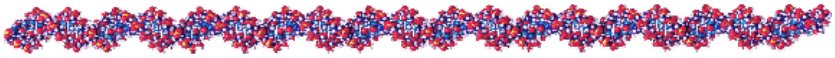
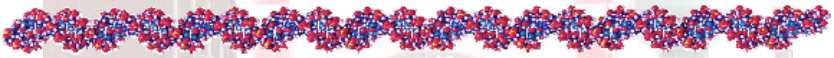


# Developing Rapid Multiplex PCR Assays with miniSTR Loci



Peter M Vallone PhD, Becky Hill MS
and John M Butler PhD
Human Identification Project
National Institute of Standards and Technology

60th Annual AAFS Meeting
Washington, D.C.
February 23rd, 2008

## NIST Disclaimer

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

Points of view are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

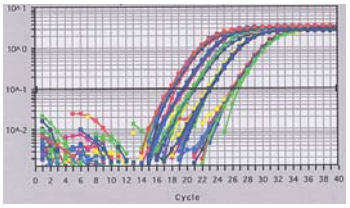
**Our publications and presentations are made available at:**
**http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm**

# Outline

- Rapid PCR
- Conditions and Parameters
- miniSTR 3plex
- Commercial Kits
- Larger Custom Multiplexes
- Conclusions

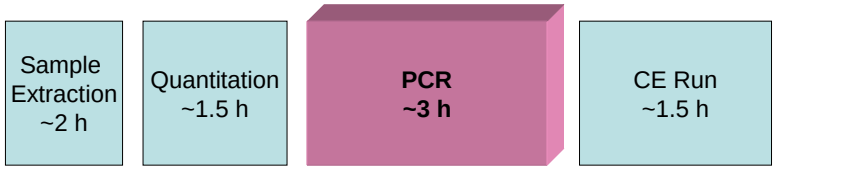
# Things That Are Rapid






# Typical STR Workflow




How can we reduce the time needed for cycling?

What happens when we alter cycling parameters?
How will existing commercial kits work?
How will different polymerases perform?
How robust will the results be?
Can we develop novel assays and further the understanding/limits of rapid multiplex PCR?

# 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
- Do not necessarily have to use CODIS 13+ loci (fewer loci or alternative loci?)

# Work Developing Integrated STR Typing Devices

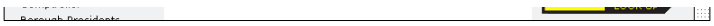
- UVA
  http://faculty.virginia.edu/landers/Home.html
- Berkeley
  http://chem.berkeley.edu/people/faculty/mathies/mathies.html
- Arizona State
  http://www.biodesign.asu.edu/centers/anb/projects/#prepsystem
- NIST
  http://www.eeel.nist.gov/812/mg.html

- Companies
  - http://www.microlabdiagnostics.com/index.html
  - http://microchipbiotech.com/
  - http://www.networkbiosystems.com/

*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*

# Rapid PCR

- What do we mean by rapid PCR?
  - Rapid hot start polymerases (save ~10min)
  - Shortening cycling hold times (5 sec vs 1 min)
  - Utilizing existing thermal cycling technology (AB 9700)
    - Eliminating 1 $^{o}$C/sec ramp rate (9600 emulation)
    - Utilize the 9700 4 $^{o}$C/sec ramp rate
  - Using commercial polymerases that are 'faster'

Obtain results in less than 45 minutes
Trying simple things first…

# Thermal Cyclers

AB 9800

http://www.appliedbiosystems.com

RapidCycler 2 Instrument
http://www.idahotech.com
Ramp rate = **10$^{o}$C/sec**

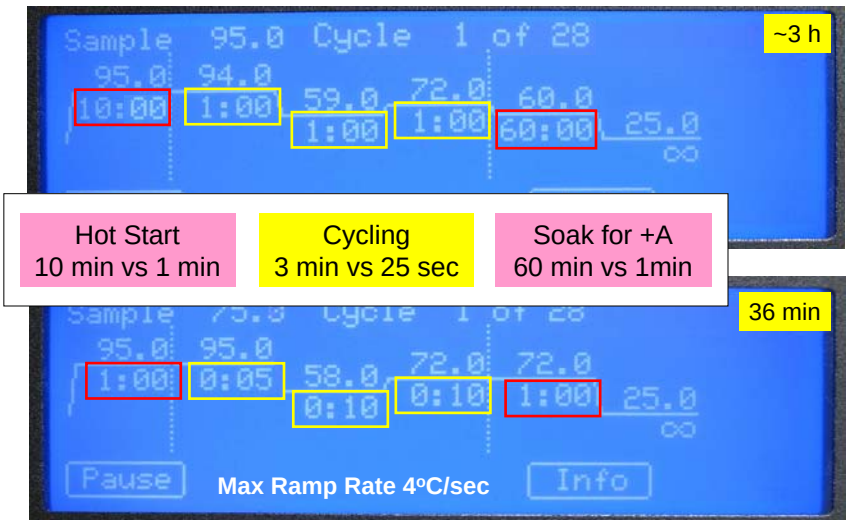
Eppendorf
Mastercycler ep
**6$^{o}$C/sec**
http://www.eppendorfna.com

