Development of Rapid Multiplex PCR Amplification Techniques

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Outline

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

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

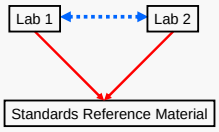
http://www.cstl.nist.gov/biotech/strbase/NIJprojects.htm

Standard Reference Materials (SRMs)

http://www.nist.gov/srm

Traceable standards to ensure accurate and comparable measurements between laboratories

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

Applications of Forensic 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

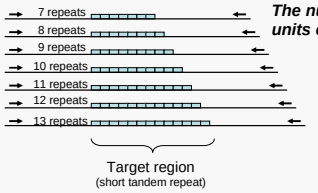
>3 million tests performed per year

Short Tandem Repeat (STR) Markers

An accordion-like DNA sequence that occurs between genes

TCCCAAGCTCTTCCTCTTCCCTAGATCAATACAGACAGAAGACA
GGTGGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
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

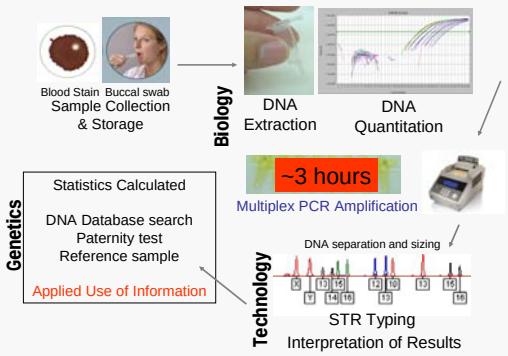
A DNA profile by itself is fairly useless because it has no context…

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**

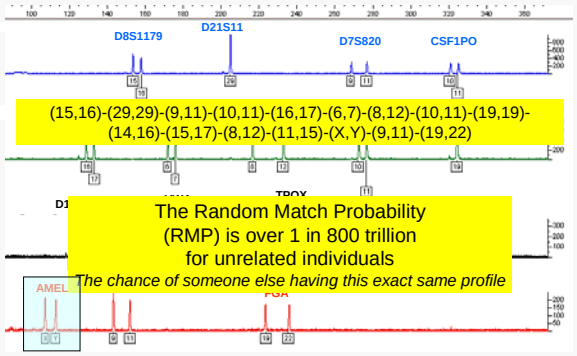
Steps in Forensic DNA Analysis

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



Identifiler STR Kit (Applied Biosystems)

Information is tied together with multiplex PCR and data analysis



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 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

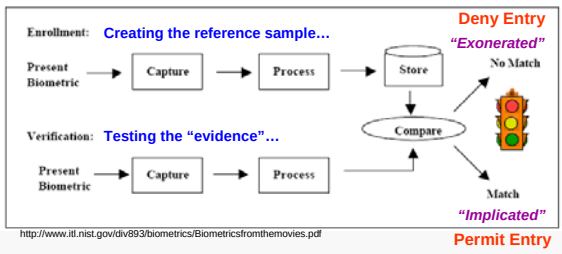
Things That Are Rapid

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

DNA as a Biometric tool

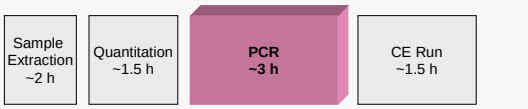
DNA within the Biometric Model



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

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

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

Thermal Cyclers

Cepheid SmartCycler
Ramp rate = 10°C/s

Purchased with FBI funding April 2009

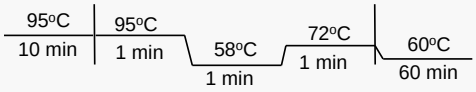
Eppendorf Mastercycler pro
Ramp rate = 6°C/sec

