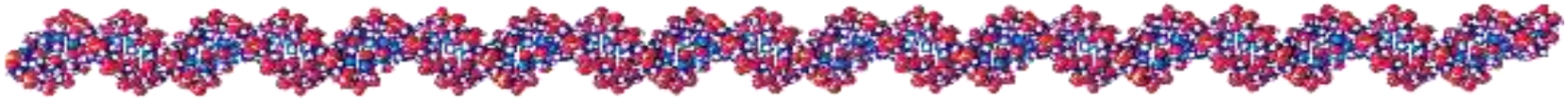


Direct PCR with PowerPlex® 18D: Performance and Concordance

Forensic Genetics
Promega Lunch Symposium
September 2, 2011

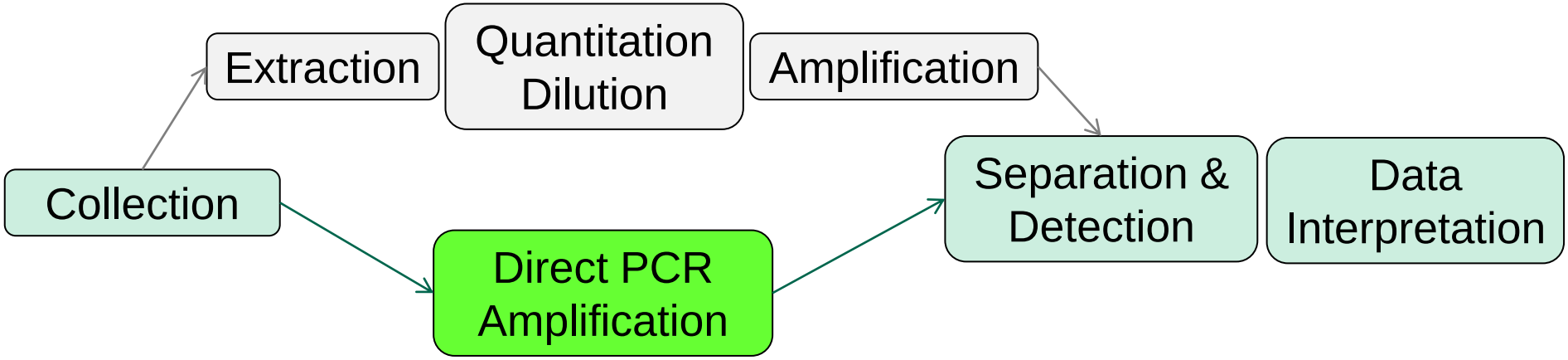
Peter M. Vallone, Carolyn R. Hill, Erica L.R. Butts
Biochemical Science Division
National Institute of Standards and Technology

Outline

- Benefits of direct PCR
- Collection media: FTA and 903 paper
- Source of DNA: Blood and buccal cells
- Direct PCR with PowerPlex 18D (PP18D)

Benefits of Direct PCR

- Sample set-up convenience: ‘punch and go’
- Amplify unpurified DNA - skip extraction and quantitation steps
- Saves time, cost, labor
- Amenable to automation (automated blood card puncher and robotics)
- Applications: offender DNA database samples, paternity samples, casework reference samples



Development of Direct PCR

- Directed towards: clinical, agricultural, and forensic applications
- PCR reagents are combination of PCR enhancers and modified/mutant DNA polymerases
- Modified polymerases are 10-100 times more tolerant of inhibitors compared to wild type *Taq* Polymerase

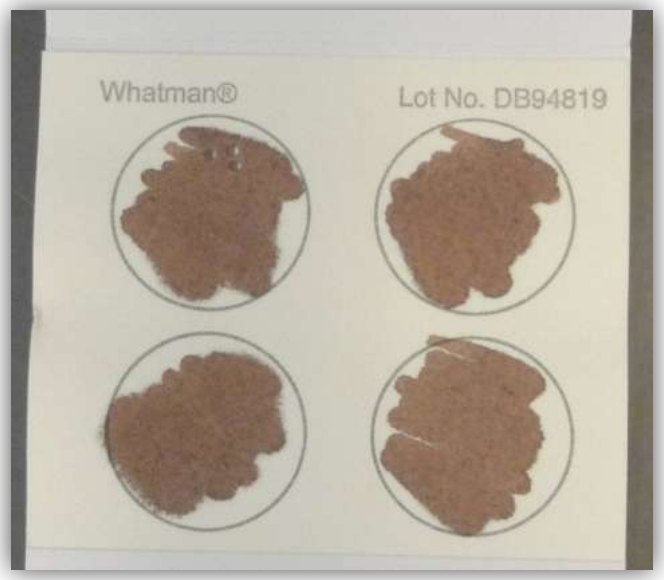
Recent commercial developments (non-STR kit)

- OmniTaq and Omni Klentaq enzymes are triple mutant DNA polymerases resistant to PCR inhibitors such as blood, serum, soil, chocolate, and milk
- Phusion® Blood Direct PCR Kit
- Clontech Direct PCR —Terra™ Polymerase Mix

Zhang et al., Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of Taq 2010 Journal of Molecular Diagnostics, 12: 152-161

Kermekchiev et al., Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples 2009 Nucleic Acids Research, 37: e40

FTA paper



- High MW DNA becomes entangled in the fibers of the paper - DNA binds to paper
- DNA collected on FTA Cards is stable for *at least 14 years* at room temperature