AB 9700

Ramp rate = **4$^{o}$C/sec**

http://www.appliedbiosystems.com

# Thermal Cycling



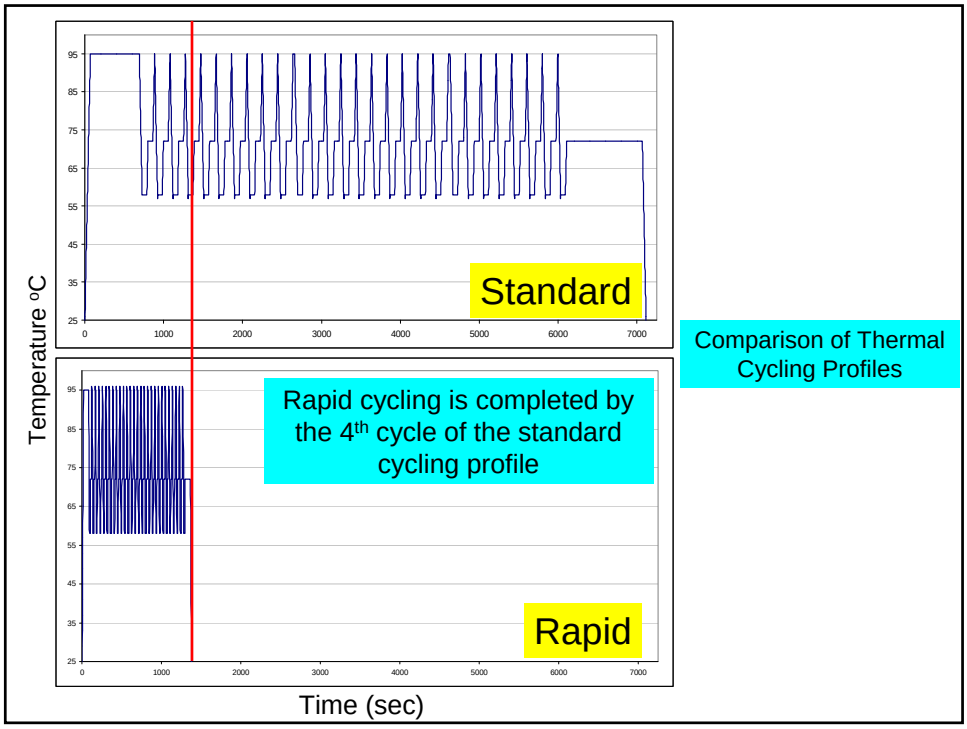

Rapid Thermal Cycling Conditions used

# 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 | Denature, annealing, elongation, Inter and intra locus balance |
| Soak | Full adenylation of PCR products |

Evaluate robustness and reproducibility




# 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 $Mg^{++}$
  - 5 color dye labeled primers
  - Separation on AB 3130
  - Not sample limited (>500 pg of DNA)

# Loci for Testing

- 26 autosomal loci characterized in our laboratory (Ms. Becky Hill last Thursday)
  - Small 3plex panels
  - Larger 26plex
- Available in kits - 13 CODIS +
- 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.

# Polymerases

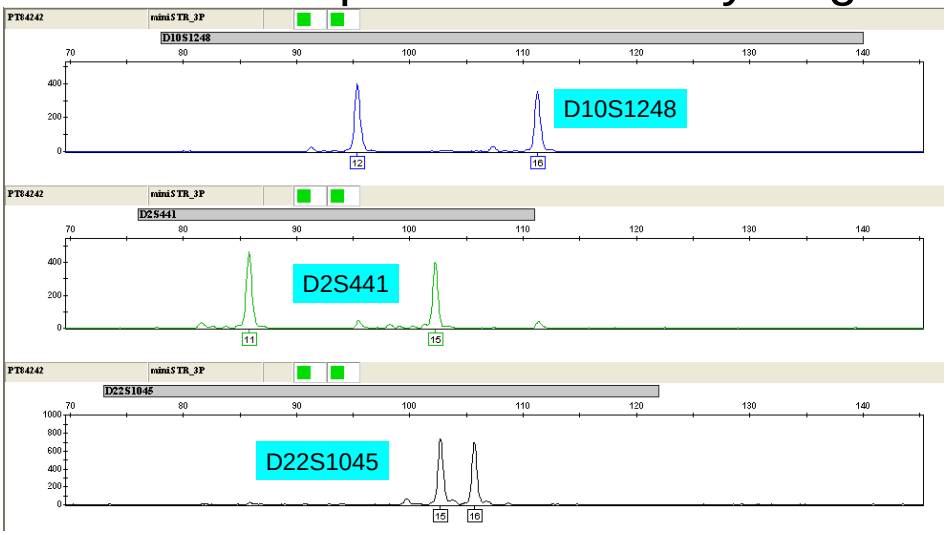
| Polymerase | Vendor | MasterMix | Hot Start |
|---|---|---|---|
| TaqGold | Applied Biosystems | no | 10 min |
| GeneAmp | Applied Biosystems | yes (2x) | 1 min |
| SpeedSTAR | Takara | no | 1 min |
| PyroStart | Fermentas | yes (2x) | 1 min |
| Qiagen Fast Cycling PCR Kit | Qiagen | yes (2x) | 5 min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

