Direct PCR Amplification of STR Loci: Protocols and Performance

NIST
National Institute of Standards and Technology
Technology Administration, U.S. Department of Commerce

Email:
peter.vallone@nist.gov

Peter M. Vallone, Carolyn (Becky) R. Hill, and Erica L.R. Butts
U.S. National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD 20899-8314

ISFG

P-315

The option to perform robust STR typing of a single-source reference sample while bypassing extraction and quantitation saves on time and reagent cost. Several commercial direct PCR kits and enzyme systems have been specifically developed for this purpose (e.g. Identifiler Direct and PowerPlex 18D). These new direct PCR typing kits contain PCR master mix components not typically found in traditional STR kits. The direct PCR master mixes are optimized to overcome PCR inhibitors commonly found in blood such as heme, immunoglobin G, and lactoferrin.

A cohort of 50 blood samples was spotted on FTA and 903 collection cards. An aliquot of each blood sample was also extracted and purified with a standard forensic protocol (Qiagen EZ1 Advanced platform) for non-direct genotyping and STR performance comparisons. Successful STR amplifications (full profile) were obtained from 1.2 mm punches of blood adhered onto FTA and 903 paper substrates without prior extraction. A series of pilot experiments involving the transfer of freshly collected buccal cells onto the paper substrates was also performed. STR profile characteristics such as N-4 stutter products, heterozygote peak height ratios, and genotype concordance (with non-direct PCR methods) were determined for each direct PCR system.

Development of Direct PCR Methods

- 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

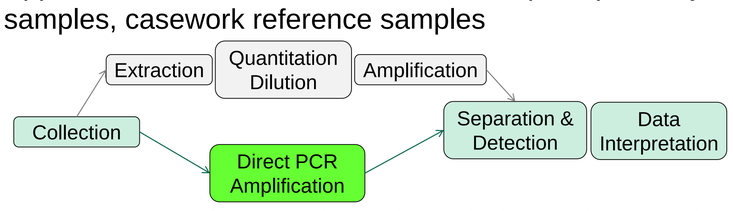
Recent commercial developments (non-STR kit)

- 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
http://www.finnzymes.com/directpcr/phusion_blood_direct_pcr_kit.html
http://www.clontech.com/US/Products/PCR_RT-PCR_Real-Time_qPCR/Direct_PCR/Terra_PCR_Direct_Polymerase_Mix

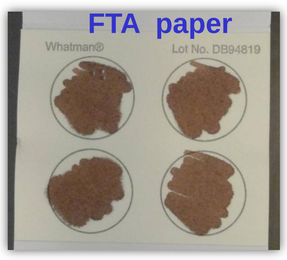
Benefits of Direct PCR Methods

- 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



For reference samples extraction and quantitation can be omitted
Total workflow time is reduced down to approximately 3-4 hours

Paper-based DNA Collection Substrates



- FTA paper
- High-purity cotton linter pulp
- Cells lyse on contact with paper
- Chemically treated with several compounds designed to kill pathogens and resist bacterial growth and DNA degradation: Tris-EDTA, sodium dodecyl sulfate, and uric acid
- High MW DNA becomes entangled in the fibers of the paper
- Blood stored on FTA cards at room temperature for *at least 14 years* has provided typeable DNA (NIST study by Margaret Kline)

- 'Schleicher & Schuell 903'
- High-purity cotton linter pulp
- No chemical added (no cell lysis)
- Used in newborn screening programs
- Support media – DNA is not bound to the paper
- Blood stored on 903 cards at room temperature for *at least 14 years* has provided typeable DNA (NIST study by Margaret Kline)

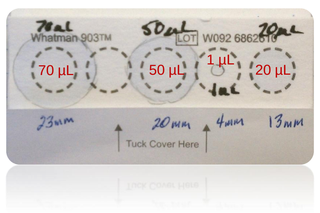
PCR Inhibitors Found in Blood and FTA Paper

- Newer PCR master mixes and DNA polymerases overcome/tolerate inhibitors present in blood and FTA paper
- FTA paper: sodium dodecyl sulfate, uric acid, EDTA
- Blood: heme, hemoglobin, lactoferrin immunoglobin G
- With optimized kits/protocols a pre-wash of FTA paper is not required

Al-Soud WA and Rådström P. Purification and characterization of PCR-inhibitory components in blood cells. J Clin Microbiol. 2001 39:485-93.
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.
*Joseph Bessetti Promega Corporation Profiles in DNA **10(1)**, 9–10 2007*

How Much DNA is in a 1.2 mm Blood Punch?