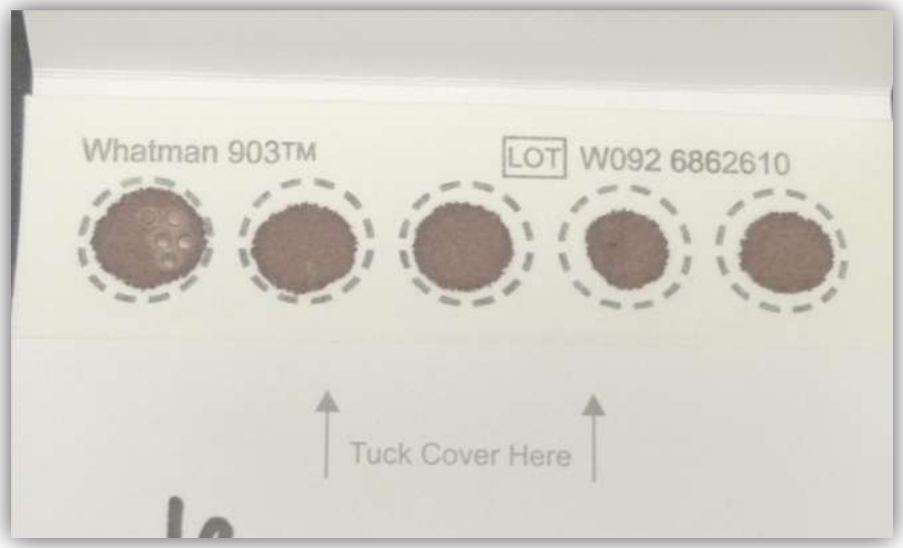
Margaret Kline NIST Study

- Inventor: Leigh Burgoyne *Patent 5,496,562 - 1999*

  Flinders University (Adelaide, South Australia)

- High-purity cotton linter pulp
- Chemically treated with several compounds designed to kill pathogens and resist bacterial growth and DNA degradation (Tris-EDTA, sodium dodecyl sulfate, and uric acid)
- Cells lyse on contact with paper

903 paper



- DNA collected on 903 Cards is stable for *at least 14* years at room temperature

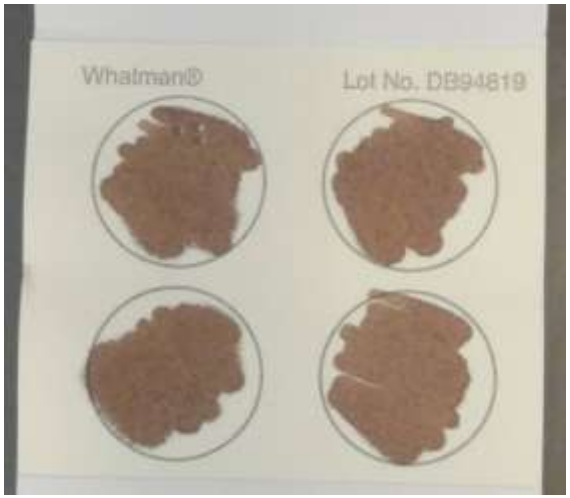
Margaret Kline NIST Study

- ‘Schleicher & Schuell 903’
- High-purity cotton linter pulp
- No chemical added
- Used in newborn screening programs
- Support media – DNA is not bound to the paper

Sources of DNA

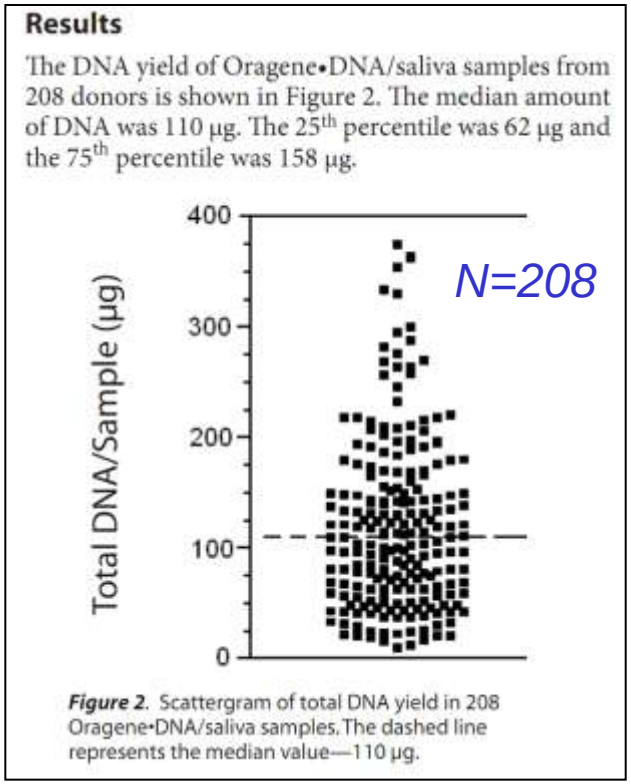
Blood

- White blood cells contain DNA
- Approximately 4,500 – 10,000 WBC per µL (varies)