Brief survey of 'fast' commercial polymerases
Others exist, these were tried first

## Initial Testing with miniSTR 3plex

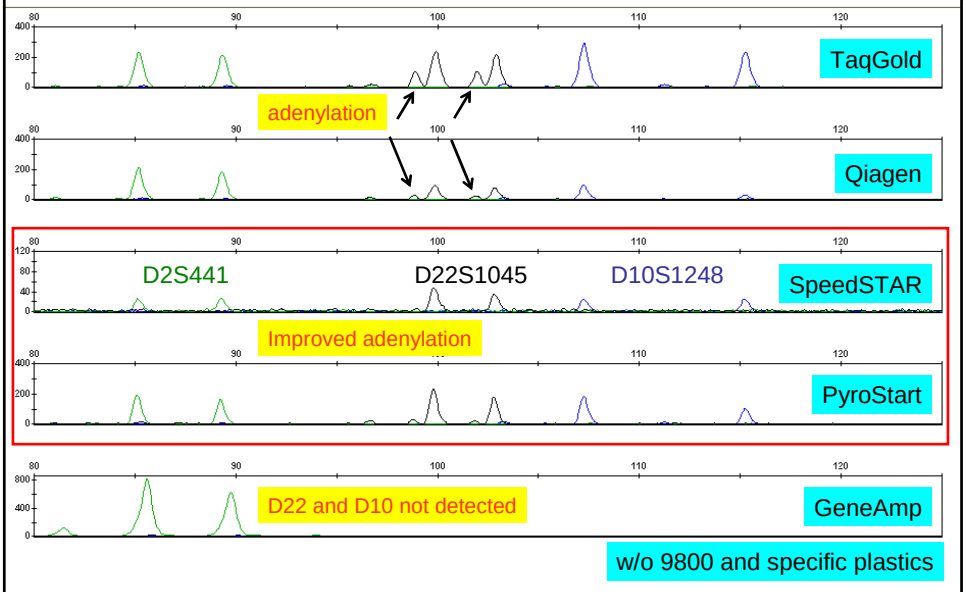
- A simple 3plex in 3 dye colors (FAM, VIC, NED)
- MiniSTR loci
  - D2S441, D10S1248 and D22S1045
  - 'European loci'
  - Amplicon size range 65-140 bp
- These loci were previously tested in a miniSTR multiplex in our lab

