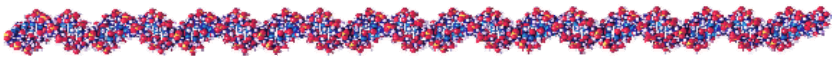
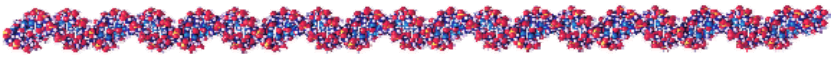


Development of Rapid Multiplex PCR Amplification Techniques



Dr. Peter M. Vallone
National Institute of Standards and Technology
Biochemical Science Division
Applied Genetics Group - Human Identification Project

Lockheed Martin BEACON Lecture Series
January 7th, 2009

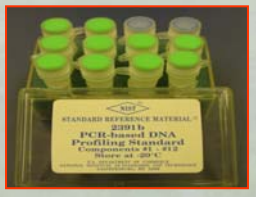
Outline

- NIST
- Forensic DNA Testing
- PCR
- Rapid PCR
  1. miniSTR 3plex
  2. Commercial Kits
  3. Larger Custom Multiplexes
  4. Alternative Thermal Cyclers

NIST History and Mission

- National Institute of Standards and Technology (NIST) was created in 1901 as the National Bureau of Standards (NBS). The name was changed to NIST in 1988.
- NIST is **part of the U.S. Department of Commerce** with a mission to develop and promote measurement, standards, and technology to enhance productivity, facilitate trade, and improve the quality of life.
- NIST supplies over 1,300 Standard Reference Materials (SRMs) for industry, academia, and government **use in calibration of measurements**.
- NIST defines time for the U.S.

$532 for 3 jars



DNA typing standard

NIST Human Identity Project Team


John Butler
(Project Leader)


Margaret Kline


Pete Vallone


Dave Duewer
Anal. Chem. Division


Jan Redman


Amy Decker


Becky Hill

Funding: **Interagency Agreement 2003-IJ-R-029 between National Institute of Justice (NIJ) and NIST Office of Law Enforcement Standards (OLES)**

National Institute of Justice
The Research, Development, and Evaluation Agency of the U.S. Department of Justice

Current Areas of Effort with Forensic DNA

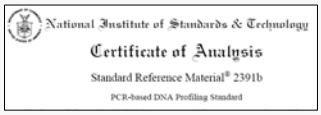
- **Standards**
  - Standard Reference Materials
  - Standard Information Resources (STRBase website)
  - Interlaboratory Studies
- **Technology**
  - Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR, **rapid PCR**
  - Assay and software development, expert system review
- **Training Materials**
  - Review articles and workshops on STRs, CE, validation
  - PowerPoint and pdf files available for download

http://www.cstl.nist.gov/biotech/strbase/NIJprojects.htm

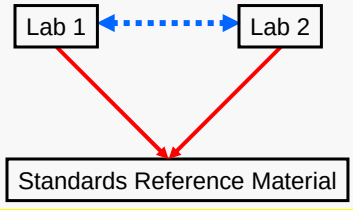
Standard Reference Materials (SRMs)

http://www.nist.gov/srm

Traceable standards to ensure accurate and comparable measurements between laboratories


National Institute of Standards & Technology
Certificate of Analysis
Standard Reference Material® 2391b
PCR-based DNA Profiling Standard

SRM 2391b – autosomal STRs
SRM 2392 &-I – mtDNA sequencing
SRM 2395 – Y-STRs
SRM 2372 – DNA quantitation
SRM 2394 – mtDNA heteroplasmy



Calibration with SRMs enables confidence in comparisons of results between laboratories

Helps meet ISO 17025 needs
for traceability to a national metrology institute

Support to the Forensic Community

…Bringing traceability and technology to the scales of justice…

- Perform beta-testing of new human identity testing products
- Provide input to
  - Scientific Working Group on DNA Analysis Methods (**SWGDAM**)
  - Department of Defense Quality Assurance Oversight Committee for DNA Analysis
  - American Prosecutor's Research Institute (**APRI**) DNA Forensics Program "Course-in-a-Box" for training lawyers
  - WTC Kinship and Data Analysis Panel (**KADAP**)
  - 2005 Hurricane Victim DNA Identification Expert Group (**HVDIEG**)
  - NIJ Expert System Testbed (**NEST**) Project

Forensic Applications of DNA Typing

- Forensic cases: matching suspect with evidence
- Paternity testing: identifying father
- Missing persons investigations
- Military DNA "dog tag"
- Convicted felon DNA databases
- Mass disasters: putting pieces back together
- Historical investigations
- Genetic genealogy

>3 million tests performed per year

Forensic DNA Testing

Probe subsets of genetic variation in order to differentiate between individuals

DNA typing must be done efficiently and reproducibly (information must hold up in court)

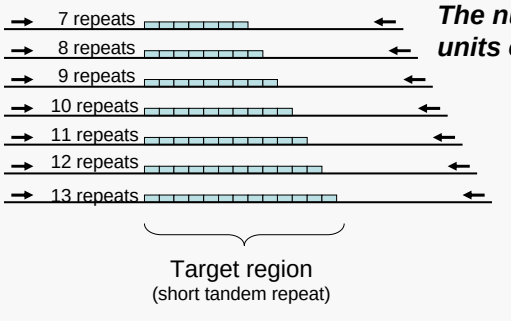
Typically, we are not looking at genes – little/no information about race, predisposal to disease, or phenotypical information (eye color, height, hair color) is obtained

Short Tandem Repeat (STR) Markers

An accordion-like DNA sequence that occurs between genes

TCCCAAGCTCTTCCTCTTCCCTAGATCAATACAGACAGAAGACA
GGTG**GATAGATAGATAGATAGATAGATAGATAGATAGATAGA**
TAGATAGATATCATTGAAAGACAAAACAGAGATGGATGATAGAT
ACATGCTTACAGATGCACAC

= 12 GATA repeats ("12" is reported)



The number of consecutive repeat units can vary between people

The FBI has selected **13 core STR loci** that must be run in all DNA tests in order to provide a common currency for DNA profiles

DNA Testing Requires a Reference Sample

A DNA profile by itself is fairly useless because it has no context…

DNA analysis for identity only works by comparison – **you need a reference sample**

Crime Scene Evidence	compared to	**Suspect(s)**
Child	compared to	**Alleged Father**
Victim's Remains	compared to	**Biological Relative**
Soldier's Remains	compared to	**Direct Reference Sample**

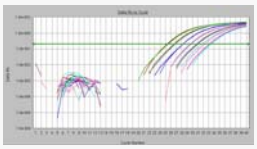
Steps in Forensic DNA Analysis

Usually 1-2 day process (a minimum of ~8 hours)

Blood Stain Buccal swab
Sample Collection & Storage

Biology

DNA Extraction

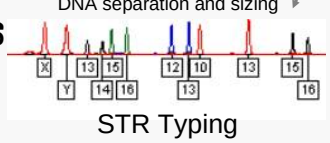


DNA Quantitation

~3 hours

Multiplex PCR Amplification

DNA separation and sizing



STR Typing
Interpretation of Results

Technology

Genetics

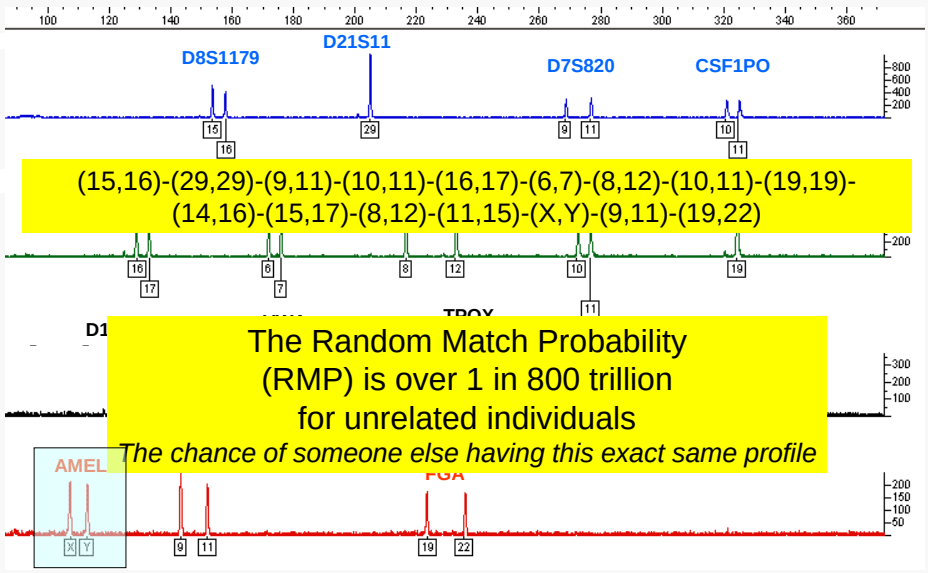
Statistics Calculated

DNA Database search
Paternity test
Reference sample

Applied Use of Information

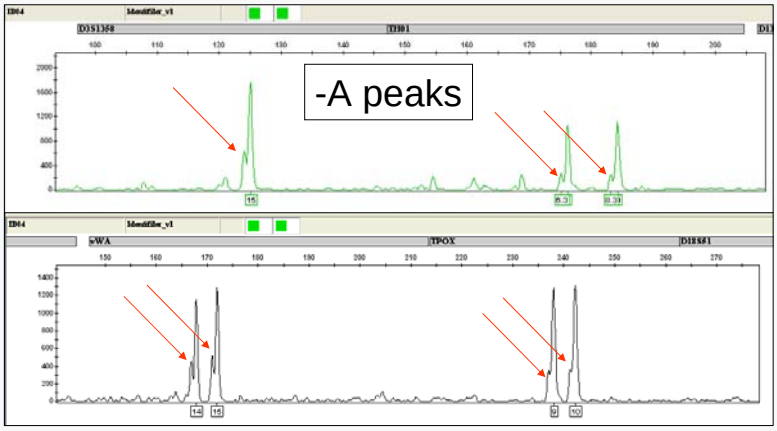
Identifiler STR Kit (Applied Biosystems)