Theoretical



- 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
- Calculate the estimated amount of cells in a 1.2 mm diameter paper punch – convert to ng of DNA

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

Experimental

- 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

Poster available for download from STRBase:
http://www.cstl.nist.gov/biotech/strbase/pub_pres/ValloneISFG2011poster.pdf

Materials and Methods

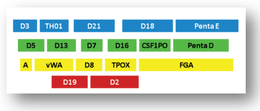
Direct PCR STR kits tested

Identifiler Direct

- Same primer sequences as Identifiler
- Enhanced master mix formulation (same polymerase as Identifiler and Identifiler Plus)
- 200 or 1000 reaction kits
- Released 2009

PowerPlex18D

- Same primer sequences as found in PowerPlex16
- Addition of D2S1338 and D19S433 loci (5-dye kit)
- Specialized rapid-direct master mix formulation
- 1.5 hour cycling time
- 200 or 800 reaction kits
- Released 2011



Identifiler Plus and PowerPlex 16 HS

- Not initially intended for direct PCR
- Contain 'enhanced' master mix components for increased inhibitor tolerance (casework)
- The protocols for direct PCR are in manual/website
- When typing PP16HS from FTA paper: Promega recommends use of *Promega PowerPlex Direct Amp Reagent* – used in place of water in the PCR master mix

AmpFℓSTR Identifiler® Plus User Guide: page 22
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/

Direct PCR Experiments Run for the 4 STR Kits

- 50 x 4 mL tubes of blood were purchased
  - 50 unique and anonymous samples – uniform comparison of samples and age of blood spots
- Blood was spotted onto FTA and 903 collection papers (25 µL)
- A single 1.2 mm punch was used for all PCR reactions
- 25 µL PCR volume (full volume)
- FTA and 903 papers were not pre-treated prior to direct amplification
- Note: Promega Direct Amp Reagent was used for PP16 HS on FTA paper experiments

In addition to the 50 blood samples: 648 extracted (non-blood) NIST population samples were typed with PP18D for genotype concordance checks