## miniSTR 3plex Standard Cycling



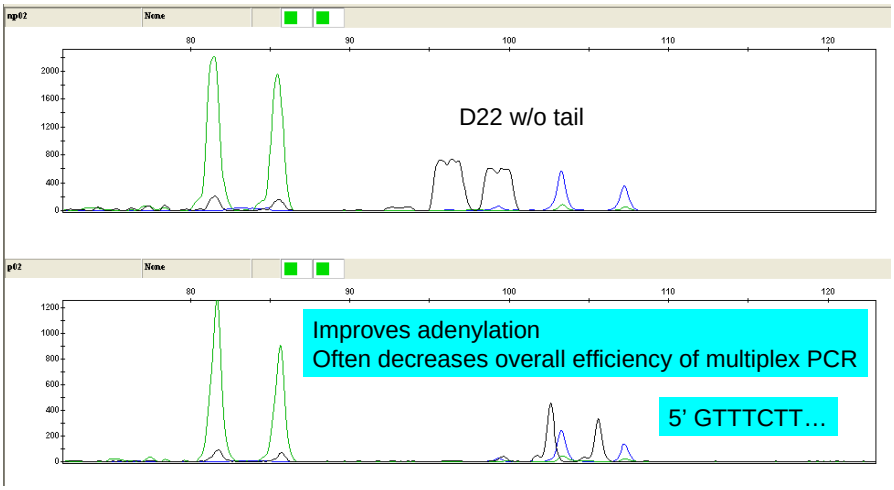

750 pg template, 28 cycles, TaqGold, ~3 hour cycling time

## Initial Rapid Testing Results




w/o 9800 and specific plastics

## Adding a Pigtail to D22S1045




2 ng template, 28 cycles, PyroStart, 36 minute cycling time

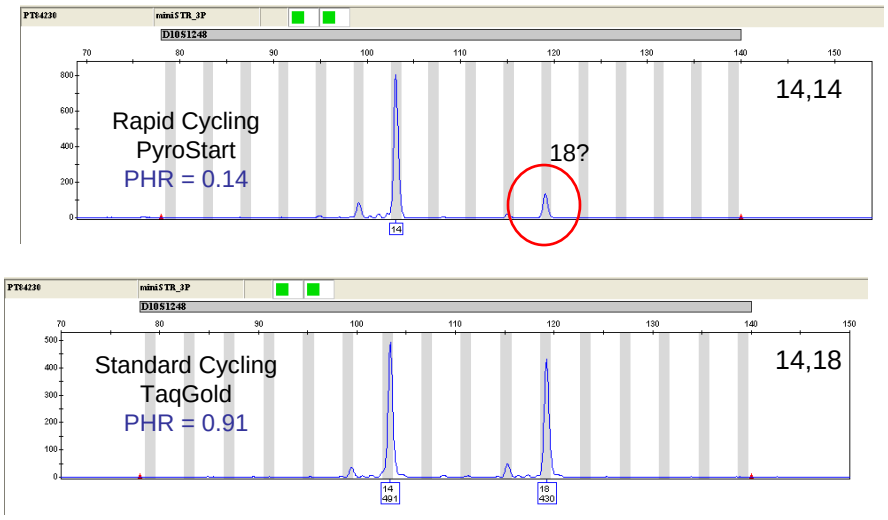
# Rapid PCR 28 cycles in 36 minutes


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

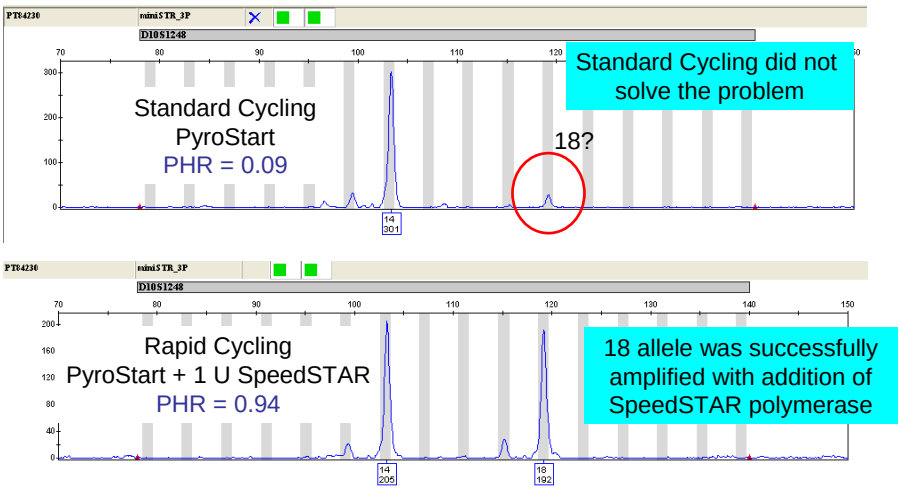
# miniSTR 3plex Concordance

- 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 severe heterozygote imbalance

# Peak Height Ratio Imbalance for D10S1248




# Peak Height Ratio Imbalance for D10S1248



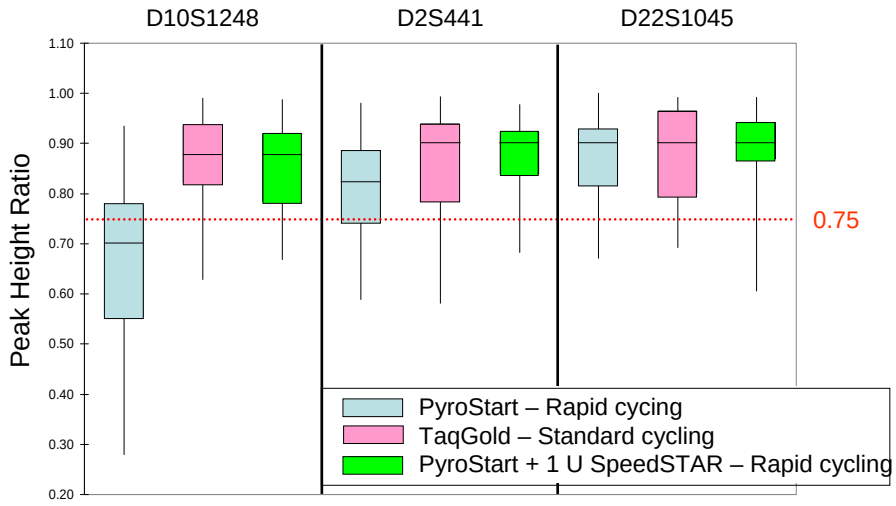
# Peak Height Ratio for 16 Samples

| Sample Name | Cycling TaqGold | Normal Pyro | Rapid Pyro+SS | Rapid | 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 extra polymerase

