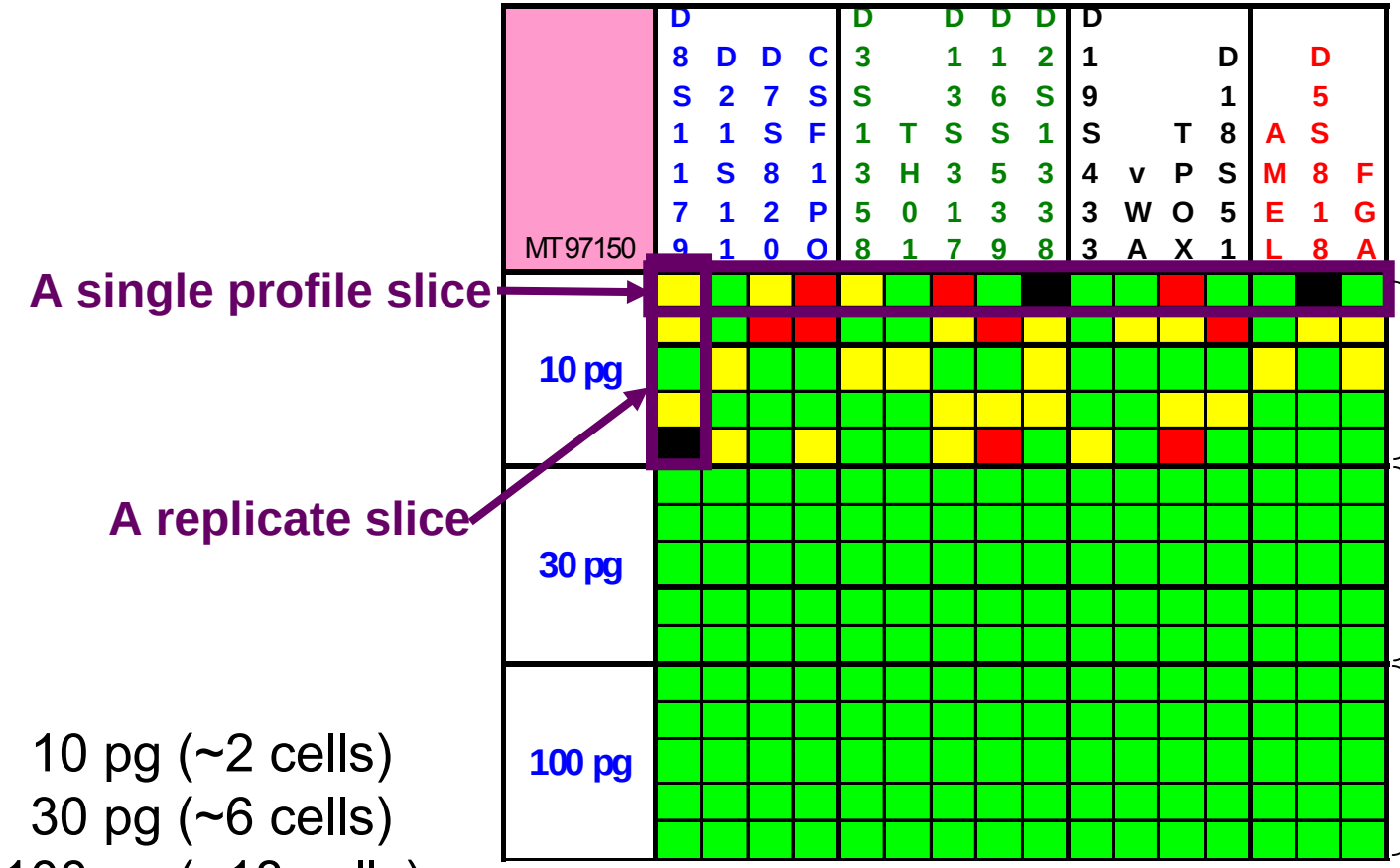


***No allelic drop-out in replicates, significant peak height imbalance**

Sensitivity Comparison

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

Tested sample is heterozygous *(possesses 2 alleles) at every locus, which permits an examination of allele dropout*



Results broken down by locus

10 pg (~2 cells)
30 pg (~6 cells)
100 pg (~18 cells)

Insertion/Deletion Assay

- Like SNPs, vary widely throughout the genome.
- Amplicons can be made small for degraded and challenged samples.
- No Stutter.
- Useful for complex paternity cases.