Buccal cells

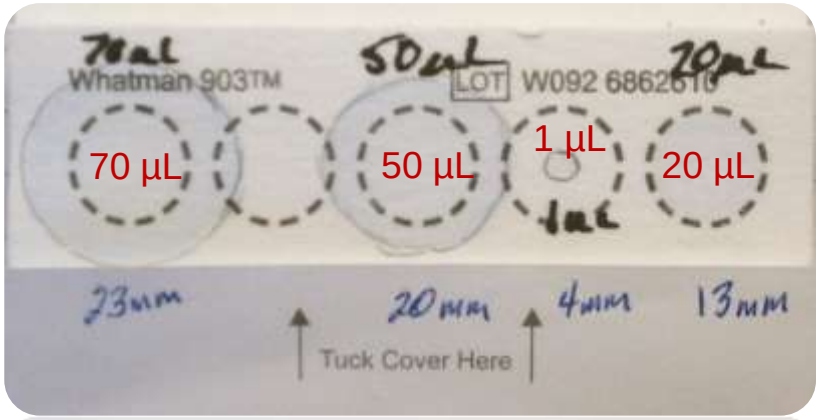
- Saliva: 100 µg/2 mL = 50 ng/µL of DNA



http://www.dnagenotek.com/pdf_files/PDWP001_DNAYield.pdf

Theoretical

How much DNA is in a 1.2 mm blood punch?



- Range: 4,500-10,000 white blood cells per µL
- Assume 4,500 WBC/µL for calculation
- $\pi \cdot r^2$ = area of a circle
- Deposit specific volume of water onto collection paper and calculate the area of the spot

Volume (uL)	WBC cells/uL	Total cells deposited	Diameter of spot (mm)	Total area mm^2	Cells/mm^2	Cells/1.2 mm punch	ng of DNA per 1.2 mm punch
70	4,500	315,000	23	415.3	759	857	5.1
50	4,500	225,000	20	314.0	717	810	4.9
20	4,500	90,000	13	132.7	678	767	4.6
1	4,500	4,500	4	12.6	358	405	2.4
Punch			1.2	1.1			

Typically ~3-5 ng of DNA per spot (1.2 mm diameter)
High range ~10 ng – assuming 10,000 WBC per µL

Measured

How much DNA is in a 1.2 mm blood punch?

- Five blood punches (1.2 mm) per sample (n=4) were collected and extracted on the Qiagen EZ1 Advanced Robot
- DNA was eluted in a 50 µL volume
- *Quantitation performed with the Qubit® 2.0 Fluorometer (Invitrogen)*

Sample	Paper	Total DNA recovered (ng) from 5 - 1.2 mm blood punches	ng of DNA recovered per punch
1	FTA	16	3.2
2	FTA	12	2.4
3	903	14	2.8
4	903	21	4.2

Post EZ1 extraction ~3 ng of DNA were recovered per 1.2 mm punch

Direct PCR Kits

Identifiler Direct

- Same primer sequences as Identifiler
- Enhanced master mix formulation (same polymerase as Identifiler and Identifiler Plus)
- 200 and 1000 reaction kits

- Released 2009

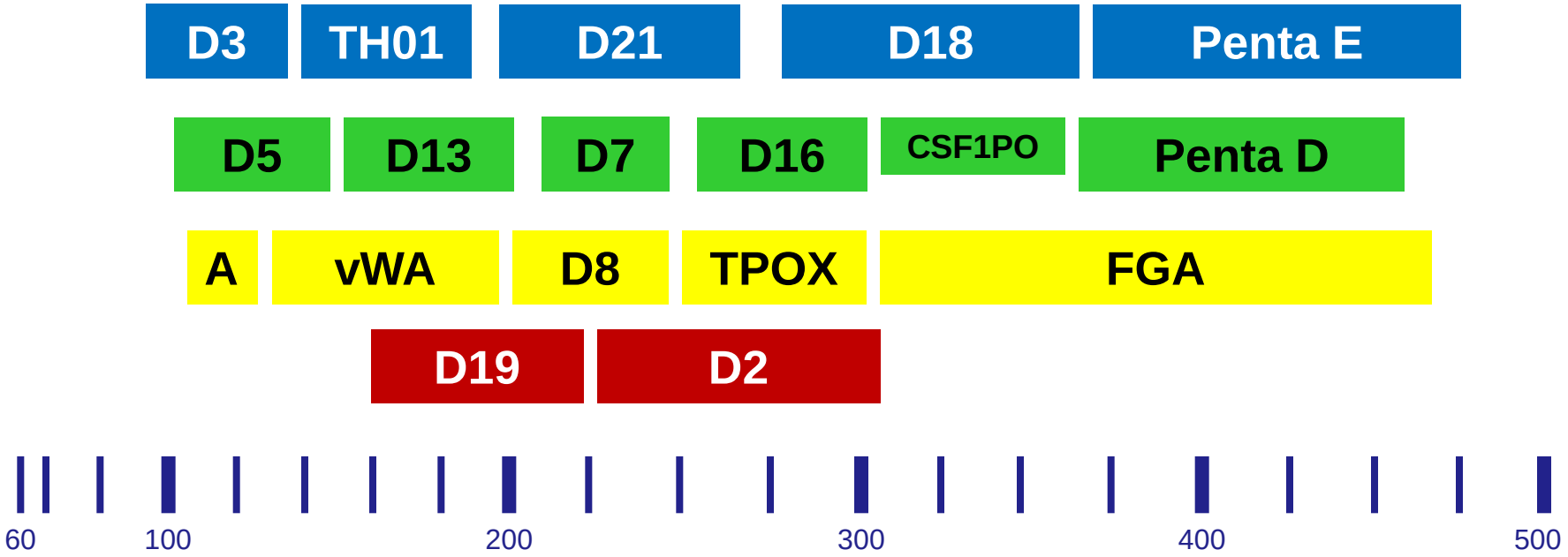
PowerPlex18D

- Same primer sequences as for the PP16 loci
- + D2S1338 and D19S433
- Specialized rapid-direct master mix formulation
- 200 or 800 reaction kit

- Released 2011

Development and Validation of the AmpFℓSTR(®) Identifiler(®) Direct PCR Amplification Kit: A Multiplex Assay for the Direct Amplification of Single-Source Samples Wang DY, Chang CW, Lagacé RE, Oldroyd NJ, Hennessy LK. J Forensic Sci. 2011 Mar 21 [Epub ahead of print]

PowerPlex 18D



Configuration of the PowerPlex ® 18D System. The PowerPlex ® 18D System contains all of the 13 CODIS loci D3S1358, THO1, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA, D8S1179, TPOX, and FGA, plus Amelogenin, Penta E, Penta D, D19S433, and D2S1338.