Samples with larger allele spreads for D10 exhibited greater imbalance
e.g. 14,16 better balance than 14,19

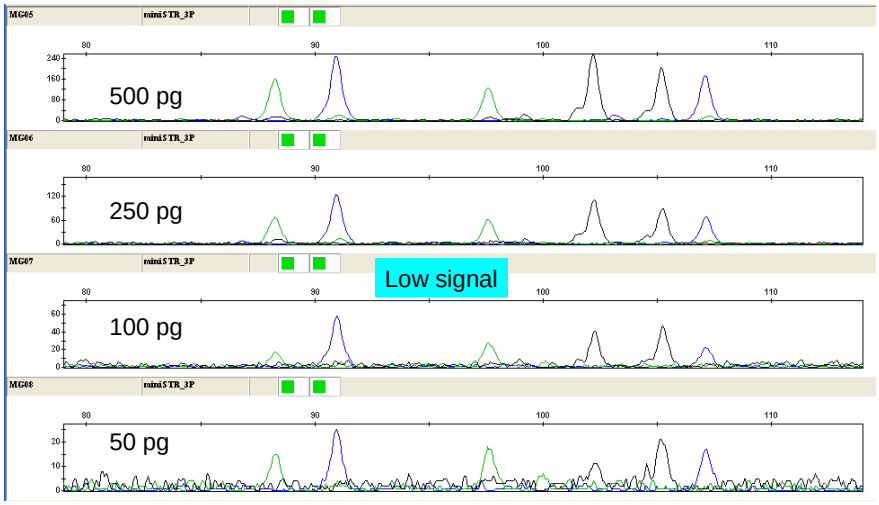
# Peak Height Ratios for 3plex Loci



## D10S1248 PHR Imbalance

- Imbalance is not solely related to amplicon size
- Improved with additional polymerase
- Not an artifact of rapid cycling conditions
- Other reasons
  - Repeat motif?
  - Primer $T_m$ ?
  - Sequence region for SNPs?

