Direct PCR Amplification of STR Loci: Protocols and Performance

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Outline

- Benefits of direct PCR
- Collection media: FTA and 903 paper
- Source of DNA: Blood and buccal cells
- STR Kits
  - PowerPlex 18D (PP18D)
  - AmpFℓSTR Identifiler Direct
  - PowerPlex 16 HS (PP16 HS)
  - AmpFℓSTR Identifiler Plus

Development of Direct PCR

- Clinical, agricultural, and forensic utility
- Combination of PCR enhancers and modified/mutant DNA polymerases
- 10-100 times more tolerant of inhibitors compared to wild type Taq Polymerase

Commercial developments

- 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

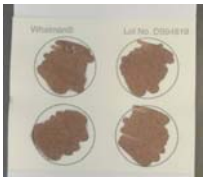
Benefits of Direct PCR

- Convenience 'punch and go'
- Unpurified DNA - skip extraction
- Save: time, cost, labor
- Amenable to automation
- Offender DNA database samples, paternity samples, casework reference samples

DNA Analysis Approaches

Steps Involved	*Traditional Protocols*	*Rapid Improvements (Direct PCR)*
Collection		
Extraction	1.5 hours (Manual)	Blood Stain
Quantitation	1.5 hours (qPCR)	
Amplification	3.5 hours	1.5 - 3 hours (Using PowerPlex 18D or Identifiler Direct for direct amplification from a 1.2mm blood punch)
Separation/ Detection	1 hour (Capillary Electrophoresis ABI 3130xl)	1 hour
Data Interpretation	Time may vary depending on software, sample quality, and analyst expertise	
Total Time	Minimum ~7.5 hours	~2 to 4 hours

FTA paper

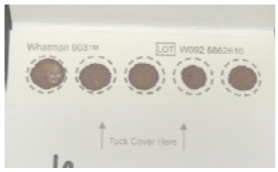


- High MW DNA becomes entangled in the fibers of the paper - DNA binds to paper
- DNA collected on FTA Cards is stable for *at least 14 years* at room temperature

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



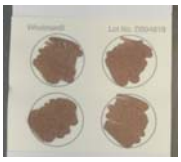
- 'Schleicher & Schuell 903'
- High-purity cotton linter pulp
- No chemical added
- Used in newborn screening programs
- Support media – DNA is not bound to the paper
- DNA collected on 903 Cards is stable for *at least 14* years at room temperature

Margaret Kline NIST Study

Sources of DNA

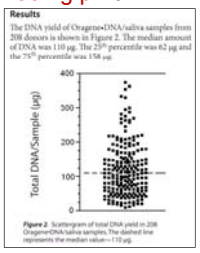
Blood

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



Buccal cells

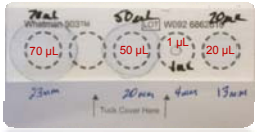
- Saliva: 100 µg/2 mL = 50 ng/µL of DNA



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

Theoretical

How much DNA is in a 1.2 mm blood punch?



- 4,500 - 10,000 white blood cells per µL
- Assume 4,500 WBC/µL
- $\pi \cdot r^2$ = area of a circle

Volume (uL)	WBC cells/uL	Total cells deposited	Diameter of spot (mm)	Total area mm^2	Cells/mm^2	Cells per 1.2 mm punch	ng of DNA
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)
High range ~10 ng – assuming 10,000 WBC per µL

Measured

How much DNA is in a 1.2 mm blood punch?

- Collect five 1.2 mm blood punches from FTA and 903 paper
- Extract DNA on the Qiagen EZ1 Advanced Robot
- Elute purified DNA in 50 µL volume
- *Quantitation performed with the Qubit® 2.0 Fluorometer (Invitrogen)*

Paper	Quant 1,2,3 (ng/uL)			Average	Total DNA recovered (ng)	ng of DNA per punch
FTA_1	0.33	0.31	0.31	0.32	16.0	3.2
FTA_2	0.25	0.24	0.23	0.24	12.0	2.4
903_1	0.28	0.28	0.28	0.28	14.0	2.8
903_2	0.42	0.43	0.41	0.42	21.0	4.2