Slide courtesy of Katie Oostdik (Promega)

Identifiler Plus and PowerPlex 16 HS kits

- Not initially intended for direct PCR
- Contain ‘enhanced’ master mix components for increased inhibitor tolerance (casework)
- These kits can also be used for direct PCR

Poster 315: *Direct PCR Amplification of STR Loci: Protocols and Performance* *(direct PCR results for Identifiler Direct, Identifiler Plus, Power Plex 16 HS and PowerPlex 18 D kits)*

PCR Inhibitors

Newer master mix and polymerase overcome/tolerate inhibitors present in blood and FTA paper

With these kits/protocols a pre-wash of FTA paper is not required

- FTA paper: sodium dodecyl sulfate, uric acid, EDTA
- Blood: heme, hemoglobin, lactoferrin immunoglobin G

[1]Al-Soud WA and Rådström P. Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol. 2001 39:485-93.
[2]Al-Soud WA, Jönsson LJ, Rådström P. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. J Clin Microbiol. 2000 38:345-50.
[3]Joseph Bessetti Promega Corporation *Profiles in DNA* **10(1)**, 9–10 2007
http://www.promega.com/resources/articles/profiles-in-dna/2007/an-introduction-to-pcr-inhibitors/

Thermal Cycling
Parameters and Times

	Hot Start	Denature	Anneal	Elongate	Cycles	Soak
PP18D	96 (2 min)	94 (10 s)	60 (1 min)		27	60 (20 min)
PP16HS	96 (2 min)	94 (30 s)	60 (30 s)	70 (45 s)	30/32	60 (30 min)
Identifiler Direct	95 (11 min)	94 (20 s)	59 (120 s)	72 (60 s)	27	60 (25 min)
Identifiler Plus	95 (11 min)	94 (20 s)	59 (3 min)		28/29	60 (10 min)

	Total Cycling Time	h:min
PP18D	1:25	
PP16HS	2:40 | 2:48	
Identifiler Direct	2:37	
Identifiler Plus	2:26 | 2:31	

Estimated cycling times on a GeneAmp 9700 (9600 emulation mode)

Experiments

- 50 unique anonymous liquid blood samples were obtained
  - Blood was spotted onto FTA and 903 collection papers
  - Uniform comparison of blood spots (age storage conditions)
- A single 1.2 mm punch was used for all PCR reactions
- 25 µL PCR volume (full volume); 28 cycles
- FTA and 903 papers were not pre-treated prior to direct amplification

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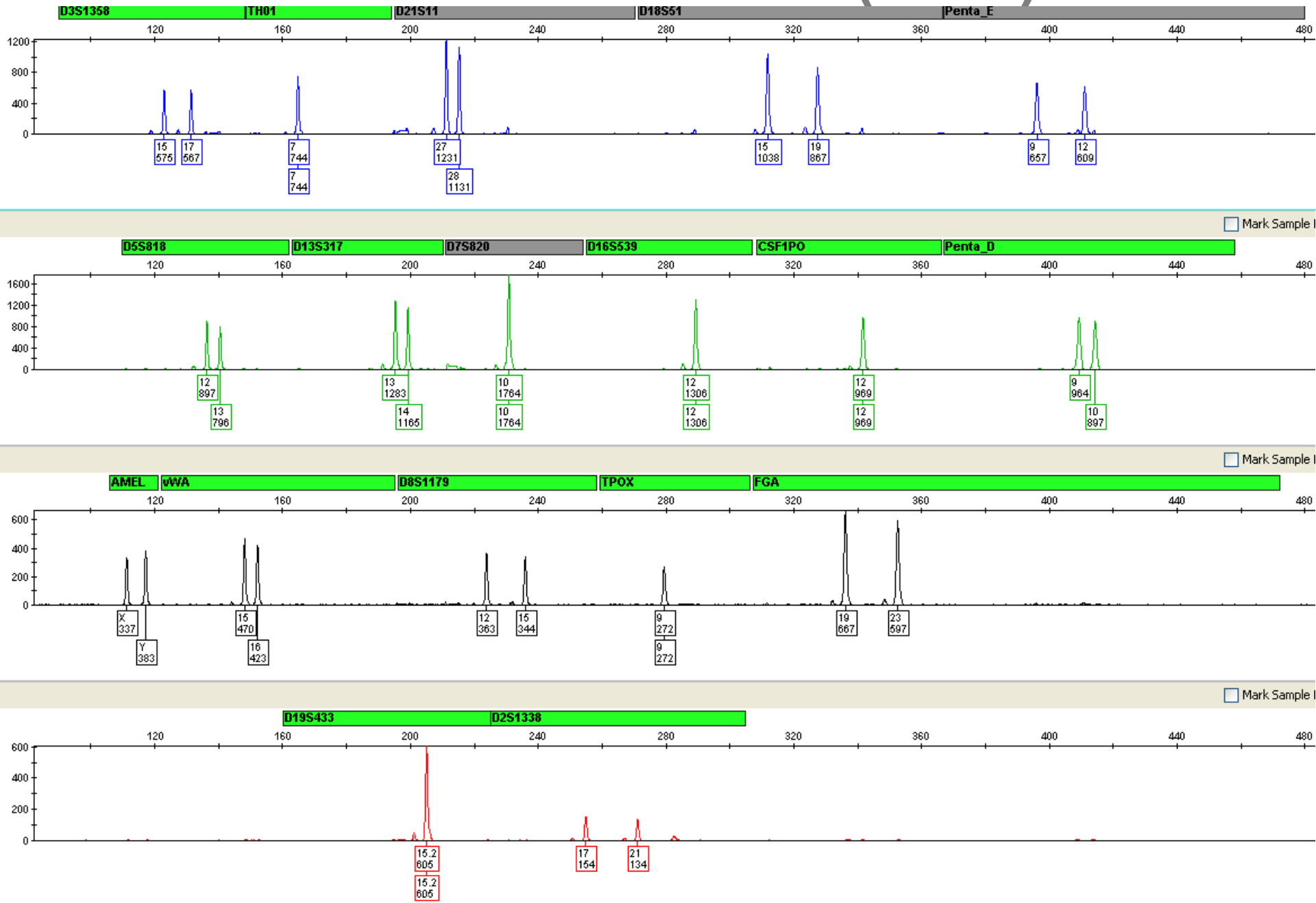
- Also typed for Identifiler Direct, Identifiler, PowerPlex 16 HS
- 648 additional extracted (non-blood) NIST population samples were typed with PowerPlex 18D for genotype concordance checks