Information is tied together with multiplex PCR and data analysis



Adenylation Efficiency or Non-Template Addition

Taq polymerase adds an extra base (dA) to the end of each PCR product
A 'soak' at 60°C for extended periods promotes full adenylation



PCR

- Polymerase Chain Reaction
- In vitro enzymatic replication
- Saiki et al., (1985) *Science* 20: 1350-1354
- Targets a specific region of a genome
- 2^N amplification (N = number of cycles)
- 50 – 10,000 base pair fragments
- Products can be used for downstream applications

A means to create billions of exact copies of a specific region of the genome

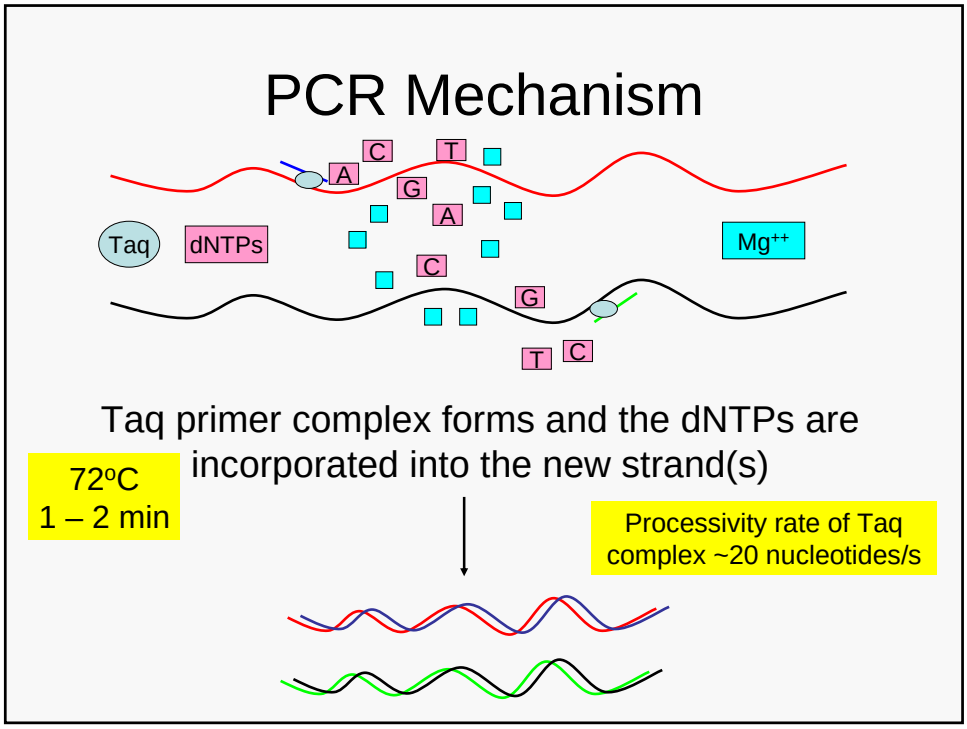
PCR Mechanism

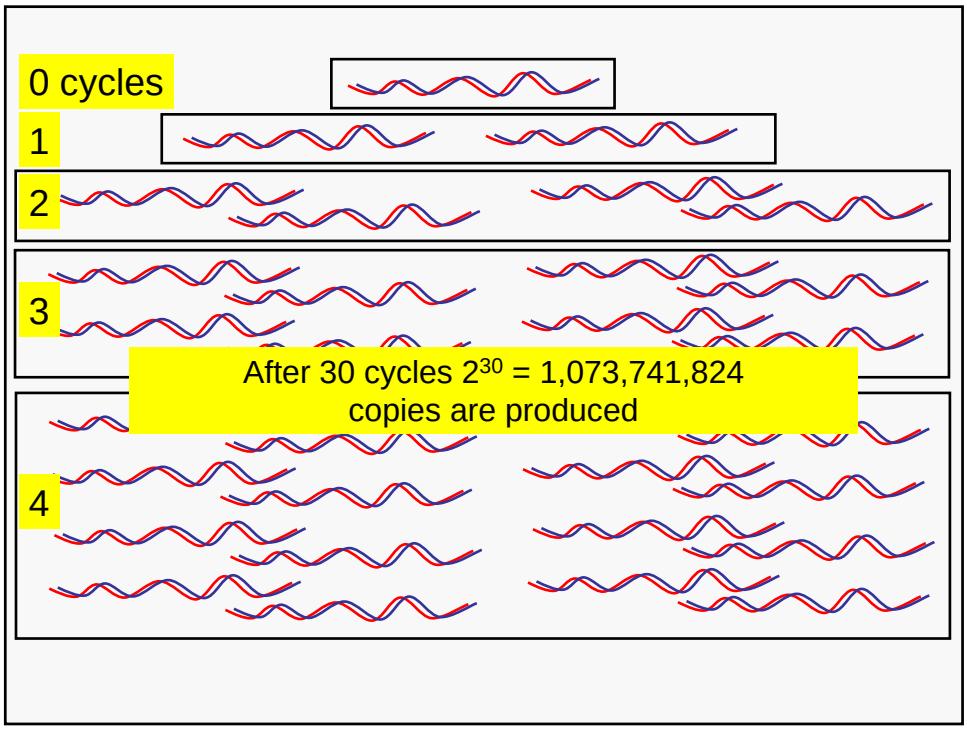
95°C
30 – 60s

Denature the genomic DNA template

~60°C
1 – 2 min

PCR primers bind to the DNA template; defines the product size


PCR Mechanism
C
T
A
G
A
Taq
dNTPs
Mg++
C
G
T
C
Taq primer complex forms and the dNTPs are incorporated into the new strand(s)
72°C
1 – 2 min
Processivity rate of Taq complex ~20 nucleotides/s


0 cycles
1
2
3
After 30 cycles 2^30 = 1,073,741,824 copies are produced
4

PCR Applications

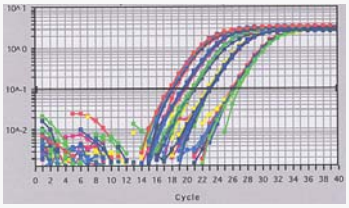
- Microbiology and Molecular Biology
  - DNA cloning, Southern blotting, DNA sequencing, Next-gen sequencing, DNA methylation assays
- Genotyping
  - forensics, pathogen detection, clinical and diagnostic applications, disease association studies, pharmacogenetics
- Real-time PCR
  - RT-PCR (reverse transcriptase) gene expression
  - Quantitation (qPCR), Genetically mod organisms

Advantages of Multiplex PCR?

- Beneficial with limited sample
  - 500 to 1000 pg of DNA
  - ~83 to 166 copies of the human genome
- Obtain more information per unit time
- Save on reagents; enzyme, buffers, labor
- Streamlines data analysis
- For forensic markers it is essential
- Coincides with high capacity instrumentation

Things That Are Rapid

3h PCR?