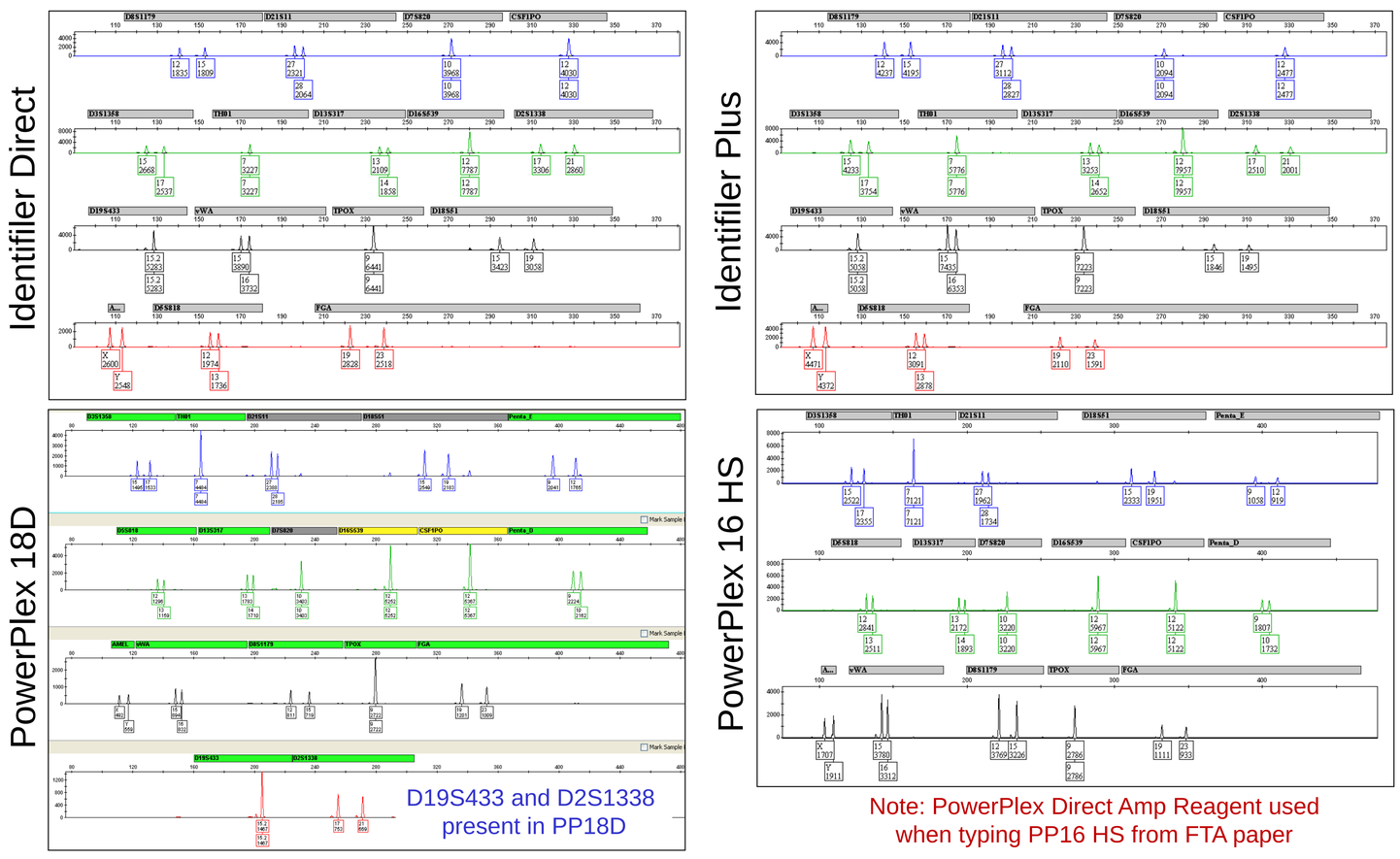
Thermal Cycling: Parameters and Times

	Hot Start	Denature	Anneal	Elongate	Cycles	Soak	Total Cycling Time
PP18D	96 (2 min)	94 (10 s)	60 (1 min)		28	60 (20 min)	1:26
PP16HS	96 (2 min)	94 (30 s)	60 (30 s)	70 (45 s)	30/32	60 (30 min)	2:40 | 2:49
Identifiler Direct	95 (11 min)	94 (20 s)	59 (120 s)	72 (60 s)	26	60 (25 min)	2:32
Identifiler Plus	95 (11 min)	94 (20 s)	59 (3 min)		28	60 (10 min)	2:26

Thermal cycling was carried out on a Applied Biosystems GeneAmp 9700 (running in 9600 emulation mode).

Fragment separation and detection was performed on an Applied Biosystems 3130xL Genetic Analyzer (36 cm array, POP4). Injection parameters: 3 kV for 5s. Data was analyzed in either GeneMapper ID v3.2, GeneMapper IDX v1.1, or GeneMapper IDX v1.2.