PowerPlex 18D (FTA)

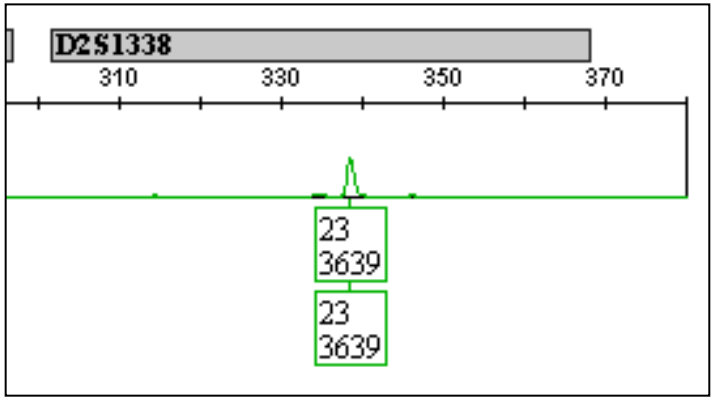
+D19S433 and D2S1338

PowerPlex 18D (903)

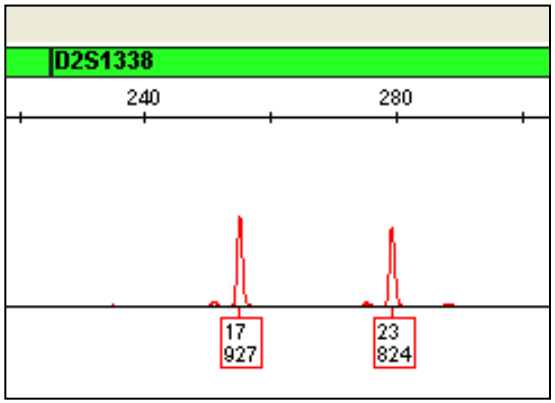


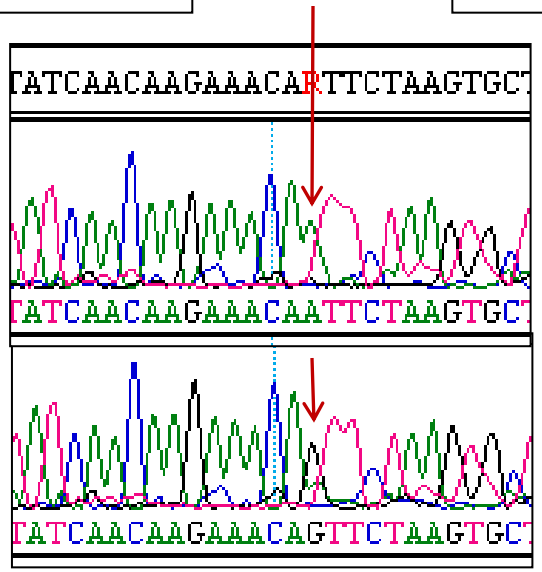
Concordance - D2S1338

Identifiler direct (23,23)



PP18D (17,23)