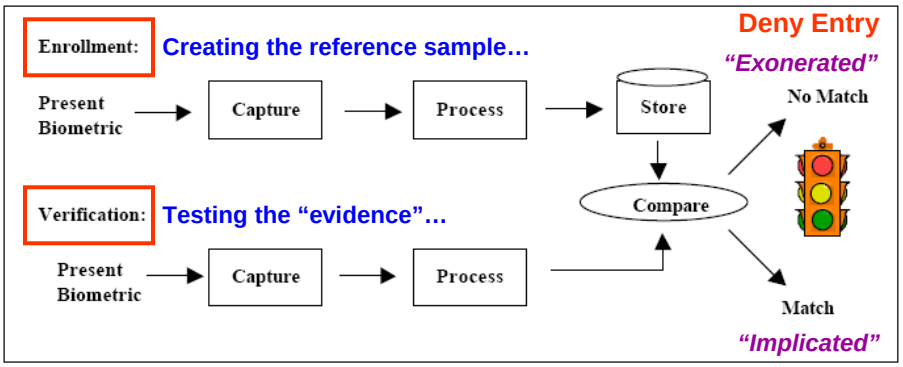


Why go Faster?
Applications for Rapid PCR

- Integrated devices ('Lab on a Chip')
- Screening at a point of interest (airport, border, crime scene, intelligence community)
- Rapid STR typing 'in the field'
  - Potential for situations/cases when a quick result is needed
  - Provide initial screening information
- Decrease overall time required for STR typing

Growing Interest in DNA for Biometrics

DNA within the Biometric Model



http://www.itl.nist.gov/div893/biometrics/Biometricsfromthemovies.pdf

Match of 13 points (each with 2 variable alleles) within DNA

String of 26 numbers (order of listing DNA results would have to be standardized)
16,17-17,18-21,22-12,14-28,30-14,16-12,13-11,14-9,9-11,13-6,6-8,8-10,10

Efforts towards Portable/Mobile DNA Devices

- NEC (Japan)
  - Poster at Promega meeting in Hollywood, CA (Oct 1-4, 2007)
  - Press release on October 15, 2007 (http://www.nec.co.jp/press/en/0710/1501.html)
- Mathies group at UC-Berkeley and Microchip Biotech
  - Publications… in *Analytical Chemistry*, *FSI Genetics*, etc.
  - http://www.microchipbiotech.com
- Landers group at UVA and MicroLab Diagnostics
  - Publications… *Proc Natl Acad Sci USA* 2006; 103:19272-19277
  - http://www.microlabdiagnostics.com
- Network Biosystems (based on Dan Ehrlich's work at Whitehead)
  - http://www.netbio.com
- NIST Michael Gaitan
  - http://www.eeel.nist.gov/812/mg.html

Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics xxx (2008) xxx–xxx
ELSEVIER
FSI GENETICS
www.elsevier.com/locate/fsig

Real-time forensic DNA analysis at a crime scene using a portable microchip analyzer

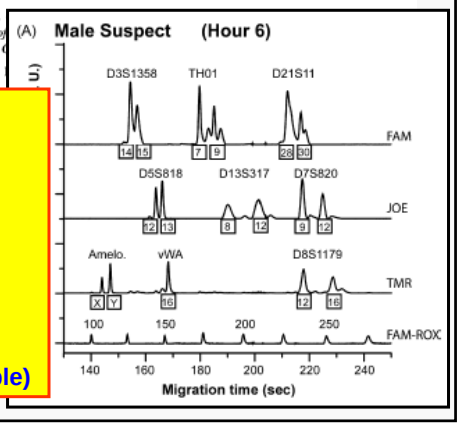
Peng Liu [a], Stephanie H.I. Yeung [a], Karin A. Crenshaw [c], Cecelia A. Crouse [c], James R. Scherer [b], Richard A. Mathies [a,b,*]

[a] UCSF/UC Berkeley Joint Graduate Group in Bioengineering,
[b] Department of Chemistry, MS 1460, University of
[c] Palm Beach County Sheriff's Office Crime Laboratory, 3228

Received 6 February 2008; received in revised form

Field Trial Results
7:00 a.m. Arrived and set up mock crime scene
7:10-7:30 a.m. Samples collected by CSI
7:30-9:30 a.m. DNA extraction
9:30-10:00 a.m. PCR set-up
10 a.m. – 12 p.m. PCR performed (2 h)
12 – 12:30 p.m. DNA separation
12:30-12:50 p.m. CODIS search of local database

6 hours from sample collection to the generation of the CODIS hit (for one sample)



Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics xxx (2008) xxx–xxx
ELSEVIER
FSI GENETICS
www.elsevier.com/locate/fsig

Real-time forensic DNA analysis at a crime scene using a portable microchip analyzer

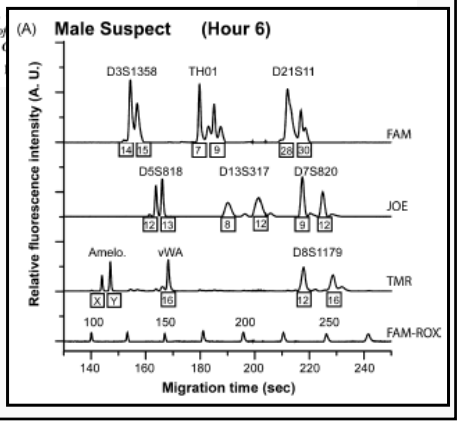
Peng Liu [a], Stephanie H.I. Yeung [a], Karin A. Crenshaw [c], Cecelia A. Crouse [c], James R. Scherer [b], Richard A. Mathies [a,b,*]

[a] UCSF/UC Berkeley Joint Graduate Group in Bioengineering,
[b] Department of Chemistry, MS 1460, University of
[c] Palm Beach County Sheriff's Office Crime Laboratory, 3228

Received 6 February 2008; received in revised form

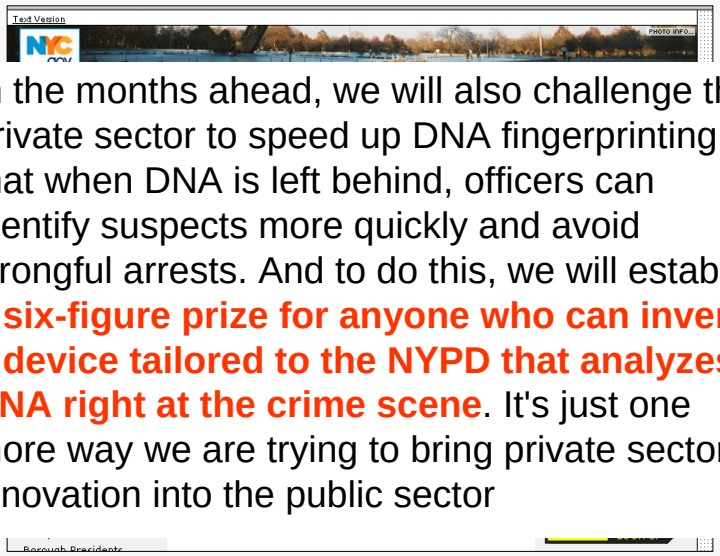
- 9plex PCR (10 μL)
- Total cycling time ~2 hours
- Using FastStart polymerase (Roche)

- Inter-locus peak height balance?
- Adenylation?



01-17-2008

NYC Prize

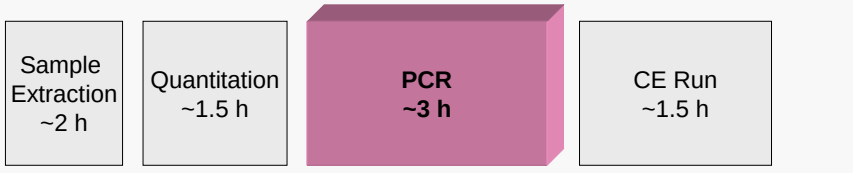


- In the months ahead, we will also challenge the private sector to speed up DNA fingerprinting so that when DNA is left behind, officers can identify suspects more quickly and avoid wrongful arrests. And to do this, we will establish **a six-figure prize for anyone who can invent a device tailored to the NYPD that analyzes DNA right at the crime scene**. It's just one more way we are trying to bring private sector innovation into the public sector

http://nyc.gov/portal/site/nycgov/?front_door=true

Typical STR Typing Workflow

Can the time required for PCR thermal cycling be reduced?



Alter thermal cycling parameters
Evaluate faster polymerases
Evaluate faster thermal cyclers
Test commercial STR typing kits

Data Review ?