Applied Biosystems 9700
Ramp rate = 4°C/s

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

95ºC 10 min → 95ºC 1 min → 58ºC 1 min → 72ºC 1 min → 60ºC 60 min

95ºC 1 min → 95ºC 5 s → 58ºC 10 s → 72ºC 10 s → 72ºC 1 min

~36 min run time

Heating/Cooling rate of ~4ºC/s

Thermal Cycling

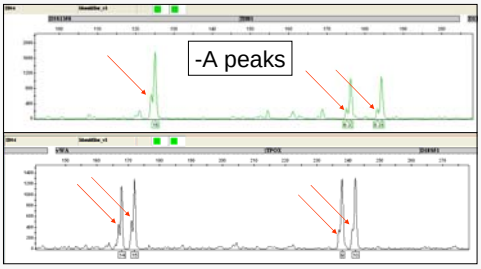
Parameter	Unit	Trad	Rapid	Difference (min)	%
Hot Start	Min	10	1	9.0	6.3
Hold	Sec	60	5/10	72.3	50.6
Soak	Min	60	1	59.0	41.2
Ramp rate	(deg/sec)	1	4	22.4	15.7
Cycles		28	28		
Time		2:58:41	0:35:38	2:23:03	

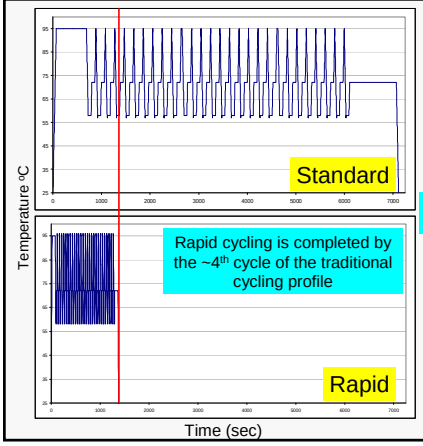
Parameter	Purpose
Hot Start	Primer Dimer, non-specific amplification
Hold	Amplification of template - Inter and intra locus balance
Soak	Complete adenylation of PCR products

Evaluate robustness and reproducibility (electropherograms)

Adenylation Efficiency or Non-Template Addition

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





Comparison of Thermal Cycling Profiles

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 – single source)

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

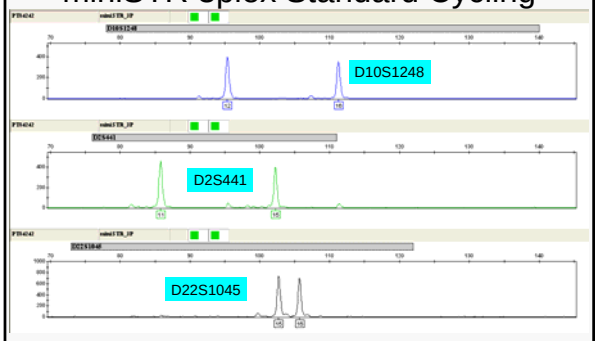
Polymerase	Vendor	MasterMix	Hot Start
TaqGold	Applied Biosystems	no	10 min
GeneAmp	Applied Biosystems	yes (2x)	1 min
SpeedSTAR	Takara	no	1 min
PyroStart	Fermentas	yes (2x)	1 min
Qiagen Fast Cycling PCR Kit	Qiagen	yes (2x)	5 min

Brief survey of 'fast' commercial polymerases

Initial Testing with miniSTR 3plex

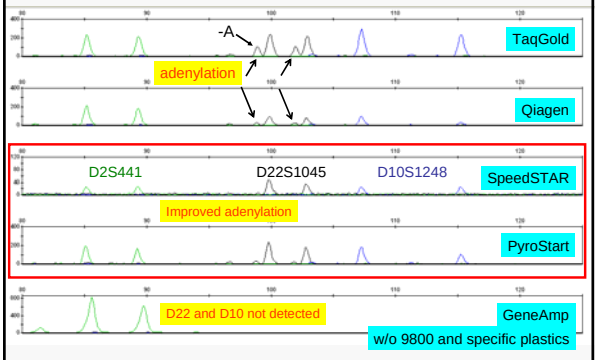
- 3 loci labeled with 3 fluorescent dyes (FAM, VIC, NED)
- MiniSTR loci (Amplicon size range 65-140 bp)
  - D2S441, D10S1248 and D22S1045
  - 'European loci' (contained in the next generation of forensic kits)
- These loci were previously tested under standard cycling conditions in a miniSTR multiplex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