## Sensitivity of miniSTR 3plex

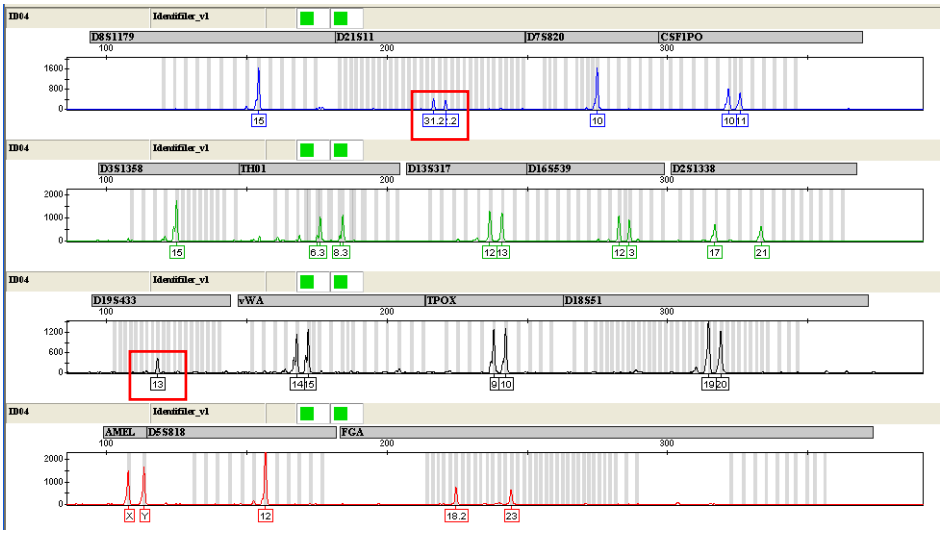



28 cycles, PyroStart polymerase, 36min

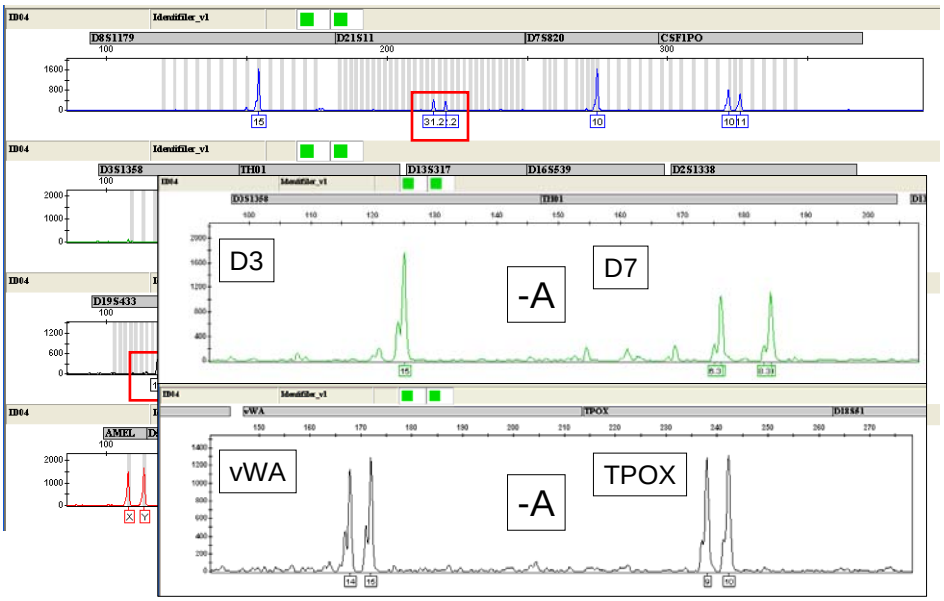
# Testing Commercials Kits

- Tested PP16, COfiler, Profiler Plus ID, Identifiler, and Minifiler primer mixes
  - 10 μL volume
  - 2 μL primer mix
  - PyroStart +1 U SpeedSTAR polymerase
  - 1 ng of template DNA
  - 28 cycles (rapid cycling parameters)
  - 36 min

# Identifiler Rapid Cycling



## Identifiler Rapid Cycling




## Evaluation…

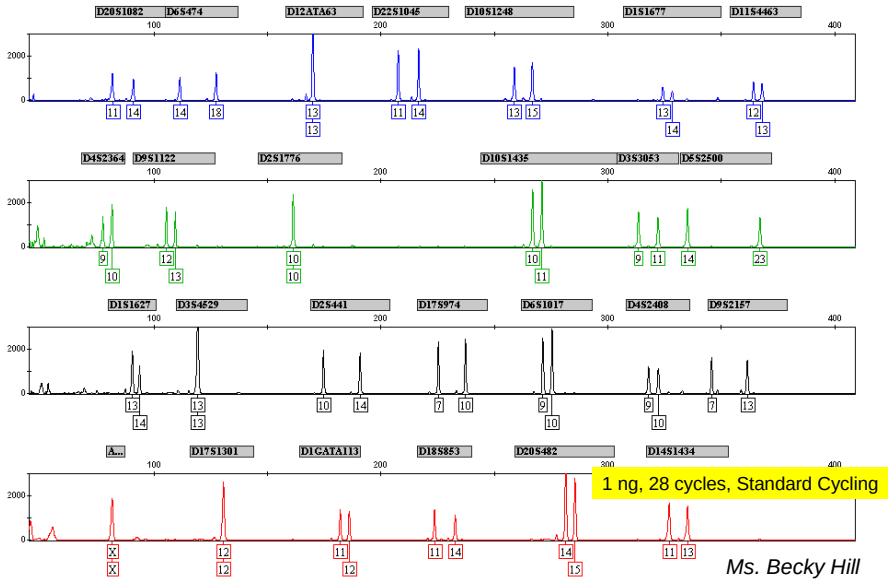
- The large 16 loci kits can be successfully amplified with a rapid thermal cycling protocol
- Additional polymerase is needed

- Which loci show imbalance?
- Stuck with poorly adenylating loci?
- Can not alter primer concentrations/sequence
- Further work to be performed: sensitivity, stutter, drop out, etc

# Further Evaluation of Our Loci

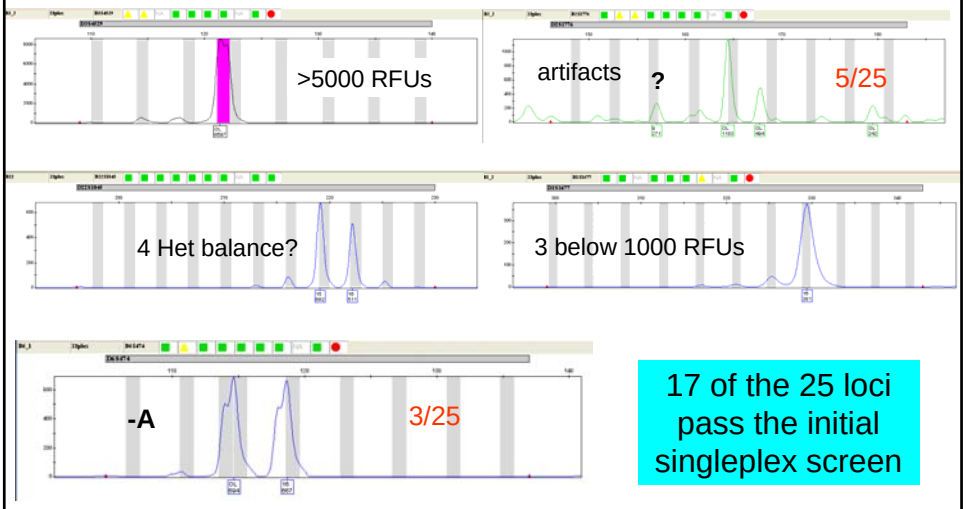
- We currently have a autosomal 26plex assay working in our lab
- Amplified (25/26) each locus in singleplex under rapid cycling conditions
- Evaluate each locus for signal, adenylation and artifacts
- Rank and test candidate loci in a rapid multiplex