DIPplex

Table 1. Discrimination power of DIPs, STRs and SNPs

	Loci	CPE/Trio *	CPI **	Population
Mentype® DIPplex	30 DIPs	0.9980	2.83×10^{-13}	German
AmpFISTR Minifiler	8 STRs	0.99976	8.21×10^{-11}	US Caucasian
AmpFISTR SEfiler	11 STRs	0.999998	7.46×10^{-14}	US Caucasian
Powerplex 16	15 STRs	0.9999994	5.46×10^{-18}	US Caucasian
Sanchez et al. 2006	52 SNPs	0.9998	5.00×10^{-21}	European

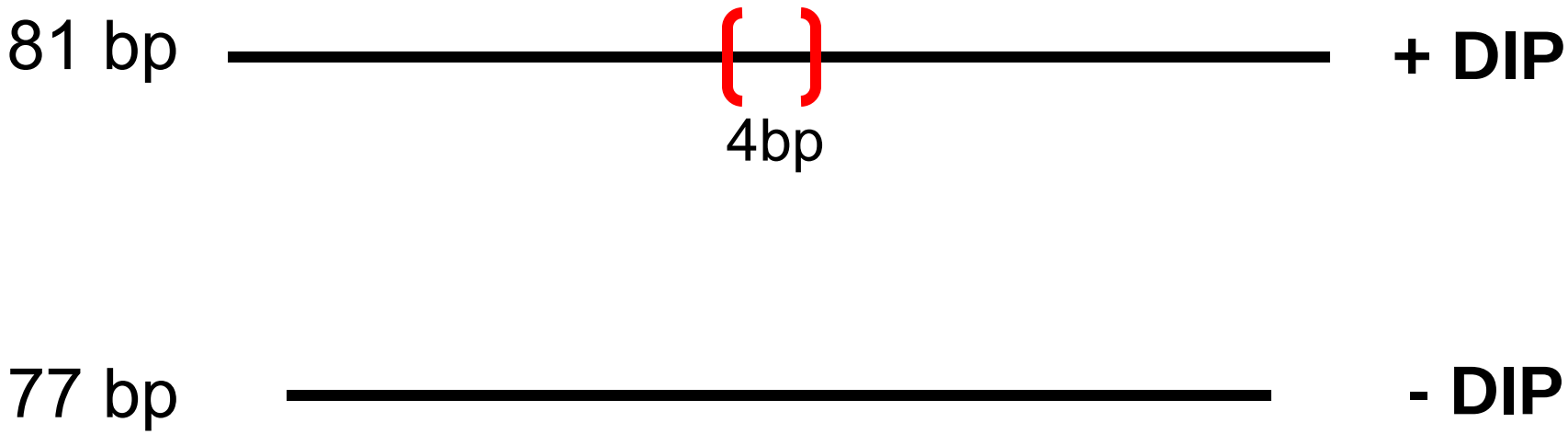
*combined probability of paternity exclusion, **combined probability of identity