Post solid phase extraction ~3 ng are recovered

Direct PCR Kits

GO DIRECT

Identifiler Direct

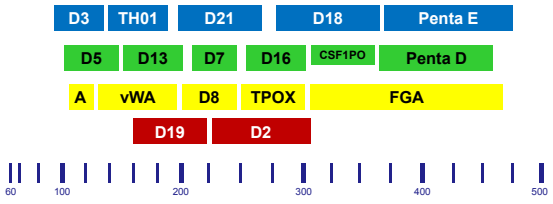
- Same primer sequences as Identifiler
- New master mix formulation and polymerase
- 1000 reactions/kit
- Released 2009

PowerPlex18D

- Same primer sequences as for the PP16 loci
- + D2S1338 and D19S433
- Specialized rapid-direct master mix formulation
- 200 or 800 reactions/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)
- Can these kits be used for direct PCR?
- Yes…
- Protocols for direct PCR in manual/website
- When typing from FTA paper: PP16 HS recommends use of Promega PowerPlex Direct Amp Reagent – used in place of water

[1]AmpFtSTR Identifiler® Plus User Guide: page 22

[2]*Wieczorek, D. and Krenke, B. Direct Amplification from Buccal and Blood Samples Preserved on Cards Using the PowerPlex® 16 HS System. 2009. http://www.promega.com/resources/articles/profiles-in-dna/2009/direct-amplification-from-buccal-and-blood-samples-preserved-on-cards-using-powerplex-16-hs/*

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

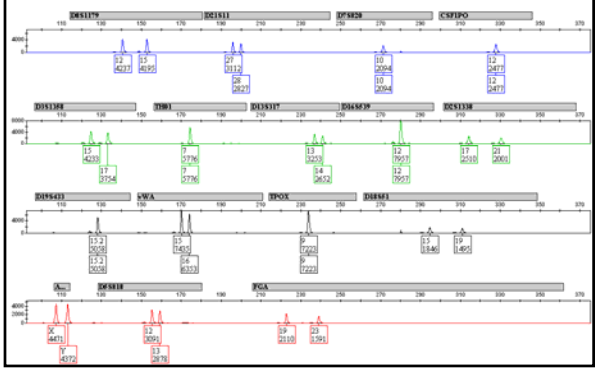
Run for the 4 STR kits

- 50 x 4 mL tubes of blood were purchased
  - 50 unique samples — Uniform comparison in terms of sample and age of blood
- Blood was spotted onto FTA and 903 collection papers
- 1.2 mm punch (single punch used for PCR)
- 25 µL PCR volume
- Cycles used in this work

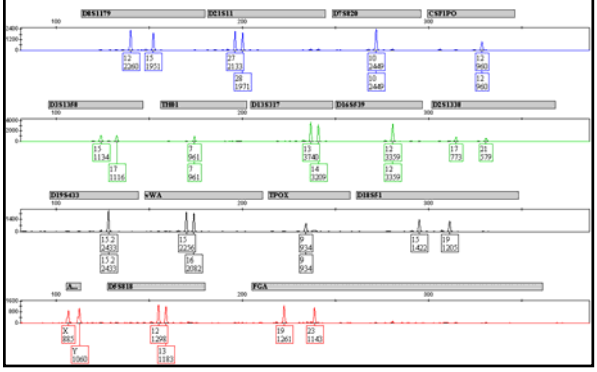
	Cycles
PP18D	28
PP16HS	30 FTA | 32 903
Identifiler Direct	26
Identifiler Plus	28

- PP16 HS + Promega Direct Amp Reagent (FTA)

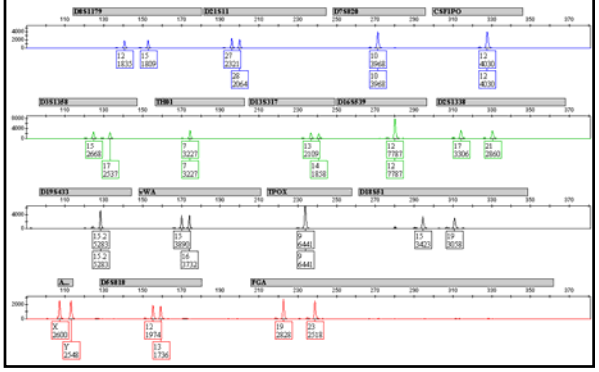
Identifiler Plus (FTA)