# 26plex Presented Last Thursday




*Ms. Becky Hill*

# Singleplex Evaluation



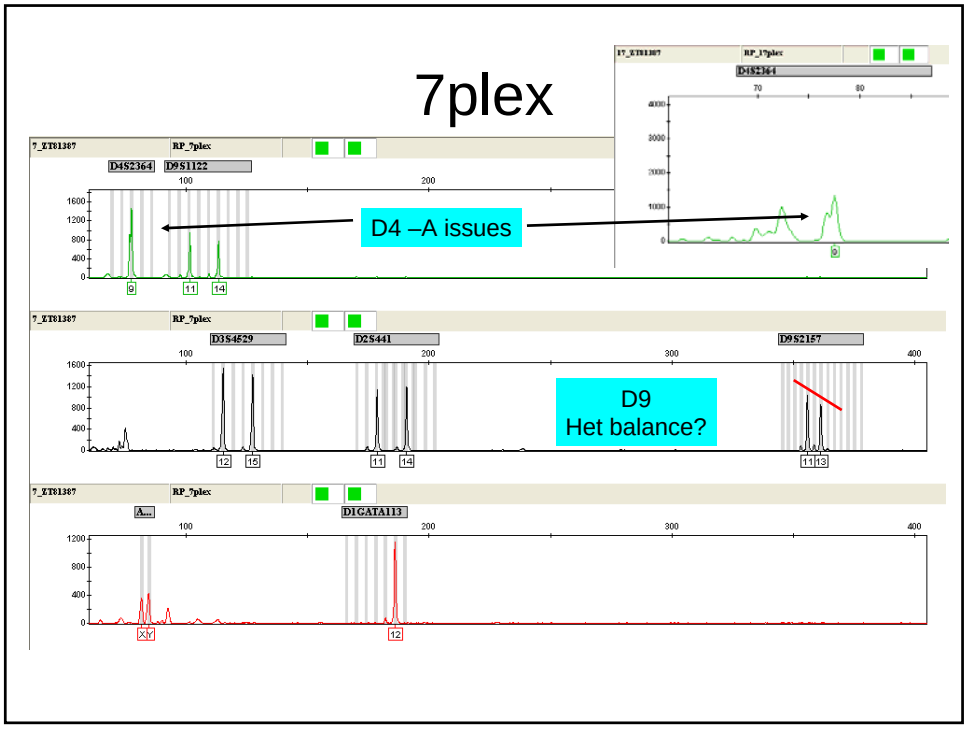

17 of the 25 loci pass the initial singleplex screen

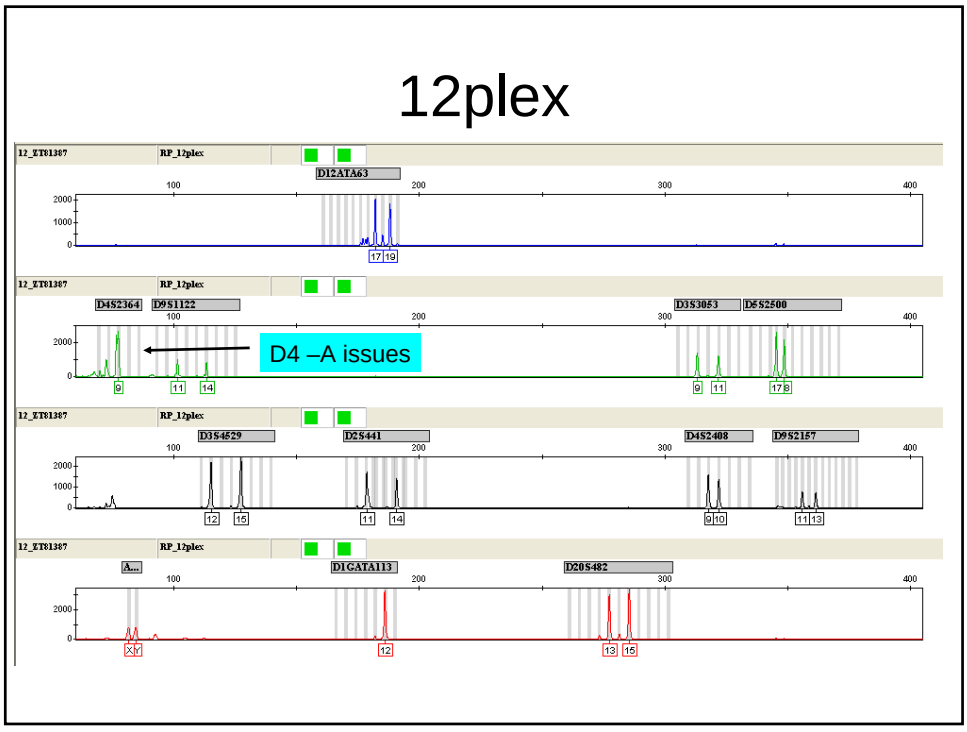
# Testing 4 Multiplexes

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

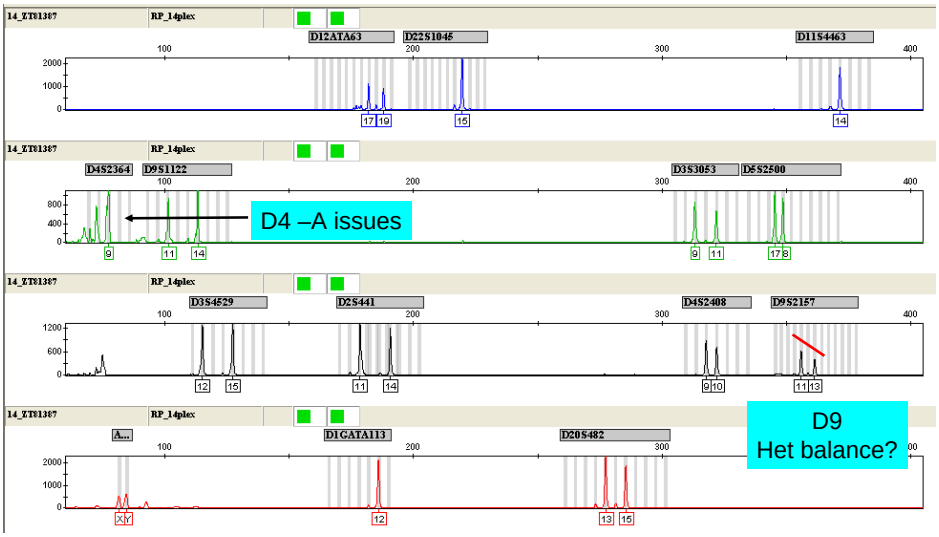
  } Subset of the 17plex

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

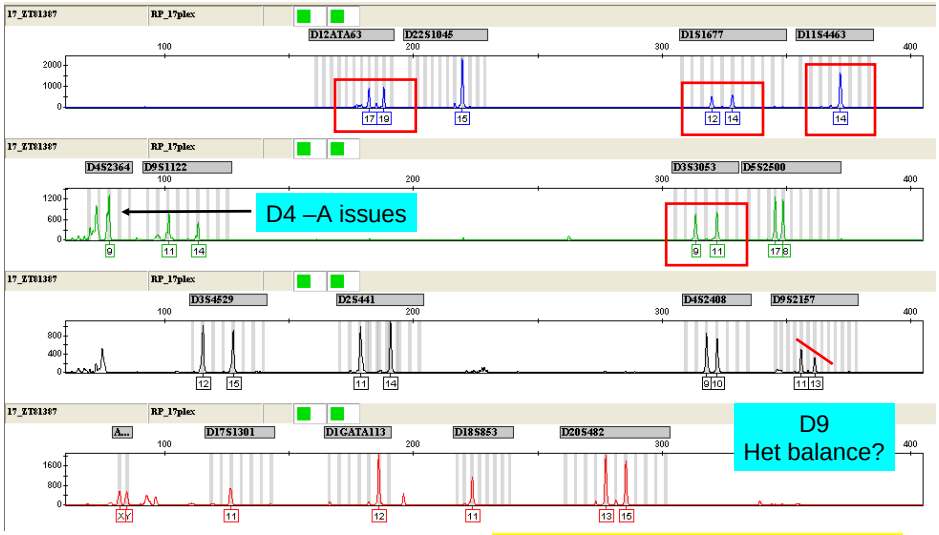

7plex
D4 –A issues
D9
Het balance?


12plex
D4 –A issues

# 14plex




# 17plex




Boxed loci have pigtailed primers

## Rapid Multiplex Concordance

- Results for the rapid multiplexes were compared with previously run assays (Standard cycling – TaqGold)
- N = 16 samples
- D4S2364 adenylation issues/artifacts
- D9S2157 severe het imbalance – allele drop out in 2 samples (13,13 vs 13,14) and (7,7 vs 7,11)
- Further evidence that heterozygote imbalance does not directly track with amplicon size

## Conclusions

- A large multiplex (17plex) can be amplified in 36 min on an AB 9700 cycler
- Must test for poor adenylation and heterozygote imbalance
- Larger multiplexes require additional polymerase to obtain complete profiles

- More rigorous testing of our larger multiplexes
- Test other/faster thermal cycling platforms
- Commercial primer mixes can be used – still needs further investigation

# Acknowledgements

John Butler
(Project Leader)

Becky Hill

Prepared primer mixes!

Margaret Kline

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