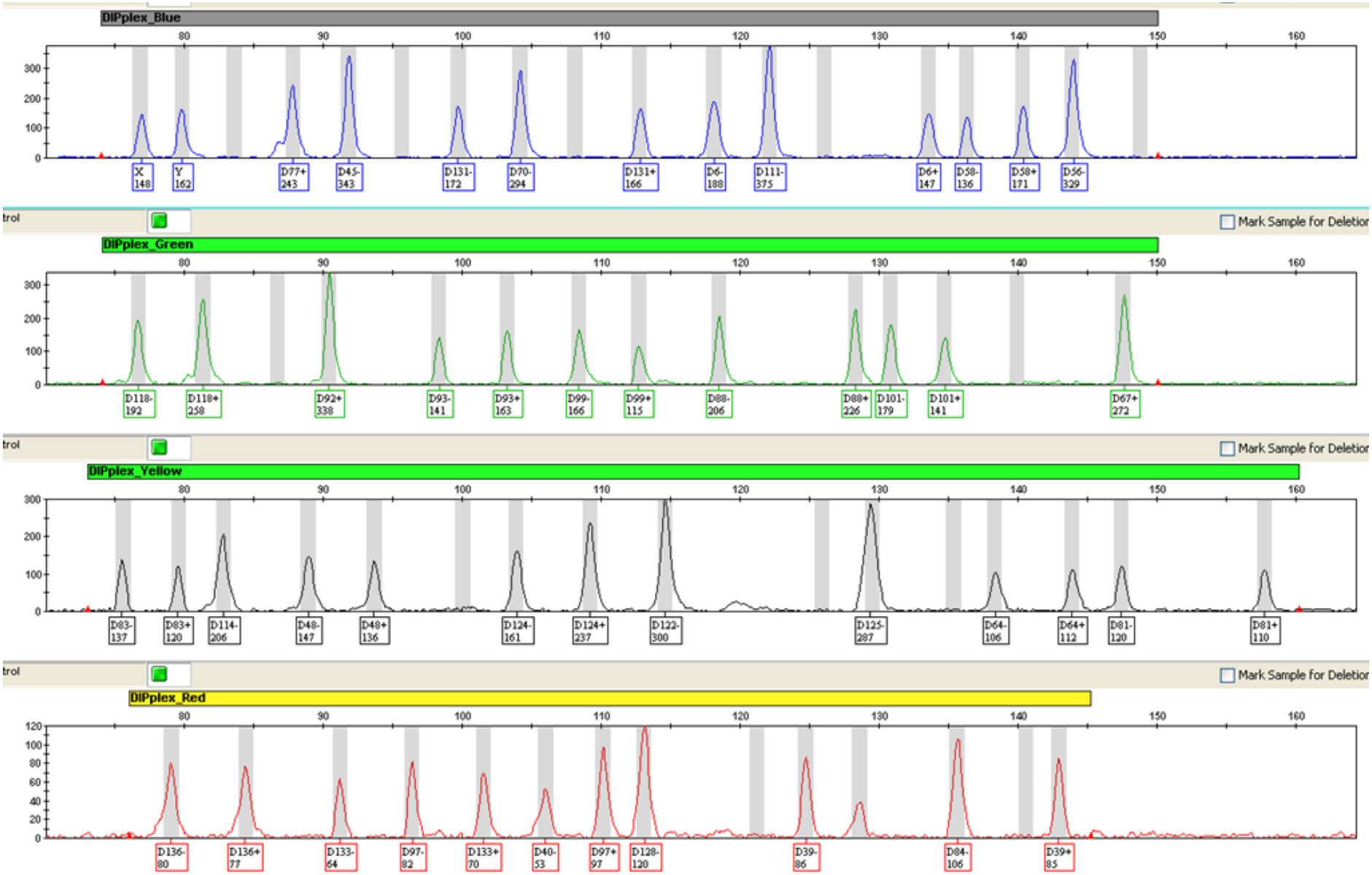
Investigator DIPplex Kit

For multiplex amplification of 30 deletion/insertion polymorphisms (also known as INDELs) plus Amelogenin