Goal: cycling in less than 45 minutes
Trying simple things first…

Thermal Cyclers

AB 9700
96 well block
0.2 μL tubes

Ramp rate = **4°C/sec**

http://www.appliedbiosystems.com

RapidCycler 2 Instrument
http://www.idahotech.com
Ramp rate = **10°C/sec**

AB 9800

http://www.appliedbiosystems.com

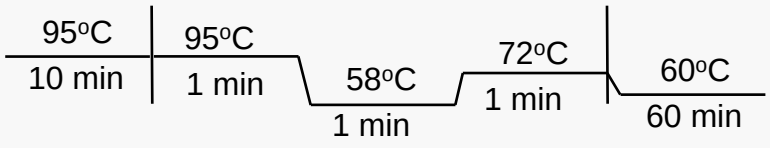
Eppendorf
Mastercycler ep
6°C/sec

http://www.eppendorfna.com

PCR Thermal Cycling Profile (Traditional)

28 - 32 cycles of PCR

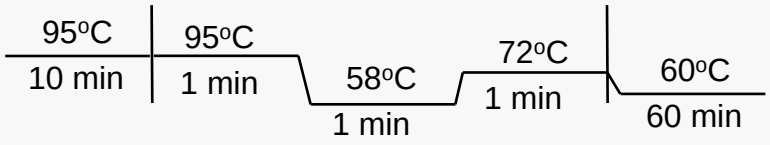
~3 hour run time

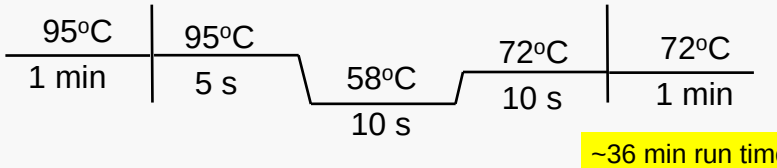


Heating and cooling of reaction takes place in a thermal block (peltier heating/cooling)
Heating/Cooling rate of ~1°C/s

PCR Thermal Cycling Profile

28-32 cycles of PCR





~36 min run time

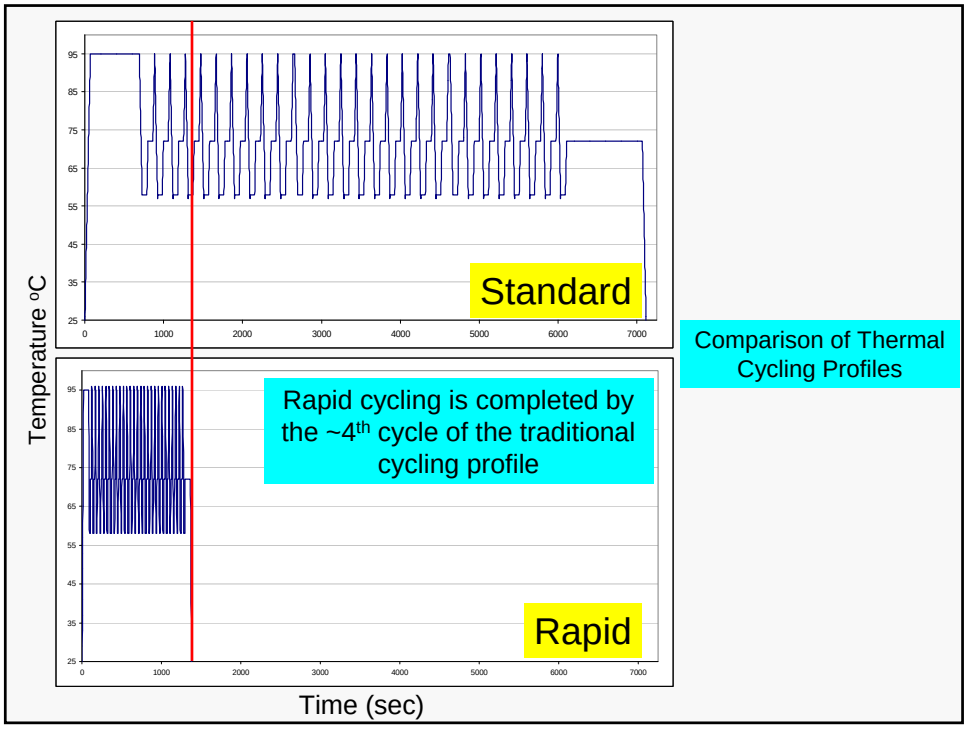
Heating/Cooling rate of ~4°C/s

Thermal Cycling

Parameter	Unit	Trad	Rapid	Difference (min)	%
Hot Start	Min	10	1	9.0	6.3
Hold	Sec	60	5/10	72.3	50.6
Soak	Min	60	1	59.0	41.2
Ramp rate	(deg/sec)	1	4	22.4	15.7
Cycles		28	28		
Time		2:58:41	0:35:38	2:23:03	

Parameter	Purpose
Hot Start	Primer Dimer, non-specific amplification
Hold	Amplification of template - Inter and intra locus balance
Soak	Complete adenylation of PCR products

Evaluate robustness and reproducibility
(electropherograms)



Initial Work/Assumptions

- Using common materials/conditions
  - AB 9700 (10 μL volume)
  - Standard plastics
  - Commercial Polymerases
  - Final primer concentration ~0.2 μM
  - ~250 μM dNTPs, 2 mM Mg^{++}
  - 4 + 1 color dye chemistry for labeling primers
  - Separation on AB 3130 (Capillary Electrophoresis)
  - Not sample limited (>500 pg of DNA)

Loci for Testing

- STR Loci present available in commercial kits (CODIS)
- 26 autosomal loci characterized in our laboratory
  - Small 3plex panels
  - Larger 26plex *Hill, C.R., Butler, J.M., Vallone, P.M. A New 26plex Autosomal STR Assay to Aid Human Identity Testing (in press JFS)*

- Existing commercial STR typing kits are not optimized for rapid PCR
- Challenge for miniaturized/integrated STR typing platforms – since they have to use a commercial kits or develop their own…

Hill, C.R., Coble, M.D., Butler, J.M. (2008) Characterization of 26 miniSTR loci for improved analysis of degraded DNA samples. J. Forensic Sci. 53(1):73-80.
Coble, M.D. and Butler, J.M. (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. J. Forensic Sci. 50: 43-53.

DNA Polymerases for Evaluation

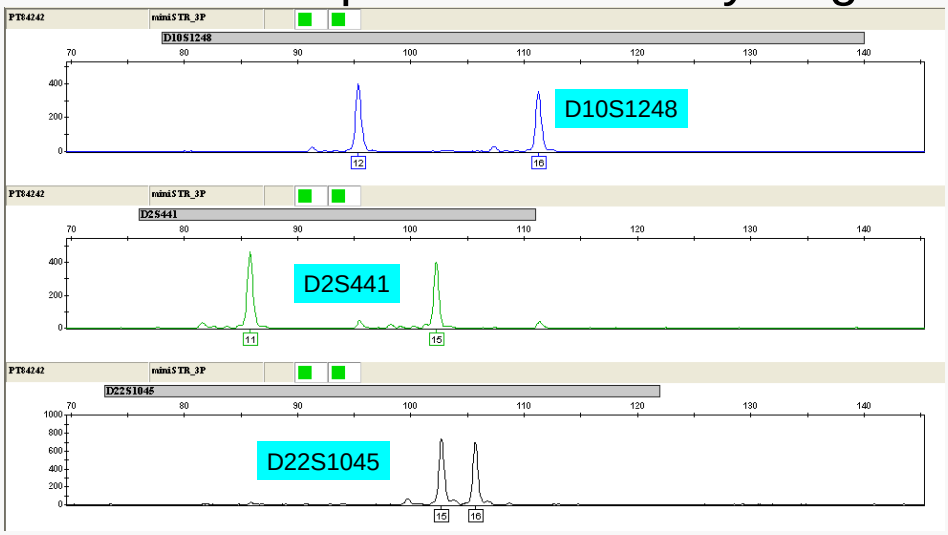
Polymerase	Vendor	MasterMix	Hot Start
TaqGold	Applied Biosystems	no	10 min
GeneAmp	Applied Biosystems	yes (2x)	1 min
SpeedSTAR	Takara	no	1 min
PyroStart	Fermentas	yes (2x)	1 min
Qiagen Fast Cycling PCR Kit	Qiagen	yes (2x)	5 min