miniSTR 3plex Standard Cycling

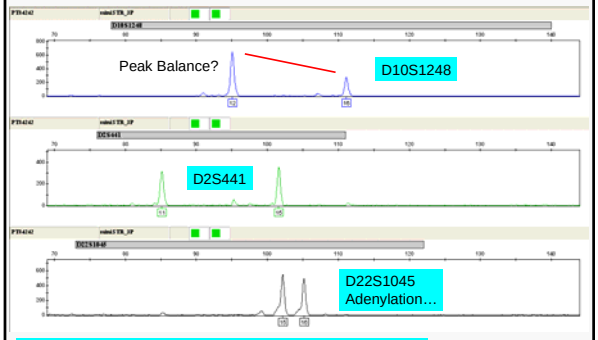


750 pg template, 28 cycles, TaqGold, ~3 hour cycling time

Initial Rapid Testing Results



Rapid PCR 28 cycles in 36 minutes

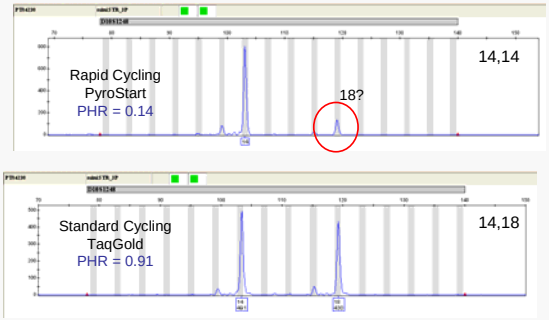


750 pg template, 28 cycles, PyroStart, 36 minute cycling time

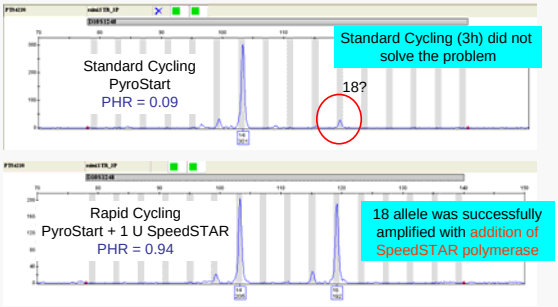
miniSTR 3plex Concordance

- 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



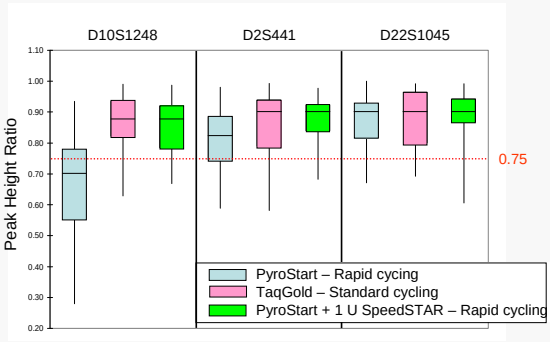
Peak Height Ratios for 16 Samples

Sample Name	Cycling 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

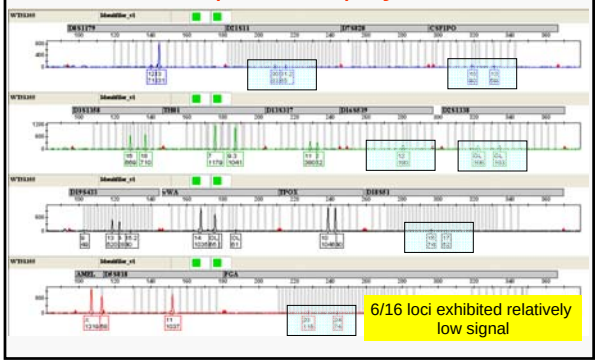
- Imbalance is not solely related to amplicon size
- Improved with additional SpeedStar polymerase
- Not an artifact of rapid thermal cycling conditions
- Other reasons
  - Repeat motif?
  - Primer T_m ?