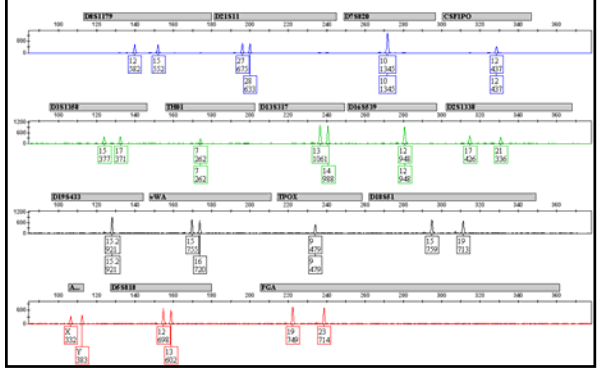
Identifiler Plus (903)



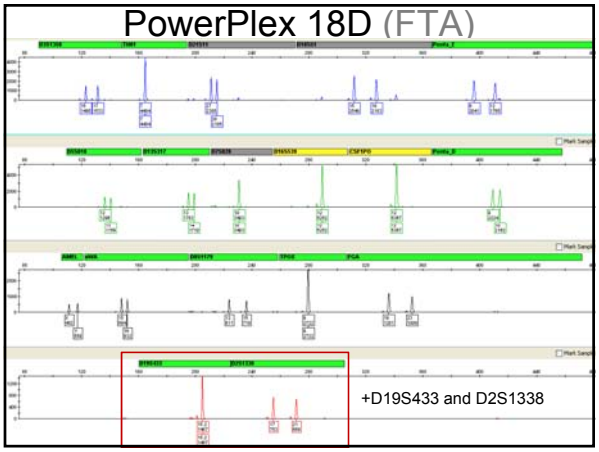
Identifiler Direct (FTA)



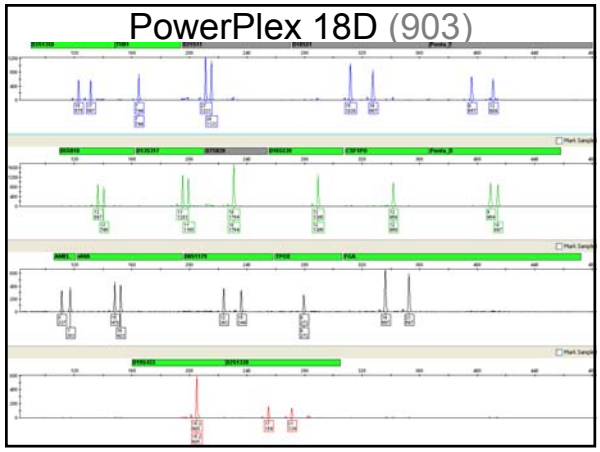
Identifiler Direct (903)



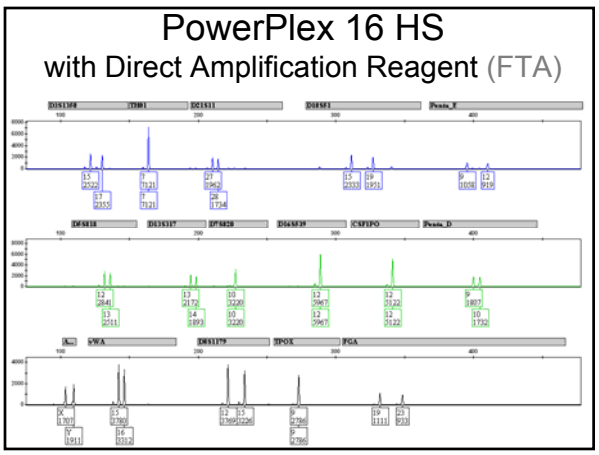
PowerPlex 18D (FTA)