Brief survey of 'fast' commercial polymerases

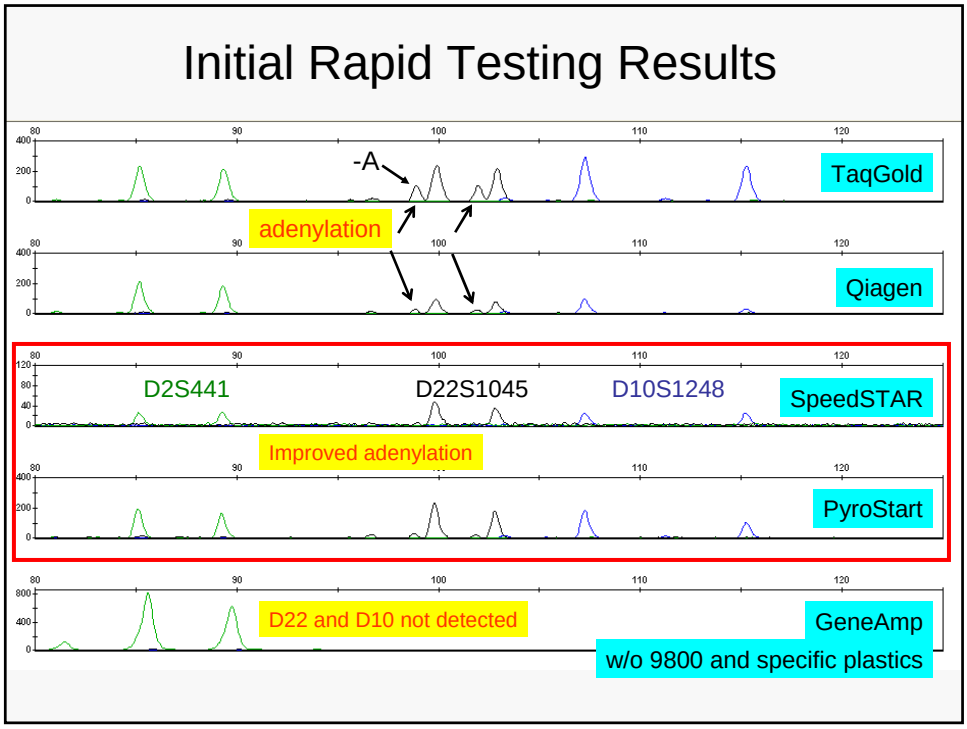
Initial Testing with miniSTR 3plex

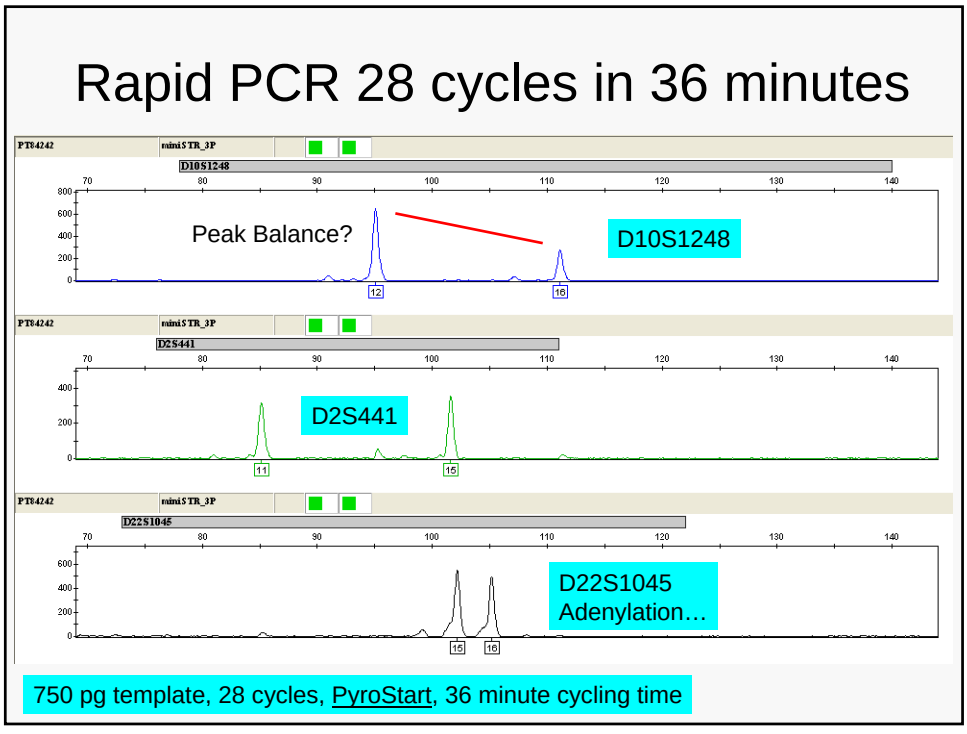
- 3 loci labeled with 3 fluorescent dyes (FAM, VIC, NED)
- MiniSTR loci (Amplicon size range 65-140 bp)
  - D2S441, D10S1248 and D22S1045
  - 'European loci' (contained in the next generation of forensic kits)
- These loci were previously tested under standard cycling conditions in a miniSTR multiplex in our lab

miniSTR 3plex Standard Cycling



750 pg template, 28 cycles, TaqGold, ~3 hour cycling time


Initial Rapid Testing Results
-A
adenylation
TaqGold
Qiagen
D2S441
D22S1045
D10S1248
SpeedSTAR
Improved adenylation
PyroStart
D22 and D10 not detected
GeneAmp
w/o 9800 and specific plastics

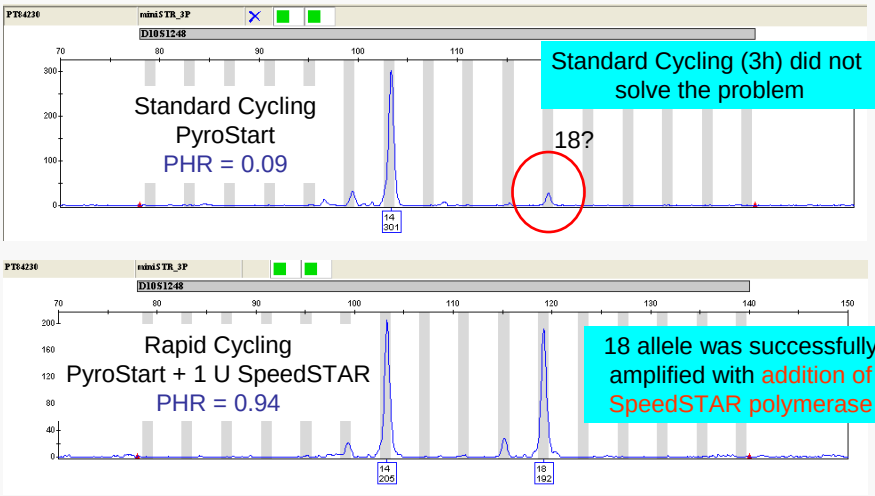

Rapid PCR 28 cycles in 36 minutes
Peak Balance?
D10S1248
D2S441
D22S1045
Adenylation…
750 pg template, 28 cycles, PyroStart, 36 minute cycling time

miniSTR 3plex Concordance

- 3plex run on a plate of samples (n = 95)
- Concordance was checked with genotypes obtained with Standard Cycling and TaqGold
- 2/285 (0.7%) of the genotype calls were discordant
- Both cases due to D10S1248 heterozygote peak height imbalance

Peak Height Imbalance for D10S1248

Peak Height Imbalance for D10S1248



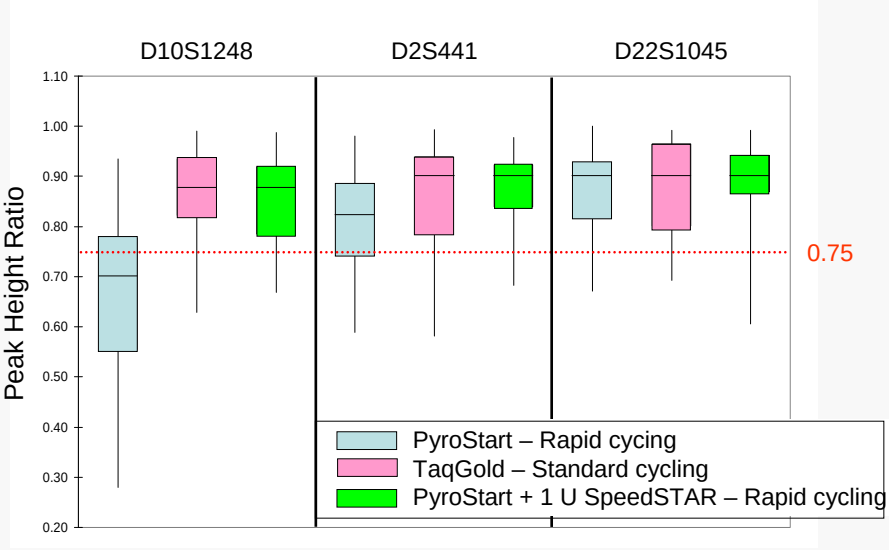
Peak Height Ratios for 16 Samples

Cycling Sample Name	Normal TaqGold	Rapid Pyro	Rapid Pyro+SS	Genotype
MT94859	0.70		0.67	**14,19**
PT84230	0.68		0.94	**14,18**
PT84243	0.63	0.28	0.73	14,17
OT05890	0.66	0.30	0.69	14,17
WT51354	0.67	0.33	0.97	14,17
UT57303	0.70	0.37	0.79	13,16
MT97172	0.70	0.37	0.87	13,16
WT51342	0.71	0.40	0.99	13,16
WT51355	0.73	0.41	0.88	13,16
ZT80865	0.74	0.41	0.91	13,16
UT57310	0.75	0.42	0.88	14,16
PT84242	0.78	0.42	0.95	12,16
PT84241	0.78	0.46	0.77	13,16
GT37862	0.78	0.47	0.87	13,16
WT51362	0.78	0.50	0.85	14,16
ZT80863	0.81	0.51	0.90	12,15
avg	0.72	0.40	0.85	
std	0.05	0.07	0.10	

- 2 samples were typed as ‘homozygous”
- 16 samples with lowest PHR values were amplified with extra polymerase
- Balance was improved with the SpeedStar polymerase