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

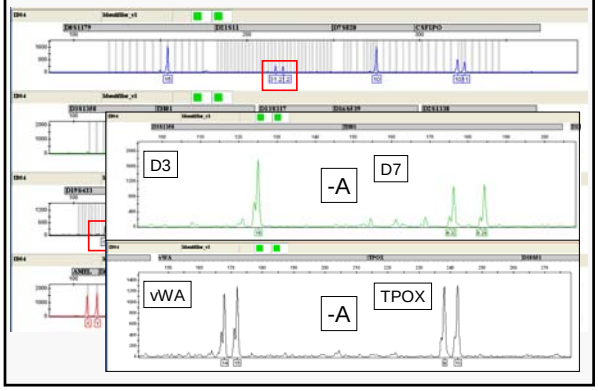


6/16 loci exhibited relatively low signal

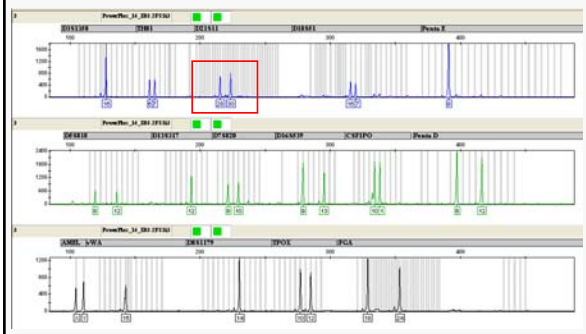
Identifiler Rapid Cycling

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

Identifiler Rapid Cycling

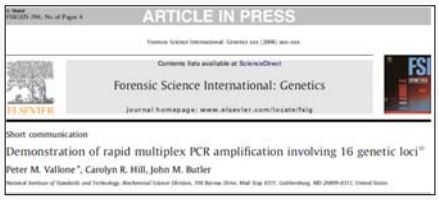


PowerPlex 16 Rapid Cycling



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

Rapid PCR Article

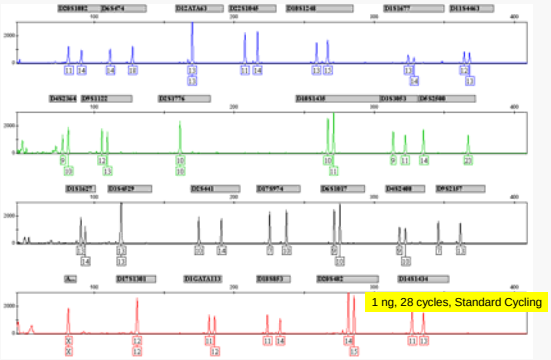


Vallone, P.M., Hill, C.R., Butler, J.M. (2008) Demonstration of rapid multiplex PCR amplification involving 16 genetic loci. *FSI Genetics* 3(1): 42-45.

Further Evaluation of NIST Loci

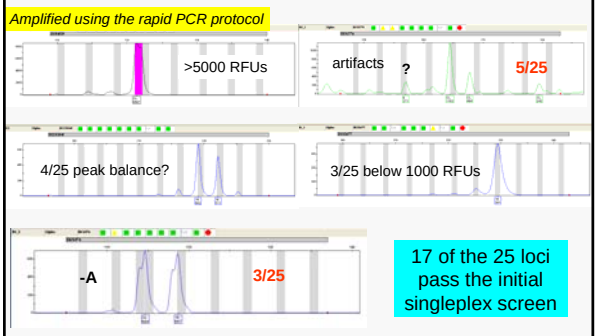
- We currently have an autosomal 26plex assay working in our lab
- Amplified (25/26) each locus in singleplex under rapid cycling conditions
- Evaluate each locus for signal intensity, full adenylation and non-specific artifacts
- Rank and test candidate loci in a rapid multiplex