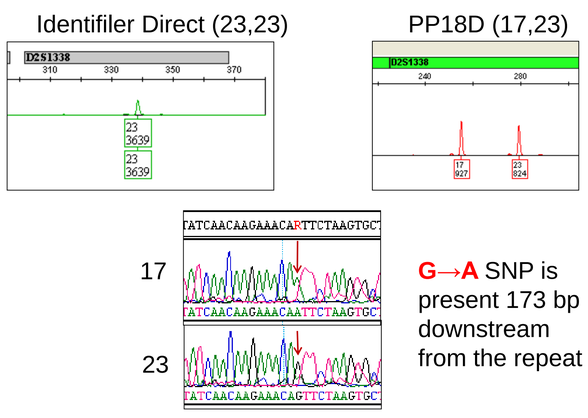
Below: example STR profiles from a 1.2 mm FTA blood punch for each kit (similar results obtained with 903 paper)
100% typing success (full profile) was obtained for the 50 blood punches on both FTA and 903 papers



N-4 stutter peaks and heterozygote peak height ratios were comparable to non-direct PCR methods. Peak height ratios for all loci were greater than 0.80 (± 0.07). Increased signal from FTA versus 903 paper was observed (30 - 50% higher). The signal increase could be expected due to more efficient cell lysis on FTA paper and did not affect data interpretation or genotyping success. Signal could be improved by increasing PCR cycles, number of punches added to the PCR, or the size of punch. However, adding more than 2 (1.2 mm) blood punches to a PCR is not recommended due to the inhibitory effect of the FTA paper and/or blood components.

Two of the 698 Samples (0.29%) Exhibited Discordant Genotypes
Allele Sequencing was Performed by Margaret Kline at NIST

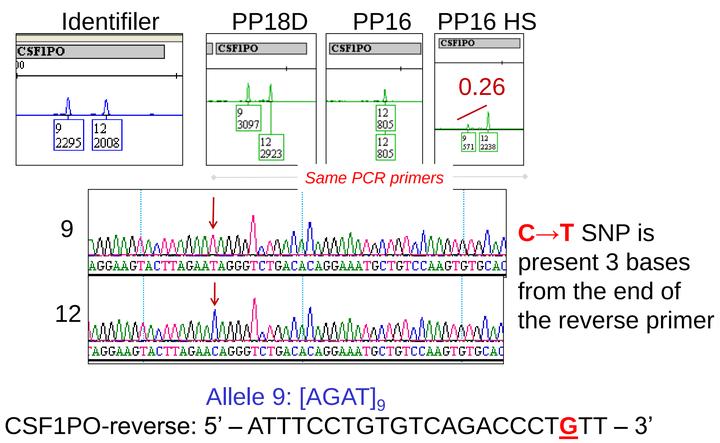
Concordance - D2S1338



Allele 17: $[TGCC]_6$ $[TTCC]_{11}$

The **G→A** SNP 173 bp downstream *could* affect the D2S1338 reverse primer binding in the Identifiler Direct kit resulting in the allelic drop out (kit PCR primers unpublished).

Concordance - CSF1PO



Allele 9: $[AGAT]_9$

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

A **C→T** SNP at the end of the CSF1PO reverse primer results in drop out of the 9 allele in PP16 and low signal (het imbalance) in PP16 HS. The PP18D primers are the same as in PP16 and PP16 HS. There may be a higher degree of mismatch tolerance with new master mixes and DNA polymerase contained in PP18D.

Conclusions

- All 4 STR kits successfully amplified 50, 1.2 mm blood punches from FTA and 903 paper substrates (full profiles): PowerPlex 18D had the fastest thermal cycling time (~1.5 h)
- Newer 'non-direct' kits can perform direct PCR effectively: PP16 HS and Identifiler Plus
- Stutter peaks and peak height ratios were comparable to non-direct PCR methods
- A trend of increased signal from FTA versus 903 paper was observed
  - This would be expected due to cell lysis on FTA paper and did not affect data interpretation
- Discordant genotype for CSF1PO (between PP16, PP16 HS and PP18D)
  - These kits contain identical PCR primers for CSF1PO
  - There seems to be a degree of mismatch tolerance with the PP18D master mix and polymerase
  - This allowed the recovery a null allele, *BUT* is this at the cost of lower specificity in the PCR?
  - May need to perform a concordance check when master mixes and/or polymerases change

Acknowledgements NIST: David Duewer and Margaret Kline. Promega: Benjamin Krenke and Kathryn Oostdik.
Funding This was supported by an interagency agreement between NIST and the FBI Biometrics Center of Excellence 'Forensic DNA Typing as a Biometric Tool'