AACARTTCT

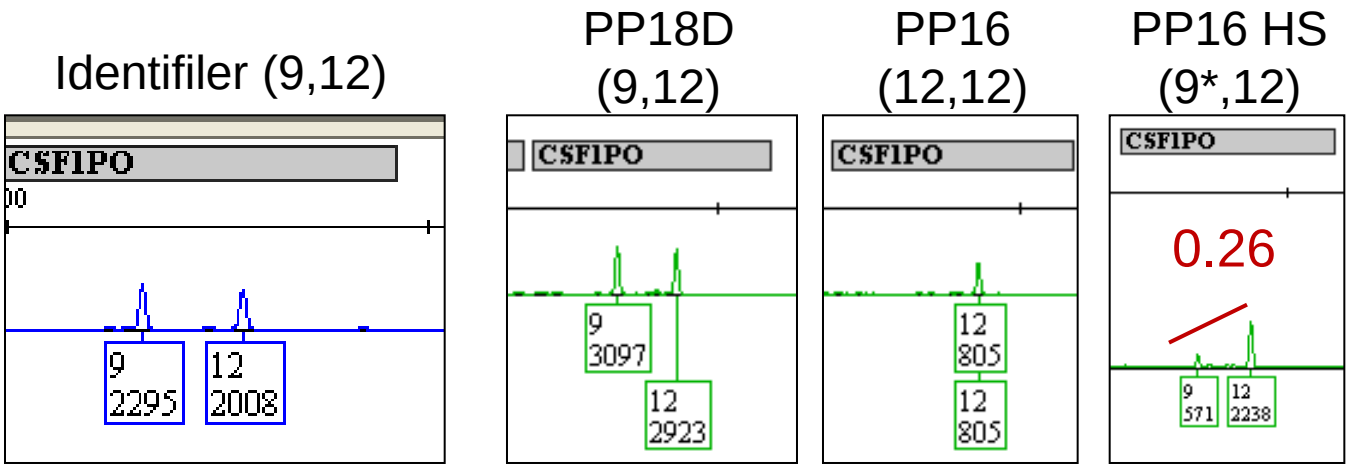
17 A

23 G

Allele 17: $[TGCC]_6$ $[TTCC]_{11}$ **G→A**

173 bp downstream - could affect Identifiler reverse primer binding

Concordance - CSF1PO



Questions:
What are the components of the PP18D kit that allow for the tolerance of primer binding site mismatches?

Possible components: Higher salt (Mg^{++}), PCR enhancers, different DNA polymerase?

Allele 9: $[AGAT]_9$ **C→T**

PP16 CSF1PO-R: 5' – ATTTCCTGTGTCAGACCCT**G**TT – 3'

Peak Height Ratios and Stutter

N = 50	PP18D	PP18D
Locus	**903**	**FTA**
AMEL	0.93	0.89
CSF1PO	0.94	0.94
D13S317	0.94	0.92
D16S539	0.93	0.94
D18S51	0.93	0.91
D19S433	0.95	0.91
D21S11	0.95	0.93
D2S1338	0.91	0.91
D3S1358	0.94	0.94
D5S818	0.92	0.90
D7S820	0.95	0.94
D8S1179	0.94	0.92
FGA	0.94	0.93
Penta D	0.93	0.92
Penta E	0.93	0.89
TH01	0.95	0.96
TPOX	0.89	0.94
vWA	0.89	0.87

Average peak height ratios (± 0.07)

Comparable to non-direct PCR peak height ratios

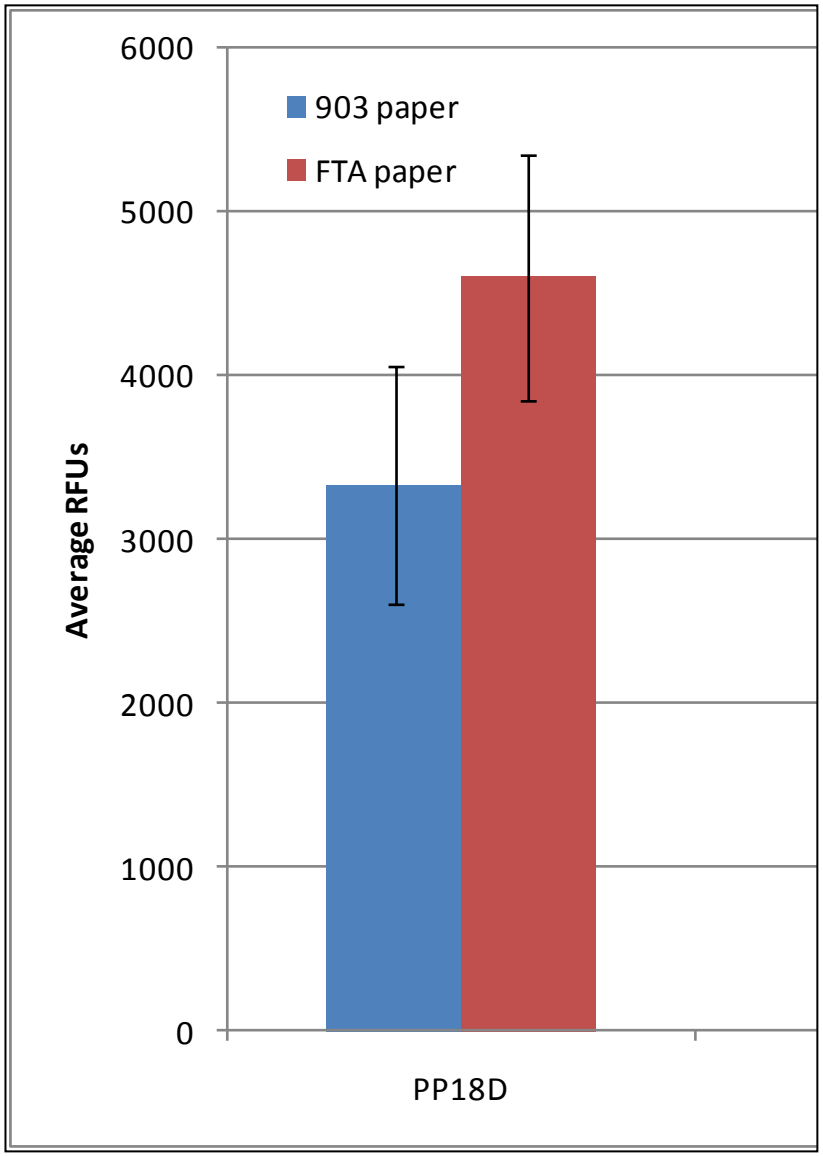
N = 50	PP18D		PP18D		Promega Validation of PP18D	
Locus	**903**	**SD**	**FTA**	**SD**	**FTA**	**SD**
CSF1PO	9.8	7.2	8.3	6.1	5.8	3.3
D13S317	7.2	4.2	6.0	3.2	6.1	3.7
D16S539	8.3	4.2	8.3	4.3	7.2	4.0
D18S51	9.5	4.3	8.2	3.5	7.2	4.3
D19S433	5.4	2.0	4.9	1.8	6.2	3.7
D21S11	8.7	2.9	7.8	2.7	8.4	4.3
D2S1338	8.1	2.2	7.5	2.4	8.1	4.7
D3S1358	9.2	1.7	9.1	2.9	8.8	4.3
D5S818	10.2	5.7	12.2	7.3	7.4	4.3
D7S820	7.5	5.7	7.3	6.1	5.1	3.7
D8S1179	6.3	2.6	6.0	2.0	6.6	4.0
FGA	7.0	2.1	6.7	2.8	7.1	4.0
Penta D	3.1	4.6	3.3	6.1	2.3	1.7
Penta E	3.3	2.1	2.6	1.5	3.8	2.7
TH01	5.2	4.4	5.5	6.1	2.3	1.3
TPOX	4.6	2.1	4.9	5.8	3.5	2.3
vWA	7.8	2.8	7.4	3.2	7.6	4.3