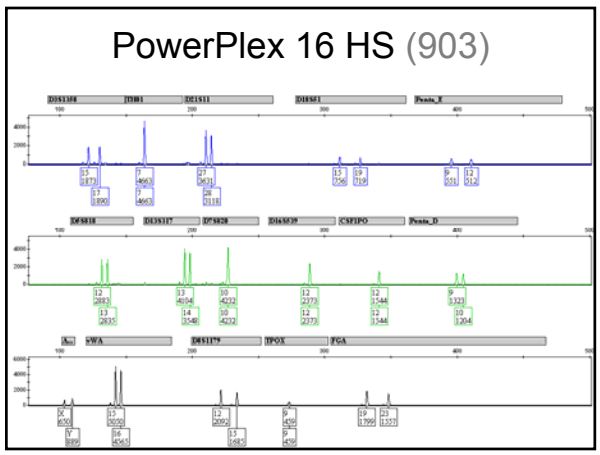
PowerPlex 18D (903)



PowerPlex 16 HS with Direct Amplification Reagent (FTA)

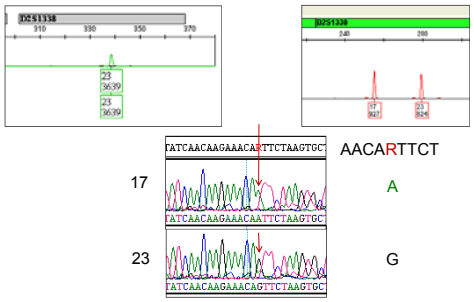


PowerPlex 16 HS (903)



Concordance - D2S1338

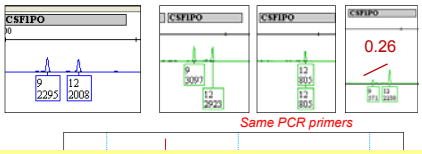
Identifiler direct (23,23) PP18D (17,23)



Allele 17: $[TGCC]_6$ $[TTCC]_{11}$ **G→A**
173 bp downstream - could affect Identifiler reverse primer binding

Concordance - CSF1PO

Identifiler PP18D PP16 PP16 HS



Same PCR primers

Questions:
What are the components of the PP18D kit that allow for the tolerance of primer binding 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'

Stutter

	Identifiler Direct		Identifiler Direct		Identifiler Plus		Identifiler Plus		PP16 HS		PP16 HS		PP18D		PP18D		Identifiler Direct		PP18D	
Locus	903	SD	FTA	SD	903	SD	FTA	SD	903	SD	FTA	SD	903	SD	FTA	SD	FTA	SD	FTA	SD
CSF1PO	5.0	1.3	4.4	1.6	4.4	1.6	4.0	2.4	8.1	6.4	8.7	4.9	9.8	7.2	8.3	6.1	4.9	1.2	5.8	3.3
D13S317																	4.9	1.5	6.1	3.7
D16S539																	5.1	1.5	7.2	4.0
D18S51																	7.4	1.8	7.2	4.3
D19S433																	6.7	1.5	6.2	3.7
D21S11																	6.5	1.3	8.4	4.3
D2S1338																	7.2	1.5	8.1	4.7
D3S1358																	7.5	1.3	8.8	4.3
D5S818																	5.8	1.4	7.4	4.3
D7S820																	4.4	1.4	5.1	3.7
D8S1179																	5.9	1.2	6.6	4.0
FGA																	6.7	1.6	7.1	4.0
Penta D									3.9	6.0	3.0	5.2	3.1	4.6	3.3	6.1			2.3	1.7
Penta E									3.7	1.8	4.1	4.2	3.3	2.1	2.6	1.5			3.8	2.7
TH01	4.9	4.0	2.2	0.8	9.7	7.3	6.3	7.0	8.8	7.5	8.4	6.7	5.2	4.4	5.5	6.1	2.2	0.9	2.3	1.3
TPOX	5.0	2.9	2.4	1.4	6.1	6.1	5.3	5.2	3.9	2.4	6.0	6.9	4.6	2.1	4.9	5.8	2.5	0.9	3.5	2.3
vWA	7.1	1.6	6.4	1.7	8.0	4.1	10.7	6.1	9.2	4.1	10.7	4.0	7.8	2.8	7.4	3.2	6.6	1.8	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