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



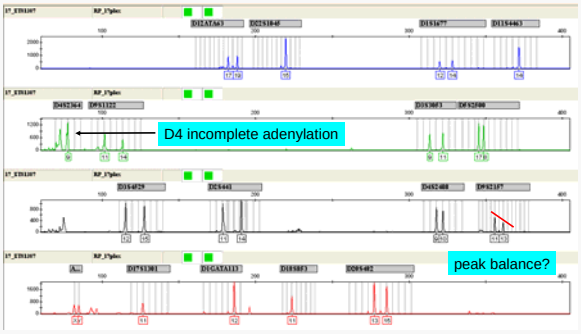
17 of the 25 loci pass the initial singleplex screen

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

17plex



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 or PCR primers redesigned

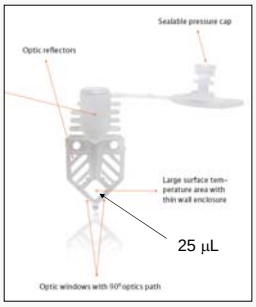
Cepheid SmartCycler and Stratagene RoboCyler 96

- Working with Dr. Daniele Podini (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

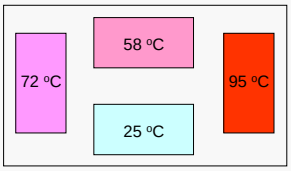
16 independent 'cells' per block

Heat 10 °C/s
Cool 2.5 °C/s



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

RoboCycler 96



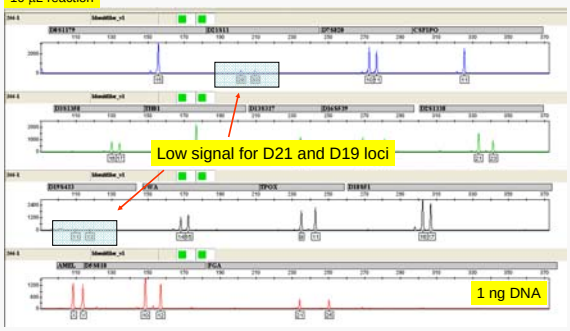
- Robotic arm moves sample tubes move from station to station
- No temperature ramping

Testing Another Polymerase

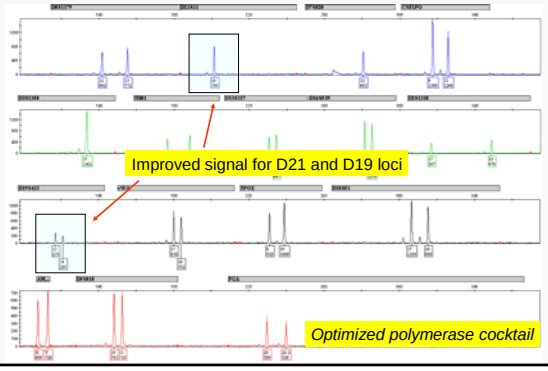
- ***Premix Ex Taq*™ (Perfect Real Time)** - Takara
- Formulated for real-time PCR
- Optimal 'cocktail' worked out
  - 2.5 µLPyroStar master mix (0.5x)
  - 2.5 µL Perfect Real Time master mix (0.5x)
  - 0.25 µL SpeedStar Enzyme
- Improvements in overall signal (esp. for D21 and D19)