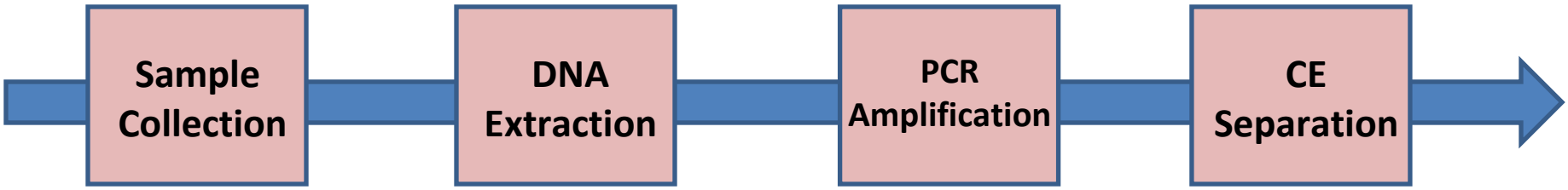
- Unique kit for amplification of deletion/insertion polymorphisms (DIPs)
- Highly suited for degraded DNA samples
- Easy interpretation with dedicated freeware DIPSorter
- Highly sensitive and no overlapping allelic ranges within a panel
- Stutter peaks are not generated during analysis

Marker/Green	-DIP [bp]*	+DIP [bp]*
HLD118	77	81




DIPplex_Blue
X 148
Y 162
D77+ 243
D45- 343
D131- 172
D70- 294
D131+ 166
D6- 188
D111- 375
D6+ 147
D58- 136
D58+ 171
D56- 329
Mark Sample for Deletion
DIPplex_Green
D118- 192
D118+ 258
D92+ 338
D93- 141
D93+ 163
D99- 166
D99+ 115
D88- 206
D88+ 226
D101- 179
D101+ 141
D67+ 272
Mark Sample for Deletion
DIPplex_Yellow
D83- 137
D83+ 120
D114- 206
D48- 147
D48+ 136
D124- 161
D124+ 237
D122- 300
D125- 287
D64- 106
D64+ 112
D81- 120
D81+ 110
Mark Sample for Deletion
DIPplex_Red
D136- 80
D136+ 77
D133- 64
D97- 82
D133+ 70
D40- 53
D97+ 97
D128- 120
D39- 86
D84- 106
D39+ 85