From independent validation studies

From this work n = 50 samples

Peak Height Ratios

	Identifiler Direct		Identifiler Direct		Identifiler Plus		Identifiler Plus		PP16 HS		PP16 HS		PP18D		PP18D	
Locus	903		FTA		903		FTA		903		FTA		903		FTA	
AMEL	0.89		0.96		0.86		0.95		0.76		0.92		0.93		0.89	
CSF1PO	0.94		0.92		0.93		0.89		0.89		0.88		0.94		0.94	
D13S317	0.96		0.91		0.94		0.89		0.92		0.90		0.94		0.92	
D16S539	0.95		0.91		0.94		0.89		0.90		0.89		0.93		0.94	
D18S51	0.94		0.89		0.91		0.82		0.90		0.87		0.93		0.91	
D19S433	0.92		0.92		0.91		0.85		n/a		n/a		0.95		0.91	
D21S11	0.94		0.93		0.94		0.90		0.90		0.90		0.95		0.93	
D2S1338	0.88		0.85		0.85		0.80		n/a		n/a		0.91		0.91	
D3S1358	0.93		0.92		0.92		0.88		0.94		0.90		0.94		0.94	
D5S818	0.95		0.93		0.95		0.90		0.94		0.90		0.92		0.90	
D7S820	0.95		0.90		0.93		0.89		0.86		0.89		0.95		0.94	
D8S1179	0.93		0.92		0.94		0.93		0.89		0.91		0.94		0.92	
FGA	0.94		0.90		0.93		0.84		0.87		0.87		0.94		0.93	
Penta D	n/a		n/a		n/a		n/a		0.88		0.86		0.93		0.92	
Penta E	n/a		n/a		n/a		n/a		0.84		0.83		0.93		0.89	
TH01	0.93		0.94		0.94		0.87		0.92		0.94		0.95		0.96	
TPOX	0.91		0.93		0.90		0.90		0.90		0.95		0.89		0.94	
vWA	0.95		0.91		0.94		0.91		0.92		0.90		0.89		0.87	

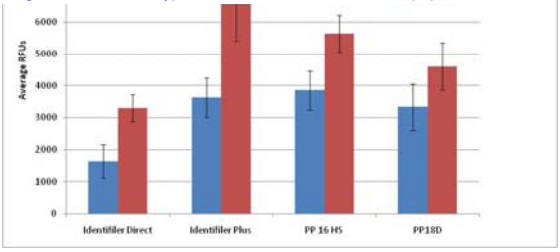
Average peak height ratios for 50 blood samples (SD 0.07)

Comparable to non-direct PCR peak heights

Average Signal Intensity

30 to 50 % increase in signal intensity when typing from a FTA blood punch compared to a 903 blood punch

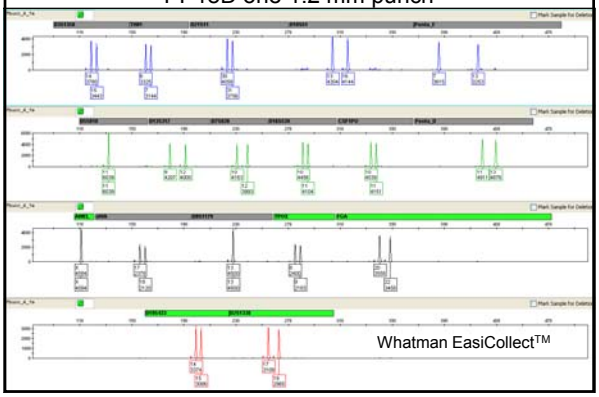
Reason: Cell lyse on contact with FTA paper
A greater amount of typable DNA is available from FTA paper versus 903