SmartCycler 5s/10s/10s 25 minutes

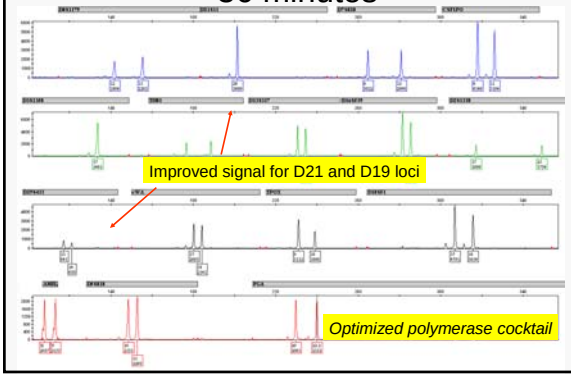
10 µL reaction



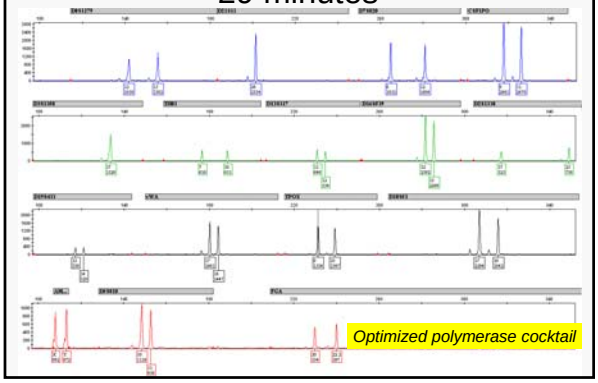
SmartCycler 5s/10s/10s 25 minutes



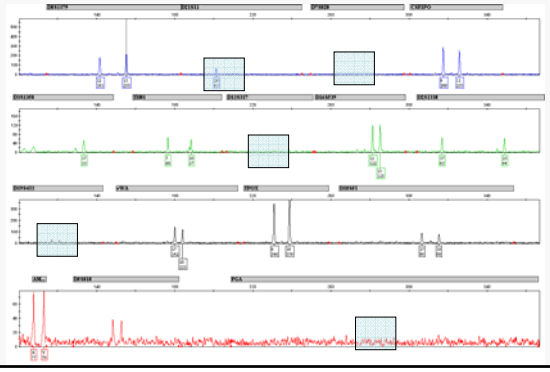
AB 9700 5s/10s/10s 36 minutes



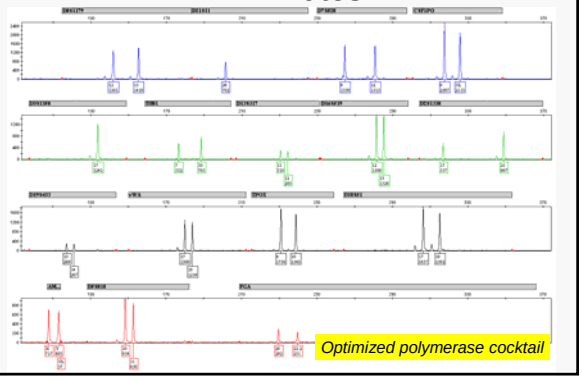
AB 9700 1s/1s/1s
29 minutes



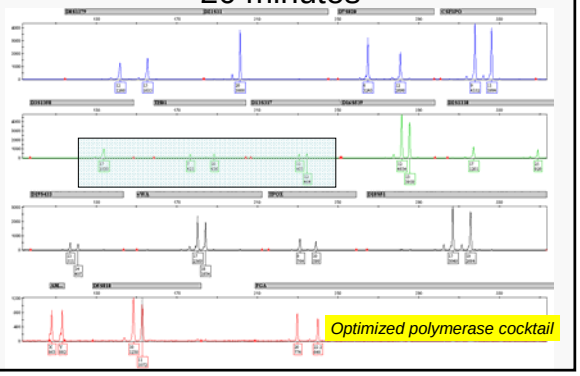
SmartCycler 1s/1s/1s
15 minutes



SmartCycler 2s/5s/4s
17 minutes



RoboCycler 10s/15s/15s
20 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

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