Sample Throughput



Swabs

LCM

Organic

Silica

Resin

Magnetic Beads

LT-DNA

New Kits

New Markers

Injection Times

Salt Removal

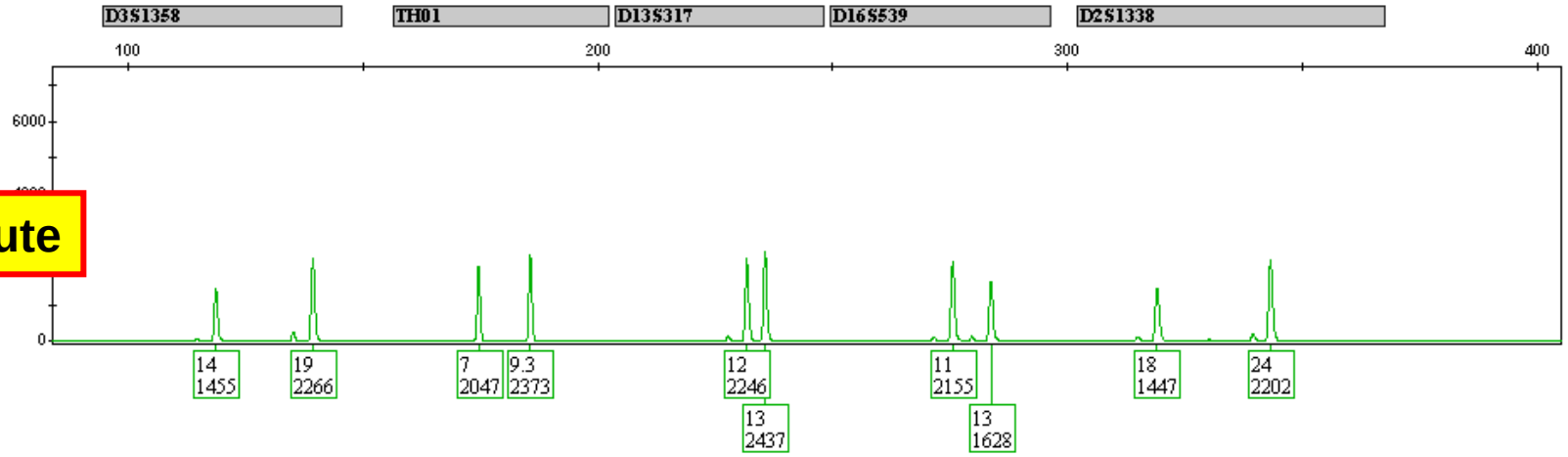
New Instrumentation

MinElute PCR Purification Kit

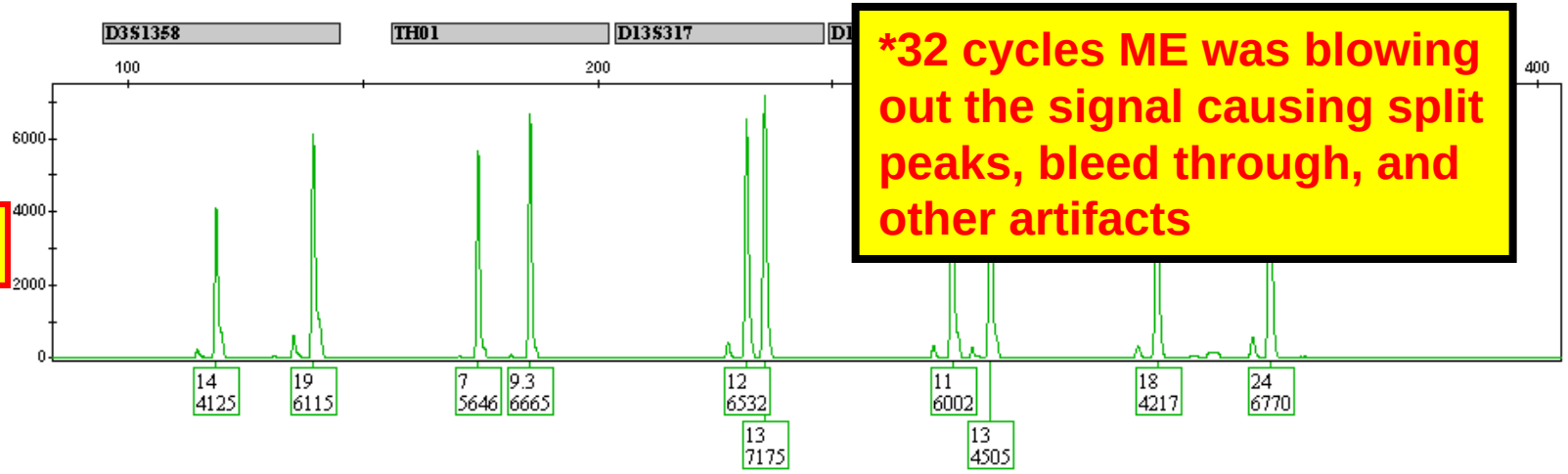
*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 100 pg