Samples with larger allele spreads for D10S1248 exhibited greater imbalance e.g. 14,16 better balance than 14,19

Peak Height Ratios for 3plex Loci



D10S1248 Peak Imbalance

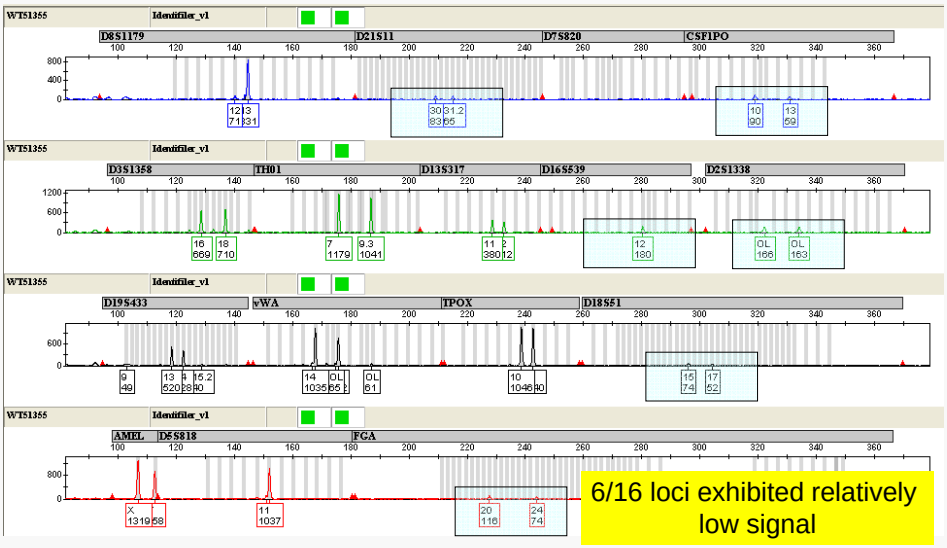
- Imbalance is not solely related to amplicon size
- Improved with additional SpeedStar polymerase
- Not an artifact of rapid thermal cycling conditions
- Other reasons
  - Repeat motif?
  - Primer T_m ?

Testing Commercials Kits

- Tested various commercial STR kits
  - 10 μL volume
  - 2 μL primer mix (commercial)
  - PyroStart +1 U SpeedSTAR polymerase
  - 1 ng of template DNA
  - 28 cycles (rapid cycling parameters)
  - 36 min

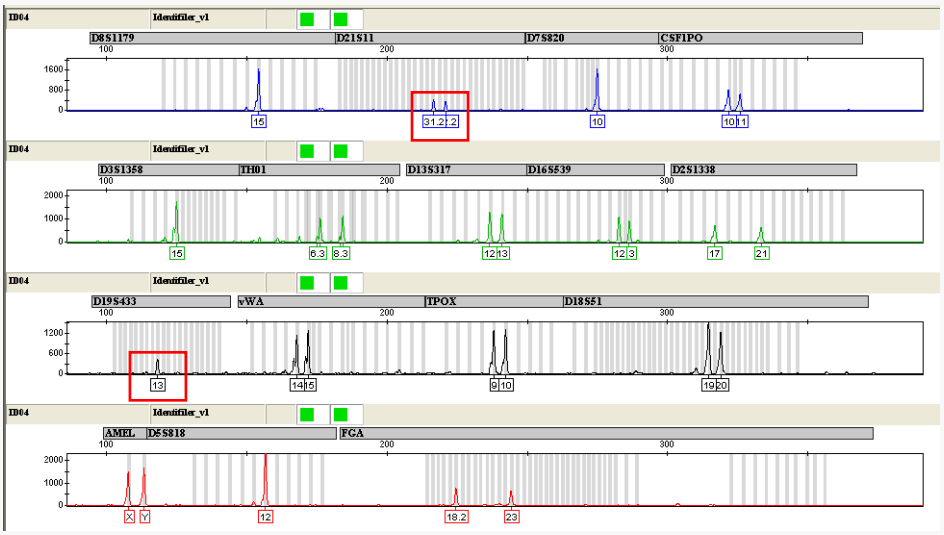
Promega Corporation → PowerPlex16
Applied Biosystems → Identifiler

Identifiler Rapid Cycling
without SpeedStar polymerase



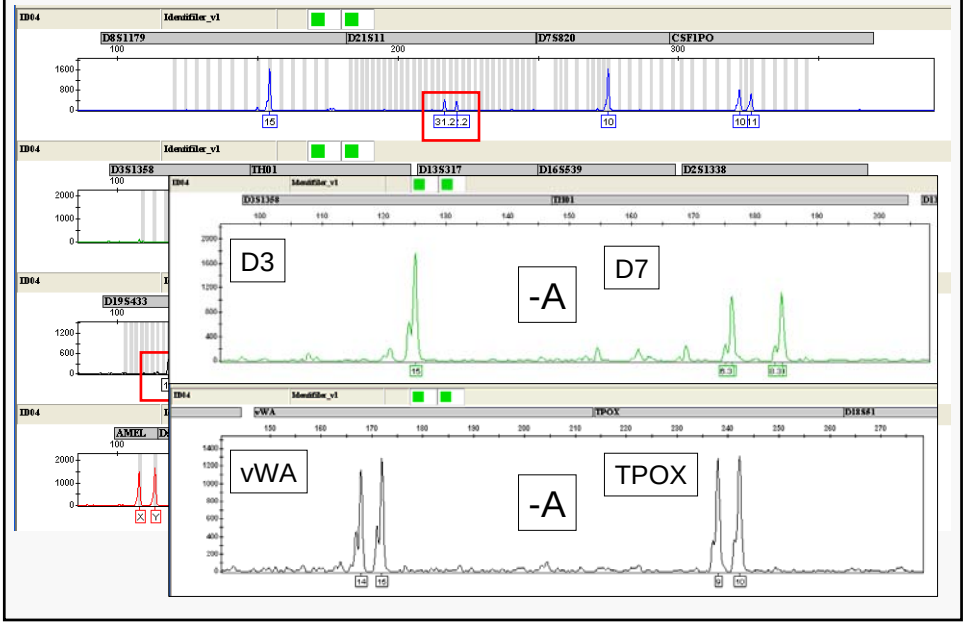
6/16 loci exhibited relatively low signal

Identifiler Rapid Cycling

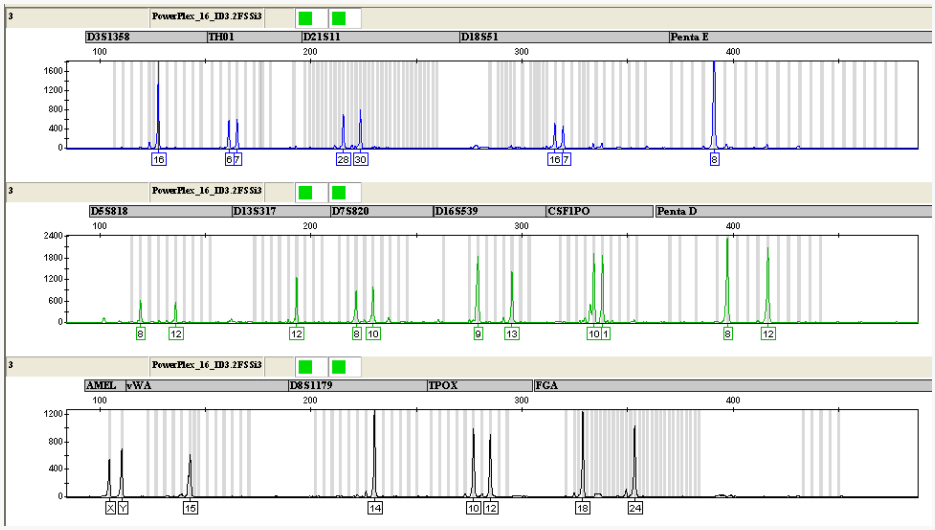


All 16 loci amplified, good balance, strong signal (except D19, D21)

Identifiler Rapid Cycling

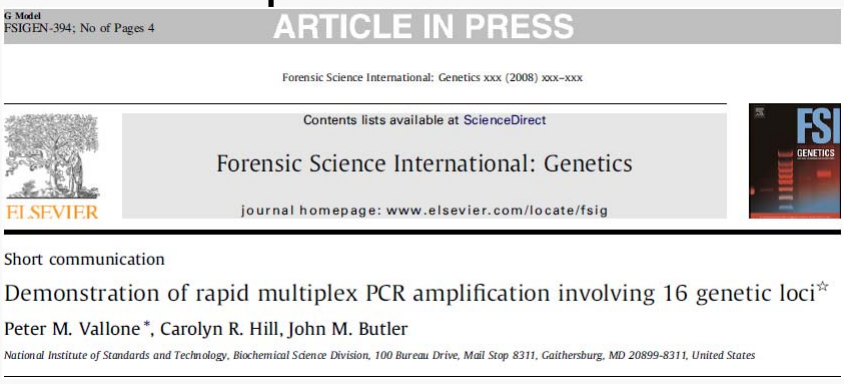


PowerPlex 16 Rapid Cycling



All 16 loci amplified, good balance, strong signal, efficient adenylation

Rapid PCR Article



G Model
FSIGEN-394; No of Pages 4

ARTICLE IN PRESS

Forensic Science International: Genetics xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Forensic Science International: Genetics