The stutter observed with the direct PCR amplification is within the error of the stutter observed in independent validation studies

No significant increase observed

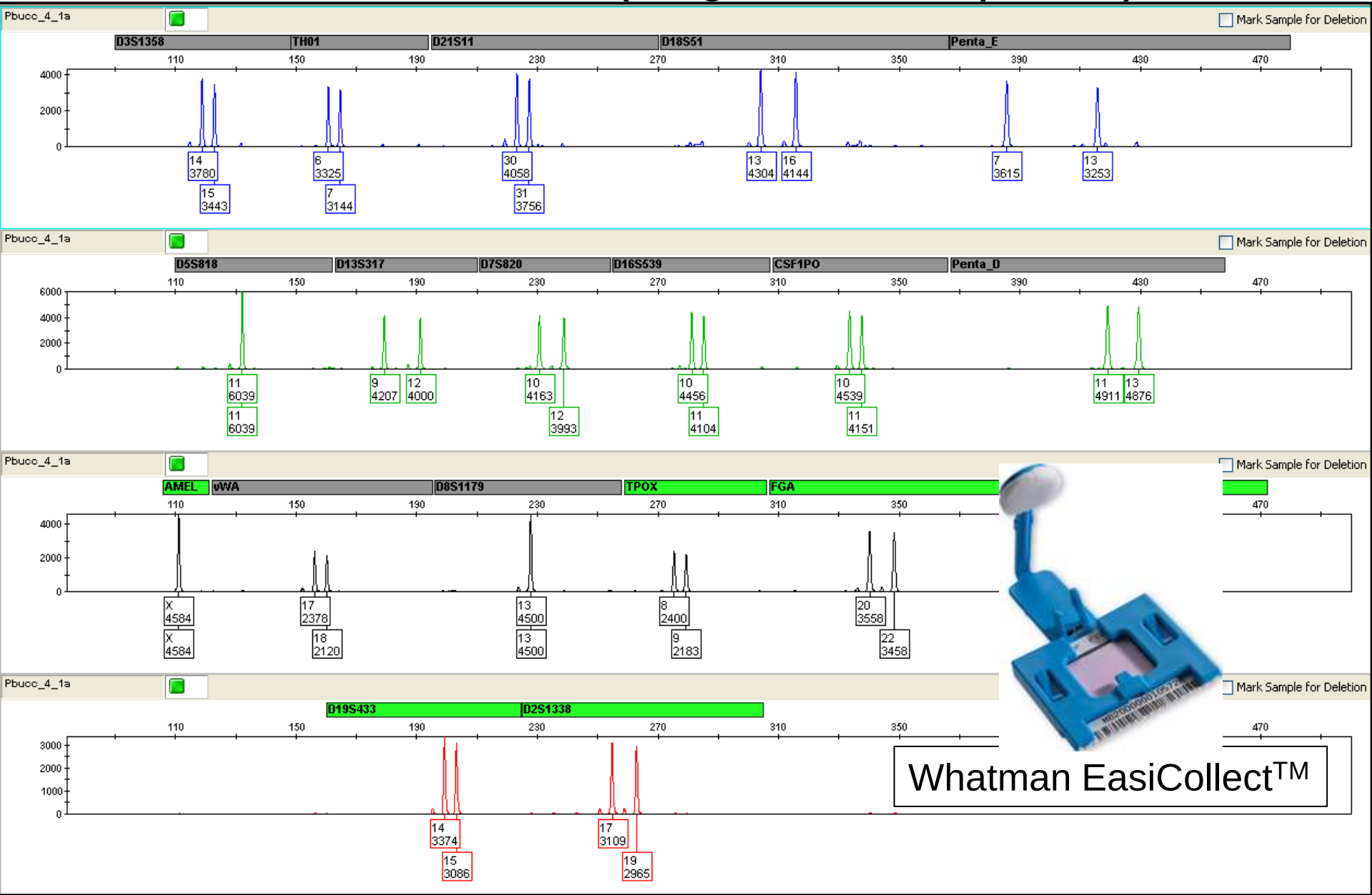
Typically not an issue with 1 ng of template DNA from a single source sample