No MinElute



MinElute



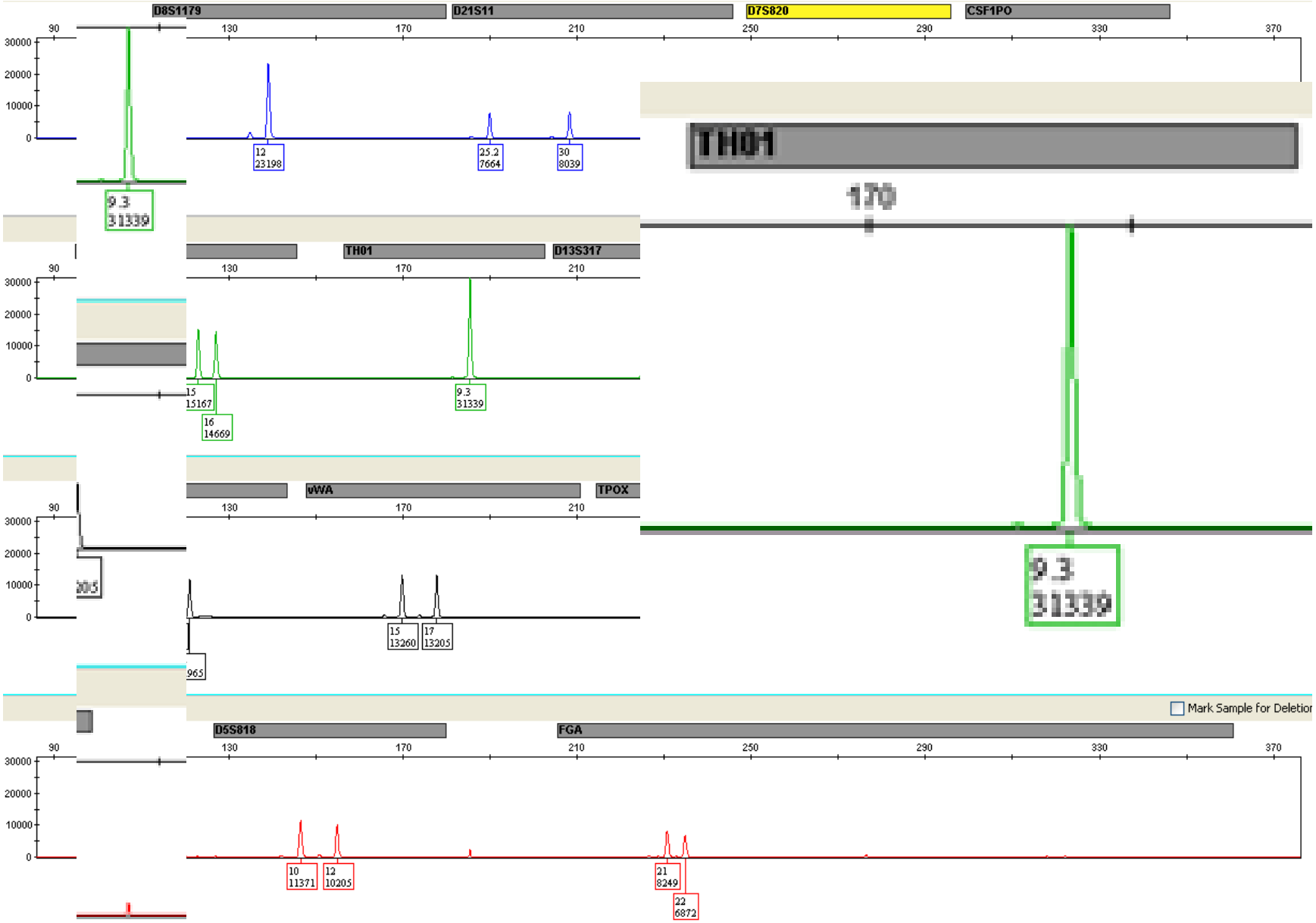
***32 cycles ME was blowing out the signal causing split peaks, bleed through, and other artifacts**