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

Short communication

Demonstration of rapid multiplex PCR amplification involving 16 genetic loci☆

Peter M. Vallone*, Carolyn R. Hill, John M. Butler

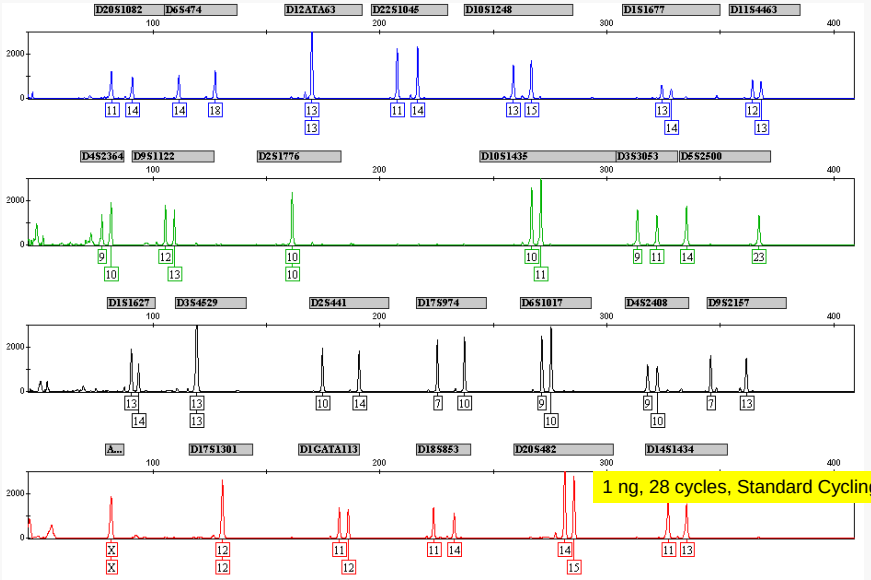
National Institute of Standards and Technology, Biochemical Science Division, 100 Bureau Drive, Mail Stop 8311, Gaithersburg, MD 20899-8311, United States

Complete concordance of STR allele calls (for 60 samples) between the rapid and standard thermal cycling protocols were observed although there was incomplete adenylation at several of the loci examined and some PCR artifacts were detected. Using less than **750 pg of template DNA and 28 cycles, STR peaks for all loci were above a 150 relative fluorescent unit (RFU) detection threshold** with fully adequate inter-locus balance and heterozygote peak height ratios of greater than 0.84.

Further Evaluation of NIST Loci

- We currently have an autosomal 26plex assay working in our lab
- Amplified (25/26) each locus in singleplex under rapid cycling conditions
- Evaluate each locus for signal intensity, full adenylation and non-specific artifacts
- Rank and test candidate loci in a rapid multiplex

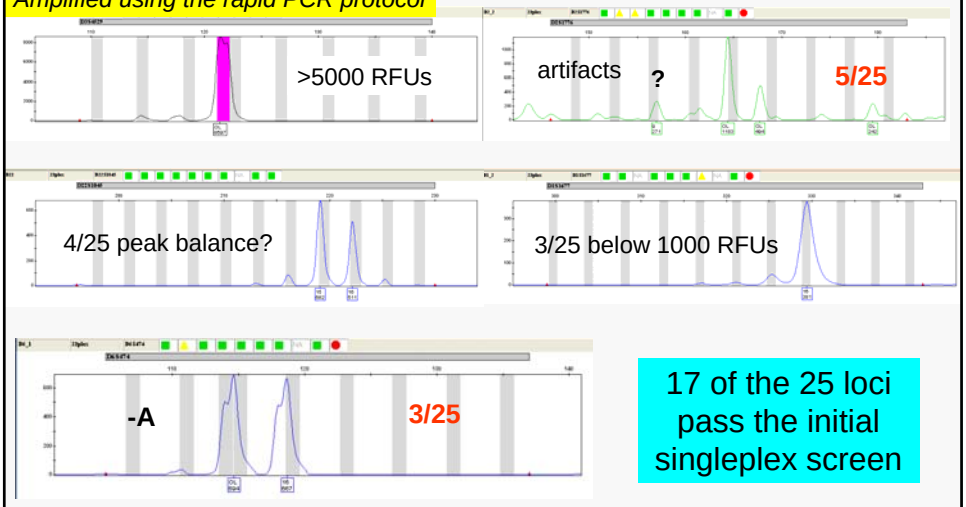
Autosomal STR 26plex



Hill, C.R., Butler, J.M., Vallone, P.M. (2008) A New 26plex Autosomal STR Assay to Aid Human Identity Testing submitted Journal of Forensic Science (in press)

Singleplex Evaluation

Amplified using the rapid PCR protocol



17 of the 25 loci pass the initial singleplex screen

Testing 4 Multiplexes

- After singleplex evaluation 4 multiplexes were tested (empirical balancing)
  - 17plex
  - 14plex
  - 12plex
  - 7plex

  } Subset of the 17plex

- Run under rapid cycling conditions
- 1 ng DNA, 28 cycles, PyroStart + 1 U SpeedSTAR

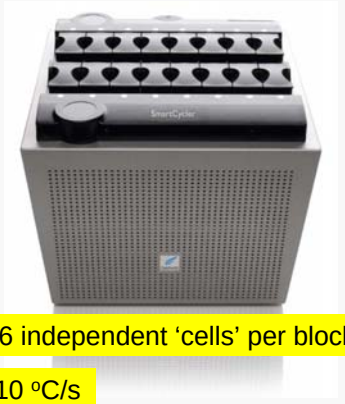
Rapid Assays Developed Using NIST Loci

- N = 16 samples
- D4S2364 adenylation issues/artifacts
- D9S2157 severe peak imbalance – allele drop out in 2 samples
- Further evidence that peak imbalance does not directly track with amplicon size
- 'Troublesome loci' can be screened out

Cepheid SmartCycler and Stratagene RoboCyler 96

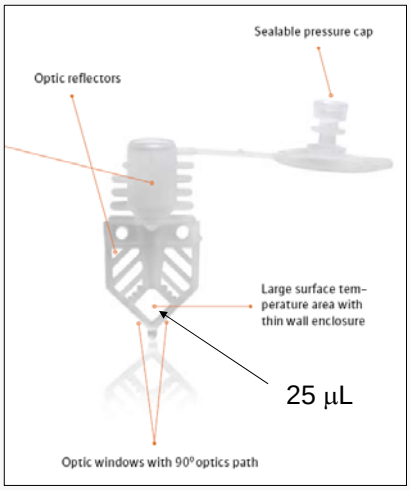
- Working with Dr. Daniele Podini (GWU)
  - Ms. Michelle Burns (NIST/GWU)
- Identifiler with rapid PCR protocols
  - Increased ramp rate
  - Shorter hold times
  - Testing other fast polymerases
  - Improved thermal transfer unique to the SmartCycler cell design