Average Signal Intensity



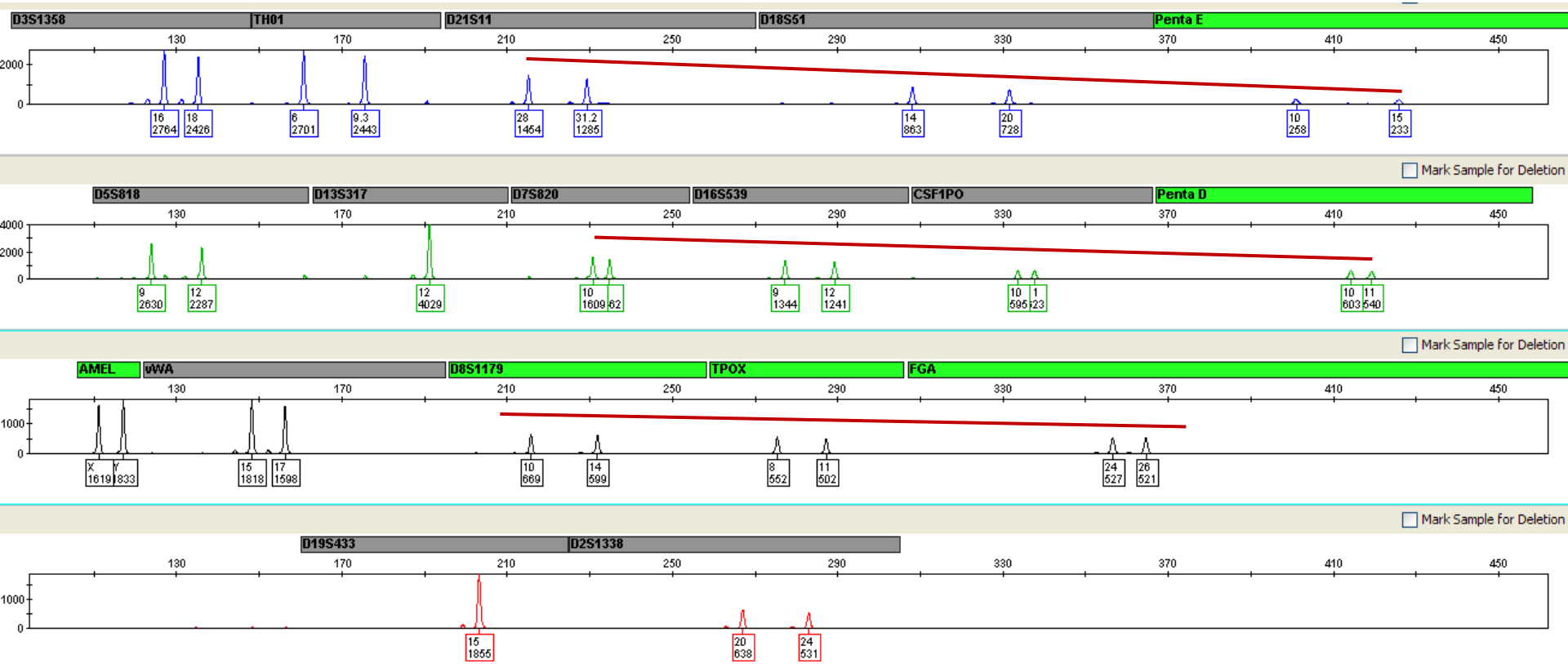
- ~27% increase in signal intensity when typing from a FTA blood punch compared to a 903 blood punch
- Reason: Cell lyse on contact with FTA paper
- A greater amount of ‘free’ DNA is available from FTA paper versus 903

Buccal cells transferred onto FTA paper
PowerPlex 18D (single 1.2 mm punch)



Whatman EasiCollect™

25 year old Blood Punch (1986) PowerPlex 18D (903 paper)



Single 1.2 mm punch stored at room temperature

Conclusions

- PowerPlex 18D successfully amplified 50 blood punches (cycling time = 1.5 h)
- Stutter peaks and peak height ratios are comparable to non-direct PCR methods
- Signal increase from FTA versus 903 paper
  - Expected due to cell lysis on FTA paper
  - Can be addressed by increasing PCR cycles or size of punch

Conclusions

- Discordant genotype for CSF1PO
  - Mismatch tolerance with the PP18D master mix and polymerase?
  - Recover a null allele *BUT* is this at the cost of lower specificity in the PCR?
  - Need to perform a concordance check when master mixes – polymerase change.

Thanks for your attention!

Questions?

Peter.Vallone@nist.gov (1-301-975-4872)

Poster: *Direct PCR Amplification of STR Loci: Protocols and Performance Poster P-315*

Acknowledgements

Margaret Kline – Allele sequencing

Ben Krenke and Katie Oostdik (Promega)

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FBI - Evaluation of Forensic DNA Typing as a Biometric Tool

NIJ – Interagency Agreement with the Office of Law Enforcement Standards

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