Signal Improvement: ~64% ~64% ~66% ~64% ~67%

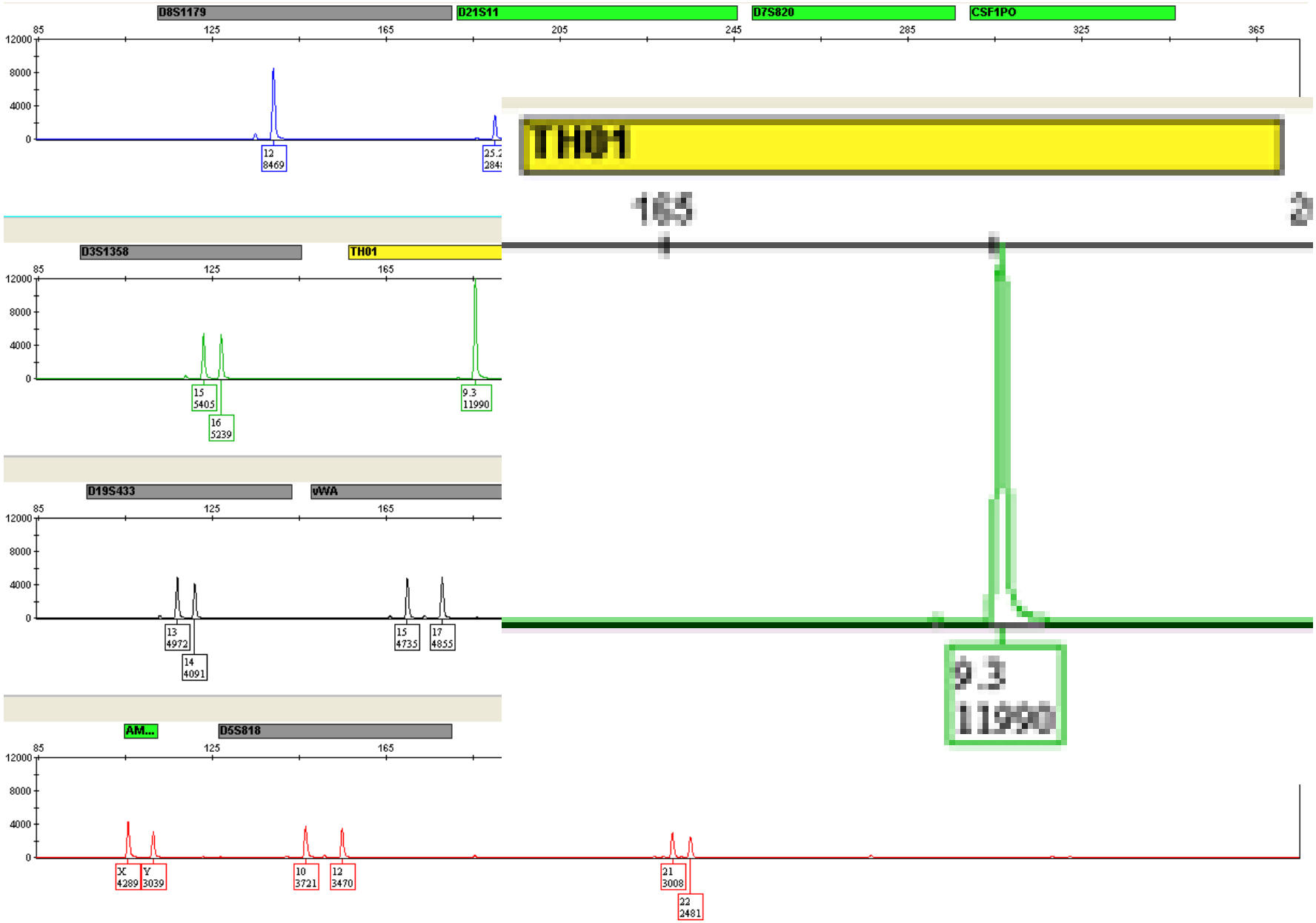
Identifiler Plus data on the 3500*xl* Genetic Analyzer (@ AFDIL)

3500*xl* – 24 Capillary Array

3500xl Default Injection, GS 500



3500xl Low Injection, GS 500



Conclusions

- Improvements in sample collection, extraction, amplification and separation are growing exponentially!
- With new kits and instrumentation, the sensitivity of detecting very low levels of DNA (authentic or not?) is improving. There should be caution that proper controls and interpretation guidelines are reflected in the analysis of the data.

Applied Genetics

The NIST Human Identity Project Team

(Forensic DNA & DNA Biometrics)

Funding from the **National Institute of Justice (NIJ)** through the NIST Office of Law Enforcement Standards and the **FBI S&T Branch** through the NIST Information Access Division

…Bringing traceability and technology to the scales of justice…

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Project Leader, Forensic DNA

Becky Hill

LT-DNA Testing
3500 testing

Pete Vallone

Project Leader, DNA Biometrics

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DipPlex Markers

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Questions?