Cepheid SmartCycler



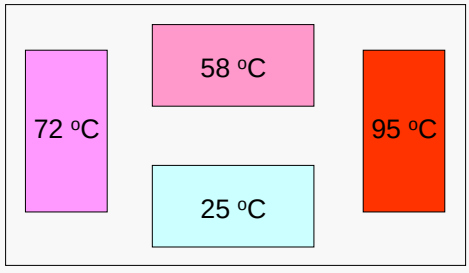
16 independent 'cells' per block

Heat 10 °C/s
Cool 2.5 °C/s



http://www.cepheid.com/systems-and-software/smartcycler-system/

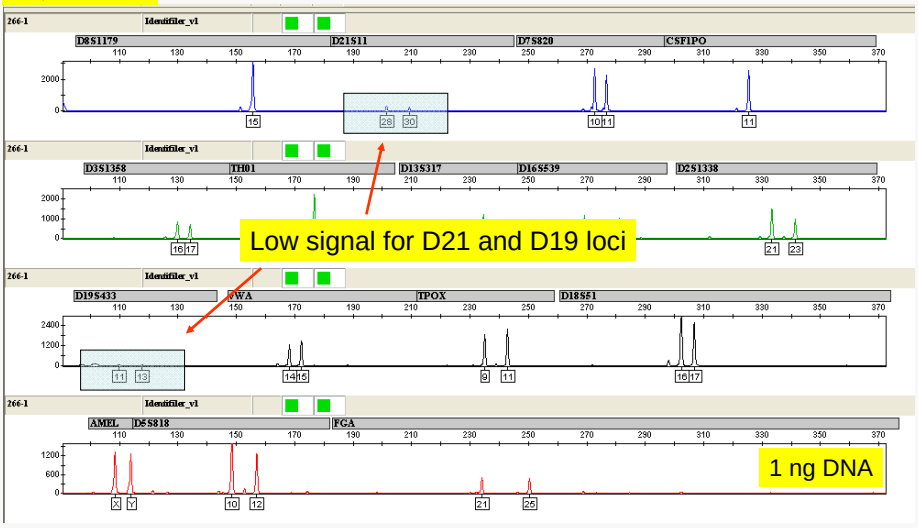
RoboCycler 96



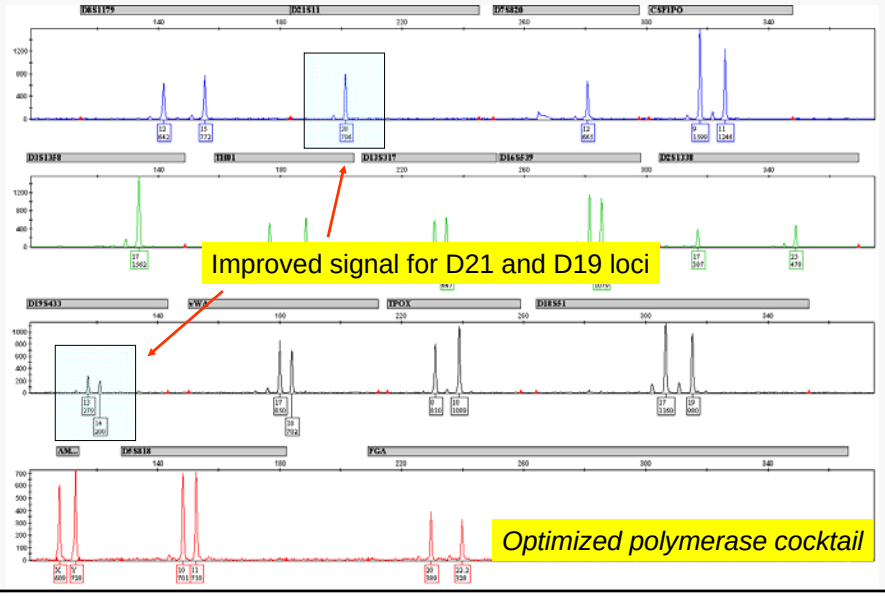
- Robotic arm moves sample tubes move from station to station
- No temperature ramping

SmartCycler 5s/10s/10s
25 minutes

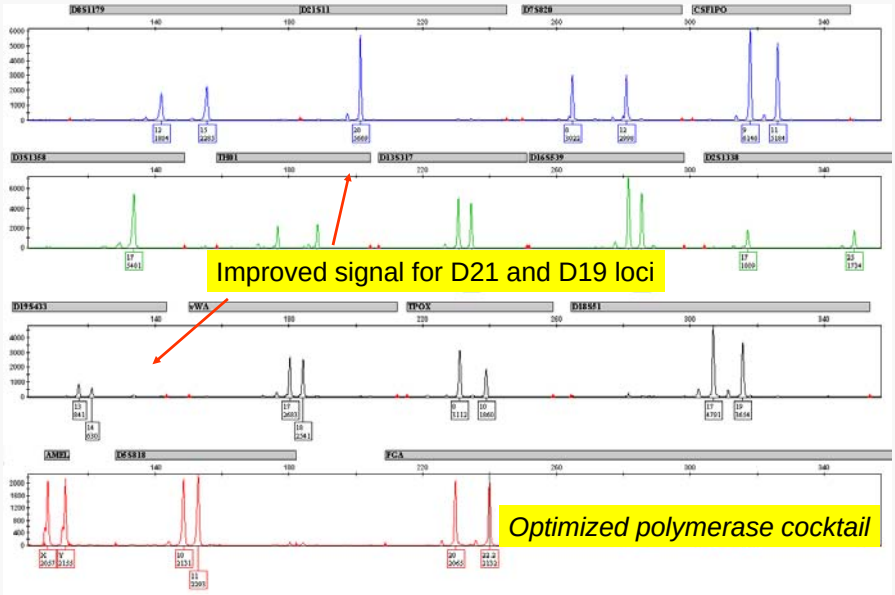
10 µL reaction



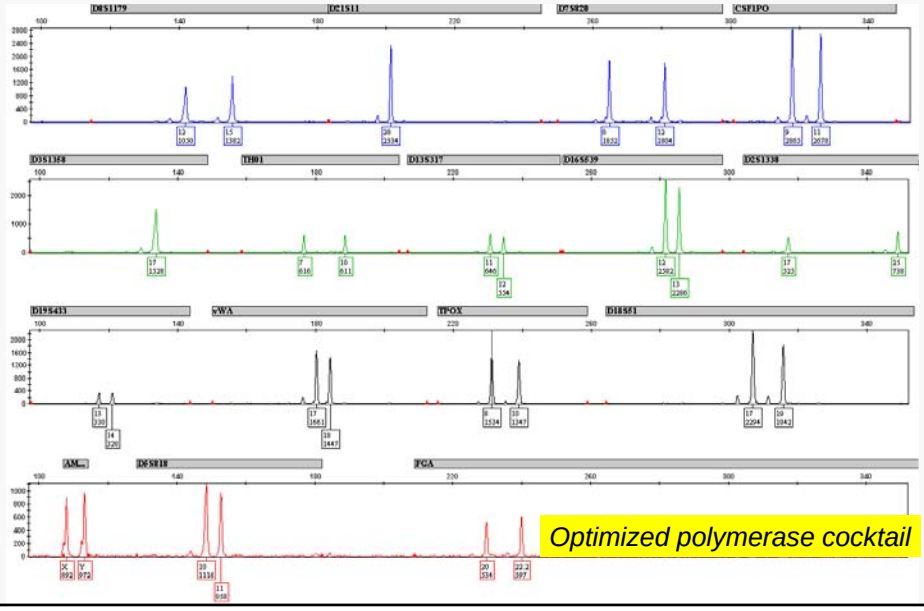
SmartCycler 5s/10s/10s
25 minutes



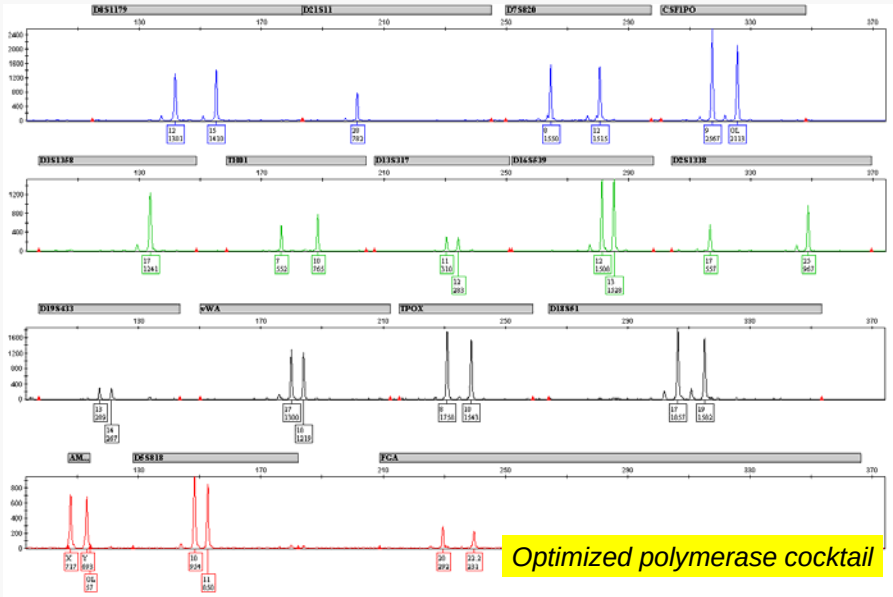
AB 9700 5s/10s/10s
36 minutes



AB 9700 1s/1s/1s
29 minutes



SmartCycler 2s/5s/4s
17 minutes



Final Conclusions

- Fast multiplex PCR amplification is possible
  - Compatible with commercial STR typing kits
  - Provides same genotypes as standard cycling
  - Some artifacts, signal imbalance, poor performing loci
- Fast (optimized) polymerases are needed
- Further work
  - Applying techniques to integrated platforms
  - Formal validation of technique
  - Sharing results with PCR community
  - Understanding the kinetics of PCR

Acknowledgements

John Butler
(Project Leader)

Becky Hill

Dr. Daniele Podini
Ms. Michelle Burns
(GWU)

Margaret Kline

Funding: **Interagency Agreement 2003-IJ-R-029 between National Institute of Justice (NIJ) and NIST Office of Law Enforcement Standards (OLES)**