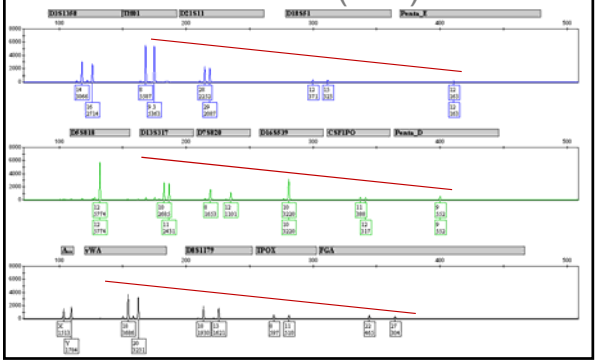
Additional Direct PCR Experiments

- Buccal sample
- Aged blood stains
- PowerPlex ESI-17
- Applied Biosystems NGM

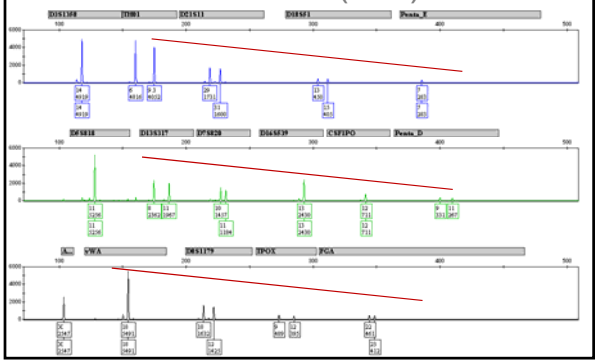
Buccal cells transferred onto FTA paper
PP18D one 1.2 mm punch



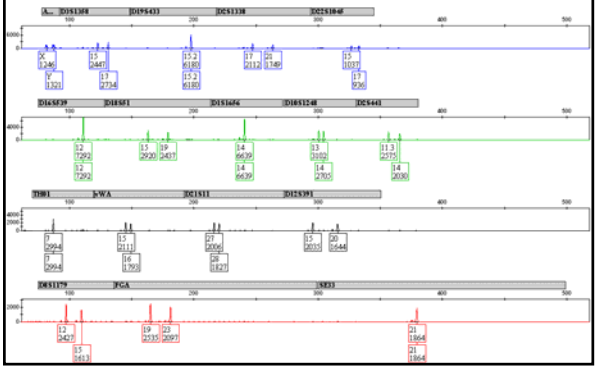
25 year old Blood punch PP16 HS (903)



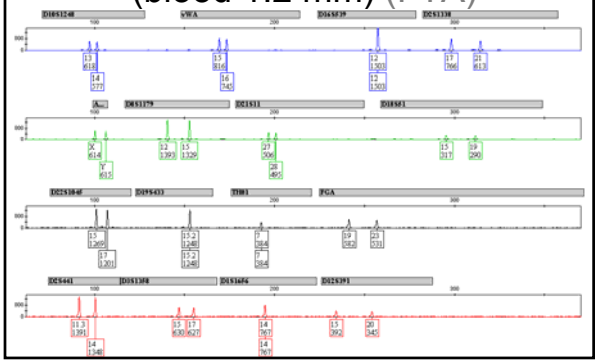
24 year old Blood punch PP16 HS (903)



PowerPlex ESI-17 (blood 1.2 mm) with Direct Amplification Reagent (FTA)



Applied Biosystems NGM (blood 1.2 mm) (FTA)



Conclusions

- All 4 STR kits successfully amplified 50 blood punches (PP18D – fastest, 1.5 h)
- Newer 'non-direct' kits can perform direct PCR effectively (PP16 HS and Identifiler Plus)
- 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

- Stutter peaks and peak height ratios are comparable to non-direct PCR methods
- Discordant genotype for CSF1PO
  - Mismatch tolerance with new master mixes 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.

Thank you for your attention!

Questions?

Peter.Vallone@nist.gov
301-975-4872

Acknowledgements
Margaret Kline – Allele sequencing
Ben Krenke and Katie Oostdik (Promega)

Outside funding agencies:
FBI - Evaluation of Forensic DNA Typing as a Biometric Tool
NIJ – Interagency Agreement with the Office of Law Enforcement Standards